Different contributions of STAT3, ERK1/2, and PI3-K signaling to cardiomyocyte hypertrophy by cardiotrophin-1[1]

Ze-jun TIAN[2], Wei CUI, Yong-jun LI, Yu-ming HAO, Jun DU, Fan LIU, Hui ZHANG, Xiu-guang ZU, Su-yun LIU, Li CHEN[3], Wei AN[3]

Department of Cardiology, Second Hospital of Hebei Medical University, Shijiazhuang 050000, China;
[3]Department of Cell Biology and Municipal Laboratory for Liver Protection, Beijing 100054, China

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ABSTRACT

AIM: To assess the contribution of signal transducer and activator of transcription 3 (JAK-STAT3) pathway, extracellular signal-regulated kinases1/2 (ERK1/2) pathway, and phosphatidylinositol 3-kinase (PI3-K) pathway to cardiomyocytes hypertrophy induced by cardiotrophin-1 (CT-1), a new member of interleukin-6 (IL-6) family of cytokines. **METHODS:** STAT3, ERK1/2, and PI3-K were assessed by Western blot analysis. Activity of ERK1/2 was also confirmed by in-gel kinase assay. Hypertrophy of cardiomyocyte was evaluated by [^{3}H]leucine incorporation and cellular protein-to-DNA ratio. **RESULTS:** CT-1 simultaneously activated phosphorylation of STAT3, ERK1/2, and PI3-K in rat cardiomyocytes. Parthenolide, an inhibitor of STAT, suppressed CT-1-induced [^{3}H]leucine incorporation by 88.3 % and protein-to-DNA ratio by 75.0 %. U0126, an MEK1/2 inhibitor, increased CT-1-induced the phosphorylation of STAT3 in a dose-dependent manner and, consistently, augmented CT-1-induced increase in [^{3}H]leucine incorporation and cellular protein-to-DNA ratio by 17.6 % and 16.3 %, respectively. Wortmannin, a PI3-K inhibitor, did not influence CT-1-induced [^{3}H]leucine incorporation and cellular protein-to-DNA ratio. **CONCLUSION:** The hypertrophic effect of CT-1 was essentially mediated by STAT3, independent of PI3-K, and negatively regulated by ERK1/2 via inhibiting the phosphorylation of STAT3. The interaction between STAT3 and ERK1/2 in CT-1-induced signaling contributes to development of cardiac hypertrophy.

INTRODUCTION

Cardiotrophin-1 (CT-1) is a novel cardiomyocyte hypertrophy-inducing factor belonging to the interleukin-6 (IL-6) family of cytokines[1-3]. This family of cytokines shares gp130 as a common signal-transducing subunit for their receptor complexes[1,4]. Binding of the CT-1 to its receptor induces the heterodimerization of a gp130, leukemia inhibitory factor (LIF) receptor and a third unique subunit[5], which leads to activation of Janus kinase (JAK) and tyrosine phosphorylation of gp130. These events result in activation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinases 1/2 (ERK1/2), and phosphatidylinositol 3-kinase (PI3-K), respectively[1,3], thus, generating three distinct signaling pathways, STAT3, ERK1/2, and PI3-K, all are implicated in the regulation

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[2] Corresponding to Dr Ze-jun TIAN. Phn 86-311-722-2944. E-mail tianzej@yahoo.com.cn.
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of development of cardiac hypertrophy[1].

The STAT family of proteins, with seven members identified in mammalian cells, is normally latent in the cytoplasm and become activated through phosphorylation on their conserved tyrosine residues (at tyrosine 705 position for STAT3) typically by JAKs. The tyrosine phosphorylation is a key event, which allows STATs to translocate into the nucleus where they regulate expression of a large number of genes. Accordingly, the tyrosine phosphorylation is recognized as a hallmark of activation of STATs. STATs are subsequently inactivated by tyrosine dephosphorylation and returns to the cytoplasm. The critical role of STAT3 pathway in the cardiomyocyte hypertrophy has been established and almost all of the evidences comes from the study on gp130.

