Growth hormone stimulates adipogenesis of 3T3-L1 cells through activation of the Stat5A/5B-PPARγ pathway

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Abstract

Growth hormone-deficient (GHD) patients show a decreased number of adipocytes, which is normalized by GH replacement, indicating an adipogenic effect of GH. However, the precise mechanisms underlying this effect remain to be clarified. In this study, we investigated the adipogenic effect of GH. GH stimulated MDI (3-isobutyl-1-methylxanthine, dexamethasone, and insulin)-induced adipogenesis of 3T3-L1 cells with early induction of peroxisome proliferator-activated receptors (PPARγ2) expression. This adipogenic effect of GH was suppressed by overexpression of Stat5A mutant (Stat5A-Y694F), a transcriptional suppressor for the GH–Stat5A/5B signaling pathway, with the reduction of PPARγ2 expression. This adipogenic effect of GH was suppressed by overexpression of Stat5A mutant (Stat5A-Y694F), a transcriptional suppressor for the GH–Stat5A/5B signaling pathway, with the reduction of PPARγ2 expression. Next, we investigated the relationship between Stat5A/5B and CCAAT/enhancer binding protein (C/EBP) in the PPARγ gene promoter. Furthermore, Stat5A/5B stimulated PPARγ-induced adipogenesis and enhanced the transcriptional activity of PPARγ. These results suggest that the GH–Stat5A/5B signaling pathway stimulates adipogenesis in cooperation with C/EBPβ or PPARγ in 3T3-L1 cells. Stat5A/5B stimulated C/EBPβ- and C/EBPδ-induced adipogenesis with enhancement of PPARγ2 expression. In addition, Stat5A/5B enhanced the transcriptional activity of C/EBPβ in the PPARγ gene promoter. To completely understand the effect of GH, C/EBPβ and C/EBPδ expression analysis was performed to screen genes affected by GH during MDI-induced adipogenesis. Among 4277 genes, 18 and 19 genes were up- and down-regulated respectively. cDNA microarray analysis indicated the up-regulation of PPARγ and the modulation of expression of genes coding for growth factors or growth factor receptors, suggesting that GH stimulates adipogenesis in association with the modulation of cell growth. Thus, the GH–Stat5A/5B signaling pathway stimulates adipogenesis through two distinct steps. In addition, cDNA microarray data provide us further insights underlying the adipogenic effect of GH.

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Introduction

Growth hormone (GH) is not only a major regulator of postnatal somatic growth, but also has an important role in the control of body composition and fat distribution, through the combination of anabolic, lipolytic, and anti-natriuretic actions (Isaksson et al. 1982, Nam & Lobie 2000, Tollet-Egnell et al. 2004, Rowland et al. 2005). Indeed, GH-deficient (GHD) patients exhibit a decreased number of adipocytes and an increased mean volume of adipocytes, and this abnormal adipose tissue composition is normalized by GH replacement (Bonnet et al. 1974, Salomon et al. 1989, Bengtsson et al. 1993). As GH replacement increases the number of adipocytes in GHD patients, this indicates a stimulatory effect of GH on adipogenesis. To date, adipogenic activity of GH has been reported in studies in vitro (Morikawa et al. 1984, Nixon & Green 1984). GH is strictly required in the conversion of preadipocytes to adipocytes and is thought to play a role in priming the cells to become responsive to insulin and insulin-like growth factor-I (IGF-I) in 3T3-F442A cells (Guller et al. 1989, Corin et al. 1990, Wabitsch et al. 1995). In other cell lines, GH also stimulates adipogenesis, although the role of GH is not exclusive (Doglio et al. 1986, Tominaga et al. 2002). However, little is known about the precise mechanisms of the stimulatory effect of GH on adipogenesis.

Adipogenesis is a well-regulated process controlled by the sequential activation of various transcription factors (Rosen & Spiegelman 2000). Adipogenic hormones, including insulin, glucocorticoids, and cAMP-producing factors, and fetal calf serum (FCS) allow growth-arrested preadipocytes to re-enter the cell cycle and undergo approximately two rounds of mitosis called mitotic clonal expansion. Both CCAAT/enhancer-binding protein (C/EBP)β and C/EBPδ are transiently induced during this period. These transcription factors induce the expression of peroxisome proliferator-activated receptor (PPARγ). PPARγ in turn stimulates the expression of
C/EBPβ. Subsequently, PPARγ and C/EBPβ positively activate the transcription of each other. Concurrently, adipogenesis enters the terminal differentiation stage, and adipocyte-specific genes including aP2, leptin, and adiponectin begin to be expressed. Although C/EBPβ and C/EBPδ have an important role in adipogenesis, mice lacking both C/EBPβ and C/EBPδ exhibited normal PPARγ expression in adipose tissue, indicating that PPARγ expression is maintained through several pathways (Tanaka et al. 1997). In addition, PPARγ null cells showed no adipogenic potential (Rosen et al. 1999). Combined together, PPARγ rather than C/EBPβ/δ has the essential role in adipogenesis.

Signal transducer and activator of transcription Stat5A and 5B, both of which have an approximately 90% identical sequence at amino acid levels, are key transducers of the GH signaling pathway (Darnell 1997). When GH binds to GH receptor (GHR), cytoplasmic tyrosine kinase Janus kinase 2 (JAK2) associates with the cytoplasmic domain of GHR and phosphorylates Stat5A/5B. Phosphorylated Stat5A/5B dimerizes, translocates into the nucleus and binds to the DNA (Piwiens-Pilipuk et al. 2002). Stat5A/5B-deficient mice showed reduced weight, similar to GH- or GHR-deficient mice, and had approximately one-fifth the epidermal fat pad of wild-type mice, indicating that GH may exert its effect on adipose tissue formation through Stat5A/5B (Teglund et al. 1998). Consistent with this, several investigators have reported the contribution of Stat5A to adipogenesis in some cell lines (Yarwood et al. 1999, Nanbu-Wakao et al. 2002, Floyd & Stephens 2003, Shang & Waters 2003, Stewart et al. 2004). However, the precise mechanisms by which Stat5A promotes adipogenesis have not been completely established. Furthermore, the role of Stat5B in adipogenesis remains to be clarified.

