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ORIGINAL

Mechanism of repression of 11β-hydroxysteroid dehydrogenase type 1 by growth hormone in 3T3-L1 adipocytes

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Abstract. 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an NADPH-dependent reductase that converts cortisone to cortisol in adipose tissue. We previously reported that GH and IGF-I decrease 11β-HSD1 activity and mRNA levels in adipocytes. Hexose-6-phosphate dehydrogenase (H6PDH) is involved in the production of NADPH, which is a coenzyme for 11β-HSD1. The aim of the present study was to clarify further the mechanism of repression of 11β-HSD1 activity by GH using linsitinib, an IGF-I receptor inhibitor. The suppression of 11β-HSD1 mRNA by IGF-I was attenuated in the presence of 1 μM linsitinib (17.2% *vs.* 53.3% of basal level, $P<0.05$). 11β-HSD1 mRNA levels in cells treated with GH in the presence of 1 μM linsitinib were not different from those in absence of linsitinib (35.9% *vs.* 33.9%). The increase in IGF-I mRNA levels with GH and 1 μM linsitinib was not different from that in the absence of linsitinib (359% *vs.* 347%). H6PDH mRNA levels were significantly decreased in cells treated with IGF-I for 8 and 24 h (55.6% and 33.7%, $P<0.05$). In the presence of 1 μM linsitinib, there was no repression of H6PDH mRNA (111.4%). H6PDH mRNA levels were significantly decreased in cells treated with GH in the absence of linsitinib for 24 h (55.9%, $P<0.05$), but not for 8 h (89.5%). The presence of 1 μM linsitinib also prevented repression of H6PDH mRNA by GH over 24 h (107.8%). These results suggest that GH directly represses 11β-HSD1 mRNA rather than acting via the IGF-I receptor, and that GH represses H6PDH through locally produced IGF-I.

Key words: Growth hormone (GH), Insulin-like growth factor-I (IGF-I), 11β-hydroxysteroid dehydrogenase type 1 (11β−HSD1), Hexose-6-phosphate dehydrogenase (H6PDH), Adipocytes

11β-HYDROXYSTEROID DEHYDROGENASE TYPE 1 (11β-HSD1) is a reductase that converts cortisone to cortisol in the liver, adipose tissue and muscles [1]. In the endoplasmic reticulum, 11β-HSD1 co-localizes with hexose-6-phosphate dehydrogenase (H6PDH), an enzyme that maintains a high NADPH (nicotinamide adenine dinucleotide phosphate)/NADP ratio and permits the reductase activity (conversion from cortisone to cortisol) of 11β-HSD1 [2]. Thus, 11β-HSD1 activity is due to not only 11β-HSD1 itself but also H6PDH.

Studies of 11β-HSD1 overexpression in transgenic mice have revealed that activation of 11β-HSD1 increases cortisol action, resulting in metabolic disorders such as visceral adiposity, insulin resistance, and dyslipidemia, which are components of metabolic syndrome [3]. Similar metabolic disorders including visceral adiposity and insulin resistance are observed in patients with adult growth hormone (GH) deficiency, and GH replacement therapy ameliorates those abnormalities in these patients [4].

It is reported that urinary tetra-metabolite ratio of cortisol and cortisone reduced in adult GH deficiency (GHD) patients treated with GH [5]. On the contrary, decreased urinary tetra-metabolite ratios in patients with acromegaly restored after trans-sphenoidal surgery [6].

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Abbreviations: 11β-hydroxysteroid dehydrogenase type 1, 11β−HSD1; hexose-6-phosphate dehydrogenase, H6PDH; nicotinamide-adenine dinucleotide phosphate, NADPH; growth hormone, GH; messenger ribonucleic acid, mRNA; insulin-like growth factor, IGF; dimethyl sulfoxide, DMSO; bovine serum albumin, BSA; Dulbecco's modified Eagle's medium, DMEM; Hanks' balanced salt solution, HBSS; calf serum, CS; fetal bovine serum, FBS.

