Adiponectin in mice with altered GH action: links to insulin sensitivity and longevity?

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Abstract

Adiponectin is positively correlated with longevity and negatively correlated with many obesity-related diseases. While there are several circulating forms of adiponectin, the high-molecular-weight (HMW) version has been suggested to have the predominant bioactivity. Adiponectin gene expression and cognate serum protein levels are of particular interest in mice with altered GH signaling as these mice exhibit extremes in obesity that are positively associated with insulin sensitivity and lifespan as opposed to the typical negative association of these factors. While a few studies have reported total adiponectin levels in young adult mice with altered GH signaling, much remains unresolved, including changes in adiponectin levels with advancing age, proportion of total adiponectin in the HMW form, adipose depot of origin, and differential effects of GH vs IGF1. Therefore, the purpose of this study was to address these issues using assorted mouse lines with altered GH signaling. Our results show that adiponectin is generally negatively associated with GH activity, regardless of age. Further, the amount of HMW adiponectin is consistently linked with the level of total adiponectin and not necessarily with previously reported lifespan or insulin sensitivity of these mice. Interestingly, circulating adiponectin levels correlated strongly with inguinal fat mass, implying that the effects of GH on adiponectin are depot specific. Interestingly, rbGH, but not IGF1, decreased circulating total and HMW adiponectin levels. Taken together, these results fill important gaps in the literature related to GH and adiponectin and question the frequently reported associations of total and HMW adiponectin with insulin sensitivity and longevity.

Key Words

- ▶ adiponectin
- ▶ high molecular weight adiponectin
- ▶ growth hormone receptor
- ▶ growth hormone
- ▶ growth hormone deficiency
- ▶ growth hormone antagonist

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Introduction

Adipose tissue, once considered a simple triglyceride storage organ, is now known as an active endocrine organ, which releases many adipokines. The most

abundant adipokine synthesized and secreted from white adipose tissue (WAT) is adiponectin (Maeda *et al.* 1996). In humans, adiponectin has been shown to decrease in

concentration as fat mass increases, unlike most other adipokines (Arita *et al.* 1999, Kern *et al.* 2003). Thus, adiponectin is considered a beneficial adipokine, showing negative correlations with many age- and obesity-related diseases and a positive correlation with longevity and insulin sensitivity (McKee Alderman *et al.* 2010, Arai *et al.* 2011). Adiponectin has also been linked to healthy phenotypes in mice, as adiponectin injections have been shown to reverse or decrease insulin resistance in several obesity prone mouse lines (Berg *et al.* 2001, Yamauchi *et al.* 2001). Further, increased adiponectin levels are associated with extended longevity in mice. That is, transgenic mice expressing high levels of human adiponectin have increased longevity (Otabe *et al.* 2007), and various mice with extended longevity have increased adiponectin (Flurkey *et al.* 2001, Combs *et al.* 2003, Berryman *et al.* 2004, Wang *et al.* 2006, 2007, Arumugam *et al.* 2007, del Rincon *et al.* 2007, Alderman *et al.* 2009, Qiao *et al.* 2011).

Adiponectin is of particular interest in mice with altered GH action, as these animals exhibit alterations in obesity, insulin sensitivity, and lifespan that break the typical patterns; that is, mice with increased GH signaling tend to be lean, insulin resistant, and short-lived, while mice with low GH signaling tend to be obese, insulin sensitive, and long-lived (Coschigano *et al.* 2000, 2003, Berryman *et al.* 2004, 2011, Liu *et al.* 2004, Olsson *et al.* 2005, List *et al.* 2009). Previous studies have shown that adult GH receptor (*Ghr*^{-/-}) knockout mice, which have essentially no GH signaling, and GH antagonist (GHA) transgenic mice, which have a reduction in GH signaling, have increased circulating total adiponectin (Berryman *et al.* 2004, Nilsson *et al.* 2005, Laron & Kopchick 2011, Masternak *et al.* 2012). Other mouse strains with decreased GH signaling, such as Ames dwarf, Snell dwarf, Lit/Lit, and Sma1 mice, also have elevated total adiponectin levels (Flurkey *et al.* 2001, Combs *et al.* 2003, Berryman *et al.* 2004, Wang *et al.* 2006, 2007, Arumugam *et al.* 2007, del Rincon *et al.* 2007, Alderman *et al.* 2009). By contrast, bGH transgenic mice have an increase in GH signaling and a decrease in circulating total adiponectin (Berryman *et al.* 2004, Nilsson *et al.* 2005, del Rincon *et al.* 2007, Wang *et al.* 2007). These mouse models of altered GH action have human clinical analogs. *Ghr*^{-/-} mice are analogous to humans affected by Laron syndrome, who have a mutation in the GH receptor (Laron & Kopchick 2011). Like *Ghr*^{-/-} mice, individuals with Laron syndrome have increased adiponectin (Kanety *et al.* 2009). Similarly, bGH transgenic mice are larger than controls with