In addition to transcription factors and signaling transducers known to be involved in adipogenesis, it is necessary to examine the expression pattern of a number of genes to completely understand the effect of GH on adipogenesis. To achieve this purpose, we performed cDNA microarray analysis to screen the gene profiles during the course of GH-mediated adipogenesis of 3T3-L1 cells.

In this study, we attempted to elucidate the role of Stat5A and 5B in the stimulatory effect of GH on adipogenesis, and demonstrated that GH exerts its effect through Stat5A and 5B, which stimulates PPARγ expression. Furthermore, we revealed that Stat5A and 5B enhance C/EBPβ, C/EBPδ, and PPARγ-induced adipogenesis. Interestingly, Stat5A and 5B enhanced the transcriptional activity of C/EBPβ/δ and PPARγ. Our results provide the evidence that the GH–Stat5A/B signaling pathway stimulates adipogenesis by two distinct steps through stimulating C/EBPβ/δ and PPARγ activities.

Materials and methods

Materials

3T3-L1 cells and C3H10T1/2 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). Growth hormone, insulin, 3-isobutyl-2-methylxanthine, and cycloheximide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dexamethasone and triiodothyronine were purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Biotin, epidermal growth factor (EGF), fetuin, and anti-β-actin antibody were purchased from Sigma. The anti-PPARγ antibody, anti-C/EBPβ antibody, and anti-C/EBPδ antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the anti-Stat5 antibody and anti-proliferating cell nuclear antigen (PCNA) antibody were from BD Transduction Laboratory, Inc. (Lexington, KY, USA).

Cell culture

3T3-L1 cells and C3H10T1/2 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS in 5% CO2 at 37°C. For the induction of adipogenesis, 3T3-L1 cells were grown to confluence. Two days after confluence, the medium was changed to differentiation medium containing 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 µg/ml insulin (MDI) in serum-free medium consisting of 5 µg/ml transferrin, 2 mM triiodothyronine, 30 ng/ml EGF, 1 µM biotin, and 200 µg/ml fetuin in DMEM (Tominaga et al. 2002). After treatment with differentiation medium, the medium was changed to maintenance medium (serum-free medium).

Oil Red O staining

On day 6, cells were stained with Oil Red O as follows. The cells were washed with PBS and fixed with 10% formalin in PBS for 20 min. After fixing, cells were washed twice with PBS and once with 60% isopropyl-alcohol. The cells were stained with 60% Oil Red O solution from a stock of 0.25 g Oil Red O in 50 ml isopropyl-alcohol for 1 h (Sigma). To quantify the incorporation of lipid, the area stained with Oil Red O was measured using an Image Pro Plus analyzer (MediaCybernetics, Inc., Silver Spring, MD, USA).

 Constructs and luciferase reporter assay

pcDNA1 Stat5A expression vector, pcDNA1 Stat5B expression vector, and β-casein promoter linked to a luciferase reporter gene (pZZ1-Luc) were kindly
provided by Yamashita et al. (2003). Stat5A-Y694F was generated by substituting phenylalanine for tyrosine at 694 amino acid and subcloned into a pcDNA1 expression vector (Invitrogen Life Technologies). The sequence of the mutant cDNA was confirmed by DNA sequence analysis. GHR cDNA (pMet-IG-mGHR) was a kind gift from Dr John J Kopchick (Ohio University, Ohio). pCMX-PPARγ expression vector and PPARγ-binding element (PPRE)-luciferase construct (PPREx3-TK-Luc) were described previously (Kliewer et al. 1994). C/EBPβ cDNA and C/EBPδ cDNA were kindly provided by Dr Shizuo Akira (Tanaka et al. 1997). PPARγ promoter fused to a luciferase construct was described previously (Ichida et al. 2004). Transient transfection was carried out using FuGENE 6 (Roche Applied Science) following the manufacturer’s protocol. The total amount of DNA added to each well was equalized using an empty vector. Luciferase assay was performed in duplicate according to the protocol of the dual-luciferase reporter assay system (Promega). Briefly, 2 days after transfection, cells were lysed and luciferase activity was determined using specific substrates in a luminometer. Transfection efficiency was normalized by co-transfection with TK-Renilla luciferase construct (Promega).

