Induction of protein synthesis in cardiac fibroblasts by cardiotrophin-1: integration of multiple signaling pathways

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Abstract

Objective: Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines and is expressed in various cardiovascular disease states. CT-1 induces cardiomyocyte hypertrophy, and protects myocytes from ischemia reperfusion injury. We sought to elucidate CT-1 signaling in cardiac fibroblasts with respect to initiation of protein synthesis. Methods: Cardiac fibroblasts were isolated from the ventricles of 200-g Sprague–Dawley rats and stimulated with CT-1 at specified concentrations with or without inhibitors of cell signaling pathways. Activation of intracellular signaling pathways was determined by Western analysis and immunocytochemistry. Protein synthesis was measured by incorporation of [3H]leucine. Results: CT-1 treatment resulted in activation of the Jak/STAT, MAPK, and Akt pathways in addition to protein synthesis regulatory proteins with resultant increase in overall protein synthesis. Analysis with phospho-specific antibodies revealed that AG490 (Jak inhibitor), PD98059 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), LY294002 (PI3-K inhibitor) and rapamycin (mTOR inhibitor) act at different levels in the signaling cascade to inhibit CT-1 induced protein synthesis. Conclusion: Cardiotrophin-1 activates the Jak/STAT, PI3K/Akt, p38 and p42/44 MAPK pathways in cardiac fibroblasts. Use of pharmacologic inhibitors reveals that each of these pathways play a role in CT-1 induced protein synthesis.

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1. Introduction

Cardiac fibroblasts and their hypersynthetic and hyper-secretory derivatives, myofibroblasts, are the most numerous cellular component of the heart and function to maintain optimal spatial placement of contractile myocytes relative to each other and of the cardiac interstitium itself. These cells play a critical role in maintenance of inter-myocyte fibrillar collagen tethers required for efficient contraction in health, but myofibroblasts in particular function to initiate wound collagen healing in response to a diverse array of pathologic insults [1,2]. These cells are governed by flexible and in some cases, rapidly inducible signaling pathways that allow for sensitive gain adjustment in regulation of protein synthesis; this is required to achieve the range of responses for normal cardiac function and for normal responsiveness to stressful stimuli. This regulation is a tightly controlled and integrated cascade that is responsive to a variety of growth factors, components of the ECM, and mechanical stress [3]. Stimulation of cardiac fibroblasts and myofibroblasts by any of these stimuli may initiate a number of diverse cellular responses, i.e. the de novo synthesis of new extracellular matrix proteins including fibrillar collagens I and III, synthesis of matrix metalloproteases with subsequent enhancement of matrix protein turnover, or fibroblast cell migration [4,5].

Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines, has been recently described as a potent inducer of cardiac myocyte growth. In myocytes, CT-1 signals via the gp130/LIFR-β receptor complex [6] with subsequent activation of the Jak/STAT, MAPK and PI3K pathways and
resultant myocyte growth and protection from ischemia-reperfusion injury [7,8]. CT-1 is elevated in the serum of patients with various cardiac disease states [9–11], and is known to induce cardiac fibroblast proliferation and collagen synthesis [12]. We and others [13] have observed elevated CT-1 expression in the infarct zone of rats undergoing coronary artery ligation, suggesting that CT-1 may play an important role in post-MI wound healing and scar formation. Thus we sought to elucidate the intracellular signaling pathways utilized by CT-1 to initiate protein synthesis.

2. Methods

2.1. Adult cardiac fibroblast isolation and culture

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines set forth by the Canadian Council on Animal Care. Fibroblasts were isolated from the hearts of 200–250-g adult male Sprague–Dawley rats as previously described [14]. Briefly, hearts were perfused with 0.1% collagenase (Worthington Biochemical, Lakewood, NJ) in Joklik’s medium for 25–35 min. Collagenase was neutralized by the addition of an equal volume of DMEM/NJ in Joklik’s medium for 25–35 min. Collagenase was neutralized by the addition of an equal volume of DMEM/F12 medium containing 10% FBS and liberated cells were collected by centrifugation. Cells were plated on 75-cm² culture flasks at 37 °C with 5% CO₂ for 3 h. Non-adherent cells (myocytes) were removed by changing the culture media and adherent cells (mainly fibroblasts) were incubated in DMEM/F12 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μM ascorbate. Fibroblasts were used for experiments after the second passage and the purity of these cells was ≥95%, using routine phenotyping methods as previously described [15]. Experiments were conducted after 24 h in serum-free medium.

