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H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

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Original Article

Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes

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Abstract

gp130-dependent signaling is known to play a critical role in the onset of heart failure. In that regard, cardiotrophin-1 (CT-1) activates several signaling pathways via gp130, and induces hypertrophy in neonatal rat cardiomyocytes. Among the mediators activated by CT-1, STAT3 is thought to be important for induction of cell hypertrophy, though its precise function in the CT-1 signaling pathway is not fully understood. In the present study, therefore, to better understand the significance of STAT3 activity in CT-1 signaling, we infected cultured cardiomyocytes with adenoviral vectors harboring a dominant-negative STAT3 mutant or one of two endogenous negative regulators of cytokine signaling via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways [suppressor of cytokine signaling (SOCS) 1 and 3] and then examined their effects on three indexes of CT-1-induced cell hypertrophy: protein synthesis, secretion of brain natriuretic peptide and changes in cell surface area. In control cells, CT-1-induced both STAT3 phosphorylation and cell hypertrophy. Overexpression of dominant-negative STAT3 mutant suppressed CT-1-induced STAT3 phosphorylation, but did not affect cell hypertrophy. On the other hand overexpression of SOCS1 or SOCS3 inhibited both CT-1-induced STAT3 phosphorylation and cell hypertrophy. CT-1 also induced phosphorylations of ERK1/2 and ERK5 in cardiomyocytes, and those, too, were suppressed by overexpression of SOCSs. CT-1-induced cell hypertrophy was suppressed by overexpression of a dominant-negative MEK5 mutant, and not by overexpression of a dominant-negative MEK1 mutant. These findings indicate that the major pathway responsible for the hypertrophic responses to CT-1 is not JAK-STAT3 pathway nor MEK1-ERK1/2 pathway, but MEK5-ERK5 pathway.

Keywords: Cardiotrophin-1; Cytokine; ERK1/2; ERK5; STAT3; Cell signaling; Hypertrophy; Cardiomyocyte

1. Introduction

Cardiotrophin-1 (CT-1) is an interleukin-6 (IL-6)-related cytokine that exerts various hypertrophic and antiapoptotic effects via the gp130-leukemia inhibitory factor (LIF) receptor complex by activating several intracellular signaling in cardiomyocytes, including the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways [1]. The receptors for IL-6-related cytokines share gp130 as a signal-transducing receptor component [1]. Its continuous activation in heart due, for example, to overexpression of IL-6 and its receptor is known to cause myocardial hypertrophy [2]. Conversely, ventricular restricted gp130-deficient mice display massive apoptosis of cardiomyocytes and are unable to achieve compensatory hypertrophy during aortic pressure overload [3]. So it is very important to elucidate the physi-

Abbreviations: BNP, brain natriuretic peptide; CT-1, cardiotrophin-1; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; GPCR, G-protein-coupled receptor; IL-6, interleukin-6; JAK, Janus kinase; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3-OH kinase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

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ological or pathophysiological role of IL-6-related cytokines in the heart.

On the other hand, little is known about the significance of the signaling pathways downstream of gp130 that mediates the phenotypic, namely hypertrophic and antiapoptotic effects of IL-6-related cytokines such as CT-1. We previously showed that CT-1 induces its antiapoptotic effects via the phosphatidylinositol 3-OH kinase (PI3K)-Akt pathway [4]. In addition, others have shown that LIF, which shares a receptor with CT-1, induces hypertrophy in cardiomyocytes via STAT3 [5], and that cardiac-specific overexpression of STAT3 leads to myocardial hypertrophy [6]. In those cases, however, the contribution made by STAT3 did not appear especially pronounced, making it unclear whether STAT3 mediated transduction in the principal pathway leading to hypertrophy. In that regard, we have shown that CT-1-induced STAT3 activation leads to upregulation of two endogenous negative regulators of cytokine signaling via JAK-STAT pathways [suppressor of cytokine signaling (SOCS) 1 and 3, also referred to as JAK-binding protein (JAB) and cytokine-inducible SH2 protein (CIS) 3/STAT-induced STAT inhibitor (SSI) 1and 3] [7–9] in the heart [10].

Finally, Kodama et al. [11] have shown that the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)1/2-ERK1/2 pathway is critically involved in LIF-induced cardiomyocyte hypertrophy. Moreover, Nicol et al. [12] recently reported that in cardiomyocytes LIF activates ERK5, a novel member of the MAPK family, and that a dominant-negative form of MEK5, the MAPK kinase directly responsible for activation of ERK5, inhibits LIF-induced elongation of cardiomyocytes.

With those as background, we hypothesized that the major molecule responsible for the hypertrophic responses to IL-6-related cytokines may not be STAT3, but ERK1/2 or ERK5. So in this study, we examined the effects of overexpressing SOCSs, dominant-negative mutant of STAT3, MEK1 or MEK5 on CT-1-induced cardiomyocyte hypertrophy with the aim of better understanding the significance of the STAT3 and MEK-ERK pathways in CT-1-induced cardiac hypertrophy.

2. Materials and methods

2.1. Materials

Recombinant rat CT-1 was prepared using a GST-fusion system (Pharmacia Biotechnology, Inc.) according to the manufacturer's instructions. Human endothelin-1 (ET-1) was purchased from Peptide Institute. Anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-p44/42 MAPK (ERK1/2), anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-phospho-ERK5 (Thr218/Tyr220) antibodies were from Cell Signaling Technology. Anti-ERK5/BMK1 antibody was from Upstate Biotechnology. Anti-SOCS3/CIS3 antibody was from Immuno-Biological Laboratories. PD98059 was from Calbiochem.

2.2. Recombinant adenoviruses

Adenoviral vectors harboring the genes for LacZ (Ad-LacZ), myc-tagged SOCS1 (AdSOCS1), myc-tagged SOCS3 (AdSOCS3), an HA-tagged dominant-negative STAT3 mutant (AdSTAT3F) in which phosphorylation-site Tyr705 was substituted with Phe [13], and Cre recombinase (AdCre) were gifts from Dr. Yasushi Hanakawa, Ehime University School of Medicine, Ehime, Japan [14]; an adenoviral vector containing the gene for a dominant-negative MEK5 mutant (AdMEK5KM) in which ATP-binding Lys106 was substituted with Met [15] was a gift from Dr. Eric N. Olson, University of Texas, Dallas, USA [12]; and an adenoviral vector harboring the gene for a dominant-negative MEK1 mutant (AdMEK1DN) in which Asp208 in the kinase subdomain VII was substituted with an Ala [16] was a gift from Dr. Seinosuke Kawashima, Kobe University, Kobe, Japan. Because of the toxic effect of SOCS1 on 293 cells used for recombinant virus production, a Cre-LoxP conditional expression system was employed to generate AdSOCS1 using the protocol described by Kanegae et al. [17]. For that reason, only AdSOCS1 was co-infected with AdCre. All adenoviral vectors harbor the cytomegalovirus enhancer and the chicken β-actin promoter.