**Generation of adenovirus**

Adenoviruses carrying Stat5A, Stat5B, Stat5A-Y694F, C/EBPβ, C/EBPδ and PPARγ were constructed using ViraPower Adenoviral Expression System (Invitrogen Life Technologies). Briefly, cDNA was inserted into TOPO pENTR vector and was recombined to the adenovirus expression plasmid pAd/CMV/V5-DEST. The pAd/CMV/V5-DEST plasmid with cDNA was digested with the PacI endonuclease and transfected with HEK293A cells. The medium supernatant containing adenovirus was collected and titrated with HEK293A cells. The medium supernatant digested with the PacI endonuclease and transfected with the pAd/CMV/V5-DEST plasmid and cDNA was recombined to the Life Technologies). Briefly, cDNA was inserted into ViraPower Adenoviral Expression System (Invitrogen Life Technologies). Briefly, cDNA was inserted into TOPO pENTR vector and was recombined to the adenovirus expression plasmid pAd/CMV/V5-DEST. The pAd/CMV/V5-DEST plasmid with cDNA was digested with the PacI endonuclease and transfected with HEK293A cells. The medium supernatant containing adenovirus was collected and titrated according to the manufacturer’s instructions. At 100% confluency, 3T3-L1 cells were infected with adenovirus at multiplicity of infection (MOI) of 500 with 0.5 confluency, 3T3-L1 cells were infected with adenovirus according to the manufacturer’s instructions. At 100% containing adenovirus was collected and titrated with HEK293A cells. The medium supernatant digested with the PacI endonuclease and transfected with the pAd/CMV/V5-DEST plasmid and cDNA was recombined to the Life Technologies). Briefly, cDNA was inserted into ViraPower Adenoviral Expression System (Invitrogen Life Technologies). Briefly, cDNA was inserted into TOPO pENTR vector and was recombined to the adenovirus expression plasmid pAd/CMV/V5-DEST. The pAd/CMV/V5-DEST plasmid with cDNA was digested with the PacI endonuclease and transfected with HEK293A cells. 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The medium supernatant containing adenovirus was collected and titrated according to the manufacturer’s instructions. At 100% confluency, 3T3-L1 cells were infected with adenovirus at multiplicity of infection (MOI) of 500 with 0.5 μg/ml poly-t-lysine (Sigma; Orlicky & Schaack 2001) in DMEM containing 2% FCS for 6 h. C3H10T1/2 cells were infected with adenovirus at MOI of 200 in DMEM containing 10% FCS.

**Western blot analysis**

To prepare whole cell lysates, cells were washed twice with ice-cold PBS and solubilized in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatinA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Lysates were centrifuged for 20 min at 4°C at 12,000 g and supernatants were collected. For cell fractionation, 3T3-L1 cells were washed twice with ice-cold PBS and solubilized in hypotonic lysis buffer (10 mM Heps (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μM dithiothreitol). Lysates were centrifuged at 12,000 g for 20 min and supernatants were used as cytosol extracts. The pellet was then lysed with hypertonic buffer (50 mM Heps (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM MgCl2, 2% glycerol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) and supernatants collected after centrifugation (12,000 g for 20 min) were used as nuclear extracts. The protein concentration was determined by the Lowry method using the DC reagent (Bio-Rad Laboratories). The same amount of samples was separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The amount of protein was checked by Ponceau S staining. Membranes were blocked in 5% BSA in Tris-buffered saline (TBS). Thereafter, the membranes were immunoblotted with anti-C/EBPβ (1:250), anti-C/EBPδ (1:250), anti-PPARγ (1:250), anti-Stat5 (1:250), anti-β-actin (1:2000), or anti-PCNA (1:5000), and developed with horseradish peroxidase-coupled anti-mouse IgG antibodies, followed by enhancement with ECL detection kits (Amersham Biosciences) or SuperSignal West Dura Extended Duration Substrate Antibodies (Pierce Chemical Co., Rockford, IL, USA). The protein bands were digitally imaged for densitometry using Scion software (Scion Corp., Frederick, MA, USA).

**RNA preparations and real-time reverse transcription-PCR (RT-PCR)**

Total RNA was prepared with RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions, and cDNA was generated using a random primer and reverse transcriptase (Superscript II, Invitrogen Life Technologies) according to the manufacturer’s instructions. Quantification of mRNA expression was carried out using a LightCycler amplification and detection system (a LightCycler-FastStart DNA Master SYBR Green I; Roche Diagnostics). GAPDH gene was used as an internal standard gene for quantification. PCR product of each gene was inserted into a pGEM T vector (Promega) and used as a standard dilution with a known number of copies. Conditions of amplification were: an initial step (94°C, 10 min) followed by 35 cycles consisting of a denaturation step (94°C, 15 s), primer annealing (55°C for PPARγ2, 57°C for glyceraldehyde-3 phosphate dehydrogenase (GAPDH), 5 s) and an extension step (72°C, 10 s). The products for PPARγ2 and GAPDH were detected at 85°C.
Specific primers for PPARγ2 (forward primer: 5′-GGTGAAACCTCTGGAGATTCC-3′; reverse primer: 5′-CAACCCATTGGTCTGCTTT-3′), GAPDH (forward primer: 5′-TGAACGGGAAGCTCTG-3′; reverse primer: 5′-TCCACCACCTGGTTCGTA-3′).

cDNA microarray analysis

The quality of extracted total RNA was confirmed with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). An IntelliGene II Mouse CHIP (Takara, Shiga, Japan), on which 4277 mouse genes were spotted, was utilized to perform the cDNA microarray analysis. Cy3- and Cy5-labeled cDNA were prepared with mRNA isolated from control cells and GH-treated cells. Both were mixed and hybridized to the CHIP. The hybridized CHIP was scanned using an Affymetrix 418 Array Scanner (Woburn, MA, USA) and ImaGene software (BioDiscovery, Los Angeles, CA, USA). The experiments were performed thrice independently. To confirm the data, RT-PCR was performed using Taq DNA Polymerase (Promega) and specific primers for interleukin-4 (IL-4) receptor (forward primer: 5′-CTGTTGGGCTTGTATTTCGTTG-3′; reverse primer: 5′-CTGGGTTCCTTGTAGGTCAC-3′), vascular endothelial growth factor (VEGF) A (forward primer: 5′-TGTGCAAGCTGCTGAACGA-3′; reverse primer: 5′-CGCTTGGCTTGTACATCTC-3′), TWIST (forward primer: 5′-ATGGACAGTCTAGAGACTCTG-3′; reverse primer: 5′-GCATTTCATGGTACTC-3′), and β-actin (forward primer: 5′-TGGAATGAGCTAAGGACTCTG-3′; reverse primer: 5′-AGAGGCATAACAGGACGACA-3′). β-Actin was used as an internal standard.