Recently, other clinical study has showed an involvement of GH in regulation of 11β-HSD1 [7]. Our previous study also indicated that 11β-HSD1 activity and messenger ribonucleic acid (mRNA) expression were suppressed by both GH and insulin-like growth factor (IGF)-I in 3T3-L1 adipocytes [8]. These findings suggest that lack of GH and IGF-I may cause activation of 11β-HSD1 in patients with adult GH deficiency, and induce metabolic disorders related to insulin resistance.

GH is an anabolic hormone that has many metabolic functions, and the biological effects of GH are mainly mediated through IGF-I. However, the biological activities of GH and IGF-I are not always the same, and GH has direct metabolic effects in bone and muscle [9, 10]. It has not been clarified whether GH regulates 11β-HSD1 activity directly or indirectly *via* locally produced IGF-I.

IGF-I acts through the IGF-I receptor, which activates AKT and ERK signaling pathways. Linsitinib is a tyrosine kinase inhibitor that inhibits autophosphorylation of the IGF-I receptor and AKT and ERK signaling [11]. In the present study, to clarify how GH regulates 11β-HSD1 activity, we investigated 11β-HSD1 and H6PDH mRNA levels in cells treated with GH and linsitinib.

Materials and Methods

Materials

3T3-L1 fibroblasts were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). GH was obtained from WAKO (Osaka, Japan), and IGF-I, insulin, dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Linsitinib was purchased from LC Laboratories (Woburn, MA, USA). Vatalanib dihydrochloride was purchased from Selleck chemicals LLC (Houston, TX, USA). Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical Co., Ltd. (Ibaraki, Japan). Calf serum (CS) and fetal bovine serum (FBS) were from PAA Laboratories GmbH (Pasching, Austria). Penicillin-Streptomycin (10000 units/mL) was obtained from Life Technologies (Carlsbad, CA, USA). Unless otherwise noted, all other chemicals and reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell culture

3T3-L1 fibroblasts were maintained in DMEM containing 10% CS and an antibiotic mixture (50 units/mL penicillin and 50 μg/mL streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. Two days after the fibroblasts had reached confluence, differentiation was induced by treating the cells with DMEM containing 25 mM glucose, 10% FBS, 1 μg/mL insulin, 1 mM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. After 4 days, the medium was replaced with DMEM containing 10% FBS, plus 1 μg/mL insulin for additional 4 days. Then the cells were incubated with DMEM containing 10% FBS for 1 day. At that time, more than 90% of the cells had morphologically differentiated into adipocytes. Prior to all experimental treatments, the cells were serum starved in DMEM containing 0.1% BSA for 24 h at 37 °C.

The differentiated 3T3-L1 adipocytes were treated with 100 nM GH or 10 nM IGF-I, or various concentrations of insulin for 24 h in the absence or presence of various concentrations of linsitinib. Basal samples (0 h) were used as a control.

To confirm the specificity of IGF-I receptor inhibition by linsitinib, vatalanib was used as other tyrosine kinase inhibitor.

Measurement of 11β-HSD1, H6PDH, and IGF-I mRNA

Total RNA was extracted and purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA was quantified by measuring absorbency at 260 nm with reference wavelength 280 nm. A total of 0.2 μg RNA was transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit and random primers (Applied Biosystems, Foster City, CA). The expression of 11β-HSD1, H6PDH and IGF-I mRNA was measured by real-time PCR analysis. A total of 2.5 μL cDNA was amplified with 1x Taqman Universal PCR Master Mix, 250 nM Taqman probe, and 450 nM of each primer (Applied Biosystems, Foster City, CA) by real-time quantitative PCR using a 7500 Real-Time PCR System (Applied Biosystems). Briefly, after incubation at 50 °C for 2 min and then at 95 °C for 10 min, 40 cycles of PCR (95 °C for 15 sec and 60 °C for 1 min) were performed. The TaqMan probe and primers for 11β-HSD1 (assay identification number Mm00476182 1.5 μL), hexose-6-phosphate dehydrogenase (H6PDH) (assay identification number Mm00557617_m1 1.5 μL), and IGF-I (assay identification number Mm00439560_

m1 1.5 μL) were assay-on-demand gene expression products (Applied Biosystems). The standard curve was generated by amplifying serial dilutions of cDNA from cell samples studied. 18S rRNA was used as the internal reference to normalize specific mRNA levels.

