

Growth Hormone Increases mRNA Levels of PPAR δ and Foxo1 in Skeletal Muscle of Growth Hormone Deficient *lit/lit* Mice

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Abstract. GH plays an important role in lipid metabolism as a partitioning hormone. PPAR δ regulates lipid oxidation in skeletal muscle and is activated by several physiological ligands including fatty acids. To investigate whether GH has an effect on the regulation of transcription of PPAR δ and other genes involved in energy metabolism in skeletal muscle, mRNA levels were studied by real-time RT-PCR in *lit/lit* mice (isolated GH deficiency) and *lit/+* mice controls (normal GH levels). Mice received either a single bolus (120 ng/g) of rat GH or vehicle, and skeletal muscle was collected 4h later. PPAR δ mRNA was increased in vehicle-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (1.67 fold, $P<0.05$). *lit/lit* mice treated with GH showed a further increase in PPAR δ mRNA levels (2.83 fold vs. vehicle-treated *lit/+* mice, $P<0.001$). mRNA levels of Foxo1 were increased in vehicle-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (1.74 fold, $P<0.05$). *lit/lit* mice treated with GH showed a further increase in Foxo1 mRNA levels (6.30 fold vs. vehicle-treated *lit/+* mice, $P<0.001$). mRNA levels of acyl CoA-oxidase showed a trend to be higher in vehicle-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice. This reached statistical significance in GH-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (2.11 fold, $P<0.05$). In summary, mRNA levels of PPAR δ and Foxo1 were increased in skeletal muscle of GH-deficient mice, and further acutely increased by GH administration. These results suggest that GH plays a relevant role in the lipid catabolism in skeletal muscle.

Key words: Growth hormone, Foxo1, PPAR-delta, Skeletal muscle

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SKELETAL muscle is a very active tissue in catabolism and utilization of fatty acids in response to physiological situations such as fasting and physical exercise. Growth hormone (GH) has marked effects on energy metabolism in skeletal muscle, inducing lipid oxidation through the direct stimulation of mitochondrial fatty acid oxidation [1]. GH administration in obese mice stimulated mRNA expression of UCP-1 and UCP-3 in the muscle [2]. Thus, GH action is strongly associated with regulation of genes involved in fatty acid oxidation.

In this study we analyzed the mRNA expression of the peroxisome proliferator-activated receptor alpha and delta (PPAR α and δ) and these downstream oxidative enzyme, acyl CoA-oxidase (ACO) in skeletal muscle in GH deficient *lit/lit* mice. PPAR δ has an important role in lipid oxidation in skeletal muscle and is activated by several physiological ligands including long chain fatty acids, prostacyclin, and retinoic acid [3–5]. Administration of PPAR δ agonist induces fatty acid β -oxidation in skeletal muscle in mice [6]. Moreover, data from transgenic mouse model allowing PPAR δ -overexpressing muscles demonstrate that PPAR δ activation enhances fatty acid catabolism by changing the myofiber composition towards a more oxidative phenotype as well as by upregulation of long chain fatty acid catabolism [7].

Another member of peroxisome proliferator-

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activated receptor concerning lipid oxidation, PPAR α transcriptionally activates the genes involved in the oxidation of fatty acid which include mitochondrial and peroxisomal β -oxidation systems and microsomal ω -oxidation system [8, 9]. It is expressed predominantly in liver and, to a lesser extent, in heart and muscle. While physiological stimuli such as fasting and physical exercise enhance fatty acid catabolism and utilization, PPAR α is not so required for such physiological adaptations of muscle metabolism, as ablation of PPAR α does not affect fatty acid oxidative capacity of mouse muscle [6]. In skeletal muscle of PPAR α knockout mouse, PPAR δ is highly expressed and can compensate for the lack of PPAR α [6].

Foxo1 is a member of the family of forkhead transcription factors that have a highly conserved DNA-binding region, termed winged helix/forkhead domain. Foxo1 has an important role in the regulation of energy metabolism in liver, muscle, and adipose tissue, where its function is inhibited by insulin and insulin-like growth factor 1 (IGF-1) [10, 11]. During starvation, Foxo1 up-regulates pyruvate dehydrogenase kinase 4 (PDK4) [12] which phosphorylates and reduces the activity of pyruvate dehydrogenase. The enhanced PDK4 activity induces inhibition of glucose oxidation in muscle sparing it for glucose-dependent tissues including brain [13]. Foxo1 stimulates fatty acid uptake and oxidation in muscle cells. PPAR α is also known to up-regulate PDK4 gene expression in liver and kidney during starvation [14].