ERK1/2 is a major member of mitogen-activated protein (MAP) kinase family. In ERK1/2 pathway, cell stimulation leads to the sequential activation of Ras, Raf, MAP kinase/ERK kinase 1/2 (MEK1/2), and ERK1/2. Activation of ERK1/2 requires dual phosphorylation on a tyrosine and a threonine residue. Up till now, the importance for ERK1/2 pathway in the hypertrophic process remains conflicting. While overexpression of constitutively active components of the ERK1/2 pathway[6] and antisense mRNA to ERK1/2[7] augmented and prevented hypertrophic changes, respectively, carbachol and adenosine triphosphate, both activate ERK1/2, did not induce the hypertrophy[8], and phenylephrine retains the ability to induce the hypertrophy despite the inhibition of ERK1/2[9]. PI3-K, which consists of an 85-kDa regulatory subunit (p85) and various catalytic subunits including p110, requires tyrosine phosphorylation of p85 for activation[10].

In the report by Railson *et al*[11], the requirements of STAT3 pathway and ERK1/2 pathway were examined using mutant strategy (transient transfection of a dominant negative STAT3 and MEK-1, respectively); in the report by Sheng *et al*[12], the requirement of ERK1/2 pathway was examined using chemical inhibitor strategy. They showed that the STAT3 pathway[11] but not ERK1/2 pathway[11,12] was required for CT-1-induced hypertrophy. Acceptation of these conclusions should be caution in the light of the fact that less study has been conducted on CT-1 compared with LIF, another member of the IL-6 family of cytokines that is also a potent hypertrophy-inducing factor, and discrepancy still exists among the studies on LIF. For example, either STAT3 pathway[13] or PI3-K pathway[14] was shown to be important for LIF-induced hypertrophy when the pathways were examined individually by mutant strategy[13] or by chemical inhibitor strategy[14]. However, when all of the three pathways were taken into account, ERK1/2 pathway was found to have more contribution than both STAT3 pathway and PI3-K pathway to the LIF-induced hypertrophy by chemical inhibitor strategy[15]. It is generally accepted that mutant strategy is superior to chemical inhibitor's one in specificity, but the need to express a mutant in cells for many hours may result in unwanted secondary effects leading to erroneous conclusions[16]. On the other hand, recent studies suggest that, in gp130-dependent intracellular signaling, the activity of STAT3 may be negatively regulated by ERK1/2[4,17,18]. Such interaction has not been identified in CT-1-induced signaling nor has its functional significance been investigated on the hypertrophic effect. Chemical inhibitor strategy is superior to mutant one when it was used to observe the possible interaction between STAT3 and ERK1/2, since it allows the observation to be practiced in dose-dependent manner. Moreover, chemical inhibitor may have therapeutic potential as anti-hypertrophic agent. In the present study, we applied chemical inhibitor strategy by taking the advantage of parthenolide, an inhibitor recently used to inhibit STAT[19]; U0126, an MEK1/2 inhibitor commonly used to block ERK1/2, and wortmannin, a widely used PI3-K inhibitor, to assess the contribution of each of the three pathways to CT-induced hypertrophy.

MATERIALS AND METHODS

Materials Cardiotrophin-1 (CT-1) was from PeproTech. Antibodies to STAT3, phosphoERK1/2, ERK1/2, IκBα, and chemical inhibitor U0126 were from Cell Signaling Technology. Antibodies to gp130, α-tubulin as well as Protein A/G agarose and Western blotting luminol reagent were from Santa Cruz Biotechnology. Antibodies to phosphotyrosine (4G10) and PI3-K p85 were from Upstate Biotechnology. Horseradish peroxidase-labeled secondary antibody was from Jackson. Parthenolide and wortmannin were from Sigma-Aldrich Co. [γ-^{32}P]ATP was from Yahui Biological and Medical Engineering Co Beijing, China. [^{3}H]Leucine was from China Institute of Atomic Energy. Bicinchoninic acid protein assay kit was from Pierce.

