Hepatic adiponectin receptor R2 expression is up-regulated in normal adult male mice by chronic exogenous growth hormone levels

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Abstract. Previous studies investigating the effects of exogenous growth hormone (GH) on the expression of adiponectin and the adiponectin receptors have generated seemingly conflicting results. Here, to determine the effects of chronic exogenous GH levels, we investigated the expression of adiponectin receptor R1 (adipor1) in skeletal muscle and adiponectin receptor R2 (adipor2) in the liver of normal C57BL/6 adult male mice 24 weeks after a single injection of rAAV2/1-CMV-GH1 viral particles. Adipor1 and adipor2 mRNA expression levels were determined by quantitative real-time reverse transcription-polymerase chain reaction. Adipor1 and adipor2 proteins were analyzed by Western blotting. Serum total and high molecular weight (HMW) adiponectin concentrations were measured by enzyme-linked immunosorbent assay (ELISA) kits for mice. The results demonstrated that the injection of rAAV2/1-CMV-GH1 within the concentration range of 1.0x10^{11} to 4.0x10^{11} viral particles significantly up-regulated hepatic adipor2 gene expression. The serum HMW adiponectin level increased significantly with the concentration range of 2.0x10^{11} to 4.0x10^{11} rAAV2/1-CMV-GH1 viral particles. The injection of 4.0x10^{11} rAAV2/1-CMV-GH1 viral particles induced a significant increase in both serum glucose and insulin concentrations (p<0.05), and the homeostasis model assessment of insulin resistance (p<0.01). This may decrease body insulin sensitivity and therefore raise the potential risk of insulin resistance. However, exogenous GH levels did not significantly affect adipor1 expression in skeletal muscle. In conclusion, chronic exogenous GH up-regulates hepatic adipor2, rather than adipor1, in vivo, and raises the serum HMW adiponectin level. However, the elevation of exogenous GH concentrations may increase the risk of insulin resistance.

Introduction

Adiponectin, an adipokine that is secreted exclusively by adipocytes, plays an important role in regulating systemic energy metabolism and insulin sensitivity in vivo (1,2). The serum adiponectin level is inversely associated with body mass index (BMI), which is relevant to metabolic syndrome. Hypoadiponectinemia is associated with insulin resistance and hyperinsulinemia (3-7). Adiponectin is present as a full-length protein of 30 kDa (fAd), which circulates in trimeric, hexameric or high molecular weight (HMW) forms. A proteolytic cleavage product of adiponectin, known as globular adiponectin (gAd), has also been shown to exhibit potent metabolic effects in various tissues (8-14). The various adiponectin oligomeric forms have distinct biological activities. HMW adiponectin is the major bioactive isoform, responsible for the insulin-sensitizing effects of adiponectin (15,16).

The effects of adiponectin are mainly mediated through the R1 and R2 adiponectin receptors (adipor1 and adipor2). In mice, adipor1 is ubiquitously expressed, but is most abundant in skeletal muscle, whereas adipor2 is predominantly found in the liver (17,18). These receptors mediate adiponectin activity mainly by participating in the activation of the adenosine monophosphate kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα) signaling pathways (18,19). Changes in their expression may influence adiponectin gene expression and ultimately influence fatty acid oxidation, glucose transport activity and insulin sensitivity (17).

Growth hormone (GH) is an effective regulator of adiponectin, and its receptors in adipocytes act through the Janus kinase 2 and p38 mitogen-activated protein kinase pathway (20-22). GH replacement therapy could be a potential alternative to future adiponectin-based therapies, since adiponectin activity is limited, despite the relatively high concentrations of plasma adiponectin (5-30 µg/ml). Further studies are required to ascertain the effects of GH on adiponectin levels and the expression of the adiponectin receptors in insulin-sensitive tissues other than adipose tissues, and to determine the mechanism(s) involved. Moreover, previous studies investigating the effects of GH on the expression of adiponectin and its receptors have generated seemingly conflicting results (20-22).
In this study, we investigated the effect of chronic exogenous GH levels on adipor1 and adipor2 expression in normal adult male mice. GH gene delivery technology was used instead of recombinant GH injection, since viral vectors produce longer-lasting effects than the recombinant protein, thus circumventing the inconvenience of the necessary exogenous subcutaneous injections. The GH1 gene [a human GH (hGH) gene, GenBank accession number NM_000515] coding sequence (cds) was transferred *in vivo* by rAAV2/1 (recombinant adeno-associated viral vectors pseudotyped with viral capsids from serotype 1) to determine the effect of chronic exogenous GH levels on adipor1 in skeletal muscle, adipor2 in liver and the serum concentrations of total and HMW adiponectin in normal male mice.

