Comparison of Low and High-dose Growth Hormone Administration on Muscle Ceramide Contents and Insulin Resistance in Rats

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ABSTRACT

We evaluated the effects of low- and high-dose growth hormone (GH) administration on muscle ceramide contents and insulin resistance in rats. 24 rats were randomly assigned to three groups: low dosage GH injection group (L/GH, n = 8), high dosage GH injection group (H/GH, n = 8) and saline injection group (CON, n = 8). GH groups received GH by subcutaneous injections (L/GH; 65 μg/kg/day, H/GH; 130 μg/kg/day, 6 days/week) for 4 weeks, while CON received saline injections that were equivalent in volume to L/GH group. At the end of 4-week treatments, plasma lipids, insulin and glucose levels were measured. Glucose transporter 4 protein (GLUT-4) and ceramide contents were determined in muscle while triglyceride contents were determined in both muscle and liver. Rate of glucose uptake was determined under submaximal and non-insulin conditions during the epitrochlearis incubation. After 4 weeks of GH injection, H/GH had significantly higher plasma FFA levels (p < 0.05), but L/GH did not have it compared to CON. Plasma insulin levels were significantly lower in H/GH and glucose level in H/GH was higher than CON group (p < 0.05). Muscle ceramide, muscle and liver triglyceride contents were significantly greater in H/GH than CON, but those were not significantly different between L/GH and CON. Glucose transport rate under submaximal insulin condition was significantly lower in H/GH group than CON group (p < 0.05), but L/GH was not different from CON. Therefore, results of this study suggested that the deterioration of muscle insulin resistance due to GH treatment is strongly associated with the elevated TG and ceramide contents due to high GH administration dosage in rats.

Keywords: Growth hormone; Muscle triglyceride, Liver triglyceride; Ceramide; Glucose transport rate; Insulin resistance.

INTRODUCTION

The over the past decades, GH administration has been accepted as anti-ageing, physical growth, and bone health treatments in medical clinics, and the physiological effects of GH are well presented in the literature (Doga et al. 2006; Henwood et al. 2002; Woodhouse et al. 2006). Notably, injection of GH is also suggested to antagonize the effects of insulin on glucose and lipid metabolism; (Brammert et al. 2003; Cartee et al. 1995; Chrisoulidou et al. 2000; Groop et al. 2005;
Ng et al. (1990) in vivo experimental investigations demonstrate that GH treatment can reduce glucose and insulin responses in animals (Cartee & Bohn, 1995; Hou et al. 2003; Ng et al. 1990). Also, the injection of GH in rats is reported to increase the triglyceride contents in both muscle and hepatic tissues and also to increase muscle insulin resistance (Park et al. 2008).

The responsible mechanism for the reported association between GH treatment and insulin resistance is not fully elucidated, but elevated levels of plasma FFA and reduced levels of GLUT-4 protein contents have been suggested (Binnerts et al. 1992; Jorgensen et al. 2004; Kim et al. 1999; Moller et al. 2003; Zisman et al. 2000). It has been widely accepted that increased plasma FFA concentrations could accelerate the increase in intramuscular triglyceride content, which might contribute to deterioration of insulin sensitivity in skeletal muscle of rats (Jung et al. 2010). Some researchers have reported a positive association between intramuscular triglyceride content and insulin resistance in muscle (Hegarty et al. 2002; Pan et al. 1997). According to previous our results, GH injection (130 μg·kg−1·day−1, 6 days·week−1) for 4 week experimental period, while CON group injected equivalent volume saline as GH group.

Tissue and blood processing

After the 8 h fasting, rats were anesthetized by sodium pentobarbital (6.5 mg·kg−1) through the intraperitoneal injection. Both right- and left-side epitrochlearis muscles were surgically removed to determine glucose transport rate in muscle (Kawano et al. 1999), while plantaris muscle and liver were excised to measure the triglyceride content. Blood samples were drawn from an abdominal aorta to determine plasma glucose, insulin, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and FFA concentrations.