2.2. Immunoprecipitation and Western blot analysis

Cells were stimulated for specified times and lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) with protease inhibitor cocktail (Sigma-Aldrich, Oakville, Ont.), 10 mM NaF, 1 mM Na₃VO₄ and 1 mM EGTA. Protein concentrations of cell lysates were determined by the BCA method. gp130 was immunoprecipitated from 100 μl cell lysate using the Immunocatcher system (Cytosignal, Irvine, CA) according to the manufacturers specification. Proteins were separated by 8–12% SDS–PAGE and transferred to PVDF membrane (Roche, Indianapolis, IN). Membranes were blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.2% Tween 20 (TBST). Proteins were visualized with ECL Plus (Amersham) after probing with primary and secondary antibodies.

2.3. Immunofluorescence

Cardiac fibroblasts were seeded onto coverslips in six-well dishes and allowed to attach overnight in medium containing 10% serum. The cells were rendered quiescent in serum free media for 24 h before being stimulated with CT-1 for specified times. The medium was removed, and cells were rinsed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 and incubated with primary antibodies, biotinylated secondary antibodies, and streptavidin FITC. Nuclei were stained with Hoechst 33342. The cells were visualized with epifluorescent microscopy with appropriate filters.

2.4. Fibroblast protein synthesis assay

Protein synthesis was determined using the methods of Wolf and Neilson [16]. Briefly, 2.5 × 10⁴ cells (counted with a hemacytometer) were loaded into each well of 24-well plates, allowed to attach overnight, and then rendered quiescent in serum free DMEM/F12 for 24 h. Cells were stimulated with CT-1 for 24 h in the presence of 2 μCi/ml [³H]leucine with or without inhibitors of signaling pathways. The culture medium was aspirated, cells rinsed twice with phosphate-buffered saline and proteins precipitated by two incubations with 10% TCA at room temperature. The precipitated protein was solubilized in 300 μl lysis buffer containing 0.5 M NaOH and 1% Triton X-100 at room temperature for 15 min. The lysate was transferred to scintillation vials, and beta emission was determined with 3 ml Ecolume scintillation fluid (ICN Pharmaceuticals) and a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA).

2.5. Reagents

Cell culture reagents were purchased from Gibco unless otherwise specified. Recombinant human CT-1 was purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-phosphotyrosine antibody, rabbit polyclonal antibodies against STAT3, STAT3 pY705 and pS727, STAT1 pY701, Akt pS73 and pT308, p70 S6 kinase pT389 and pT411/pS424, mTOR pS2448, 4E-BP1 pS65, eIF4E pS409, S6 ribosomal protein pS235/236, Mnk1 pT197/202, and goat anti-rabbit HRP-linked secondary antibody were purchased from Cell Signaling (New England Biolabs, Mississauga, Ont.). Polyclonal JAK1 pYpY1022/1023 and JAK2 pYpY1007/1008 antibodies were from Biosource International (Camarillo, CA). JAK1 and gp130 polyclonal antibodies and JAK2 antisera were purchased from Upstate (Lake Placid, NY). Antibodies against Erk1/2 pT204, p38 MAPK pY182, JNK1/2 pT183/pY185 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Inhibitors of cell signaling pathways were from Calbiochem (San...
Diego, CA) and were solubilized in DMSO. l-[3,4,5-\textsuperscript{3}H(N)]-leucine was from Perkin Elmer Life Sciences (Boston, MA). Other laboratory grade reagents were purchased from Sigma-Aldrich Canada (Oakville, Ont.).

### 2.6. Statistics

Data are expressed as mean±standard error and are compared using one-way Student’s t-test. A P-value of ≤0.05 was considered significant.