2.3. Cardiomyocyte culture and adenovirus infection

Ventricular myocytes were prepared from 1-day-old Wistar rats using a Percoll gradient as previously described [18]. The investigation conforms to the Guiding Principles in the Care and Use of Animals (American Physiological Society). After collecting the myocytes from the gradient, they were preplated on noncoated dishes for 1 h, after which the unattached cells were collected; this cell population consisted of >97% myocytes as assessed by immunofluorescence with anti-rat sarcomeric actin antibody (DAKO Japan Co., Ltd.). The myocytes were then plated on gelatin-coated dishes in serum-containing medium for 24 h. The medium was then replaced with serum-free medium, and the cells were infected for 24 h with one of the recombinant adenoviruses at a multiplicity of infection (MOI) of 10 viral particles per cell. Under these conditions, >99% of the myocytes were infected (assessed by X-gal staining or immunocytochemistry with anti-tag antibodies). Thereafter, the cells were treated with 10^{-9} mol/l CT-1 or 10^{-8} mol/l ET-1 for the indicated times. In some cases, the MEK inhibitor PD98059 was applied for 30 min prior to addition of CT-1 or ET-1.

2.4. Western blot analysis

After incubating the cells with CT-1 or ET-1 for the indicated times, they were washed with ice-cold phosphate-buffered saline (PBS) and lyzed with lysis buffer (Cell Signaling Technology). The resultant whole-cell protein extracts were subjected to 10% SDS-PAGE, and the resolved proteins were electrophoretically transferred onto polyvinylidene dif-

luoride membranes (Bio-Rad Laboratories). The membranes were then blocked with 5% skim milk (Difco Laboratories) and probed with the indicated antibodies.

2.5. Analysis of protein synthesis in cultured cells

Protein synthesis in cultured cardiomyocytes was evaluated using [^{3}H]-leucine incorporation as an index. Following incubation with CT-1 or ET-1, the cells were cultured for 24 h, after which 3 μCi of [^{3}H]-leucine (Amersham Life Science) was added for an additional 24 h. After washing twice with ice-cold PBS, the cells were incubated in 10% trichloroacetic acid for 30 min at 4 °C. The resultant precipitate was solubilized in 0.2 N NaOH for >4 h, and the radioactivity was measured in a liquid scintillation counter.

2.6. Radioimmunoassay for brain natriuretic peptide

Levels of brain natriuretic peptide (BNP) in medium conditioned for 48 h by cardiomyocytes after stimulation with CT-1 or ET-1 was measured using a specific radioimmunoassay as previously described [19].

2.7. Statistical analysis

Data are presented as mean ± standard deviations (S.D.) of results of four independent experiments. Unpaired Student's *t*-tests were used to determine significant differences between two groups, and ANOVA with post hoc Fisher's tests was used to determine significant differences among three or four groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effects of SOCSs and STAT3F on CT-1-induced STAT3 phosphorylation

First, to determine whether SOCSs are the true endogenous negative regulators of CT-1 signaling in our cultured cardiomyocytes, Western blot analysis was carried out using samples from CT-1-treated cardiomyocytes probed with anti-SOCS3 antibody. As shown in Fig. 1A, upregulation of SOCS3 protein was confirmed in CT-1-treated cardiomyocytes. We previously showed that CT-1 induced tyrosine phosphorylation of STAT3 in cardiomyocytes, and that the response peaked within 5–15 min of CT-1 application [20]. Next, therefore, we examined the effects of overexpressing SOCS1, SOCS3 and STAT3F on levels of STAT3 tyrosine phosphorylation after 10 min of CT-1 stimulation. AdSOCS1, AdSOCS3 and AdSTAT3F did not have an influence on the basal phosphorylation of STAT3 (Fig. 1B). As shown in Figs. 1B, and 1C, CT-1 induced substantial phosphorylation of STAT3 in cardiomyocytes infected with AdLacZ. This effect was completely blocked in cardiomyocytes infected with AdSOCS1 or AdSOCS3, and significantly inhibited in

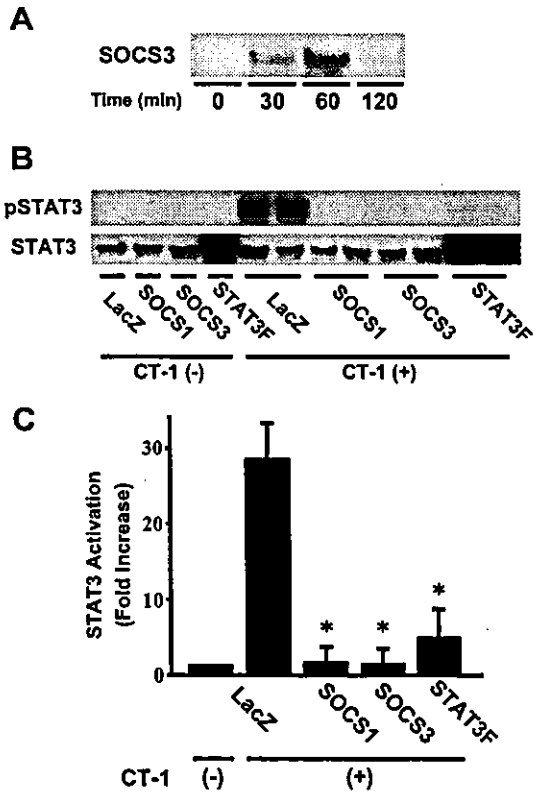

Fig. 1. SOCS3 expression induced by CT-1 and effects of SOCS1, SOCS3 and STAT3F on CT-1-induced phosphorylation of STAT3. (A) Representative Western blots showing the time course SOCS3 protein expression in CT-1-treated cardiomyocytes without adenovirus infection. (B) Representative Western blots showing the effects of SOCS1, SOCS3 and STAT3F on CT-1-induced phosphorylation of STAT3. Cardiomyocytes infected with AdLacZ, AdSOCS1, AdSOCS3 or AdSTAT3F were treated with 10^{-9} mol/L CT-1 for 10 minutes, after which cell lysates were harvested. (C) Phospho-STAT3 (pSTAT3) and STAT3 were measured densitometrically from immunoblots like those in panel B. The ratio of pSTAT3 to STAT3 was normalized to that in AdLacZ-infected cardiomyocytes without CT-1 treatment, which was assigned a value of 1. Only in regard to AdSTAT3F-infected group, the ratio to the average STAT3 of the other adenovirus-infected groups was used. Values are means ± S.D. (n = 8) of four independent experiments, each experiment performed with two distinct samples; *$P < 0.01$ vs. LacZ with CT-1.