Statistical analysis

All data are expressed as the mean ± s.e.m. Results were analyzed for statistically significant differences using Student’s t test or ANOVA followed by Bonferroni multiple comparison post hoc test (Stat View software, SAS Institute, Cary, NC, USA). Statistical significance was set at P < 0.05.

Results

GH stimulates adipogenesis of 3T3-L1 cells

In order to investigate the effect of GH on adipogenesis, 3T3-L1 cells were treated with 50 ng/ml GH in the presence of 10% FCS as the first experimental condition. GH could not induce adipogenesis, indicating that GH by itself has little effect on the initiation of adipogenesis (Fig. 1A). Next, we treated 3T3-L1 cells with differentiation medium for 2 days, and 50 ng/ml GH were added to both differentiation and maintenance media to examine the effect of GH. Since GH is intrinsically contained in FCS, serum-free medium was utilized (Smith et al. 1988). On day 6, the area of Oil Red O staining did not show any significant difference between cells treated with or without GH (Fig. 1B). Treating cells with differentiation medium for 2 days is the most commonly used protocol for adipogenesis (Rubin et al. 1978). In this condition, most cells were strongly induced to differentiate into adipocytes even in serum-free medium (Fig. 1B). We speculated that such a potent induction protocol is likely to mask the effect of GH on adipogenesis. In order to detect the effect of GH, we modified the induction protocol by reducing the incubation time with differentiation medium. When cells were treated with differentiation medium for 3 h followed by incubation with maintenance medium, addition of GH to both differentiation and maintenance media showed the significant initiation of adipogenesis when compared with control (Fig. 1B and C). The effect of GH was in a dose-dependent manner and 50 ng/ml GH showed the maximum effect on adipogenesis (Fig. 1D). Although more than 50 ng/ml GH showed the trend of decrease in GH effect, there was no significant difference.

GH stimulates adipogenesis of 3T3-L1 cells through the early induction of PPARγ2 expression, not C/EBPβ and C/EBPδ

To examine whether GH has any effects on the expression of C/EBPβ, C/EBPδ and PPARγ, we determined the expression of these transcription factors under the culture condition in which GH shows its stimulatory effect on adipogenesis. Although both C/EBPβ and C/EBPδ were transiently induced after 3 h adipogenic induction, GH did not affect the expression of C/EBPβ and C/EBPδ (Fig. 2A). PPARγ has two isoforms which are generated by alternative splicing of mRNA (PPARγ1 and PPARγ2). Whereas PPARγ1 is expressed in several tissues including adipose tissue, PPARγ2 is specifically expressed in adipose tissue (Vidal-Puig et al. 1997). PPARγ1 was expressed in 3T3-L1 preadipocytes, and the expression of PPARγ1 increased gradually as adipogenesis proceeded (Fig. 2A). PPARγ2 was not expressed in 3T3-L1 preadipocytes, and the expression of PPARγ2 increased gradually as adipogenesis proceeded, and was induced earlier in cells treated with GH when compared with cells without GH (Fig. 2A and B). Quantitative RT-PCR analysis also showed a significant up-regulation of PPARγ2 mRNA expression in the cells treated with GH (Fig. 2C). To examine whether this enhanced PPARγ expression is due to increased synthesis or decreased degradation, 3T3-L1 cells were transfected with PPARγ expression vector and cultured with or without GH for 16 h. After incubation with GH, 10 μg/ml cycloheximide was added to inhibit
de novo protein synthesis, and whole cell lysates were collected at indicated periods. Western blot analysis of PPARγ showed no difference in the degradation rate between cells treated with or without GH (Fig. 2D). These findings suggest that GH exerts its effect through the up-regulation of PPARγ synthesis.

**GH facilitates the nuclear translocation of Stat5 during the early stage of adipogenesis**

To address whether Stat5A and 5B are implicated in GH-mediated adipogenesis, we first examined the intracellular localization of Stat5 by subcellular fractionation. Consistent with the previous report (Richter et al. 2003), localization of Stat5 in the nucleus significantly increased in cells treated with GH at 3, 6, and 12 h (Fig. 3A and B). Immunofluorescence experiments also showed nuclear localization of Stat5 as early as 1 h after GH stimulation (data not shown). As shown in Fig. 3B, in 3T3-L1 cells treated with GH, nuclear Stat5 expression increased at 3 h, significantly decreased thereafter and increased after 24 h. Similar to our data, Nanbu-Wakao et al. (2002) reported the tyrosine phosphorylation of Stat5 during adipogenesis using 3T3-L1 cells treated with standard 2-day MDI in the presence of 10% FCS. In contrast, in 3T3-L1 cells treated without GH, nuclear Stat5 expression increased after 24 h, indicating that Stat5 is activated independent of GH in this condition (Fig. 3B, open columns). Component of maintenance medium or growth factor(s) produced by 3T3-L1 cells during adipogenesis may be involved in Stat5 activation. Indeed, EGF, which is a component of maintenance medium, significantly increased the nuclear Stat5 localization under the same conditions. Since EGF can induce Stat5 tyrosine phosphorylation, the effect of EGF may be due to Stat5 phosphorylation, which could not be assessed in this study.
Figure 2 Effect of GH on the expression of C/EBPβ, C/EBPδ, and PPARγ. 3T3-L1 cells were induced to differentiate by 3-h incubation with differentiation medium followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. (A) Nuclear extracts were collected at the indicated periods and then subjected to western blot analysis of PPARγ, C/EBPβ, C/EBPδ and PCNA. Earlier induction of PPARγ2 expression was noted in the cells treated with GH. (B) The protein expression of PPARγ2 was quantified by densitometry from four independent experiments. Densities were normalized against the density of PCNA. (C) Quantitative RT-PCR analysis of mRNA expression of PPARγ2. Total RNA was extracted 3 h after adipogenic induction. Data were normalized by the expression of GAPDH. (D) Degradation of PPARγ. Whole cell lysates of 3T3-L1 cells transfected with PPARγ expression vector were subjected to western blot analysis of PPARγ and β-actin at the indicated periods after the addition of 10 μg/ml cycloheximide. No difference in degradation rate was noted between cells incubated with or without GH. LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein. Statistically significant differences were: *P < 0.05 and †P < 0.01.
medium, was reported to activate Stat5 (Ruff-Jamison et al. 1995). Combined together, nuclear Stat5 expression might be regulated by the combination of GH-dependent activation in the early stage of adipogenesis and GH-independent activation in the later stage of adipogenesis. Although the exact reason why levels of nuclear Stat5 fluctuate in this way in cells with GH and the outcome of this fluctuation are not clear, GH is thought to function at an early stage of adipogenesis. In conclusion, GH facilitates the nuclear translocation of Stat5 and the GH–Stat5 signaling pathway is possibly involved in the stimulatory effect of GH in the early stage of adipogenesis.