In the present study, we used mouse model with chronic obesity and decreased GH signaling due to genetic alteration. In this murine model, we have determined the expression levels of genes involved in fatty acid oxidation in skeletal muscle. Additionally, GH was administered to GH deficient mice to evaluate the acute effects of GH on the mRNA expression of these genes.

Materials and Methods

Animals and tissue collection

Male 3 month old mice were used in these experiments. The C57BL/6J-Ghrhr *lit/lit* mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The *lit/lit* mouse, which has an inactivating mutation at the GHRH receptor, is an excellent model

of isolated GH deficiency with the expected dwarf phenotype and decreased circulating GH concentrations [15, 16]. In the present study, homozygous *lit/lit* mice were used for the model of isolated GH deficiency without other pituitary hormone deficiencies and heterozygous *lit/+* mice were used as controls having normal GH action. The experimental *lit/lit* and *lit/+* mice were maintained under standardized conditions of temperature ($21 \pm 2^\circ\text{C}$), humidity (30–65%), and with lights on between 0600 and 2000. The mice had free access to a 5K52 diet (crude fat not less than 6.0%, LabDiet, St. Louis, MO) and water. To investigate the acute effects of GH administration, *lit/lit* mice and *lit/+* mice were injected with 120 ng/g BW recombinant rat GH (rrGH; gift from Genentech Inc., South San Francisco, CA) sc in a total of 100 μL of 0.9% NaCl, or vehicle (100 μL of 0.9% NaCl) once at 0800–1000 ($n = 5$ –6 per group). Skeletal muscle (quadriceps) was harvested 4 h after GH injection, frozen immediately in liquid nitrogen and stored at -80°C .

All animal procedures were approved by the University of Virginia Institutional Animal Care and Use Committees.

Serum insulin measurement

Blood was drawn by cardiac puncture when the animals were sacrificed. Serum was centrifuged at 4°C and stored at -80°C . Serum insulin concentration was assayed by radioimmunoassay [17] in duplicate and the effective range of the value was 1.24–40 ng/ml. Insulin assay was performed in the Immunoassay Core Facility of the University of Virginia Diabetes and Endocrinology Research Center.

Quantitation of mRNA expression

Total muscle RNA was prepared using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH), treated with DNase I (Qiagen, Valencia, CA), and quantified using RiboGreen[®] RNA quantitation kit (Molecular Probes, Eugene, OR). Primers were designed using Primer3 software [18] or were published previously [6, 19, 20]. The PCR primers used were as follows: IGF-I, forward, 5'-gtgtggaccgaggggcttttacttc-3', reverse, 5'-gcttcagtggggcacagtacatctc-3'; Foxo1, forward, 5'-aaccagctcaaatgctagtaccatc-3', reverse, 5'-cagaaggttctccatgttttctgga-3'; PPAR δ , forward, 5'-gctgctgcagaagatggca-3', reverse, 5'-cactgca

tcattctgggcatg-3'; PPAR α , forward, 5'-acgatgctgtcctcttgatg-3', reverse, 5'-gtgtgataaagccattgccgt-3'; acyl CoA-oxidase, forward, 5'-gaactccagataattggcaccta-3', reverse, 5'-agtgtttccaagcctcgaa-3'; SREBP-1c, forward, 5'-atcggcgcggaagctgtcgggtagcgtc-3', reverse, 5'-actgtcttggttgatgatgagctggagcat-3'; 18S ribosomal RNA primers were purchased from Ambion (Austin, TX).

To measure mRNA levels, two step RT-PCR was performed. Five hundred nanograms of total muscle RNA were reverse transcribed in a total volume of 10 μ L using the iScript cDNA Synthesis Kit (Bio Rad). For cDNA measurement 2X QuantiTect master mix from QuantiTect SYBR[®] Green RT-PCR kit (Qiagen), primers, and 5 μ L of the diluted cDNA (1 : 20) were run for 40 cycles in a total volume of 20 μ L (denaturation: 15 sec at 94°C, annealing: 40 sec at 62°C and extension: 45 sec at 72°C), using the iCycler iQ Real-Time PCR detection system instrument and software (Bio-Rad, Hercules, CA). All results were standardized against the expression of 18S ribosomal RNA, which was analyzed simultaneously. Data were obtained as C_T values (cyclic threshold—the cycle number at which logarithmic PCR plots cross a calculated threshold line). The ΔC_T was calculated by subtracting the C_T for 18S from the C_T for target genes. The relative mRNA abundance was calculated using the $2^{-\Delta C_T}$ formula and reported as fold difference.