high serum levels of insulin-like growth factor 1 (IGF1) and are comparable to untreated human acromegalic individuals (Olsson *et al.* 2005). As in bGH mice, adiponectin levels in individuals with acromegaly are decreased (Lam *et al.* 2004).

There are several unresolved issues related to adiponectin in mice with altered GH action. First, *Ghr*^{-/-}, GHA, and bGH mice exhibit major changes in the amount of WAT with advancing age, which may influence adipokine secretion over time (Coschigano *et al.* 2000, 2003, Bartke 2003, Magon 2009, Palmer *et al.* 2009, Berryman *et al.* 2010, List *et al.* 2011). Secondly, little is known about the relative secretory contribution of individual WAT depots to circulating adiponectin in these mice that are known to have preferential accumulation of fat in specific depots. Thirdly, no previous studies on mice with altered GH action have differentiated between the total and high-molecular-weight (HMW) forms of adiponectin. As the HMW form is considered to have the predominant bioactivity in terms of insulin sensitivity (Pajvani *et al.* 2004, Fisher *et al.* 2005, Hara *et al.* 2006, Kadowaki *et al.* 2006, Lara-Castro *et al.* 2006, Trujillo & Scherer 2006, von Eynatten *et al.* 2008, Wang *et al.* 2008), studies that have assessed only total adiponectin levels may be misleading as they do not reflect the abundance of the more bioactive form. Finally, measurement of adiponectin levels in other mouse models that have more moderate alterations in the GH axis would support the link between GH action and circulating adiponectin levels. Thus, the current study includes several additional models: HiGH mice, which have a moderate (two- to threefold) increase in circulating GH; AOiGHD mice, which have adult-onset isolated GH deficiency; and Ames dwarf mice, which are deficient in GH, TSH, and prolactin (Masternak *et al.* 2010, Gahete *et al.* 2011, Luque *et al.* 2011). Additionally, inclusion of mice injected with GH or IGF1 allows us to determine the differential effects of GH and IGF1 on circulating adiponectin. Therefore, the major goals of this study were to determine the circulating levels of total and HMW adiponectin throughout life in bGH, GHA, and *Ghr*^{-/-} mice, evaluate circulating adiponectin in other mouse lines with altered GH action, determine the depot of origin of normal and increased circulating adiponectin, examine the effects of acute GH exposure to genetically normal mice that have not been chronically exposed to altered GH levels, and establish differential effects of GH and IGF1 on circulating adiponectin.

Materials and methods

Animals and sample collection

bGH, GHA, and *Ghr*^{-/-} mice For the majority of experiments, three genetically modified animal models were used: GH receptor knockout (*Ghr*^{-/-}) mice, GHA mice, and bGH transgenic mice, all of which have been previously described (Chen *et al.* 1991, Zhou *et al.* 1997, Berryman *et al.* 2004). These three mouse strains were either produced on a pure C57BL/6J background or backcrossed more than ten generations into C57BL/6J mice. These animals were bred and housed up to four animals per cage at the Ohio University animal facility with a 10 h light:14 h darkness cycle. After weaning, mice had *ad libitum* access to standard rodent chow (ProLab RMH 3000, PMI Nutrition International, Inc., St Louis, MO, USA) throughout the study. Animals were fasted for 12 h overnight before whole blood collection from the tail tip using heparinized capillary tubes. Blood was centrifuged at 4000 g for 10 min at 4 °C to separate and isolate plasma, which was stored at -80 °C until time of analysis. Tissue samples were dissected after killing by cervical dislocation, flash-frozen in liquid nitrogen, and stored at -80 °C until further processing. Animal protocols for these mice were approved by Ohio University's Institutional Animal Care and Use Committee.