### Materials and methods

Construction and production of the rAAV2/1 vector containing GH1. The rAAV2/1 vector containing GH1 was constructed and produced as previously described (23). Briefly, GH1 was cloned from a polymerase chain reaction (PCR) product using the primers 5'-GcGcGgATTCGcCgCc CATgGcTACgGcTCCcGG-3' (forward) and 5'-CTgGtCT CGAgCAgGgcCcAGCtGcCcCTc-3' (reverse) (EcoRI and SalI restriction sites are indicated by underlining) from the template of a pUC19 plasmid DNA containing GH1 (Xinxing Medical University, Henan Province, P.R. China). The GH1 DNA fragment (677 bp, including the 651-bp cds) was digested with SalI and EcoRI and inserted into the SalI and EcoRI sites of the pHSNV2.0 vector (AGTC Gene Technology Co. Ltd., P.R. China). rAAV2/1 production and purification were performed as previously described (24). The viral genome particle titer (~1.0x10^12 v.g./ml) was determined by a quantitative DNA dot blot method (25).

Animal treatment. Thirty C57BL/6 male mice (10-12 weeks old) were obtained from the Institute of Laboratory Animal Sciences, CAMS & PUMC (P.R. China). These were randomly divided into five groups as follows: a high-dose (HD) group (n=6) injected with 4.0x10^11 rAAV2/1-CMV-GH1 viral particles by a single intravenous injection into the tail vein; a middle-dose (MD) group (n=6) injected with a single dose of 2.0x10^11 rAAV2/1-CMV-GH1 viral particles; a low-dose (LD) group (n=6) injected with a single dose of 1.0x10^11 rAAV2/1-CMV-GH1 viral particles; a control (Ctl) group (n=6) injected with a single dose of rAAV2/1 empty vectors; and a placebo (Plac) group (n=6) injected with a single dose of placebo (saline). The mice were sacrificed 24 weeks after the injection of rAAV2/1-CMV-GH1 or empty vector.

Body composition. Before sacrifice, the body length (BL), naso-anal length (N-A) and total body weight (TBW) were measured at week 24 using a daily calibrated electronic balance (Scout Pro Balance; Ohaus, USA) and an electronic digital caliper (Control, USA). The BMI was calculated according to the BMI formula: BMI = TBW (g)/[BL (cm)^2]. The weight of the subcutaneous fat pad (SF), visceral fat (VF) and lean mass (LM) was measured separately using a precision electronic balance (AV264; Ohaus). After removal of the skin, the weight of the perirenal and epididymal fat pads was summed for VF measurement, while the sum of the fat pads from the interscapular and axillary region, thighs and inguinal region was used for SF measurement. LM was measured by weighing animals deprived of tail, skin, adipose tissue and internal organs. The LM, VF and SF weights of each animal were normalized to TBW by calculating the percentage as follows: [weight (g)/TBW (g)] x 100%.

Sample preparation. Animals were fasted overnight then anesthetized with a single dose of intraperitoneal sodium phenobarbital (45 mg/kg body weight). Serum samples were collected from the abdominal aorta into plain microcentrifuge tubes (1.5 ml) and allowed to clot at room temperature for 60 min for serum formation. This was followed by centrifugation at 3,000 x g for 15 min at 4˚C. The serum was removed, aliquoted and stored at -80˚C. After blood collection, liver and gastrocnemius muscle was quickly dissected, flash-frozen with liquid nitrogen and stored at -80˚C.

Animal experiments were consistent with the NIH animal care and use guidelines (National Research Council Guide for Care and Use of Laboratory Animals, 1996, National Academy of Science). All efforts were made to minimize the number of animals used and their suffering.

**PCR, semi-quantitative PCR and real-time quantitative PCR analysis.** Total DNA in liver and gastrocnemius muscle was extracted from the tissue using the Puregene DNA Isolation kit (Qiagen, Germany). A 286-bp fragment of the rAAV genome was amplified using a sense primer of the inverted terminal repeat (ITR) region and an antisense primer of the cytomegalovirus (CMV) promoter (26). A 677-bp fragment of the GH1 gene was amplified using primers as described above. The PCR procedure consisted of 1 min of denaturation at 94˚C followed by 30 cycles of 30 sec at 94˚C, 30 sec at 57˚C and 4 min at 72˚C, and finally extension for 5 min at 72˚C. Aliquots (6 µl) of the 25 µl final PCR volume were analyzed by electrophoresis on 2.0% agarose gels.