Statistical Analysis

Data are the means \pm SE (n = 5–6 per group). Groups were compared by Student's *t*-test. The values are presented as a relative ratio of control values.

Results

Body weights and serum levels of insulin after GH administration

The homozygous *lit/lit* mice were, as previously described [21], obese and smaller than heterozygous *lit/+* mice. *lit/+* mice weighed 25.2 g (SD 3.2), whereas *lit/lit* mice weighed 13.6 g (SD 1.2).

The results of serum insulin levels at baseline in these experimental rats were reported before. Serum insulin levels were markedly decreased in *lit/lit* mice compared to *lit/+* mice. The values for *lit/lit* mice

were below the sensitivity levels of the assay (<1.24 ng/ml), whereas the mean concentration in *lit/+* mice was 2.55 ng/ml ($P < 0.01$) [22]. At 4 h after GH administration, insulin levels in *lit/+* mice were not different from those in vehicle-treated *lit/+* mice, and those in *lit/lit* mice were still below the limit of detection.

IGF-I mRNA levels in skeletal muscle

IGF-I mRNA levels in muscle were decreased in *lit/lit* mice compared with those in *lit/+* mice (45% of *lit/+* mice, $P < 0.05$). 4 h after GH administration, IGF-I expression increased to 2.7 fold ($P < 0.01$) in *lit/+* mice, and there was a trend to increase in IGF-I mRNA levels in *lit/lit* mice ($P = 0.06$) (Table 1).

Effect of GH on PPAR δ PPAR α , acyl CoA-oxidase (ACO), Foxo1 and SREBP-1c gene expression

PPAR δ mRNA was increased in vehicle-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (1.67 fold, $P < 0.05$). *lit/lit* mice treated with GH showed a further increase in PPAR δ mRNA levels (2.83 fold vs. vehicle-treated *lit/+* mice, $P < 0.001$; 1.69 fold vs. vehicle-treated *lit/lit* mice, $P < 0.05$) (Fig. 1A). mRNA levels of PPAR α showed no difference between *lit/lit* and *lit/+* mice, and no changes after GH treatment in *lit/lit* mice in skeletal muscle (Fig. 1B).

mRNA levels of ACO showed a trend to be higher in vehicle-treated *lit/lit* compared to vehicle-treated *lit/+* mice. This reached statistical significance in GH-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (2.11 fold, $P < 0.05$) (Fig. 2A).

mRNA levels of Foxo1 were increased in vehicle-treated *lit/lit* mice compared to vehicle-treated *lit/+* mice (1.74 fold, $P < 0.05$). *lit/lit* mice treated with GH

Table 1. IGF-I expression increased to 2.7 fold in *lit/+* mice, and there was a trend to increase in IGF-I mRNA levels in *lit/lit* mice 4 h after GH administration.

Mice		IGF-I mRNA in muscle (fold of control)
<i>lit/+</i>	Vehicle treated	1.0
	GH treated	2.7**
<i>lit/lit</i>	Vehicle treated	0.45*
	GH treated	1.40

* $P < 0.05$, ** $P < 0.01$, vs. vehicle treated *lit/+* mice.