cardiomyocytes infected with AdSTAT3F. The apparently augmented expression of STAT3 in cardiomyocytes infected with AdSTAT3F reflects the cross-reaction of anti-STAT3 antibody with overexpressed STAT3F, and is indicative of the efficiency of the protein expression in cardiomyocytes transfected using these recombinant adenoviral vectors.

3.2. Effects of SOCSs and STAT3F on CT-1-induced cardiomyocyte hypertrophy

We next examined the effects of overexpressing SOCS1, SOCS3 or STAT3F on CT-1-induced hypertrophy of cardiomyocytes. For comparison, ET-1, a G-protein-coupled receptor (GPCR) agonist, was also used to induce a distinct form of cardiomyocyte hypertrophy [21]. We first evaluated CT-1- and ET-1-induced [^{3}H]-leucine incorporation as an index of protein synthesis. As shown in Fig. 2A, CT-1 significantly increased [^{3}H]-leucine incorporation in cardiomyocytes

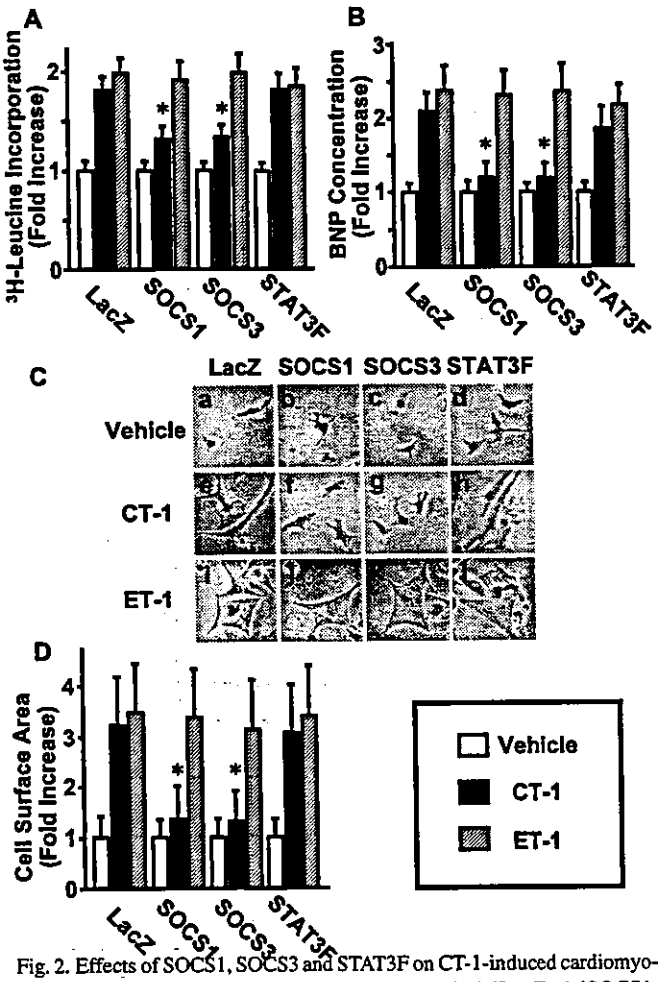

Fig. 2. Effects of SOCS1, SOCS3 and STAT3F on CT-1-induced cardiomyocyte hypertrophy. Cardiomyocytes infected with AdLacZ, AdSOCS1, AdSOCS3 or AdSTAT3F were treated with vehicle (open bars), 10^{-9} mol/L CT-1 (solid bars) or 10^{-8} mol/L ET-1 (hatched bars). The fold increase is relative to vehicle-treated cells of each adenovirus-infected group. (A) ^{3}H-leucine incorporation by cardiomyocytes during the period from 24 to 48 hours after treatment. (B) BNP concentration in the cultured media 48 hours after treatment. (C) Phase-contrast photographs of cultured cardiomyocytes taken 48 hours after treatment with vehicle *(a-d)*, CT-1 *(e-h)* or ET-1 *(i-l)*. (D) Cell surface areas were analyzed using NIH Image software. A total of 100 cells were examined for each group. The experiments were repeated four times independently, and each was performed with six distinct samples (A, B). Values are means ± S.D. (n = 24 in A and B, n = 100 in D); $^*P < 0.05$ vs. LacZ with CT-1.

infected with AdLacZ. This effect was significantly inhibited by infection with AdSOCS1 or AdSOCS3 but, somewhat surprisingly, not by infection with AdSTAT3F. Infection with AdSOCS1, AdSOCS3 or AdSTAT3F had no effect on ET-1-induced [^{3}H]-leucine incorporation.

The effects of AdSOCS1, AdSOCS3 and AdSTAT3F on BNP secretion from cardiomyocytes paralleled their effects on [^{3}H]-leucine incorporation (Fig. 2B)—i.e. infection with AdSOCS1 or AdSOCS3 significantly inhibited CT-1-induced BNP secretion, whereas infection with AdSTAT3F did not. ET-1-induced BNP secretion was unaffected by AdSOCS1, AdSOCS3 or AdSTAT3F.

CT-1- and ET-1-induced cardiomyocyte hypertrophy was also examined by evaluating their effect on cell shape and surface area. Consistent with an earlier report [21], CT-1 induced a characteristic hypertrophy in cardiomyocytes infected with AdLacZ (Fig. 2Ce)—i.e. it elicited pronounced increases in cell length, but did not significantly affect the width. ET-1, by contrast, elicited increases in both cell length and width (Fig. 2Ci). Infection with AdSOCS1 or AdSOCS3 (Fig. 2Cf, g and 2D), but not AdSTAT3F (Fig. 2Ch and 2D), inhibited CT-1-induced changes in cell shape and size. ET-1-induced changes in cell shape and size were unaffected by AdSOCS1, AdSOCS3 or AdSTAT3F (Fig. 2Cj-l and 2D).