Statistical analysis

Results are expressed as means ± SEM. For comparisons, data were analyzed using a two-way or three-way ANOVA followed by Fisher's PSLD post-hoc test. Differences were considered to be statistically significant at $P<0.05$. Calculations were performed using Stat-View ver. 5.0 (SAS Institute Inc., Cary, NC).

Results

Effects of GH, IGF-I and insulin on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes

11β-HSD1 mRNA levels were low in preadipocytes and increased throughout the process of adipogenesis (100 ± 9% to 3471 ± 729% mRNA levels on the tenth day after induction of differentiation, $P<0.05$). Therefore, the cells on the tenth day after induction of differentiation were considered fully differentiated adipocytes and used in this study.

Cells were treated with GH (100 nM) or IGF-I (10 nM) for 8 and 24 h and levels of 11β-HSD1 mRNA were measured (Fig. 1). 11β-HSD1 mRNA levels decreased significantly after 24 h of exposure to GH or IGF-I to 23.0 ± 1.8% and 21.9 ± 1.2% of the basal median levels, respectively ($P<0.05$).

Adipocytes were treated with various concentrations of insulin for 24 h and levels of 11β-HSD1 mRNA were measured (Fig. 2). The levels of 11β-HSD1 mRNA were not changed by insulin treatment.

Effects of IGF-I on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes treated with linsitinib or vatalanib

Adipocytes were treated with IGF-I (10 nM) and linsitinib for 24 h and then levels of 11β-HSD1 mRNA were measured (Fig. 3). 11β-HSD1 mRNA levels with 0.1 and 1 μM linsitinib were 22.9 ± 2.0% and 53.3 ± 2.8% of basal levels, respectively. 11β-HSD1 mRNA levels with 1 μM linsitinib were significantly higher than those in the absence of linsitinib (17.2 ± 1.3%, $P<0.05$).

On the other hand, 11β-HSD1 mRNA levels with IGF-I and 0.1 or 1 μM vatalanib treatment were 28.4 ± 3.5 and 37.2 ± 6.1% of basal levels, respectively. The

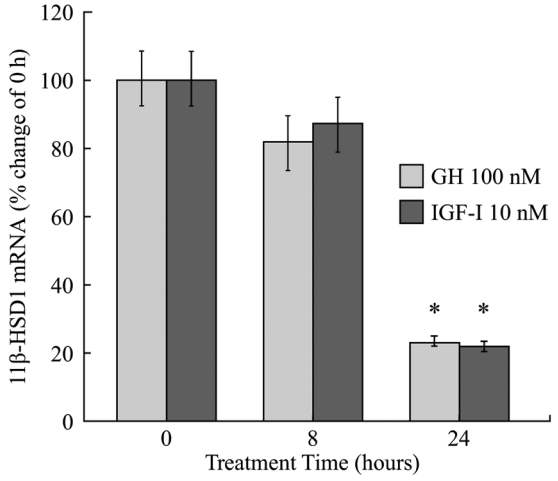

Fig. 1 Effects of GH and IGF-I on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with GH (100 nM) or IGF-I (10 nM) for 8 and 24 h. Quantitative real-time PCR was performed and levels of 11β-HSD1 mRNA were normalized to those for 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=4. *, $P<0.05$ compared with untreated controls (0 h).