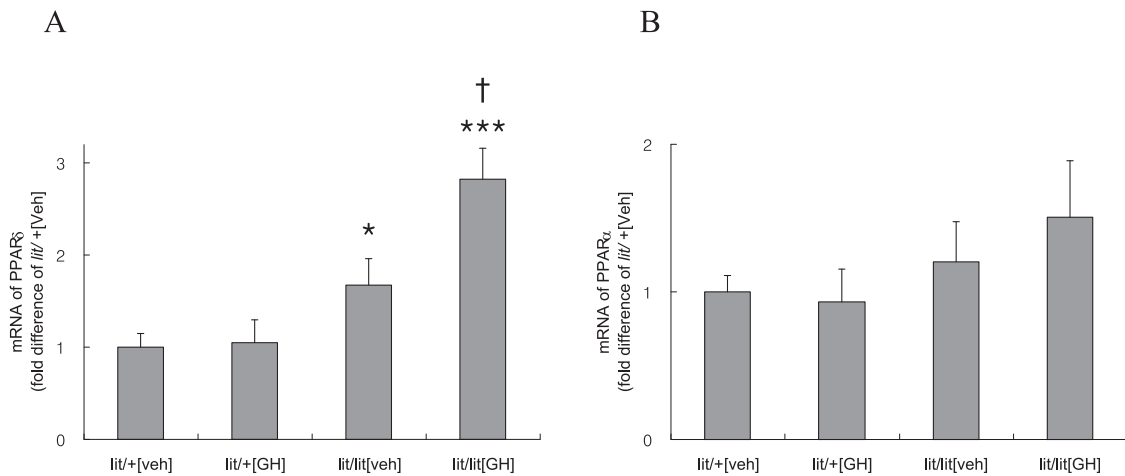


Fig. 1. mRNA levels of PPAR δ (A) and PPAR α (B) in *lit/+* and *lit/lit* mice 4 h after treatment with GH or vehicle. * P <0.05, *** P <0.001 vs. *lit/+*[vehicle]; † P <0.05 vs. *lit/lit*[vehicle].

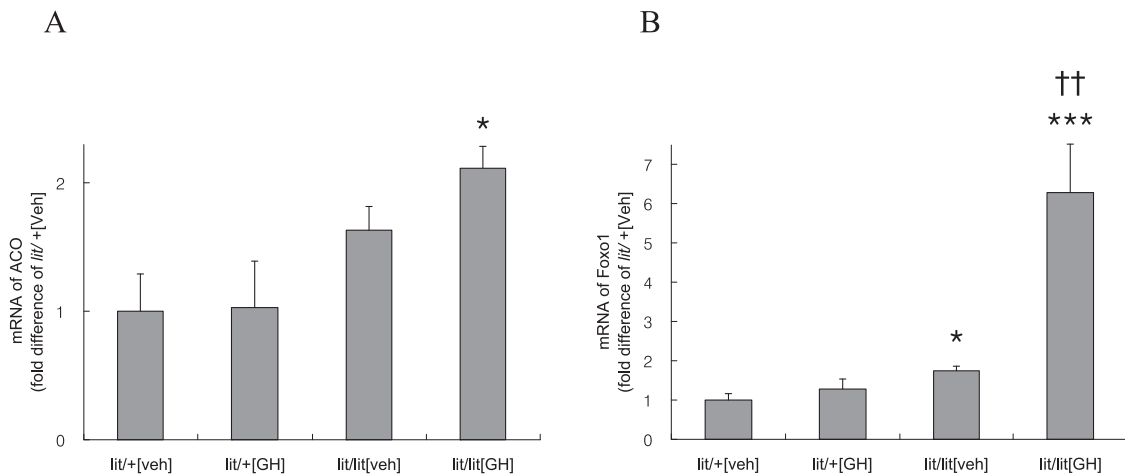


Fig. 2. mRNA levels of acyl-CoA oxidase (A) and Foxo1 (B) in *lit/+* and *lit/lit* mice 4 h after treatment with GH or vehicle. * P <0.05, *** P <0.001 vs. *lit/+*[vehicle]; †† P <0.01 vs. *lit/lit*[vehicle].

showed a further increase in Foxo1 mRNA levels (6.30 fold vs. vehicle-treated *lit/+* mice, P <0.001; 3.61 fold vs. vehicle-treated *lit/lit* mice, P <0.01) (Fig. 2B).

mRNA levels of sterol regulatory element binding protein-1c (SREBP-1c), a family of “master” transcription factors, which regulates lipogenesis and are increased in liver of GH deficient mice, showed no difference between *lit/lit* and *lit/+* mice, and no changes after GH treatment in *lit/lit* mice (Fig. 3).

Discussion

The present study demonstrates that mRNA levels

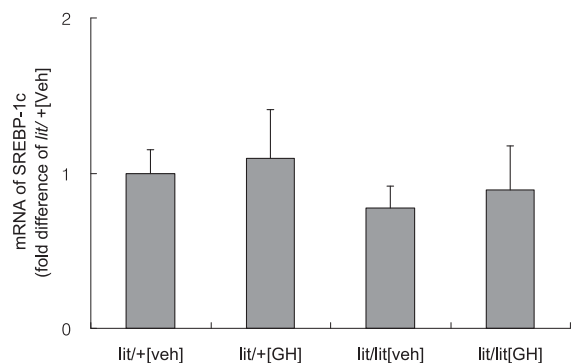


Fig. 3. mRNA levels of SREBP-1c in *lit/+* and *lit/lit* mice 4 h after treatment with GH or vehicle.