3.3. Effects of SOCSs and STAT3F on CT-1-induced ERK phosphorylation

Collectively, the results presented so far indicate that inhibition of STAT3 alone does not inhibit CT-1-induced cardiomyocyte hypertrophy. Only when SOCSs are used to inhibit other signaling pathways is the hypertrophy inhibited. In that regard, CT-1 is known to also induce activation of ERK1/2 in cardiomyocytes [1], while LIF, another IL-6-related cytokine, has been reported to induce activation of ERK5 in cardiomyocytes [12]. With that in mind, we examined the effects of overexpressing SOCS1, SOCS3 or STAT3F on CT-1-induced activation (phosphorylation) of ERK1/2 and ERK5. AdSOCS1, AdSOCS3 and AdSTAT3F did not affect the basal phosphorylation of ERK1/2 nor ERK5 (Fig. 3D). We found that CT-1-induced phosphorylation of both ERK1/2 and ERK5 that peaked within ~10 min (Fig. 3A–C), and these effects were significantly inhibited by infection with AdSOCS1 or AdSOCS3, but not AdSTAT3F (Fig. 3D–F). Thus, the effects of overexpressing SOCS1, SOCS3 or STAT3F on CT-1-induced ERK activation differed from the effects on STAT3 activation, but paralleled the effects on cardiomyocyte hypertrophy.

3.4. Effects of PD98059 on CT-1-induced ERK phosphorylation and on CT-1-induced cardiomyocyte hypertrophy

The MEK1 inhibitor PD98059 [22] was recently shown to also inhibit MEK5, the specific upstream activator of ERK5, though at a somewhat higher concentration [23]. Likewise, we found that PD98059 concentration-dependently inhibited CT-1-induced phosphorylation of both ERK1/2 and ERK5, but that it was a more potent inhibitor of the former (Fig. 4A–C). PD98059 also inhibited both CT-1- and ET-1-induced [^{3}H]-leucine incorporation and BNP secretion (Fig. 4D, E). It is noteworthy that the potency of the inhibitory effect of PD98059 on the activities of CT-1 and ET-1 paralleled the potency of its effect on the phosphorylation of ERK5 and ERK1/2, respectively.

3.5. Effects of dominant-negative MEK1 and MEK5 mutants on CT-1-induced cardiomyocyte hypertrophy

The results obtained with PD98059 were confirmed when we examined the effects of overexpressing dominant-negative

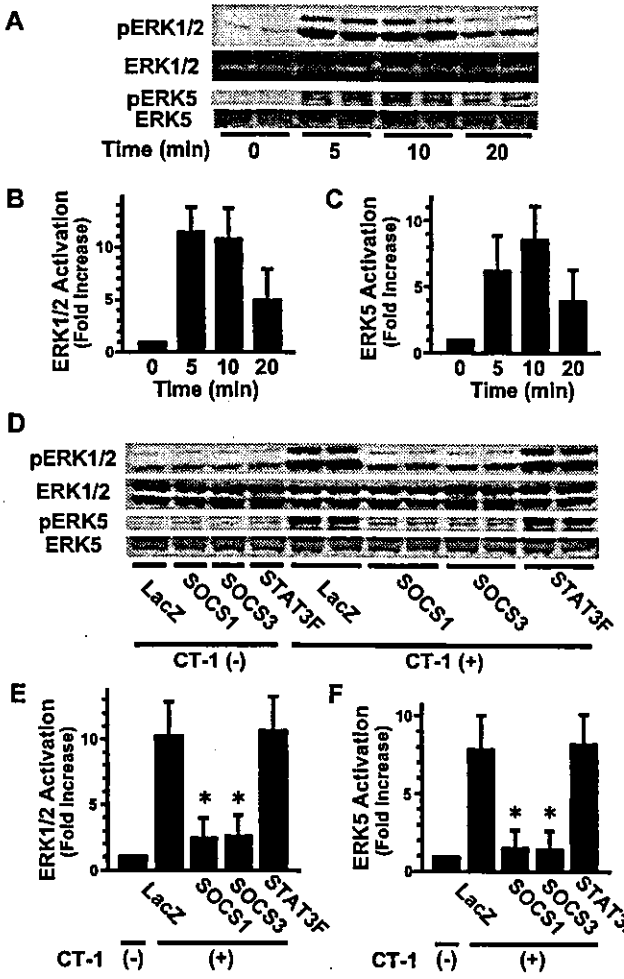

Fig. 3. Time course of CT-1-induced phosphorylation of ERK1/2 and ERK5, and effects of SOCS1, SOCS3 and STAT3F on CT-1-induced phosphorylation of ERK1/2 and ERK5. (A) Representative Western blots showing the time course of CT-1-induced phosphorylation of ERK1/2 and ERK5. Cardiomyocytes infected with AdLacZ were stimulated with 10^{-9} mol/L CT-1 for the indicated times, after which cell lysates were harvested. (B) Phospho-ERK1/2 (pERK1/2) and ERK1/2 were measured densitometrically from immunoblots like those in panel A. The ratio of pERK1/2 to ERK1/2 was normalized to that in cardiomyocytes without CT-1 treatment, which was assigned a value of 1. (C) Phospho-ERK5 (pERK5) and ERK5 were analyzed as panel B. (D) Representative Western blots showing the effects of SOCS1, SOCS3 and STAT3F on CT-1-induced phosphorylation of ERK1/2 and ERK5. Cardiomyocytes infected with AdLacZ, AdSOCS1, AdSOCS3 or AdSTAT3F were treated with 10^{-9} mol/L CT-1 for 10 min, after which the cell lysates were harvested. (E) Phospho-ERK1/2 (pERK1/2) and ERK1/2 were measured densitometrically from immunoblots like those in panel D. The ratio of pERK1/2 to ERK1/2 was normalized to that in AdLacZ-infected cardiomyocytes without CT-1 treatment, which as assigned a value of 1. (F) Phospho-ERK5 (pERK5) and ERK5 were analyzed in the same way as panel E. Values are means ± S.D. (n = 8) of four independent experiments, each experiment performed with two distinct samples; $^*P < 0.05$ vs. LacZ with CT-1.