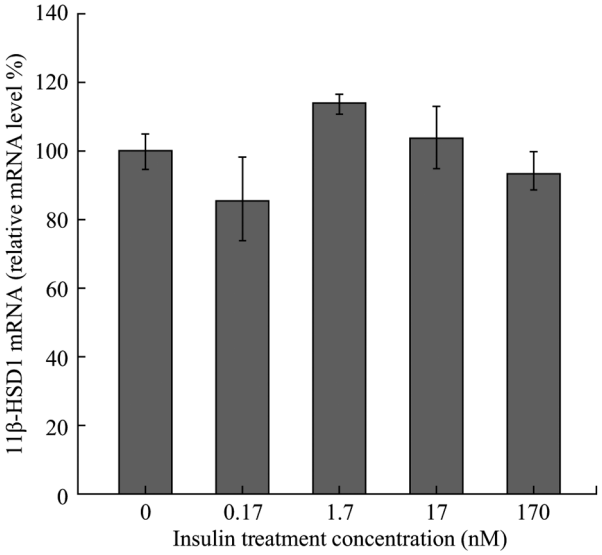

Fig. 2 Effects of insulin on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes
Fully differentiated 3T3-L1 adipocytes were treated with various concentrations of insulin for 24 h. Quantitative real-time PCR was performed and levels of 11β-HSD1 mRNA were normalized to those for 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 nM as 100%. Values are means ± SEM. n=4. *, $P<0.05$ compared with untreated controls (0 nM).

levels were not significantly different from those in the absence of vatalanib (25.7 ± 6.5%, Fig. 4A).

Effects of GH on 11β-HSD1 and IGF-I mRNA levels in 3T3-L1 adipocytes treated with linsitinib

Adipocytes were treated with GH (100 nM) and linsitinib for 24 h and then levels of 11β-HSD1mRNA were measured (Fig. 5A). 11β-HSD1 mRNA levels with 0.1 and 1 μM linsitinib treatment were 38.7 ± 2.7 and 35.9 ± 3.4% of basal levels, respectively. The levels were not significantly different from those in the absence of linsitinib (33.9 ± 1.9%). Furthermore, 11β-HSD1 mRNA levels with GH and 0.1 or 1 μM vatalanib treatment were 19.1 ± 1.7 and 25.9± 1.7% of basal levels, respectively. The levels were not significantly different from those in the absence of vatalanib (21.4 ± 2.3%, Fig. 4B).

IGF-I mRNA levels in cells treated with GH for 8 and 24 h were significantly increased (672 ± 71%, 357 ± 34% of basal levels, respectively, $P<0.05$).

IGF-I mRNA levels increased with GH in the presence of 0.1 and 1 μM linsitinib (303 ± 29%, and 359 ± 29% of basal levels, respectively). The levels were not significantly different from those in the absence of linsitinib (347 ± 31%, Fig. 5B).

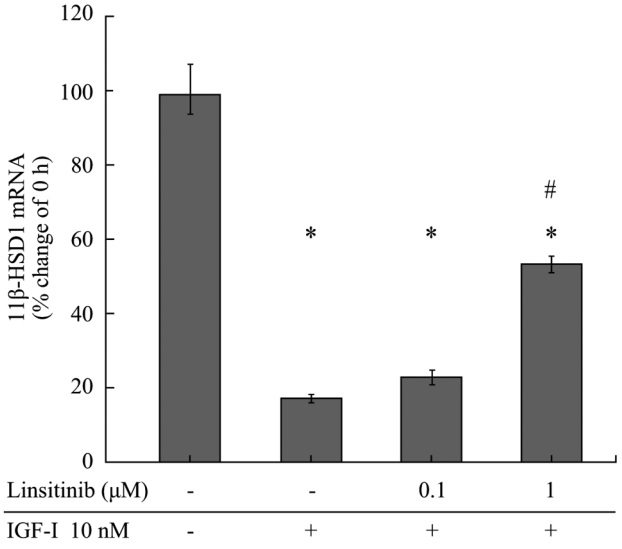

Fig. 3 Effects of IGF-I on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes treated with linsitinib
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with IGF-I (10 nM) for 24 h with DMSO (0.2%) or 0.1 or 1 μM linsitinib. Quantitative real-time PCR was performed and levels of 11β-HSD1 mRNA were normalized to those of 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=5. *, $P<0.05$ compared with untreated controls (0 h). #, $P<0.05$ compared with IGF-I (10 nM) without linsitinib.