**Stat5A-Y694F suppresses the stimulatory effect of GH in MDI-induced adipogenesis, while Stat5A and 5B enhance MDI-induced adipogenesis**

In order to examine whether the adipogenic effect of GH is mediated through Stat5A and/or 5B, we used a point mutant of Stat5A (Stat5A-Y694F; Nanbu-Wakao et al. 2002). First, we examined the effect of Stat5A-Y694F on the transcriptional activity of Stat5A and 5B in 3T3-L1 cells by a luciferase reporter assay using a β-casein gene promoter luciferase construct containing a Stat5 response element. In the presence of GH, Stat5A and 5B showed 21- and 7-fold enhancement of transcriptional activity respectively. Co-expression of Stat15A-Y694F showed approximately 80 and 70% suppression of the transcriptional activity of Stat5A and 5B respectively, thus indicating that Stat5A-Y694F functions as a transcriptional suppressor for both the GH–Stat5A and the GH–Stat5B signaling pathways in 3T3-L1 cells (Fig. 4A).

Next, we explored the effect of overexpression of Stat5A-Y694F, Stat5A, and Stat5B in the adipogenic effect of GH using adenovirus gene transfer system. Two days after infection, the cells were induced to differentiate by 3-h incubation with differentiation medium followed by the incubation with maintenance medium, and GH was added to both differentiation and maintenance media for 3 h followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. (A) Lysates from cytosol extracts and nuclear extracts were prepared at the indicated periods and then subjected to western blot analysis of Stat5, β-actin (cytosol extracts), and PCNA (nuclear extracts). (B) The protein expression of nuclear Stat5 was quantified by densitometry from three independent experiments. Densities were normalized against the density of PCNA. Statistically significant differences were: *P<0.001, †P<0.01, and ‡P<0.05.
Figure 4 Involvement of the GH–Stat5A and the GH–Stat5B signaling pathway in MDI-induced adipogenesis. (A) Overexpression of Stat5A-Y694F suppressed the transcriptional activity of Stat5A and 5B. 3T3-L1 cells were transfected with pZZ1 (0.1 μg), TK-Renilla reporter plasmid (5 ng), GH receptor expression vector (0.2 μg), Stat5A or 5B expression vector (0.05 μg), and Stat5A-Y694F expression vector (0.2 μg). The cells were treated with or without 500 ng/ml GH in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining Renilla luciferase activity. Experiments were repeated independently five times. (B–E) Stat5A-Y694F suppressed the stimulatory effect of GH with the reduction of PPARγ2 expression, while Stat5A and 5B enhanced MDI-induced adipogenesis with the enhancement of PPARγ2 expression. 3T3-L1 cells were infected with LacZ, Stat5A, 5B, or Stat5A-Y694F adenovirus. After 2 days, the cells were induced to differentiate with differentiation medium for 3 h followed by incubation with maintenance medium, and 50 ng/ml GH were added to both differentiation and maintenance media. On day 6, the cells were stained with Oil Red O (B) and the stained area was measured (C). Whole cell lysates were collected 9 h after adipogenic induction and then subjected to western blot analysis of PPARγ, Stat5, and β-actin (D). The protein expression of PPARγ2 was quantified by densitometry from three independent experiments. Densities were normalized against the density of β-actin (E). Statistically significant differences were: *P<0.0001, †P<0.01, and ‡P<0.05.
maintained media. Overexpression of Stat5A-Y694F significantly suppresses the stimulatory effect of GH during MDI-induced adipogenesis with reduction of PPARγ expression (Fig. 4B–E), indicating the involvement of the GH–Stat5A/5B signaling pathway in GH-mediated adipogenesis with modulation of PPARγ expression. In addition, western blot analysis of PPARγ expression in the cells infected with Stat5A or 5B in a GH-dependent manner (Fig. 4D and E). Oil Red O staining at day 6 showed that overexpression of Stat5A or 5B stimulated MDI-induced adipogenesis, however, GH-dependent manner was not noted (Fig. 4B and C).

**Stat5A/5B stimulates C/EBPβ- and C/EBPδ-induced adipogenesis**

Since PPARγ is induced by C/EBPβ and C/EBPδ during adipogenesis, we examined the relationship between Stat5A/5B and C/EBPβ/δ. Overexpression of Stat5A or 5B in 3T3-L1 cells did not induce adipogenesis, indicating that Stat5A/5B by itself has little effect on the initiation of adipogenesis (Fig. 5A). When C/EBPβ or C/EBPδ was overexpressed in 3T3-L1 cells and adipogenic program was initiated, co-expression of Stat5A or 5B further enhanced C/EBPβ- and C/EBPδ-induced adipogenesis (Fig. 5A and B). Consistent with this result, Stat5A and 5B enhanced PPARγ2 expression in 3T3-L1 cells induced by C/EBPβ or C/EBPδ (Fig. 5C and D). These findings demonstrate that Stat5A/5B is involved in adipogenesis in cooperation with C/EBPβ/δ. To support this notion, we investigated the effect of Stat5A and 5B on the transcriptional activity of C/EBPβ/δ in the PPARγ gene promoter in 3T3-L1 cells. Overexpression of Stat5A and 5B showed 145±16 and 118±7% enhancement of C/EBPβ/δ transcriptional activity respectively (Fig. 5E).