MEK1 or MEK5 mutants on CT-1-induced phosphorylation of ERKs and cardiomyocyte hypertrophy. As shown in Fig. 5, infection with AdMEK1DN inhibited both the basal phosphorylation of ERK1/2 and CT-1-induced phosphorylation of ERK1/2, but did not have an influence on the phosphorylation of ERK5. On the other hand, infection with AdMEK5KM inhibited both the basal phosphorylation of

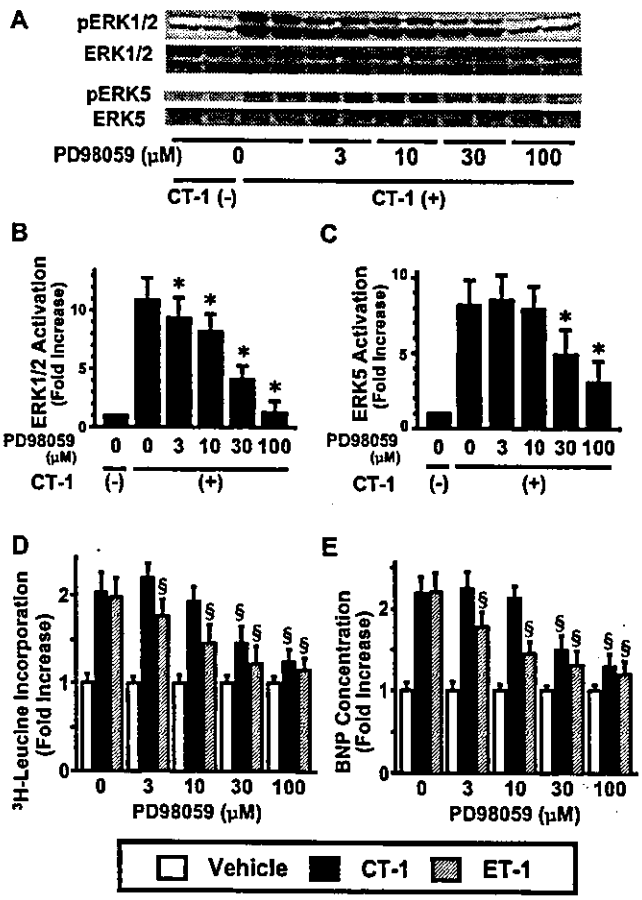

Fig. 4. Effects of PD98059 on CT-1-induced phosphorylation of ERK1/2 and ERK5 and CT-1- and ET-1-induced cardiomyocyte hypertrophy. Cardiomyocytes cultured with the indicated concentration of PD98059 were treated with vehicle (open bars), 10^{-9} mol/L CT-1 (solid bars) or 10^{-8} mol/L ET-1 (hatched bars). (A) Representative Western blots of phospho-ERK1/2 (pERK1/2) and phospho-ERK5 (pERK5) in lysates harvested after 10 minutes of CT-1 treatment. (B) pERK1/2 and ERK1/2 were measured densitometrically from immunoblots like those in panel A. The ratio of pERK1/2 to ERK1/2 was normalized to that in cardiomyocytes without CT-1 treatment, which as assigned a value of 1. (C) pERK5 and ERK5 were analyzed in the same way as panel B. (D) ^{3}H-leucine incorporation by cardiomyocytes during the period from 24 to 48 hours after treatment. (E) BNP concentration in the cultured media 48 hours after treatment. Fold increase is relative to vehicle-treated cells in each concentration group. The experiments were repeated four times independently, and each was performed with two distinct samples (A-C) or with sis distinct samples (D, E). Values are means ± S.D. (n = 8 in A to C, n = 24 in D and E); $^*P < 0.05$ vs. PD98059 (-) with CT-1, §$P < 0.05$ vs. LacZ with CT-1 or ET-1, correspondently.

ERK5 and CT-1-induced phosphorylation of ERK5, but did not have an influence on the phosphorylation of ERK1/2. And as shown in Fig. 6, infection with AdMEK5KM but not AdMEK1DN significantly inhibited CT-1-induced increases in [^{3}H]-leucine incorporation, BNP secretion and cell surface area. Conversely, infection with AdMEK1DN but not AdMEK5KM inhibited the ET-1-induced effects.

4. Discussion

The aim of the present study was to assess the significance of the STAT3 and MEK-ERK pathways in CT-1-induced

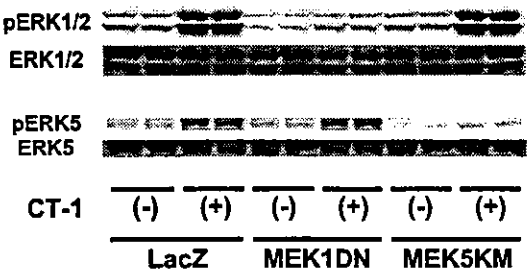

Fig. 5. Effects of dominant-negative MEK1 (MEK1DN) and MEK5 (MEK5KM) mutants on CT-1-induced phosphorylation of ERK1/2 and ERK5. Representative Western blots of phospho-ERK1/2 (pERK1/2) and phospho-ERK5 (pERK5) in lysates harvested after 10 minutes of CT-1 treatment.

hypertrophy of cultured neonatal rat ventricular myocytes. Among several pathways activated by IL-6-related cytokines in cardiomyocytes, the STAT3 pathway is reportedly important for mediating cardiomyocyte hypertrophy [5,6,24]. In our study, however, a dominant-negative STAT3 mutant did not inhibit CT-1-induced cardiomyocyte hypertrophy, although it suppressed activation (phosphorylation) of STAT3. We can not explain strictly the discrepancy of our results from previous study [5], but we think that it may be attributable to the difference of culture conditions, for example the degree of fibroblasts contamination. We have confirmed the existence of STAT3 in cardiac fibroblasts and its phosphorylation induced by CT-1 (data not shown). With the results in our study, the major pathway leading from CT-1 binding to the gp130 complex to cardiomyocyte hypertrophy is not via STAT3. On the other hand, two recently identified [7–9] endogenous negative regulators of cytokine signaling via JAK-STAT pathways, SOCS1 and SOCS3, significantly inhibited both STAT3 phosphorylation and the hypertrophic effects of CT-1, which is consistent with an earlier report [14]. In addition, SOCS1/3 and dominant-negative STAT3 mutant had the same influence on the hypertrophic effects of LIF, another member of IL-6-related cytokines (data not shown), indicating that these differential effects of SOCSs vs. dominant-negative STAT3 are not CT-1 specific, but shared with other members of IL-6-related cytokines. The key question then was what gp130-dependent signaling pathway do SOCSs suppress to inhibit CT-1-induced hypertrophy?