Cell culture Preparation of primary neonatal ventricular cardiomyocytes from 1- to 2-d-old Sprague-

Dawley rats (Experimental Animal Center of Hebei Medical University, grade II) were isolated by enzymatic dissociation and collected on the basis of differential adhesiveness[20,21].

Preparations of cellular extracts The cardiomyocytes were starved by incubation in media containing low serum (0.5 % FCS) for 2 d followed by incubation in serum-free media for 2 h, then changed to fresh serum-free media containing the indicated concentrations of CT-1 in the absence or presence of indicated chemical inhibitor(s) for the indicated time durations. Cellular extracts were prepared as described previously[20]. In brief, the cells were washed with cold PBS (pH 7.4) containing 1 mmol/L sodium orthovanadate, scraped off the dish, harvested with lysis-buffer [20 mmol/L Tris-HCl (pH 7.4), 1 % NP-40, 0.1 % SDS; 150 mmol/L NaCl, 1 mmol/L edetic acid, 10 mg/L aprotinin; 1mmol/L sodium orthovanadate; and 0.5 mg/L PMSF], homogenized using Dounce homogenizers at 4 ºC and checked microscopically for cell lysis. The homogenates were centrifuged at 100 000×*g* for 30 min at 4 ºC and the supernatant containing the total cellular extracts was collected. Protein concentrations were measured by bicinchoninic acid protein assay.

Immunoprecipitation and Western blot analysis[20,22] Cellular extracts were incubated with antibodies to gp130, STAT3, or PI3-K p85 overnight at 4 ºC. Immunocomplexes were collected by incubating with 50 μL of protein A/G agarose for 4 h. The immunoprecipitates were washed with TBS buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5 mmol/L PMSF, 1 mmol/L sodium orthovanadate], eluted with 25 μL of sample buffer [62.5 mmol/L Tris (pH 6.8), 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol], and heated at 95 ºC for 5 min. Proteins were separated on 5 % -8 % SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5 % nonfat dry milk for 2 h at room temperature. The membranes were incubated overnight at 4 ºC with anti-phosphotyrosine antibody at a 1:1000 dilutions and then incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 h. Proteins were detected by enhanced chemiluminescence. For reprobing, the membranes were incubated in stripping buffer [2 % SDS, 62.5 mmol/L Tris-HCl (pH 6.8), 100 mmol/L β-mercaptoethanol] for 30 min at 50 ºC, rinsed with TBST and detection was restarted using the same antibodies as used for immunoprecipitation at a 1:1000 to 1:2000 dilution, to ensure equal loading of the proteins. For detection of the threonine/tyrosine phosphorylation of ERK1/2, the cellular extracts obtained were directly separated on 10 % SDS-PAGE, immunoblotted with anti-phospho ERK1/2, and reprobed with anti-ERK1/2 antibodies (1:1 000 each). IκBα was separated on 10 % SDS-PAGE, detected by antibody to anti-IκBα (1: 500) and, for loading control, to anti-α-tubulin (1:500).

In-gel ERK kinase assay[20,23] Equal amounts of cellular protein were loaded on a 1 g/L myelin basic protein-containing 10 % SDS-polyacrylamide gel. After electrophoresis, SDS was removed by incubating the gel twice for 1 h each with 50 mmol/L Tris (pH 8.0) containing 20 % 2-propanol, followed by 50 mmol/L Tris (pH 8.0) containing 5 mmol/L dithiothreitol (buffer A). Denaturation and renaturation of the proteins were performed by incubating the gel, respectively, in buffer A containing 6 mol/L guanidine for 1 h and buffer A containing 0.04 % Tween-20 at 4 ºC for 16 h. The gel was pre-incubated with kinase assay buffer [40 mmol/L HEPES (pH 8.0), 2 mmol/L dithiothreitol, 0.1 mmol/L egtazic acid, and 5 mmol/L magnesium acetate] at room temperature for 30 min, followed by another incubation at the same temperature, in the kinase assay buffer containing 370 MBq/L [γ-^{32}P]ATP for 90 min. The reaction was stopped by washing the gel with 5 % trichloroacetic acid solution containing 1 % sodium pyrophosphate and then the gel was dried and exposed to X-ray film.