Biochemical measurements

Blood was added to 0.1 ml EDTA (24 mg/ml, pH 7.4) and centrifuged for 10 min at 1,000 g, and the plasma was separated. Plasma glucose was determined with a glucose analyzer (Model YSI-23A, Yellow Springs Instruments, Yellow Springs, OH, USA) and plasma insulin was measured via radioimmunoassay (Linco, St. Charles, MO) by using a double antibody procedure (Morgan et al. 1962). Plasma FFA was determined using Noma's method (Noma et al. 1973). Plasma triglyceride, total cholesterol, and HDL-C concentrations were determined by an enzymatic method.

Muscle and liver triglyceride content

Plantaris muscle samples were homogenized as 1:20 dilution in 50 mM potassium phosphate and 1 mM EDTA (pH 7.4), while liver samples were homogenized with 1:15 dilution. Lipid was determined by following guidelines.
described by Burton and Anderson (Burton et al. 1985). After centrifugation (1,000 g at 4°C for 10 min), the lipid-containing phase was evaporated to dryness with N2 gas, leaving a small lipid pellet that was then resuspended in 0.5 ml of 4% ethanoic KOH and heated for 20 min at 75°C to hydrolyze the triglyceride into fatty acyl and glycerol. The addition of 1 ml of 0.15 M MgSO4 and subsequent centrifugation (1,000 g at 4°C for 10 min) allowed for precipitation of the fatty acyl units, leaving only glycerol, which was determined by an enzymatic method.

**Skeletal muscle GLUT-4 content**

GLUT-4 protein was assayed in plantaris muscle homogenates by use of quantitative Western blotting procedures, which involved digitizing autographs and using a heart reference standard, as reported previously (Park et al. 2008).

**Skeletal Muscle Ceramide Content**

Ceramide content was assayed in plantaris muscle as reported previously (Park 2008). Plantaris muscle was homogenized in buffer (0.25M sucrose, 25mM KCl, 50mM Tris, 0.5mM EDTA; pH 7.4). After homogenization, lipid extraction was made by adding a chloroform, methanol, and buffer water mixture maintained at the ratio of 0.5:1.0:0.4 and 1.0:1.0:0.9 (v/v/v) before and after dilution, respectively. Lipid extraction and ceramide quantification were by following methods described by Bielawska et al. (2001). The silica gel was then exposed to X-ray film at -20 °C for 12 hours. Then, ceramide 1-[32P] phosphate’s activity was measured for one minute per silica gel using a liquid scintillation counter (Wallac 1409 DSA; PerkinElmer Inc., Waltham, MA, USA).

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### Table 1: Biochemical characteristics after 4-week GH injection

<table>
<thead>
<tr>
<th>Variable</th>
<th>CON (n=8)</th>
<th>L/GH(n=8)</th>
<th>H/GH(n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUCOSE(mmol/L)</td>
<td>10.05±0.58</td>
<td>9.31±0.52</td>
<td>8.37±0.16</td>
</tr>
<tr>
<td>INSULIN(pmol/L)</td>
<td>6.60±0.69</td>
<td>7.78±0.83</td>
<td>11.46±1.74</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>0.58±0.04</td>
<td>0.63±0.05</td>
<td>0.88±0.06*</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>2.06±0.06</td>
<td>2.14±0.06</td>
<td>2.23±0.07</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.39±0.04</td>
<td>0.44±0.03</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>HDL-C(mmol/L)</td>
<td>0.79±0.04</td>
<td>0.92±0.07</td>
<td>0.85±0.04</td>
</tr>
</tbody>
</table>

CON, Control group; L/GH, Low GH injection group; H/GH, High GH injection group; FFA, free fatty acids; TC, total cholesterol; TG, Triglycerides; HDL-C, high-density lipoprotein cholesterol. Data are show as means ± SE. *p<0.05 vs CON.
Glucose transport rate assay

Epitrochlearis muscles were subjected to glucose transport rate assay, which was performed by using non-metabolized 3-O-methyl-d-glucose (3-MG), as described previously (Jung and Kang, 2013). After pre-incubation, epitrochlearis muscle was incubated in 2 mL of Krebs–Ringer buffer containing 4 mmol·L⁻¹ [3H]3MG (2.2 Ci·mL⁻¹) and 16 mmol/L d-[1,14C]-mannitol (0.2 Ci·mL⁻¹) at 30°C for 10 min. Epitrochlearis was then digested with 1 mol·L⁻¹ NaOH at 80°C for 10 min, neutralized with 1 mol·L⁻¹ HCl, and then centrifuged at 1,600 rpm for 4 min at the micro centrifuge. Radioactivity in the supernatant was measured by dual-label liquid scintillation counting and then glucose transport rate was (Young et al. 1997).
Statistical Analysis
Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). Values are expressed as means ± standard error (Mean ± SE). A significant difference was considered to exist when p < 0.05. One-way ANOVA was used to see the change among three groups after handling. Least significant difference was used for post-hoc analysis.