Among the possibilities are the STAT1 pathway [25], the PI3K-Akt pathway [26] and the MEK1/2-ERK1/2 pathway [11,16], all of which appear to be involved in LIF-induced cardiomyocyte hypertrophy. The first two are easily ruled out. We found that dominant-negative STAT3 inhibited CT-1-induced phosphorylation of not only STAT3 but also STAT1 (data not shown), most likely because STAT3 and STAT1 share docking sites for JAK1/2 [1]. Therefore, if CT-1 acted via STAT1, we would expect its effects to have been inhibited by the dominant-negative STAT3 mutant. As for the PI3K-Akt pathway, we previously showed that this pathway is largely responsible for CT-1's antiapoptotic effects rather than cell hypertrophy [4].

This leaves the MEK-ERK pathways. PD98059 is known as a specific inhibitor of MEK1 [22], but some reports suggest that it also inhibits MEK5 at a somewhat higher concentration [23,27]. Consistent with those results, we found that at concentrations <30 μM PD98059 selectively inhibits evoked ERK1/2 phosphorylation and ET-1-induced cardiomyocyte hypertrophy. At concentrations ≥30 μM, however, PD98059 also inhibits ERK5 phosphorylation and CT-1-induced cardiomyocyte hypertrophy. On the other hand, it is also known that PD98059 loses specificity at the higher concentrations. So we added the examinations with dominant-negative MEK1 or MEK5 mutant to confirm our hypothesis acquired from the examinations with PD98059. Dominant-negative MEK1 mutant partially inhibited ET-1-induced hypertrophy, but had not effect on CT-1-induced hypertro-

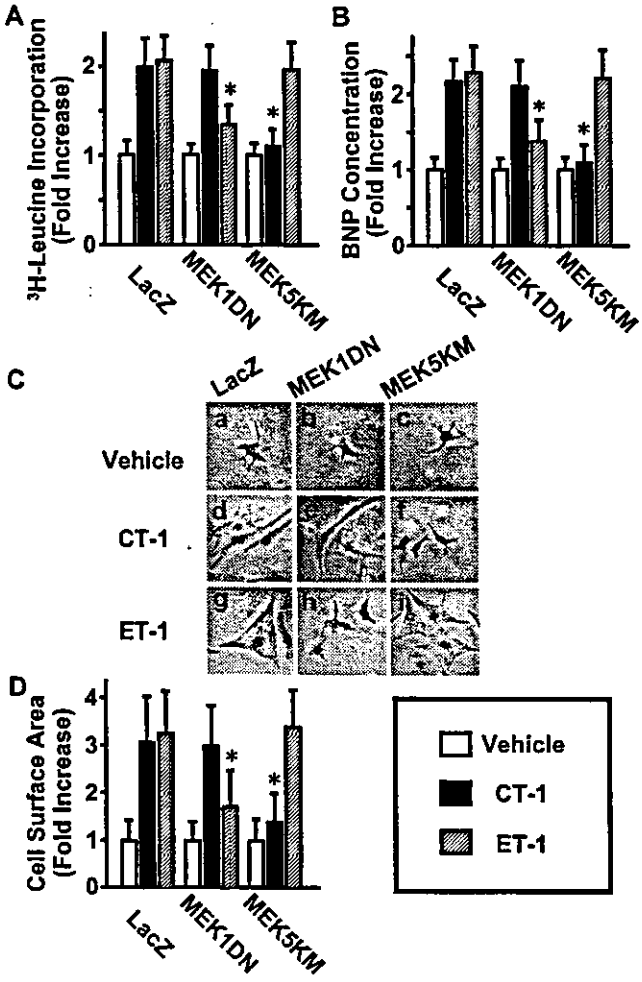

Fig. 6. Effects of dominant-negative MEK1 (MEK1DN) and MEK5 (MEK5KM) mutants on CT-1-induced cardiomyocyte hypertrophy. Cardiomyocytes infected with AdLacZ, AdMEK1DN or AdMEK5KM were treated with vehicle (open bars), 10^{-9} mol/L CT-1 (solid bars) or 10^{-8} mol/L ET-1 (hatched bars). The fold increase is relative to vehicle-treated cells in each adenovirus-infected group. (A) ^{3}H-leucine incorporation by cardiomyocytes during the period from 24 to 48 hours after treatment. (B) BNP concentration in the cultured media 48 hours after treatment. (C) Phase-contrast photographs of cultured cardiomyocytes obtained 48 hours after treatment with vehicle *(a-c)*, CT-1 *(d-f)* or ET-1 *(g-i)*. (D) Cell surface areas were analyzed using NIH Image software. A total of 100 cells were examined for each group. The experiments were repeated four times independently, and each was performed with six distinct samples (A, B). Values are means ± S.D. (n = 24 in A and B, n = 100 in D); *$P < 0.05$ vs. LacZ with CT-1.

phy, whereas dominant-negative MEK5 mutant almost completely inhibited CT-1-induced hypertrophy, but had no effect on ET-1-induced hypertrophy. We therefore conclude that CT-1-induced cardiomyocyte hypertrophy is mediated mainly via a MEK5-ERK5 pathway, while ET-1-induced hypertrophy is at least partially via a MEK1/2-ERK1/2 pathway. The fact that SOCSs inhibited CT-1-induced phosphorylation of ERK5 indicates they suppress hypertrophic responses to CT-1 through inhibition of a MEK5-ERK5 pathway. These conclusions are further supported by the findings that gp130-deficient [28] and ERK5-deficient mice [29] show similar embryonic cardiovascular defects, suggesting ERK5 is an important mediator situated downstream of gp130 during cardiovascular development.

CT-1-induced cardiomyocyte hypertrophy is distinct from that induced by GPCR agonists including ET-1 and angiotensin II [21]. Pathophysiological significance of GPCR agonists in the heart is undoubted because of the clinical usefulness of angiotensin-converting-enzyme (ACE) inhibitors [30–33]. On the other hand, there is little clinical evidence concerning IL-6-related cytokines including CT-1. With regard to mouse models, however, IL-6-related cytokines are known to induce concentric hypertrophy via gp130 in in vivo heart [2]. Although here we have shown the significance of the MEK5-ERK5 pathway in cardiomyocyte hypertrophy induced by CT-1, a member of IL-6-related cytokines, activated MEK5 has been shown to induce eccentric, not concentric, hypertrophy in in vivo heart [12]. That is to say, activation of only a MEK5-ERK5 pathway can not account for the cardiac phenotype induced by IL-6-related cytokines. Several signaling pathways including JAK-STATs, MEK-ERKs and PI3K-Akt pathways probably cooperate and express the cardiac phenotype induced by IL-6-related cytokines. So it is necessary to investigate more detailed role of not only a MEK5-ERK5 pathway, but also the other signaling pathways downstream of gp130 in the cardiomyocyte or in the heart stimulated with IL-6-related cytokines. Furthermore, it is expected to elucidate the clinical significance of IL-6-related cytokines in the heart.