Measurement of myocyte hypertrophy Cardiomyocyte hypertrophy was evaluated by [^{3}H]leucine incorporation (index of protein synthesis) and by cellular protein normalized to DNA (index of cell size)[24]. The cells were starved for 24 h and stimulated with or without CT-1 (0.1 nmol/L) in the presence or absence of parthenolide (50 μmol/L, 2 h) and/or U0126 (10 μmol/L, 1 h) or wortmannin (10 nmol/L, 0.5 h), *etc*. For measurement of [^{3}H]leucine incorporation[25], 37 MBq/L [^{3}H]leucine was added to the culture media at the time of adding CT-1 and the cells were cultured for additional 24 h. Thereafter, cultures were rinsed with PBS, incubated with 10 % trichloroacetic acid on ice for 30 min, and lysed with 0.2 mol/L NaOH for 4 h at room temperature. Radioactivity was counted with a liquid scintillation counter. For measurement of protein-to-DNA ratio, the cells were cultured for 24 h and then protein and DNA contents were assayed as described[26].

Statistic analysis The data were expressed as mean±SD. Differences among groups were assessed by one-way ANOVA with least significant difference

(LSD) analysis using SPSS 11.5. $P<0.05$ was considered significant.

RESULTS

Tyrosine phosphorylation of gp130 induced by CT-1 in cardiomyocytes CT-1 2 nmol/L induced tyrosine phosphorylation of gp130 in a time-dependent manner at 1, 5, 10, 20, and 30 min (Fig 1).

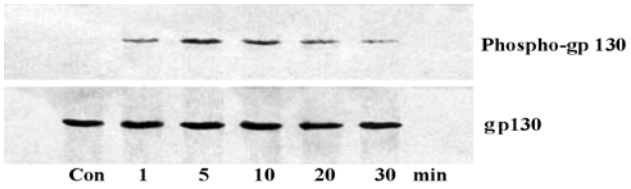

Fig 1. Tyrosine phosphorylation of gp130 induced by CT-1 at 2 nmol/L in cardiomyocytes.

Effect of parthenolide, U0126, and wortmannin on CT-1-induced phosphorylation of STAT3, ERK1/2, and PI3-K p85 CT-1 simultaneously induced the tyrosine phosphorylation of STAT3, the threonine/tyrosine phosphorylation of ERK1/2, and the tyrosine phosphorylation of PI3-K p85 in cardiomyocytes. Parthenolide (50 μmol/L, 2 h), an inhibitor of STAT, decreased the phosphorylation of STAT3 and increased the phosphorylation of ERK1/2 but did not affect the phosphorylation of PI3-K p85. U0126 (10 μmol/L, 1 h), a MEK1/2 inhibitor, completely inhibited the phosphorylation of ERK1/2 and noticeably increased the phosphorylation of STAT3 but did not influence the phosphorylation of PI3-K p85. Wortmannin (10 nmol/L, 0.5 h), a PI3-K inhibitor, attenuated the phosphorylation of PI3-K p85 without, if any, effect on the phosphorylations of both STAT3 and ERK1/2 (Fig 2).

Effect of ERK1/2 inhibition on STAT3 activation After pre-treatment with U0126 2-10 μmol/L for 1 h and subsequent incubation with CT-1 0.1 nmol/L for 5 min, the activity of ERK1/2 was decreased in concentration-dependent manner (Fig 3A), in contrast, the tyrosine phosphorylation of STAT3 was increased correspondingly. The maximal activation of STAT3 reached at U0126 8 μmol/L (Fig 3B).