**Stat5A/5B stimulates adipogenesis in cooperation with PPARγ**

Although Stat5A/5B enhanced PPARγ expression in C/EBPβ- and C/EBPδ-induced adipogenesis of 3T3-L1 cells, this does not exclude the possibility that Stat5A/5B has any effects on PPARγ activity. To examine this, we overexpressed PPARγ in 3T3-L1 cells. Overexpression of PPARγ induced adipogenesis, and co-expression of Stat5A or 5B stimulated PPARγ-induced adipogenesis (Fig. 6A–C). To further confirm whether Stat5A/5B have any effects on PPARγ activity, a luciferase reporter assay using PPARγ-binding element (PPRE)-luciferase construct was performed. The GH–Stat5A and the GH–Stat5B signaling pathways showed the trend of enhancement of the transcriptional activity of PPARγ in 3T3-L1 cells (Fig. 6D). However, due to the large variation in the data, there was no significant difference. We speculated that this outcome might be due to low transfection efficiency in 3T3-L1 cells. Therefore, to add further evidence, we investigated this effect using a multipotent mesenchymal cell line, C3H10T1/2, in which high transfection efficiency was achieved. In C3H10T1/2 cells, overexpression of Stat5A or 5B also stimulated PPARγ-induced adipogenesis and the GH–Stat5A and the GH–Stat5B signaling pathways significantly enhanced the transcriptional activity of PPARγ by 145±10 and 191±3% in the presence of TGZ respectively (Fig. 6A, B, and D). These findings indicate that Stat5A/5B functions by enhancing the transcriptional activity of PPARγ.

**cDNA microarray analysis**

Although Stat5A/5B has been reported to have an important role in adipose tissue formation, and our data suggest that GH is likely to exert its effect through Stat5A/5B, there are possibly other pathways through which GH exerts its effect on adipogenesis. In fact, some genes have been reported to be induced in response to GH during adipogenesis in 3T3-F442A cells (Gurland et al. 1990, Clarkson et al. 1995, 1999, Shang et al. 2002, Huo et al. 2006). To investigate the mechanisms by which GH stimulates adipogenesis, we analyzed the gene expression profiles of 3T3-L1 cells treated with GH during MDI-induced adipogenesis using cDNA microarray analysis. Adipogenesis was induced with the incubation of differentiation medium in the presence or absence of GH, and total RNA was extracted 3 h after adipogenic induction. As we intended to detect the genes that affect PPARγ expression, we set the point of RNA extraction as 3 h after induction when PPARγ2 expression was not detected by western blot analysis. Gene expression was compared between cells treated with or without GH. cDNA microarray analysis was performed thrice, and genes which showed an increment or decrement of more than 1·5-fold in the fluorescence level at least twice were selected as up- and down-regulated genes respectively. The number of up- and down-regulated genes was 18 and 19 among the 4277 spotted genes respectively (Tables 1 and 2). Among the genes associated with adipogenesis, PPARγ was up-regulated, supporting our findings that GH induces PPARγ expression during adipogenesis.

In addition, the expression of genes coding for growth factors or growth factor receptors was altered. Among them, mRNA levels of IL-4 receptor, VEGF-A, and TWIST were confirmed using semiquantitative RT-PCR analysis (Fig. 7). Since the expression of these genes was altered by MDI-treatment alone, GH is thought to function under the influence of MDI-treatment. So far, we have not been able to identify
Figure 5 Stimulation of C/EBPβ- or C/EBPδ-induced adipogenesis by Stat5A and 5B. 3T3-L1 cells were infected with LacZ, C/EBPβ or C/EBPδ adenovirus together with Stat5A or 5B adenovirus, and cultured for 6 days in DMEM containing 10% FCS. (A and B) The cells were stained with Oil Red O (A) and the stained area was measured (B). (C and D) Whole cell lysates were collected and subjected to western blot analysis of PPARγ, C/EBPβ, C/EBPδ, Stat5, and β-actin (C). The protein expression of PPARγ2 was quantified by densitometry from three independent experiments. Densities were normalized against the density of β-actin (D). (E) Enhancement of the transcriptional activity of C/EBPβ/δ in the PPARγ gene promoter by Stat5A and 5B. 3T3-L1 cells were transfected with PPARγ gene promoter luciferase construct (0.1 μg), TK-Renilla reporter plasmid (5 ng), GH receptor expression vector (0.2 μg), C/EBPβ expression vector (0.025 μg), C/EBPδ expression vector (0.025 μg), and Stat5A or 5B expression vector (0.05 μg). The cells were treated with 500 ng/ml GH in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining Renilla luciferase activity. Experiments were repeated independently seven times. Statistically significant differences were: *P<0.01 and †P<0.05.
Figure 6 Stat5A and 5B stimulate adipogenesis in association with PPARγ. (A–C) 3T3-L1 cells and C3H10T1/2 cells were infected with PPARγ adenovirus together with or without Stat5A or 5B adenovirus and cultured for 6 days in DMEM containing 10% FCS. The cells were stained with Oil Red O (A) and the stained area was measured (B). Whole cell lysates were collected and subjected to western blot analysis of PPARγ, STAT5, and β-actin (C). (D) Stat5A and 5B enhance the transcriptional activity of PPARγ. 3T3-L1 cells and C3H10T1/2 cells were transfected with 3×PPRE-Luc (0.1 μg), TK-Renilla reporter plasmid (5 ng), GH receptor expression vector (0.2 μg), Stat5A or 5B expression vector (0.05 μg), and PPARγ expression vector (0.05 μg). The cells were treated with either 500 ng/ml GH or 1 μM troglitazone (TGZ), or both in DMEM for 16 h. Luciferase activity was measured using cell extracts and normalized by determining Renilla luciferase activity. Experiments were repeated independently five times. Statistically significant differences were: *P < 0.0001, †P < 0.001, ‡P < 0.01, and §P < 0.05. ns, not significant.
the genes that are regulated by GH independent of MDI-treatment during GH-mediated adipogenesis.