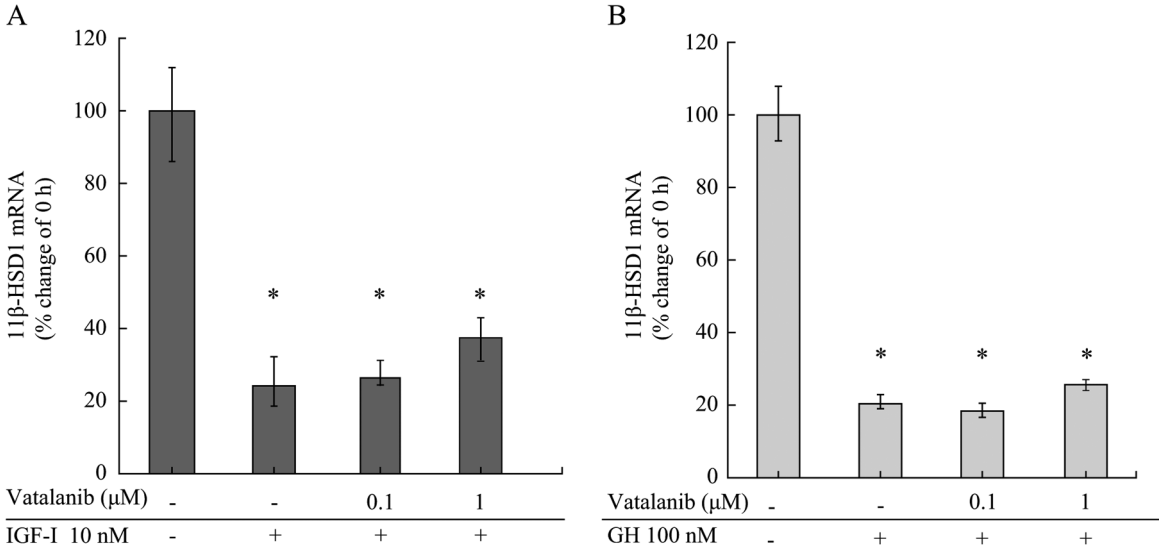

Fig. 4 Effects of IGF-I and GH on 11β-HSD1 mRNA levels in 3T3-L1 adipocytes treated with vatalanib
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with IGF-I (10 nM) (A) and GH (100 nM) (B) for 24 h with DMSO (0.2%) or 0.1 or 1 μM vatalanib. Quantitative real-time PCR was performed and levels of 11β-HSD1 mRNA were normalized to those of 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=4. *, $P<0.05$ compared with untreated controls (0 h).