Finally, our finding that the MEK5-ERK5 pathway plays a critical role in CT-1-induced cardiomyocyte hypertrophy raises the question, what is the role of STAT3 in cardiomyocytes? Kunisada et al. [6] reported that STAT3 transduces a protective signal against doxorubicin-induced cardiomyopathy, but we detected no related phenotypes in cardiomyocytes overexpressing a dominant-negative STAT3 mutant. It may be that the key role played by STAT3 in cardiomyocytes involves the induction of SOCSs [10] and subsequent inhibition of the MEK5-ERK5 pathway.

Acknowledgments

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Transgenic Mice Overexpressing Des-Acyl Ghrelin Show Small Phenotype

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Ghrelin, a 28-amino acid acylated peptide, displays strong GH-releasing activity in concert with GHRH. The fatty acid modification of ghrelin is essential for the actions, and des-acyl ghrelin, which lacks the modification, has been assumed to be devoid of biological effects. Some recent reports, however, indicate that des-acyl ghrelin has effects on cell proliferation and survival. In the present study, we generated two lines of transgenic mice bearing the preproghrelin gene under the control of chicken β-actin promoter. Transgenic mice overexpressed des-acyl ghrelin in a wide variety of tissues, and plasma des-acyl ghrelin levels reached 10- and 44-fold of those in control mice. They exhibited lower body weights and shorter nose-to-anus lengths, compared with control mice. The serum GH levels tended to be lower, and the serum IGF-I levels were significantly lower in both male and female transgenic mice than control mice. The responses of GH to administered GHRH were normal, whereas those to administered ghrelin were reduced, especially in female transgenic mice, compared with control mice. These data suggest that overexpressed des-acyl ghrelin may modulate the GH-IGF-I axis and result in small phenotype in transgenic mice. (*Endocrinology* 146: 355–364, 2005)

GHRELIN, AN ACYLATED peptide of 28 amino acids, was identified as an endogenous ligand for the GH secretagogue (GHS) receptor (GHS-R) (1). The major site of production of ghrelin is the stomach and it is also expressed in the hypothalamus (2–5). Plasma ghrelin levels are regulated by acute feeding states. They rise by fasting and are rapidly suppressed by feeding (3, 6–8). Secretion of ghrelin is also regulated by chronic feeding states. Plasma ghrelin levels are elevated in patients with anorexia nervosa and food-restricted animals and are reduced in obese subjects (3, 6–10). These data suggest the possible involvement of ghrelin in energy homeostasis. In fact, ghrelin stimulates food intake in animals and humans and exhibits anticachectic effect in cancer-bearing mice (8, 11–13).

Exogenously administered ghrelin strongly stimulates GH release in a clear dose-dependent manner *in vivo* (1, 2, 14–16). The site of ghrelin action on GH release is not well known to date. The GHS-R is reported to be expressed in the pituitary as well as hypothalamus (17–19). Previous studies indicate that ghrelin binds to membranes from the pituitary and stimulates GH release from cultured pituitary cells (1, 20), suggesting that the pituitary is one of the sites of ghrelin actions. The stimulatory effect of GHSs and ghrelin on GH secretion, however, is more prominent *in vivo* than *in vitro*, and intact GHRH signaling is essential for the effect (1, 21). Hexarelin, one of the potent GHSs, cannot efficiently stimulate GH release in patients with GHRH receptor deficiency (22). Moreover, as we demonstrated, ghrelin has a synergistic action with GHRH. Even a low dose of ghrelin can highly augment GH release by GHRH (23). These data indicate a critical role of the hypothalamus in the stimulatory effect of ghrelin on GH secretion. The strong potency of ghrelin suggests its role as a physiological regulator of GH secretion (1, 2, 14–16). The issue, however, is currently controversial. One recent study (24), using a GHS antagonist, revealed that circulating ghrelin in peripheral blood may not play a role in generating pulsatile GH secretion. Moreover, deletion of ghrelin impairs neither growth nor appetite, indicating that ghrelin is not critically required for GH secretion (25). Another study (26), however, demonstrated that the attenuation of the GHS-R expression *in vivo* results in reduction in food intake and growth, suggesting a physiological role of the ghrelin-GHS-R system in the secretory regulation of GH.

The acylation of ghrelin is assumed to be essential for its actions (1). Des-acyl ghrelin, which lacks the fatty acid modification and circulates at 10-fold higher concentration than acylated ghrelin (1, 3, 27), is devoid of any endocrine activities including GH release, based on previous studies (1, 28). Recent studies (29, 30), however, indicated that des-acyl ghrelin may share with acylated ghrelin the modulation of neoplastic cell proliferation and cardiovascular cell survival *in vitro*. Moreover, one study shows that des-acyl ghrelin may offset the inhibitory effect of acylated ghrelin on insulin secretion (28). Although previous studies indicated that several tissues and cell lines produce des-acyl and/or acylated ghrelin (3, 27, 31, 32), the mechanism by which ghrelin is acylated is also unknown to date.

In the present study, we generated transgenic mice bearing the preproghrelin gene under the control of a cytomegalovirus immediate early enhancer and a modified chicken β-

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Abbreviations: BMI, Body mass index; GHS, GH secretagogue; GHS-R, GHS receptor.

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actin promoter, designated CAG promoter (33, 34). This promoter sequence has been demonstrated to have high activity in cultured cells and transgenic mice (33, 34). Transgenic mice in the present study overexpressed des-acyl ghrelin in plasma and a wide variety of tissues and showed small phenotype. Here we show that des-acyl ghrelin may modulate endogenous ghrelin action and alter the GH-IGF-I axis in transgenic mice.

Materials and Methods

All procedures in animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. The procedures were performed in accordance with the principle and guidelines established by the committee.