**Discussion**

Consistent with the clinical features of GHD patients, several reports have suggested a stimulatory effect of GH on adipogenesis (Morikawa et al. 1984, Nixon & Green 1984). While GHD patients show a decreased number of adipocytes, they can form adipose tissue (Bonnet et al. 1974, Salomon et al. 1989, Bengtsson et al. 1993). These findings suggest that GH is not a major regulator, but an important modulator for adipogenesis. In this study, GH alone could not induce adipogenesis of 3T3-L1 cells. However, GH could stimulate adipogenesis when agents with adipogenic action, such as MDI, were added. These findings support the suggestion that GH stimulates adipogenesis by modulating the adipogenic program.

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of C/EBPβ in response to GH in 3T3-L1 cells during adipogenesis. Lysates from nuclear extracts were separated with SDS-PAGE and immunoblotted with C/EBPβ antibody, showing no band representing the faster migrating forms of C/EBPβ indicated in the previous reports (Liao et al. 1999, Piwien-Pilipuk et al. 2001; data not shown). Our data, together with previous studies, demonstrated that GH stimulates adipogenesis of 3T3-L1 cells through the enhancement of PPARγ expression, not C/EBPβ/δ expression (Tomitaka et al. 2002). However, the mechanisms by which GH enhances PPARγ expression remain to be determined.

GH mediates its action through various pathways, such as Stat, mitogen activated protein kinase (MAPK), and phosphatidylinositol 3 kinase (PI3K) (Piwien-Pilipuk et al. 2002). Among these signaling molecules, Stat5 was reported to be involved in adipose tissue formation (Teglund et al. 1998). Therefore, we examined the effect of Stat5A/5B on GH-mediated adipogenesis. To investigate the role of Stat5A/5B in the adipogenic effect of GH, Stat5A-Y694F was utilized. Overexpression of Stat5A-Y694F suppressed the stimulatory effect of GH in MDI-induced adipogenesis with the reduction of PPARγ2 expression, indicating that GH mediates its adipogenic effect through Stat5A/5B PPARγ. However, the stimulatory effect of GH was not completely diminished by overexpression of Stat5A-Y694F. This is probably because remaining endogeneous Stat5A/5B activity mediates some GH action and/or because factors other than Stat5A/5B may be involved in this stimulatory effect of GH. In addition, overexpression of Stat5A and 5B enhanced MDI-induced adipogenesis. Although PPARγ2 expression at an early stage of adipogenesis was enhanced in a GH-dependent manner, Oil Red O staining 6 days after induction showed no GH-dependent enhancement (Fig. 4C). This finding was partly explained by the Stat5 activation independent of GH over 24 h after adipogenic induction (Fig. 3A and B). Western blot analysis in Fig. 4D and E was performed 9 h after adipogenic induction, when the cells in the absence of GH showed no nuclear translocation of Stat5. In this condition, GH significantly induced the PPARγ2 expression. Therefore, the up-regulation of PPARγ2 in the cells with GH could be explained by the difference of nuclear Stat5 expression. While Oil Red Staining in Fig. 4B and C was performed 6 days after induction, Stat5 is translocated into nucleus even in the cells without GH. GH-independent activation of Stat5, especially in the culture system where Stat5 is overexpressed, may have been enough to stimulate MDI-induced adipogenesis even in the absence of GH.

Although Stat5A is reported to be necessary for GH-dependent adipogenesis in 3T3-F442A cells (Yarwood et al. 1999, Shang & Waters 2003), as far as we know, our study is the first report investigating the
adipogenic effect of the GH–Stat5A signaling pathway in 3T3-L1 cells. Nanbu-Wakao et al. (2002) reported that the overexpression of Stat5A stimulates adipogenesis and Stat5A-Y694F partially suppresses adipogenesis of 3T3-L1 cells, and Stewart et al. (2004) reported that Stat5 activator can replace the requirement of FCS in 3T3-L1 adipogenesis. However, the effect of GH on adipogenesis was not demonstrated in these reports. In addition to the adipogenic effect of the GH–Stat5A signaling pathway, we also revealed the adipogenic effect of the GH–Stat5B signaling pathway. As Floyd & Stephens (2003) reported that Stat5B cannot stimulate adipogenesis in both BALB/c and NIH-3T3 cells treated with MDI, this difference may come from the use of different cells.