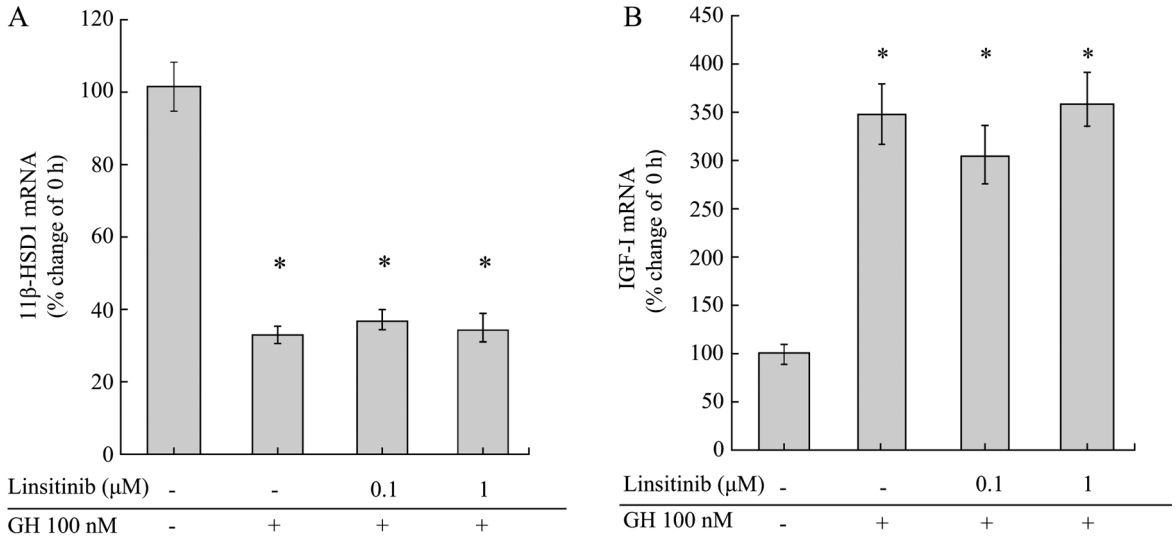

Fig. 5 Effects of GH on 11β-HSD1 and IGF-I mRNA levels in 3T3-L1 adipocytes treated with linsitinib
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with GH (100 nM) for 24 h with DMSO (0.2%) or 0.1 or 1 μM linsitinib. Quantitative real-time PCR was performed and levels of 11β-HSD1 (A) and IGF-I (B) mRNA were normalized to those of 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=5. *, $P<0.05$ compared with untreated controls (0 h).

Effects of GH and IGF-I on H6PDH mRNA levels in 3T3-L1 adipocytes

Adipocytes were treated with GH (100 nM) or IGF-I (10 nM) for 8 and 24 h in the presence or absence of linsitinib.

H6PDH mRNA levels in cells treated with IGF-I in the absence of linsitinib for 8 and 24 h were significantly decreased (55.6 ± 5.4% and 33.7 ± 3.9% of basal levels, respectively, $P<0.05$, Fig. 6). In the presence of 1 μM linsitinib, H6PDH mRNA was 111.4 ± 6.0% of basal levels, and this value was significantly higher than in the absence of linsitinib (29.2 ± 2.5%, $P<0.05$, Fig. 7A).

H6PDH mRNA levels in cells treated with GH in the absence of linsitinib for 24 h were significantly decreased (55.9 ± 3.5% of basal levels, $P<0.05$, Fig. 6), but not after 8 h (89.5 ± 12.0%). At 24 h, suppression of H6PDH mRNA levels by GH was significantly weaker than that by IGF-I. In the presence of 1 μM linsitinib, H6PDH mRNA levels were 107.8 ± 11.0% and this value was significantly higher than in the absence of linsitinib (61.5 ± 3.3%, $P<0.05$, Fig. 7B).

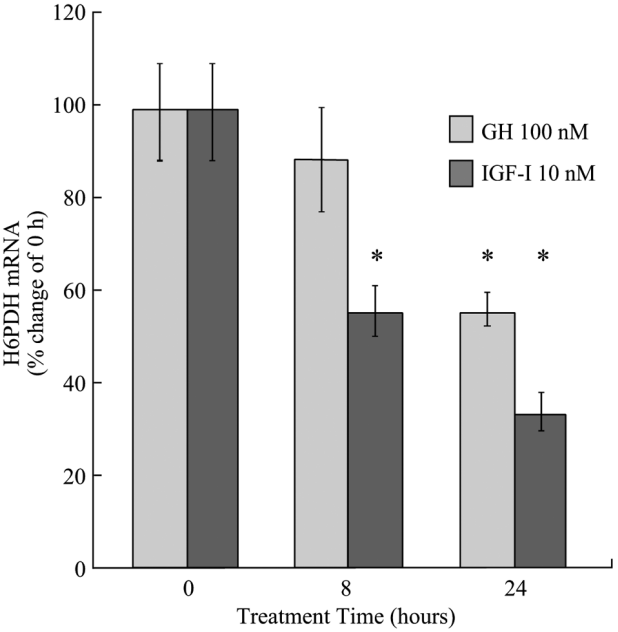

Fig. 6 Effects of GH and IGF-I on H6PDH mRNA levels in 3T3-L1 adipocytes
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with GH (100 nM) or IGF-I (10 nM) for 8 and 24 h. Quantitative real-time PCR was performed and levels of H6PDH mRNA were normalized to those of 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=4. *, $P<0.05$ compared with untreated controls (0 h).