Effect of CT-1 on IκBα degradation in cardiomyocytes The quantities of IκBα remained at a level comparable to that of control in a 60-min period of observation following treatment with CT-1 0.1 nmol/L, indicating that CT-1 at this concentration did not induce

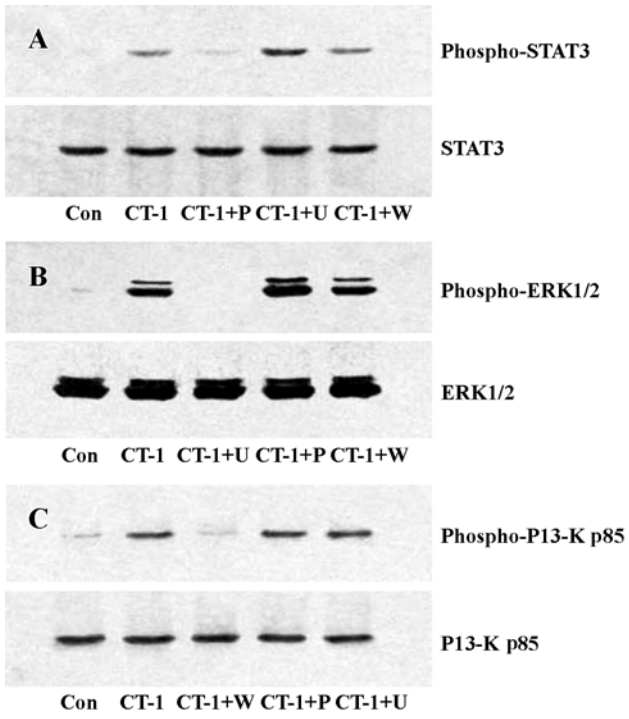

Fig 2. Effect of parthenolide, U0126, and wortmannin on CT-1-induced phosphorylation of STAT3, ERK1/2, and PI3-K. P, parthenolide (50 μmol/L, 2 h); U, U0126 (10 μmol/L, 1 h); W, Wortmannin (10 nmol/L, 0.5 h). Representative blots were from three separate experiments.

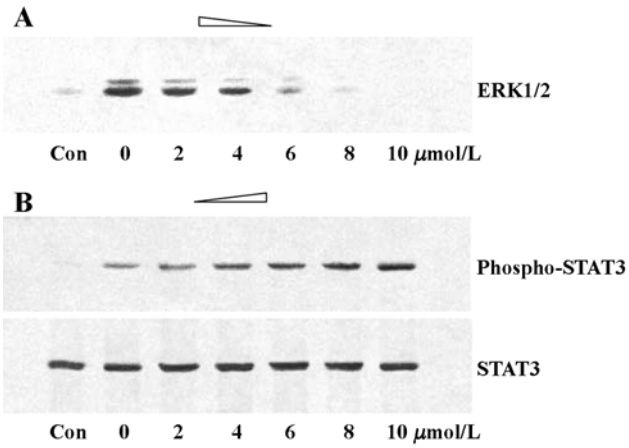

Fig 3. Effect of U0126 (2-10 μmol/L) on the phosphorylation of STAT3 induced by CT-1 (0.1 nmol/L, 5 min) in cardiomyocytes.

IκBα degradation. However, the degradation of IκBα was induced by CT-1 at or higher than 10 nmol/L after 45 min (Fig 4).