of genes involved in lipid oxidation are increased in skeletal muscle of GH deficient *lit/lit* mice and are further increased by GH administration. The mechanism of increased mRNA levels of PPAR δ in GH deficient *lit/lit* mice is not clearly explained. Skeletal muscle is a major site of lipid catabolism. Genes involved in fatty acid catabolism in skeletal muscle are up-regulated during starvation and exercise [23, 24]. In the state with chronic GH deficiency, systemic delivery of free fatty acids is increased, which might be an explanation of increased mRNA levels of PPAR δ in muscle of *lit/lit* mice. GH has been shown to stimulate mitochondrial fatty acid oxidation acutely [1]. It stimulates the transcription of fatty acid-binding proteins in the liver [25] and increases the transcription and activity of medium-chain acyl-CoA dehydrogenase [26], one of the several acyl-CoA dehydrogenases catalyzing the first step of β -oxidation. In the present study, mRNA expression of PPAR δ was rapidly and significantly increased 4h after GH administration in GH deficient *lit/lit* mice, supporting that GH has an acute effect on fatty acid oxidation in skeletal muscle. Moreover, mRNA levels of ACO, a peroxisomal enzyme involved in fatty acid oxidation were changed parallel to those of PPAR δ . Lack of GH effect on the expression of PPAR δ and Foxo1 in GH sufficient *lit/+* mice might result from the use of lower, more physiological dose of rrGH than that used in previously study.

It has been documented that PPAR α plays a crucial role in fatty acid homeostasis and is considered the primary regulator that protects against abnormal accumulation of neutral lipids in liver and cardiac muscle [27]. PPAR α is also known to stimulate fatty acid utilization and mRNA expression of genes involved in fatty acid oxidation in primary human skeletal muscle cells [28]. In the present study, in contrast to PPAR δ expression, mRNA levels of PPAR α in skeletal muscle of GH deficient mice, were not increased compared to *lit/+* GH sufficient mice, nor altered after GH administration. This difference of mRNA expression between PPAR α and PPAR δ can be explained, at least partly by the fact that PPAR δ is several-fold more abundant than PPAR α in mouse skeletal muscle, unlike in liver and heart [6]. Additionally, no change of expression of SREBP-1c, a family of "master" transcription factors which regulates fatty acid and cholesterol synthesis, was demonstrated in skeletal muscle of mouse with variable levels of GH.

The present study demonstrates that mRNA levels of Foxo1 are parallel with those of PPAR δ in skeletal muscle of GH deficient mice. In GH deficient *lit/lit* mice, serum concentrations of insulin/IGF-I and mRNA levels of IGF-I in skeletal muscle were lower than control *lit/+* mice. Insulin and IGF-1 have previously shown to critically inhibit Foxo1 function through PI3K/Akt pathway [10, 11]. When Akt1 activity is suppressed, protein synthesis is inhibited [29]. Furthermore, Foxo1 becomes dephosphorylated, thereby transcriptionally activating muscle proteolytic enzymes [30, 31]. Taken together, these can be one of possible explanations why Foxo1 mRNA is increased in muscle of GH deficient *lit/lit* mice and why muscle mass decreases in GH deficient state.

Recent studies have revealed that in fasting state, an increase in fatty acid oxidation in muscle induced by the activation of PPAR α and PPAR δ [32, 33] activates PDK4, which phosphorylates and inactivates the pyruvate dehydrogenase complex. This inactivation of pyruvate dehydrogenase complex that catalyzes carbohydrate oxidation in muscle by PDK4-induced PDC inhibition results in increased activation of the Foxo1 [12, 34]. Though the direct mechanism of increase of PPAR δ expression by GH administration is not explained, further strikingly increase of muscular Foxo1 expression 4h after GH administration in GH deficient *lit/lit* mice can be attributable to up-regulation of PPAR δ .

In summary, mice with chronic GH signaling deficiency, accompanied by obesity, showed increased activation of genes as PPAR δ , Foxo1 and these downstream enzyme ACO, involved in lipid oxidation, though these data are lacking the results of corresponding proteins. Furthermore, acute GH administration was followed by a further increase in expression of these oxidative genes. These suggest that GH plays a relevant role in energy metabolism and stimulate fatty acid catabolism in skeletal muscle.

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