#### 3. Results

#### 3.1. Cardiotrophin-1 activates diverse signaling pathways

We examined CT-1 induced activation of intracellular signaling pathways in cardiac fibroblasts. CT-1 treatment induced rapid phosphorylation of gp130 and phosphorylation of Jak1 and Jak2 (Fig. 1). CT-1 induced tyrosine and serine phosphorylation of STAT3 and tyrosine phosphor-

Fig. 1. (A) Phosphorylation of gp130 was investigated by immunoprecipitating gp130 from cell lysates of CT-1 treated or non-treated cells and probing with anti-phosphotyrosine antibody. To verify equal protein loading between different lanes, the membrane was stripped and probed for gp130. (B) Jak1 and Jak2 phosphorylation was analyzed by Western analysis. Equal protein loading was verified by stripping the membranes and probing for total Jak1 and total Jak2. Representative Western blots from three separate experiments are shown.
ylation of STAT1 (Fig. 2A). Phosphorylation of STAT5 and STAT6 was not observed (data not shown). CT-1 treatment also induced phosphorylation of ERK 1/2, p38 MAPK and JNK (Fig. 2B). CT-1 induced phosphorylation of STAT3 was accompanied by nuclear accumulation of STAT3 (Fig. 3).

3.2. CT-1 induces protein synthesis in cardiac fibroblasts

To determine the impact of CT-1 treatment on cardiac fibroblast protein synthetic function, we measured uptake of $^3$H-labeled leucine. CT-1 treatment of cardiac fibro-

![Western Blot Images]

Fig. 2. (A) CT-1 induces phosphorylation of STAT1 and STAT3. To verify equal protein loading, the membranes were stripped and probed for total STAT3. (B) CT-1 induces phosphorylation of ERK1/2, p38 MAPK and pJNK. To verify equal protein loading, the membranes were stripped and probed for actin. Representative Western blots from three separate experiments are shown.
blasts caused a dose dependent increase in protein synthesis as evidenced by increased incorporation of \[^3\text{H}\]leucine compared to controls (Fig. 4).

3.3. CT-1 activates protein synthesis regulatory proteins

The activity of translational regulatory proteins is modified by phosphorylation. We used phospho-specific antibodies to determine if CT-1 directly influenced the activity of these regulatory proteins. Stimulation of cardiac fibroblasts with CT-1 induced a modest increase in phosphorylation of Akt at threonine 308 and serine 473, and increased phosphorylation of p70 S6 kinase at threonine 389 and threonine 421/serine 424, as well as phosphorylation of Mnk1, eIF4E, 4E-BP1 and S6 ribosomal protein (Fig. 5).

We then determined the effect of pharmacologic inhibitors on the activation of this signaling cascade. AG490, an inhibitor of JAK2 [17], suppressed CT-1 induced phosphorylation of Akt at both sites, p70 S6 kinase T389, Mnk1, eIF4E and 4E-BP1, but did not significantly affect CT-1 induced activation of ERK 1/2, p38 MAPK, mTOR, p70 S6 kinase T421/S424, or S6 ribosomal protein. AG490 in

Fig. 3. CT-1 induces nuclear accumulation of STAT3. Cells were stimulated with CT-1 for 5 min and immunostained with anti-STAT3 antibody. Nuclei were identified by staining with Hoechst 33342 (lower panels). Representative images are shown from three separate experiments.