Effect of parthenolide, U0126, and wortmannin on CT-1-induced cardiomyocytes hypertrophy CT-1 0.1 nmol/L increased [^{3}H]leucine incorporation by 47.2 %

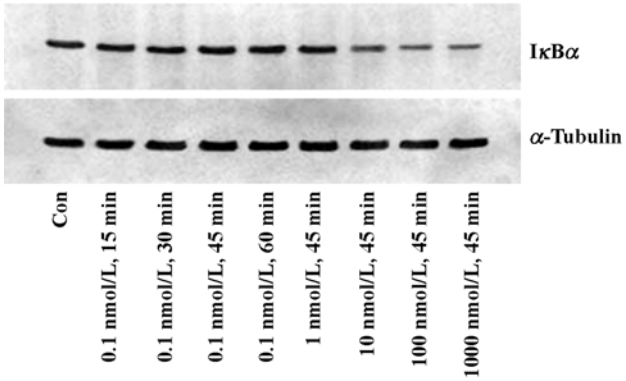

Fig 4. Effect of CT-1 on IκBα degradation. Representative blots from 3 separate experiments.

and protein-to-DNA ratio by 38.7 % compared with CT-1-untreated cells, respectively (P<0.01). Parthenolide (50 μmol/L, 2 h) inhibited the increase in [^{3}H]leucine incorporation and protein-to-DNA ratio induced by CT-1 by 88.3 % and 75.0 %, respectively (P<0.01). U0126 (10 μmol/L, 1 h) further elevated the increase in [^{3}H]leucine incorporation and protein-to-DNA ratio induced by CT-1 by 17.6 % and 16.3 % (P<0.05). Parthenolide reversed the changes of [^{3}H]leucine incorporation and protein-to-DNA ratio by U0126 to levels comparable to that produced by parthenolide alone. Wortmannin (10 nmol/L, 0.5 h) did not affect both [^{3}H]leucine incorporation and protein-to-DNA ratio under the same conditions (P>0.05, Fig 5).

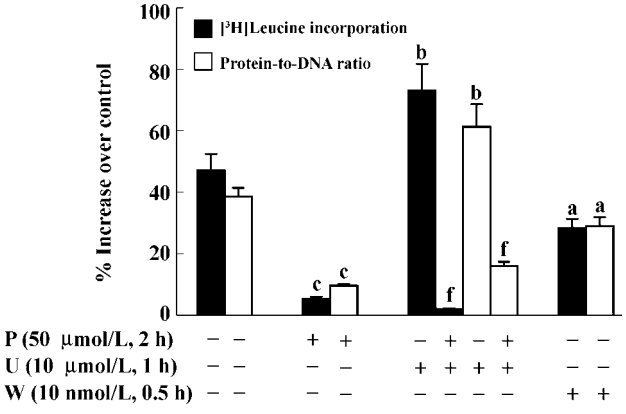

Fig 5. Effect of parthenolide, U0126, and wortmannin on CT-1-induced cardiomyocytes hypertrophy. [a]P>0.05, [b]P< 0.05, [c]P<0.01 *vs* CT-1-treated group. [f]P<0.01 *vs* U0126-treated group.

DISCUSSION

The major finding of the present study is that, in CT-1-induced cardiomyocyte hypertrophy, STAT3 pathway played a predominant role, ERK1/2 pathway served as a negative regulator via inhibiting the activity of STAT3, and PI3-K pathway was not involved in this process.

There are two concerns in explaining the results produced by parthenolide in the present study. The first one is the possible contribution by NF-κB. Our results showed that this concern could be overlooked. By extension, one would speculate that, since the targets of parthenolide include both STAT3, which is essentially required for the CT-1-induced hypertrophy[11], and NF-κB, which is critically involved in the CT-1-induced cardiac protection[27], then the two cardiac events induced by CT-1, hypertrophy and protection, may be coordinated. The plausibility of such coordination is further strengthened by the observation that STAT3 itself may transduce not only a hypertrophic signal but a protective signal by inhibiting reduction of cardiac contractile genes and inducing cardiac protective factors, respectively[28]. Another concern is which signaling molecule(s) would be acted on by parthenolide. It has been hypothesized that parthenolide act on JAKs in IL-6-family of cytokine-induced response[19]. This hypothesis, however, is not supported by the observations that parthenolide increased the phosphorylation of ERK1/2 besides its expected effect of inhibiting the phosphorylation of STAT3 in our study and in a report by Legendre *et al*[29]. For JAKs are located upstream both STAT3 and ERK1/2 in gp130-dependent signaling so that inhibition of JAKs would render either STAT3 or ERK1/2 inactive. Further experiments will be required to examine whether parthenolide inhibits STATs by acting on STATs themselves, and if it is evidenced, then its enhancing effect on the activation of ERK1/2 will be additionally explained.