Importantly, our findings also suggested the possibility that PPARγ mediates the adipogenic effect of the GH–Stat5A/5B signaling pathway. To elucidate the role of PPARγ in the adipogenic effect of the GH–Stat5A/B signaling pathway, we first investigated the relationship between Stat5A/5B and C/EBPβ/δ. In the present study, Stat5A and 5B stimulated C/EBPβ- and C/EBPδ-induced adipogenesis with the enhancement of PPARγ expression. Since Stat5A and 5B enhanced the transcriptional activity of C/EBPβ/δ in the PPARγ gene promoter, this suggests that Stat5A and 5B stimulate adipogenesis by inducing PPARγ expression in cooperation with C/EBPβ/δ. Next, we investigated the relationship between Stat5A/5B and PPARγ. Stat5A and 5B stimulated PPARγ-induced adipogenesis and enhanced the transcriptional activity of PPARγ, indicating that Stat5A and 5B stimulate adipogenesis by reinforcing the transcriptional activity of PPARγ. In contrast to our results, Stat5A and 5B have been reported to inhibit the transcriptional activity of PPARγ in COS1 and HEK293 cells (Richer et al. 2003, Shipley & Waxman 2004). These results were repeatable in our hands (data not shown). These findings indicate that the effect of Stat5A/5B on the transcriptional activity of PPARγ is dependent on the cell type and Stat5A/5B up-regulates the transcriptional activity of PPARγ in cell lines with adipogenic potential, including 3T3-L1 and C3H10T1/2 cells. Taken together, the GH–Stat5A/5B signaling pathway stimulates adipogenesis through two distinct steps; one is transcriptional activation of C/EBPβ/δ in the PPARγ gene promoter and the other is direct activation of PPARγ activity.

Although we mainly focused on the postreceptor events in this study, it is important to consider the role of GHR to completely understand the role of GH. GHR has the truncated splice variants, which inhibit full-length GHR in a dominant-negative manner (Ross et al. 1997). In 3T3-L1 cells, full-length GHR is expressed 100 times as much as the truncated form of GHR, and expression of full-length GHR increases as adipogenesis proceeds, while the truncated form does not (Iida et al. 2003). We performed RT-PCR analysis and obtained similar findings (data not shown). Considering the abundance of full-length GHR expression and the finding that expression level of full-length GHR increases in parallel with adipogenesis, dominant-negative effect of truncated variants of GHR has minimal effect of GH-mediated adipogenesis in 3T3-L1 cells. We have to consider the effect of GH-binding protein (GHBP), because expression of GHBP increases in parallel with adipogenesis (Zou et al. 1997). GHBP has been demonstrated to compete with GHR for ligand binding in the extracellular space in vitro (Lim et al. 1990); however, other aspects of GHBP function have been reported (Bick et al. 1994, Graichen et al. 2003). GHBP is also localized intracellularly, both attached to intracellular membranes and soluble in the cytoplasm and nucleoplasm (Lobie et al. 1992, Frick et al. 1994), and intracellular GHBP functions as a transcriptional enhancer in GH-stimulated Stat5-mediated transcription (Graichen et al. 2003). Therefore, we cannot exclude the possibility that GH stimulates adipogenesis partly through intracellular GHBP and further investigations are needed to clarify this.

Other genes have also been reported to be involved in the adipogenic effect of GH (Clarkson et al. 1999, Shang et al. 2002). IGF-I has been reported to stimulate adipogenesis of 3T3-L1 cells (Smith et al. 1988). As the production of IGF-I is up-regulated in response to GH in various cells (Pwien-Plipuk et al. 2002), there arises the possibility that GH exerts its adipogenic effect through production of IGF-I. To address this, we examined IGF-I expression by semiquantitative RT-PCR analysis. IGF-I expression could not be detected in 3T3-L1 preadipocytes, while it was detected in 3T3-L1 adipocytes (data not shown). When analyzed 3 h after adipogenic induction, IGF-I expression was not detected in cells treated with or without GH (data not shown). These results indicate the low possibility of IGF-I involvement in the stimulatory effect of GH in the early stage of adipogenesis.

Next, we analyzed the expression of genes at an early stage using cDNA microarray analysis (Tables 1 and 2). The expression of IL-4 receptor transiently increases during the early stage of adipogenesis of 3T3-L1 cells treated with MDI, and the expression pattern of IL-4 receptor was reported to be very similar to that of C/EBPβ (Hua et al. 2004). Our cDNA microarray analysis demonstrated that the expression of IL-4 receptor increased in cells treated with GH, suggesting the possibility that IL-4 signaling participates in the adipogenic effect of GH. Considering the expression pattern of IL-4 receptor, IL-4 signaling may be involved in clonal expansion. However, no proof of adipogenic effect of IL-4 signaling was reported (Hua et al. 2004).
VEGF-A expression was also up-regulated in cells treated with GH. Adipose tissue mass can be regulated by its vasculature (Rupnick et al. 2002). In addition, reciprocal interaction between adipogenesis and angiogenesis is crucial in the formation of adipose tissue, and VEGF-A was reported to be involved in this interaction (Fukumura et al. 2003). VEGF-A is expressed in adipocytes but not in preadipocytes both in vitro and in vivo (Soukas et al. 2001). As GH is important in the regulation of angiogenesis, it may induce adipogenesis through the promotion of angiogenesis. Other than VEGF-A, the expression levels of growth factors and their receptors were altered in response to GH. Although their contribution to adipogenesis was unclear, these results suggest that GH stimulates adipogenesis in association with cell growth modulation. In addition to growth factors, the expression of TWIST, a basic helix-loop-helix transcription factor, was down-regulated. The down-regulation of TWIST may have an impact on adipogenesis because TWIST is involved in osteogenesis and chondrogenesis (Bialek et al. 2004, Reinhold et al. 2006), and adipocytes, osteoblasts, and chondrocytes originate from the same precursor stem cells, mesenchymal stem cells.

In summary, our data demonstrate that GH stimulates adipogenesis under adipogenic induction through the enhancement of PPARγ expression, and both Stat5A and 5B are involved in the adipogenic effect of GH. In addition, we provide evidence that Stat5A and 5B function in association with C/EBPβ, C/EBPδ, and PPARγ resulting in the promotion of adipogenesis.

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