Fig. 4. CT-1 increases protein synthesis. Protein synthesis was measured by incorporation of \[^3\text{H}\]leucine. Results are displayed as mean±S.E.M. Samples from five separate experiments were analyzed in triplicate. *P<0.05 versus control.
the absence of CT-1 induced phosphorylation of p70 S6 kinase T^{421/424} and to a lesser extent, S6 ribosomal protein, a phenomenon that was reproducible, but unexplainable. PD98059, an inhibitor of MEK 1/2 [18], had the greatest inhibitory effect on basal and CT-1 induced phosphorylation of ERK 1/2, Mnk1, and eIF4E, but had a lesser effect on CT-1 induced phosphorylation of Akt, p70 S6 kinase, and Mnk1, a finding that supports previous work demonstrating that p38 MAPK can activate Akt [20], p70 S6 kinase [21] and Mnk1 [22]. As expected, LY294002, an inhibitor of PI3K [18], suppressed basal and CT-1 induced phosphorylation of Akt, mTOR, p70 S6 kinase, Mnk1, eIF4E, 4E-BP1 and S6 ribosomal protein. LY294002 also attenuated activation of ERK 1/2 and p38 MAPK, suggesting that PI3K participates in CT-1 induced activation of these signaling mediators. Rapamycin, an inhibitor of FRAP/mTOR [18], decreased basal and CT-1 induced phosphorylation of p70 S6 kinase and S6 ribosomal

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**Fig. 5.** CT-1 increases phosphorylation of protein synthesis regulatory proteins. Membranes were probed with phospho-specific antibodies. To verify equal protein loading, the membranes were stripped and probed for actin. Representative blots from three separate experiments are shown.
Fig. 6. Incubation with inhibitors of signaling pathways depresses phosphorylation of translational regulatory proteins. Cardiac fibroblasts were pre-incubated with inhibitors for 15 min, then stimulated with CT-1 for an additional 15 min. Cell lysates were separated by SDS–PAGE and membranes were probed with phospho-specific antibodies. The control lane represents cells incubated with 0.1% DMSO alone. To verify equal protein loading, the membranes were stripped and probed for actin. Representative blots from three separate experiments are shown.
protein phosphorylation, and had a lesser effect on 4E-BP1 phosphorylation (Fig. 6). These phosphorylation events are compared to 0.1% DMSO control (lane 1, Fig. 6).

3.4. Inhibitors of signaling pathways reduce CT-1 induced protein synthesis

To determine if the inhibitory effect of these pharmacologic inhibitors impacted CT-1 induced protein synthesis, we again measured CT-1 induced [3H]leucine uptake in the presence of inhibitors of the Jak/STAT, PI3K, p42/44 and p38 MAPK and mTOR pathways. CT-1 induced protein synthesis could be suppressed by co-incubation with AG490, PD98059, SB203580, LY294002 or rapamycin (Fig. 7).

4. Discussion

We have demonstrated that CT-1 stimulation of cultured primary cardiac fibroblasts leads to the activation of multiple pathways with subsequent induction of protein synthesis in these cells. Specifically, this study demonstrates for the first time that CT-1 mediated phosphorylation of glycoprotein 130 (gp130) is attended by activation of protein synthesis regulatory proteins including Akt/PKB, p70 S6 kinase, eIF4E, 4E-BP1 and S6 ribosomal protein with concomitant induction of protein synthesis in primary cardiac fibroblasts. These events were paralleled by strong characteristic activation of the Janus-kinase signal transducer and activator of the transcription (Jak-STAT) pathway. We confirmed that CT-1 induced Jak1 and Jak2 phosphorylation as well as STAT1 and STAT3 phosphorylation occurs within minutes of CT-1 treatment of fibroblasts in vitro. The Y705 phosphorylation site of STAT3 and the Y701 site of STAT1 are requisite for nuclear translocation of these transcription factors, whereas the S727 site of STAT3 is required for optimal translational activity [23]. The S473 phosphorylation site of Akt may be an autophosphorylation site, and both S473 and T308 phosphorylation are required for maximal activation of Akt [24,25]. Increased kinase activity of p70 S6 kinase is accompanied by phosphorylation at T389, while the role of the T421/S424 site is unclear [21]. Thus, we suggest that CT-1 signaling activates a characteristic series of gp130-dependent phosphorylation cascades in primary cardiac fibroblasts to subserve the hypersecretory nature of these cells.