RESULTS

Biochemical Characteristics
Biochemical characteristics after 4 weeks of GH treatments are showed in Table 1. Insulin concentrations of H/GH had lower than that of CON (p < 0.05) and plasma glucose concentrations of H/GH had higher than that of CON (p < 0.05). However, plasma glucose and insulin levels were not different between L/GH and CON. H/GH had significantly higher FFA values than the CON (p < 0.05), but FFA level was not different between L/GH and CON. There were no statistically significant differences in plasma triglyceride, total cholesterol, and HDL-C values among all three groups.

Muscle and liver triglyceride content
Triglyceride contents in the muscle and liver of H/GH had significantly higher than those in the CON, respectively (Figure 1). However, triglyceride contents in muscle and liver were not different between L/GH and CON.

Skeletal muscle GLUT-4 content
There was no significant difference contents among all three groups in GLUT-4 protein (Figure 2).

Glucose transport assay
Glucose transport rate in epitrochlearis muscle of H/GH had a lower under submaximal insulin concentrations than did the CON, but the glucose transport rate was not significantly different between L/GH and CON. But, there was no significant difference among three groups under non-insulin concentration (Figure 3).

Skeletal Muscle Ceramide Content
Ceramide content in muscle of H/GH was significantly higher than that of the CON (Figure 4). However, the ceramide contents were not significantly different between L/GH and CON.

DISCUSSION
In this investigation, we found a decreased glucose transport rate under submaximal insulin concentration and also an increased triglyceride contents in both muscle and liver at only high dosage GH injected rats. However, in low dosage GH injected rats, a glucose transport rate and triglyceride contents in muscle and liver was not significantly affected by GH injection compared to control rats. In addition, plasma FFA and glucose levels in high dosage GH injected rats were significantly elevated in comparison to control rats, but those in low dosage GH injected rats did not. Hence, the significant result of this experiment is that the insulin sensitivity and triglyceride content of skeletal muscle in rats would be insignificantly affected by low dosage of GH injection.

It has been reported that chronic GH injection in rats significantly elevated intramuscular and hepatic triglyceride contents and also deteriorated insulin sensitivity in epitrochlearis muscle (Park et al. 2008). Thus, the authors suggested that intramuscular triglyceride increase due to GH administration could partly related to the deterioration of insulin resistance. Also, the elevated intramuscular triglyceride content in itself might not affect muscle insulin sensitivity, but rather acts as a source for lipid derivatives, such as diacylglycerol, ceramides or long chain fatty acid acyl-CoA, that directly affect the intracellular insulin-signaling process and, thus, insulin sensitivity (Straczkowski et al. 2007; Summers. 2006; Summers et al. 1998). According to the results of Summer’s study (Summers. 2006), it has been reported that the muscle ceramide content may affect insulin signaling process at the level of intracellular protein kinase B (PKB)/Akt in muscle (Schmitz-Peiffer et al. 1999). Hence, we suggested that the decreased glucose transport activity due to GH treatment may partly related to the elevations of intramuscular triglyceride storage and ceramide content. In this investigation, we observe that muscle triglyceride storage and ceramide contents are only significantly elevated in high dosage GH injected rats, but they are not
occurred in low GH dosage injected rats. Thus, the increased muscle ceramide content due to GH treatment may probably depend upon amount of GH injected dosage.