Discussion

We previously reported that GH and IGF-I repress 11β-HSD1 activity and expression of 11β-HSD1

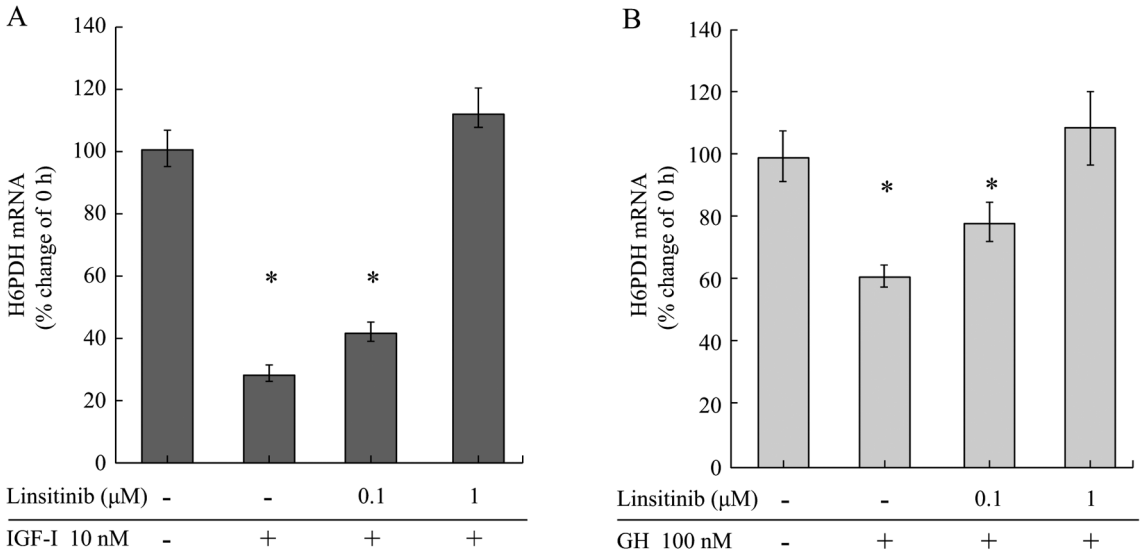

Fig. 7 Effects of IGF-I and GH on H6PDH mRNA levels in 3T3-L1 adipocytes treated with linsitinib
3T3-L1 adipocytes that had undergone induction of adipogenesis for 10 days were treated with IGF-I (10 nM) (A) and GH (100 nM) (B) for 24 h with DMSO (0.2%) or 0.1 or 1 μM linsitinib. Quantitative real-time PCR was performed and levels of H6PDH mRNA were normalized to those of 18S rRNA. The results are shown as relative amounts, taking the mean of the group at 0 h as 100%. Values are means ± SEM. n=5. *, $P<0.05$ compared with untreated controls (0 h).

mRNA [8]. In this study, to further elucidate the mechanisms of 11β-HSD1 regulation by GH, we investigated whether GH regulates 11β-HSD1 activity directly or indirectly *via* locally produced IGF-I using the IGF-I receptor inhibitor linsitinib.

First, we confirmed that 11β-HSD1 mRNA levels increased throughout the process of adipogenesis. Due to these increased levels, we decided to use fully differentiated adipocytes on the tenth day after induction of differentiation for our experiments. Levels of 11β-HSD1 mRNA were decreased by GH and IGF-I in these fully differentiated adipocytes as we previously reported [8]. The repression of 11β-HSD1 mRNA by IGF-I treatment was partially restored by linsitinib. The possible cause of partially but not fully restorization might be due to the dose of linsitinib, but is not clarified. In contrast, GH decreased 11β-HSD1 mRNA levels regardless of IGF-I receptor inhibition by linsitinib. These data suggested that GH directly represses 11β-HSD1 mRNA independent of IGF-I action.