Total RNA extracted from the liver and gastrocnemius muscle was isolated and purified with TRIzol reagent (Invitrogen, USA) and the NucleoSpin® RNA clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically at 260 nm, then 5 ng was subjected to semiquantitative reverse transcription (RT)-PCR using the primers for the GH1 gene (Table I). Thermal cycling was performed as follows: 94˚C for 5 min, 35 cycles at 94˚C for 30 sec, annealing for 35 sec and 72˚C for 30 sec, and a final step of 72˚C for 5 min. Aliquots (6 µl) of the 25 µl final PCR volume were analyzed by electrophoresis on 2.5% agarose gels.

Quantification of adipor1 mRNA in skeletal muscle and adipor2 mRNA in liver was performed using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science) according to the manufacturer's instructions. The PCR reaction was repeated for 40 cycles after initiation with 10 min of denaturation at 94˚C; each cycle included denaturation at 94˚C for 15 sec, annealing for 35 sec and primer extension at 72˚C for 15 sec. The primers and annealing temperature for adipor1 (GenBank accession no.: NM_028320.3) and adipor2 (GenBank accession no.: NM_197985.3) are shown in Table I. Melting curves and 1.5% agarose gel electropho-
Resins were produced to validate the specificity of the amplified products. The comparative threshold cycle (cT) method was used to calculate amplification fold. The expression level of β-actin was used as a reference to normalize the RNA expression levels. The experiment was performed in triplicate for each gene and the average expression value was computed for subsequent analysis. The results were compared using the 2-ΔΔcT method (27) and are expressed as the log2 mean ± SD.

Western blot analysis. Soluble protein was extracted from liver and gastrocnemius muscle using a protein extraction reagent (Pierce, USA). The final protein concentrations were determined using a BCA assay (Pierce) according to the manufacturer's instructions. Supernatants of the protein extracts were resolved on 12% SDS-PAGE gels, followed by electrophoretic transfer to nitrocellulose membranes. The nitrocellulose membranes were then pre-treated with 1.0% non-fat dried milk in 50 mM Tris (pH 8.0) and incubated overnight with antibodies against hGH (rabbit polyclonal IgG), adiponectin receptor 1 (mouse monoclonal antibody) or adiponectin receptor 2 (mouse monoclonal antibody) (1:500, 1:400 and 1:400, respectively) (Santa Cruz, USA). The bound primary antibody was detected using biotinylated rabbit anti-mouse antibody (Zhongshanjinqiao, P.R. China) and visualized using 3'-3'-diaminobenzidine tetrahydrochloride. Detection was performed using an ECL chemiluminescent kit (Amersham Pharmacia Biotech, UK) according to the manufacturer’s instructions.

Serum hormone and blood glucose analysis. Serum hGH concentrations were determined by the Roche hGH ELISA assay kit (26). Enzyme-linked immunosorbent assay (ELISA) kits for mouse were used to elevate the mice serum hormone levels, including insulin-like growth factor 1 (IGF-1) (ADL, USA) and insulin (Otsuka Life Science, Japan), according to the manufacturer’s recommendations. Serum concentrations of total and HMW adiponectin were measured using immunoassays purchased from ALPCO Diagnostics (USA). Serum samples were pre-treated as previously described to determine HMW adiponectin levels (28). The assay included quality controls provided by the manufacturer. Each sample was assayed in duplicate. The mouse fasting blood glucose level was estimated using the method of Sasaki et al (29).

We used the homeostatic model to assess insulin resistance, where the homeostasis model assessment of insulin resistance (HOMA-IR) = blood glucose (µU/ml) x blood insulin (mmol/l)/22.5 (30-32).

Statistical analysis. Gray-scale analyses for Western blotting were performed using Image-Pro Plus version 6.0 (Media Cybernetics Inc., USA). Data were reported as the arithmetic means ± SD of 3-6 animals in each group. Statistical analysis was performed by ANOVA with post hoc analysis using Bonferroni’s method using the SPSS statistical package 13.0 (SPSS Inc., USA). Data were considered statistically significant at p<0.05.