It has been suggested that plasma FFA level due to GH injection is positively related to insulin resistance in rats (Krag et al. 2007; Segerlantz et al. 2003). In this investigation, the elevated FFA levels is only observed in H/GH group, but they are not observed in L/GH group. Many prior studies suggested that an elevation in plasma FFA level might be a major responsible factor for the deterioration of insulin affinity in skeletal muscle and adipocytes (Boden et al. 2002; McGarry 2002). Elevation of plasma FFA levels by GH injection has been proposed to be strongly connected to insulin resistance (Johansen et al. 2003; Jorgensen et al. 2004; Moller et al. 2003). According to the results of the previous studies, the lipolytic activities of GH might stimulate the gene expression after binding to the GH receptor on plasma membrane and then subsequent elevating activities of JAK2 tyrosine kinase and adenyl cyclase, and then stimulating cAMP production, activating the hormone-sensitive lipase by increasing plasma FFA level (Louveau et al. 2004; Richelsen et al. 1999; Yip et al. 1999). In accordance with the glucose-FFA cycle proposed by Randle et al. (Randle et al. 1963), the elevated plasma FFA levels may reduce the glucose uptake in insulin sensitive tissues such as skeletal muscle and adipocyte. In here, skeletal muscle is major storage space for 70 - 80% of insulin-mediated transported carbohydrate in the whole body. Thus, the skeletal muscle is considered to be the major body tissue of insulin resistance (Ivy et al. 1999). The mechanism by which increased plasma circulating FFA levels suppressed the insulin-mediated glucose uptake of skeletal muscle may involve the intracellular signaling pathway of insulin responsible for GLUT-4 translocation to muscle plasma membrane, including glycogen synthase activity, PI3K activity or IRS-1 phosphorylation (Boden 2003; Cartee & Bohn. 1995; Christopher et al. 1998; Jessen et al. 2005). In addition, a reverse relation between insulin sensitivity and triglyceride content has been observed in human skeletal muscle by biopsy (Pan et al. 1997), computed tomography (Goodpaster et al. 2000) and magnetic resonance spectroscopy (Virkamaki et al. 2001). Triglyceride level of the skeletal muscle has been considered as an important factor between insulin resistance in skeletal muscle and circulating FFA level in blood, and the suggested mechanism involves intra-myocellular increase of diacylglycerol, activation of myoplasmic long-chain fatty acyl CoA, or protein kinase C (Hegarty et al. 2003; Stannard et al. 2004).

In this investigation, we reported that GLUT-4 protein contents were not significantly different among the L/GH, H/GH and CON groups. In the previous study, GLUT-4 protein was considered to be the most important protein of glucose uptake in skeletal muscle, and GLUT-4 content protein was strongly related to the rate of glucose uptake in skeletal muscle incubation (Henriksen et al. 1990). Thus, any reduction in the rate of glucose transport in high GH-treated rats could seem to require a decrease in GLUT-4 contents of muscle. But, in this study, no reduction of muscle GLUT-4 contents was observed in the high GH-treated rats. This observation suggests that the deflection in the insulin signaling pathway appeared prior to the process of GLUT-4 translocation. This observation is consistent with the previous report that GLUT-4 contents in muscle were unaltered in GH-injected rats (Hou et al. 2003), and supports the result that alterations of GLUT-4 contents in muscle may not be responsible for the deterioration of insulin-mediated glucose uptake in high-dose GH-injected rats.

It is reasonable that high-dose GH-injected rats only appear the deterioration of muscle insulin resistance of rats due to increased muscle triglyceride and ceramide contents. Increase in plasma FFA concentrations due to high GH treatment develops the elevation of intramuscular triglyceride content. Increased muscle triglyceride and ceramide contents are responsible for the deterioration of insulin resistance in skeletal muscle of rats. Our results in this investigation also observed that reduced glucose transport rate in the high GH-treated rats occurred without the significant change in GLUT-4 protein content, and was directly related to the triglyceride and ceramide contents of the skeletal muscle in rats. To our knowledge, a significant finding of the current study is that this would be the first observation to particularly examine the association among muscle triglyceride level, muscle ceramide content and glucose transport rate after the
different dosage of GH administration. The current investigation is well designed to eliminate all possible confounding influences using a randomized control design. Further investigations could be necessary to examine our findings in humans.

In conclusion, we found that the elevation of muscle insulin resistance due to GH treatment is strongly associated with the dosage of GH administration in rats. In addition, the contributing factor for insulin resistance in skeletal muscle might be that GH treatment elevates triglyceride content and ceramide level in skeletal muscle, and an increased triglyceride and ceramide contents are positively related to the insulin resistance of muscle in rats.

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