Levels of H6PDH mRNA, which is involved in 11β-HSD1 activity, were repressed by GH and IGF-I after 24 h. The repression of H6PDH mRNA was restored by either GH or IGF-I treatment in the presence of linsitinib. GH increased IGF-I mRNA levels independent of linsitinib treatment. Repression of H6PDH after treatment with GH was not observed at 8 h, and repression by GH was weaker than that by IGF-I at 24 h. These data suggest that GH represses H6PDH through locally produced IGF-I. Although we did not measure IGF-I levels in a culture medium, GH stimulated IGF-I mRNA levels. Furthermore, in our preliminary study, the value of H6PDH by Western immunoblotting (WIB) was decreased after 24 h by GH or IGF-I (74.0%, 66.8% of basal levels, respectively). In this study, we did not investigate 11β-HSD1 activity, however, the repression of H6PDH by locally produced IGF-I might play some role in the repression of 11β-HSD1 activity by GH *in vivo*.

Linsitinib inhibits autophosphorylation of insulin receptor as well as IGF-I receptor. It might be possible that IGF-I repress 11β-HSD1 mRNA *via* insulin receptor. However insulin did not repress 11β-HSD1 mRNA in this system, suggesting that linsitinib might attenuate repression of 11β-HSD1 by IGF-I *via* IGF-I receptor. We have investigated other tyrosine kinase such as vatalanib which did not related to IGF-I receptor pathway. Vatalanib did not attenuate repression of 11β-HSD1 by IGF-I in adipocytes.

The regulation of 11β-HSD1 differs among species or tissues [1], although many parts of 11β-HSD1 regulation have not been clear yet. In rodent adipose tissue,

some studies suggested that glucocorticoids upregulate [12] and PPARγ downregulates [13] on 11β-HSD1 and a main factor directly regulating 11β-HSD1 transcription in adipose tissues is C/EBPβ [14]. On the other hand, H6PDH catalyzes the initial step of pentose phosphate pathway within a lumen of the endoplasmic reticulum. Fan *et al*. indicated that glucose is one of the regulators of H6PDH [15]. In the present study, as we used glucose free-medium before stimulating cells, we could ignore the effects of glucose. Our study suggested that IGF-I could one of regulators of H6PDH in an autocrine/paracrine manner.

We found that GH regulates 11β-HSD1 mRNA directly, and H6PDH mRNA *via* locally produced IGF-I. However this study did not clear the repression of 11β-HSD1 activity and how much related to GH and IGF-I. It is very interesting to know the pathophysiological role in the different mechanisms of regulation of 11β-HSD1 and H6PDH mRNA by GH, and further study will be required.

In this study, we measured mRNA levels but not activity and protein levels of 11β-HSD1 and H6PDH. To clarify the regulation of these activities by GH or IGF-I, further studies will be required.

In summary, GH directly repressed 11β-HSD1 mRNA and repressed H6PDH *via* locally produced IGF-I (Fig. 8).

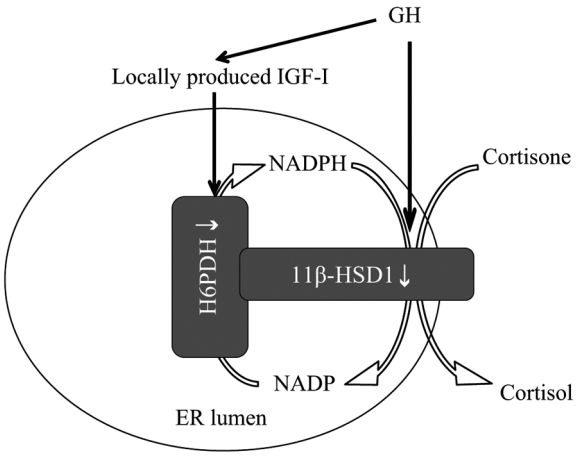

Fig. 8 A diagram of the regulation of 11β-HSD1 activity by GH 11β-HSD1 is a reductase that converts cortisone to cortisol in the endoplasmic reticulum. H6PDH supply NADPH which is coenzyme for 11β-HSD1. GH and IGF-I repress 11β-HSD1 activity [8].

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Disclosures

None of the authors have any potential conflicts of interest associated with this research.

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