### Table I. PCR primers and conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH1</td>
<td>5'-GAACCTGGCAGAAGGCCACT-3' 5'-AGGGTCTGGGCCATAGA-3'</td>
<td>56.0</td>
<td>215</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>5'-CTTTACTGTCCTCCCCACAGC-3' 5'-GACAAAGCCTCAGGATAG-3'</td>
<td>57.4</td>
<td>196</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>5'-GGATCTGGAGTGATCGTGTG-3' 5'-ACCTGGTCACACGAGACACC-3'</td>
<td>56.1</td>
<td>218</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GAAGTGTGACGACATCG-3' 5'-GCCTAGAAGCATTTGCGTG-3'</td>
<td>60.0</td>
<td>282</td>
</tr>
</tbody>
</table>

### Table II. Body composition 24 weeks after the injection of rAAV2/1-CMV-GH1.

<table>
<thead>
<tr>
<th>Group</th>
<th>TBW (g)</th>
<th>BMI (g/cm²)</th>
<th>LM (%)</th>
<th>SF (%)</th>
<th>VF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td>28.20±2.20</td>
<td>0.30±0.03</td>
<td>41.59±7.69</td>
<td>1.44±0.12</td>
<td>0.53±0.10</td>
</tr>
<tr>
<td>Plac</td>
<td>28.81±2.12</td>
<td>0.31±0.03</td>
<td>40.98±7.10</td>
<td>1.42±0.11</td>
<td>0.51±0.10</td>
</tr>
<tr>
<td>LD</td>
<td>29.55±2.30</td>
<td>0.29±0.02</td>
<td>42.53±6.62</td>
<td>1.29±0.08</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td>MD</td>
<td>31.85±2.36</td>
<td>0.28±0.02</td>
<td>44.43±5.89</td>
<td>1.19±0.10</td>
<td>0.40±0.05</td>
</tr>
<tr>
<td>HD</td>
<td>32.35±2.52</td>
<td>0.27±0.02</td>
<td>47.91±7.06</td>
<td>1.16±0.11</td>
<td>0.38±0.04</td>
</tr>
</tbody>
</table>

SD, standard deviation; TBW, total body weight; BMI, body mass index; LM, lean mass; SF, subcutaneous fat pad; VF, visceral fat; Ctrl, control; Plac, placebo; LD, low-dose; MD, middle-dose; HD, high-dose. *p<0.05 and **p<0.01 vs. the Ctl group. n=6 for each group.
Results

Validation of \(GH1\) gene transfer in vivo. In liver and skeletal muscle tissues transduced with rAAV2/1-CMV-GH1 vector 24 weeks after infection, PCR analysis demonstrated both a 286-bp band corresponding to the ITR sequence of the CMV promoter sequence and a 677-bp band corresponding to the \(GH1\) gene. In tissues transduced with empty vector, only the 286-bp PCR product was observed (Fig. 1A). A 215-bp band for the \(GH1\) gene was detected by real-time quantitative RT-PCR in the gastrocnemius muscle and the liver of the \(GH1\)-treated mice (Fig. 1B). A 22-kDa band for the hGH was detected in the \(GH1\)-treated mice by Western blot analysis 24 weeks after injection (Fig. 1C). This indicated that the enhanced expression of hGH in skeletal muscle and liver was sustained for at least half a year. The mice serum levels of IGF-1 were significantly increased in the MD (\(p<0.05\)) and HD (\(p<0.01\)) groups 24 weeks after injection (Table II). Total serum adiponectin was not significantly altered in the \(GH1\)-treated mice. However, serum glucose and insulin concentrations were significantly increased in the HD group only (\(p<0.05\)) 24 weeks after injection (Table II). As a result, the HOMA-IR of the HD group also significantly increased (\(p<0.01\)).

Mouse body weight, composition and serum lipid profile. Mouse body weight and composition changes 24 weeks after injection are shown in Table III. In the present study, mouse body weight increased significantly and body fat mass percentage (both SF and VF) decreased significantly in the MD and HD groups 24 weeks after the injection of rAAV2/1-CMV-GH1. The LM percentage increased and the BMI decreased significantly in the HD group (\(p<0.05\)) (Table III).