Initiation of protein synthesis is a tightly regulated process that culminates in the positioning of a charged ribosome at an initiation codon [26]. The ribosome does not bind directly to the mRNA 5’ cap, but is directed there by the concerted action of a large number of eukaryotic translation initiation factors (eIFs). In this regard, eIF4E and eIF4G are important components of a trimeric complex eIF4F, and it is known that the levels of eIF4F fluctuate with varying translational rates. Growing or stimulated cells contain high levels of eIF4F, whereas starved or quiescent cells contain low levels of eIF4F. The phosphorylation status of eIF4E also correlates with the degree of transla-
tional activity, since the phosphorylated form has a greater affinity for cap structures [27]. Formation of eIF4F is regulated at least in part by a family of translation repressors, the eIF4E binding proteins (4E-BPs). Binding of 4E-BP1 to eIF4E prevents association of eIF4E and eIF4G, thereby preventing formation of the initiation complex. Regulation of 4E-BP1 is accomplished through phosphorylation: hypophosphorylated 4E-BP1 binds tightly to eIF4E, whereas phosphorylation of 4E-BP1 releases eIF4E and allows formation of the initiation complex [28]. 4E-BP1 is the primary target of FRAP/mTOR kinase activity [28], while the MAPK-related kinase Mnk1 is the most likely candidate kinase in regulation of eIF4E phosphorylation [29]. Mnk1 can be activated by either p38 MAPK or p42/44 MAPK [22]. FRAP/mTOR is a target of Akt/PKB, which is activated primarily by PI3 kinase [30], but can also be activated by p38 or p42/44 MAPK [20]. Thus integration of the PI3K and MAPK pathways is implicit at the level of initiation of translation. The current study demonstrates that these events occur in cardiac fibroblasts in response to CT-1 signaling and culminate in augmented protein synthesis.

It is well known that the JAK-STAT pathway does not typically operate autonomously in various cell types [31] and the current study provides direct evidence that an array of intrinsic pathways contributes to modulation of net protein synthesis in cardiac fibroblasts. While members of the IL-6 superfamily of cytokines classically signal through the Jak/STAT pathway [23], we have shown that the PI3K/Akt/PKB/FRAP-mTOR axis and p38 and p42/44 MAPK pathways are also operating in these cells. We found that five different pharmacologic inhibitors were capable of inhibiting protein synthesis. As seen in Fig. 6, these inhibitors act at distinct levels in the signaling cascade. Our interpretation of how these pathways are integrated to produce protein synthesis is shown in Fig. 8. This synopsis shows typical activation of Jaks, leading to phosphorylation

Fig. 8. Scheme of CT-1 signaling in rat cardiac fibroblasts. The CT-1 signal is transduced by activation of the classic Jak/STAT pathway and is paralleled by activation of the PI3Kinase/Akt/mTOR cascade and the p38 and p42/44 MAPK pathways. We suggest that these pathways converge for initiation of translation.
and nuclear translocation of STAT proteins. There is concomitant activation of the p38 and p42/44 MAPK pathways which have multiple secondary effects. These include activation of Mnk1 and eIF4E, as well as p70 S6 kinase and Akt. While this is not the classical activation pathway for Akt and p70 S6 kinase, it has been previously described [20,21]. PI3-kinase is known to play a central role in cardiac fibroblast growth [32,33], and our study has confirmed the importance of this signaling mediator with respect to protein synthesis. PI3-kinase is activated by Jak2 [31], with sequential activation of Akt/PKB, FRAP/mTOR, p70 S6 kinase, S6 ribosomal protein and 4E-BP1.

A large body of evidence exists to support the hypothesis that IL-6 and CT-1 (and other IL-6 family cytokines) induce cardiac myocyte hypertrophy. Recent work has indicated that CT-1 may participate in cardiac fibrosis post-MI and the current study provides evidence for signaling pathways that may underlie the augmented synthesis of matrix component proteins in heart tissues populated by fibroblasts. In summary, we have demonstrated that CT-1 treatment of cardiac fibroblasts induces protein synthesis in a manner that involves activation of not only the Jak/STAT pathway, but also MAPK pathways and the PI3K/Akt pathway. Our results contribute to a growing body of evidence demonstrating the role of CT-1 in myocardial remodeling that occurs in response to various cardiovascular disease states.

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