Plasmid construction and generation of transgenic mice

The full-length mouse preproghrelin cDNA (1) and the pCAGGS expression vector including the CAG promoter (34) were kindly donated by Professor Masayasu Kojima (Division of Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Japan) and Professor Junichi Miyazaki (Department of Nutrition and Physiological Chemistry, Osaka University School of Medicine, Osaka, Japan), respectively. Plasmid pCAGGS-ghrelin was constructed by inserting the mouse preproghrelin cDNA into the unique *Eco*RI site between the CAG promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. The DNA fragment was excised from its plasmid by digestion with *Sal*I and *Hind*III and then purified and microinjected into the pronuclei of fertilized eggs obtained from BDF1 female mice (Charles River Japan, Yokohama, Japan) as reported previously (35). Founder transgenic mice were identified by PCR analysis and bred with C57BL/6 mice (Japan CLEA, Osaka, Japan). Mice were housed in air-conditioned animal quarters, with the lights on between 0800 and 2000 h and were given standard rat chow (CE-2, 352 kcal per 100 g, Japan CLEA) and water *ad libitum*.

Measurement of total and acylated ghrelin levels in tissue samples

Tissues such as the stomach, cerebrum, heart, and kidney were removed from 8-wk-old mice under anesthesia with diethyl ether. Each sample was diced and boiled for 7 min in a 5-fold volume of water. The solution was adjusted to 1.0 M acetic acid and 20 mM hydrogen chloride after boiling, and the tissue was homogenized. The supernatant was obtained after centrifugation at 10,000 rpm for 30 min. Tissue ghrelin levels were measured using two kinds of RIAs, C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin as reported previously (9, 27). C-RIA and N-RIA recognize total (acylated plus des-acyl ghrelin) and acylated ghrelin, respectively (9, 27).

Measurement of plasma total and acylated ghrelin levels

Blood samples were collected from the inferior vena cava of mice under anesthesia with diethyl ether. The samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan; 1000 kallikrein inactivator U/ml) and centrifuged at 4 C. For N-RIA, hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after the separation of plasma. Plasma ghrelin was measured as reported previously (1, 3, 27). Briefly, the samples were subjected to a Sep-Pak C18 cartridge and C-RIA and N-RIA were carried out.

Measurement of body weights and lengths, organ weights, and daily food intake

Body weights of control and transgenic mice were measured weekly, beginning at 3 wk of age. Body lengths of 8- and 52-wk-old mice were measured by manual immobilization and extension of mice to the nose-to-anus lengths, always by the same individual. Body mass indexes (BMIs = weight/(nose-to-anus lengths)^2) were calculated in 8- and 52-wk-old control and transgenic mice (36, 37). Organs such as the pituitary, stomach, cerebrum, heart, liver, kidney, spleen, pancreas, and epididymal fat were removed from 8-wk-old mice under anesthesia with diethyl ether and weighed. Daily food intake was monitored for 3 wk, beginning at 5 wk of age.

Measurement of blood glucose, serum total protein, total cholesterol, and hormones

To examine the nutritional conditions, blood glucose and serum total protein and total cholesterol levels were measured. Eight-week-old control and transgenic mice were used. Four hundred microliters of blood samples were collected from the tail vein of mice for blood glucose levels at 1000 h after 12 h fasting. Then the mice were anesthetized with diethyl ether, and 400 μl of blood samples were collected from the inferior vena cava for serum total protein, total cholesterol, and hormone levels. Blood glucose, serum total protein, and total cholesterol levels were measured by the glucose oxidase method with a reflectance glucometer (One Touch II; Lifescan, Milpitas, CA), BCA protein assay reagent kit (Pierce, Rockford, IL), and Amplex red cholesterol assay kit (Molecular Probes, Eugene, OR), respectively. Serum GH and IGF-I levels were measured with EIA kits (SPI-BIO, Bonde, France, and Diagnostic Systems Laboratories Inc., Webster, TX, respectively). Serum insulin and plasma ACTH levels were measured with EIA kits (Morinaga, Tokyo, Japan) and ACTH-RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), respectively. Serum TSH, LH, and FSH levels were measured with EIA kits (Amersham Biosciences, Buckinghamshire, UK).

Effects of GHRH and ghrelin on serum GH levels

Human GHRH and rat ghrelin were purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan) and Peptide Institute, Inc. (Osaka, Japan), respectively. Male and female 8-wk-old control and Tg 10–1 mice were used under no anesthesia. Control and transgenic mice were housed in the same cage and tested on the same day. Forty mice were divided into five groups for blood sampling. Eight mice in the same group were used for each blood sampling. Control and transgenic mice were iv injected with human GHRH (60 μg/kg) or rat ghrelin (40 μg/kg). Four hundred microliters of blood samples were collected from the inferior vena cava of mice 0, 10, 20, 30, and 60 min after the injection. Serum GH levels were measured with an EIA kit (SPI-BIO).

Real-time PCR analysis of preproghrelin, GH, GHRH, somatostatin, and GHS-R mRNAs

Total RNAs from tissues, such as the stomach, small intestine, cerebrum, hypothalamus, pituitary, liver, kidney, lung, heart, and skeletal muscle, were extracted using the acid guanidinium thiocyanate-phenol-chloroform method (38). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript II RT (Life Technologies, Inc., St. Louis, MO) with random hexamers according to the manufacturer's instructions. Taqman-PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using VIC-labeled fluorogenic probes specific for preproghrelin, GH, GHRH, somatostatin, or GHS-R transcript, or the internal standard glyceraldehyde-3-phosphate dehydrogenase. Oligo primers and probes (Table 1) were chosen using the Primer Express software (Applied Biosystems). The PCR was performed using Taqman Universal PCR Mastermix (Applied Biosystems) to which primers and probes were added (final concentrations 400 and 200 nM, respectively). All samples were run in triplicate in 96-well plates in the ABI Prism 7700 sequence detector according to the manufacturer's standard protocol. For the primer sets, serial dilutions were conducted with different cDNA preparations to confirm the kinetics of the PCR. There was no significant difference in glyceraldehyde-3-phosphate dehydrogenase mRNA levels among experimental groups.

Effects of continuous infusion of des-acyl ghrelin on the GH-IGF-I axis and body weights

Rat des-acyl ghrelin was purchased from Peptide Institute, Inc. Des-acyl ghrelin was dissolved in saline at a concentration of 700 μg/ml and stored in osmotic minipumps (DURECT Corp., Cupertino, CA). The