Discussion

Previous studies have shown paradoxical results concerning the effect of GH on adiponectin and its receptors. Fasshauer et al (2004) (20) and Xu et al (2004) (21) reported that GH is a positive regulator of the expression of adiponectin and its receptors in vitro. However, Nilsson et al (2005) (22) demonstrated that GH induced a decrease in adiponectin receptor gene expression in cultured human adipose tissue and reduced the serum adiponectin level in GH transgenic mice.

In the present study, we investigated the effects of chronic exogenous GH on the expression of adipor1 in skeletal muscle and adipor2 in the liver of normal male adult mice 24 weeks post infection.
after the injection of rAAV2/1-cMV-GH1 particles. HMW adiponectin is the most active adiponectin isoform in the mediation of insulin and blood glucose levels (33). The ratio of HMW to total adiponectin, $S_a$, is a more sensitive marker of insulin resistance and metabolic syndrome than the serum HMW or the total adiponectin concentration (34). Therefore, we examined HMW adiponectin, total adiponectin concentrations and $S_a$. Our results demonstrated that chronic exogenous GH treatment significantly up-regulated hepatic adipor2 gene expression, rather than adipor1 skeletal muscle expression, in vivo. Serum HMW adiponectin and $S_a$ were also increased in the GH1-treated mice, which may be due to the hepatic adipor2 up-regulation induced by the elevation of exogenous GH concentration.

Several studies have demonstrated the mechanisms by which adiponectin decreases insulin resistance. In brief, fAd augments the insulin-induced inhibition of glucose output in liver cells in vivo and in vitro, whereas gAd most potently increases fatty acid metabolism in muscle cells (2,35,36). In addition, adipor1 is thought to be a receptor for gAd, whereas adipor2 is a receptor for both gAd and fAd (10). These receptors mediate the insulin-sensitizing effects of adiponectin by participating in the activation of the AMPK and PPARα signaling pathways (36,37). Excess exogenous GH treatment in vivo may result in diabetogenic effects and, to a certain extent, insulin resistance, as seen in patients with GH-secreting pituitary adenomas (38). In the present study, the up-regulation of adipor2 and serum HMW adiponectin may be a compensating mechanism whereby insulin sensitivity is at least partially restored.

A high-dose injection of rAAV2/1-CMV-GH1 (4.0x10^{11} viral particles) induced a significant increase in both serum glucose and insulin (p<0.05), although HMW adiponectin was significantly increased in the HD (p<0.05) group. As a result, a notable increase in HOMA-IR was seen in the HD group (p<0.01). The decrease in body insulin sensitivity induced by chronic exogenous GH may increase the potential risk of insulin resistance. In this case, the increase in HMW adiponectin may be insufficient to counterbalance the decrease in body insulin sensitivity induced by high-dose GH. Meanwhile, the serum adiponectin level is inversely associated with body fat percentage (4). Chronic exogenous GH treatment decreased the body fat percentage and increased body weight, which may in turn affect serum adiponectin and insulin levels in mice. The rise in serum HMW adiponectin level and $S_a$ may also be due directly to the change in body weight.

### Table III. Serum adiponectin, glucose and insulin levels after the injection of rAAV-CMV-GH1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean value ± SD for each group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Ad (µg/ml)</td>
</tr>
<tr>
<td>Ctl</td>
<td>9.10±0.46</td>
</tr>
<tr>
<td>Plac</td>
<td>9.08±0.56</td>
</tr>
<tr>
<td>LD</td>
<td>9.30±0.61</td>
</tr>
<tr>
<td>MD</td>
<td>9.37±0.55</td>
</tr>
<tr>
<td>HD</td>
<td>9.48±0.62</td>
</tr>
</tbody>
</table>

SD, standard deviation; total Ad, total adiponectin; HMW Ad, HMW adiponectin; HOMA-IR, homeostasis model assessment of insulin resistance; Ctl, control; Plac, placebo; LD, low-dose; MD, middle-dose; HD, high-dose. \(a\)p<0.05 and \(b\)p<0.01 vs. the Ctl group. N=6 for each group.
composition induced by the chronic exogenous GH level. It appears that the molecular mechanisms by which GH affects the expression of adiponectin and the adiponectin receptors is suppressed by the dosage of rAAV2/1-CMV-GH1 treatment.

In conclusion, our results showed that chronic exogenous GH up-regulates hepatic adipor2 gene expression and increases both HMW and S forms of adiponectin. This may decrease body insulin sensitivity and therefore increase the risk of insulin resistance.

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References