The molecular basis of the antagonist nature in STAT3-ERK1/2 interaction in CT-1-induced signaling delineated in the present study is unclear; however, some recent findings in gp130-related investigation shed a light on it. Mutation at tyrosine 759 (Y759) in gp130 leads to enhancement of STAT3 activation and its responsible biological actions[30-32]. Y759 is a recruitment motif for both the protein-tyrosine phosphatase 2 (SHP2), which mediates signals to ERK1/2[17,30], and the suppressor of cytokine signaling 3 (SOCS3), which is a natural inhibitor of STAT3[33]. The biological consequences of Y759 mutation described above may result from either lack of negative regulation by SHP2 or,

alternatively, lack of negative feedback regulation by SOCS3[34]. It is conceivable that it is SHP2 that is responsible for the antagonist nature of the STAT3-ERK1/2 interaction in the present study. This explanation seems also applicable to several findings in gp130-related studies. For example, expression of constitutively active MEK1 inhibited activation of STAT3 and over-expression of ERK2 potentiated inhibition of STAT3 DNA binding in IL-6 induced response[34]. Inversely, neurite outgrowth induced by gp130 stimulation in PC12 cells is totally dependent on ERK1/2 activation, while STAT3 activation induced by gp130 stimulation inhibits it[35]. 'Knock-in' mice with a COOH-terminal gp130ÄSTAT mutation that deletes all STAT-binding sites showed a sustained gp130-mediated ERK1/2 activation[36]. Interestingly, Hirano *et al*[17] has recently proposed a 'Signal Orchestration' hypothesis, which was based on the behaviors of ERK1/2 and STAT3 in gp130-dependent signaling, that contradictory signals can be generated from a cytokine receptor through its distinct cytoplasmic regions in a given target cell, and the balance between the contradictory signals determines the final output. However, few studies have so far concerned STAT3-ERK1/2 interaction in CT-1-induced signaling. Sheng *et al*[12] reported that CT-1 (1 nmol/L) alone or CT-1 (1 nmol/L) plus PD98059 (10 μmol/L, a MEK1 inhibitor that is structurally unrelated to U0126) produced an equivalent increase in atrial natriuretic factor mRNA and hypertrophic morphological changes in cardiomyocytes. The inability to observe the augmentation of the hypertrophic changes by MEK1 inhibition in this study may be due to CT-1 being used at 1 nmol/L, the dose that induced the maximal hypertrophic activity by CT-1[2] thus leaving little room for the augmentation to be manifested.

The negative regulation of STAT3 by ERK1/2 in CT-1-stimulated response delineated in this study may assist CT-1 in exerting adequate hypertrophic effect. Cardiac hypertrophy can be either beneficial when it occurs in the early setting which compensate cardiac function, or harmful when it occurs in the setting of pathological remodeling which compromises cardiac function. Interestingly, ERK1/2 act as a negative regulator of STAT3 to prevent excessive differentiation of hepatocytes[37]. Relevantly, an antagonistic nature of interaction between ERK1/2-dependent proliferation and STAT3-dependent differentiation has been found in MDCK epithelial cells[38] and in myeloid precursors[39, 40]. These findings prompt us to hypothesize that ERK1/2 may act as a negative regulator of the STAT3 activity to prevent overt cardiac hypertrophy. Further study is needed to investigate whether the STAT3-ERK1/2 interaction in CT-1-induced signaling is regulated by the stage of heart failure. Greater understanding of CT-1-induced signaling will lead to increased insight into the regulation of cardiomyocyte hypertrophy by CT-1 and also may lead to novel therapeutic approaches to heart failure.

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