Administration of GH and IGF1 C57BL/6J mice were purchased from The Jackson Laboratory at 4 weeks of age and put onto a high-fat (HF) diet (D12492; Research Diets, New Brunswick, NJ, USA). Based on previous studies, it is known that adiposity differences in chow-fed GH-injected mice are minimal, while effects of GH injection on body composition are more robust in HF-fed mice (List 2010, Ding *et al.* 2011). Additionally, the changes in circulating adiponectin caused by the relatively short-term exposure to GH/IGF1 in injected mice are expected to be less dramatic than the changes seen in mice with genetic modulations in the GH axis. Thus, to make potential differences in adipokine levels between groups more apparent, this study used HF-fed mice for GH and IGF1 injections. Mice were kept on a HF diet for 16 weeks and housed two to three per cage with a 10 h light:14 h darkness cycle. Purified rbGH, a gift from Monsanto (St Louis, MO, USA), and rhIGF1, a gift from Tercica, Inc. (Brisbane, CA, USA), were diluted in PBS. At 5 months of age, mice were injected s.c. twice daily with 5.0 µg rbGH/g body weight, 2.5 µg IGF1/g body weight, or both for 3 weeks. Whole blood was collected through ocular

bleeding after a 12-h overnight fast. Plasma was isolated as described earlier. GH and IGF1 injection studies were conducted at Ohio University and were approved by Ohio University's IACUC.

HiGH and AOiGHD mice Mice with elevated endogenous GH levels (referred to as HiGH mice; Gahete *et al.* 2011) show a selective knockout of both the *Insr* and *Igf1r* only in the GH-producing cells of the anterior pituitary, which results in a threefold increase in circulating GH levels, a 20% increase in circulating IGF1, and a modest (10%) increase in body weight compared with controls. Floxed mice served as controls. AOiGHD mice (Luque *et al.* 2011) have a selective destruction of the Cre-inducible diphtheria toxin receptor (iDTR) expressing GH-producing cells of the anterior pituitary following treatment with diphtheria toxin (DT) at 12 weeks of age, which leads to a 60 and 20% reduction in circulating GH and IGF1 levels respectively. DT-treated iDTR mice, not expressing the rGHPcre transgene, served as controls. Both HiGH and AOiGHD mice were created on a C57BL/6 background.

HiGH mice, AOiGHD mice, and their respective controls were housed two to four mice per cage with a 12 h light:12 h darkness cycle. Mice were weaned onto standard rodent chow (Formulab Diet, Purina Mills, Inc., Richmond, IN, USA). Based on previous studies, it is known that HiGH and AOiGHD mice show few phenotypic differences from controls when fed a low-fat diet. Thus, to make potential differences between groups more apparent, at 12 weeks of age, mice were switched to a HF diet (HF-fat, #12492; Research Diets). *Ad libitum* access to food and water was allowed throughout the study. At 6.5 months of age, mice were killed by decapitation and trunk blood and tissues were collected under fed conditions. The HiGH and AOiGHD studies were conducted at the Jesse Brown VA Medical Center (JBVAMC; Chicago, IL, USA) with the approval of the JBVAMC and University of Illinois at Chicago IACUC.

Ames dwarf mice Ames dwarf mice (*Prop1^{df}/Prop1^{df}*) and normal (+/+ or +/*Prop1^{df}*) littermate controls were produced on a heterozygous genetic background and housed four to five per cage with a 12 h light:12 h darkness cycle. To date, there is no evidence that heterozygous animals for Ames dwarfism differ from WT animals. Mice were fed a standard chow *ad libitum* (Rodent Laboratory Chow 5001; LabDiet, PMI Feeds, Inc., St Louis, MO, USA). At 6 months of age, fasted whole blood was collected via cardiac puncture following isoflurane administration at Southern Illinois University. Animal protocols for these

mice were approved by the Southern Illinois University Animal Care and Use Committee.

Body composition

A quantitative NMR machine was used to analyze body composition within 1 week of blood collection in bGH, GHA, and *Ghr*^{-/-} mice (Minispec, Bruker Optics, Billerica, MA, USA or Echo MRI whole-body composition analyzer; Echo Medical Systems, Houston, TX, USA).

Leptin and insulin levels

Leptin was quantified using the Quantikine Mouse Leptin Immunoassay distributed by R&D Systems (Minneapolis, MN, USA; catalog number SMOB00). Insulin was quantified using the Mouse Insulin ELISA distributed by ALPCO Diagnostics (Salem, NH, USA; catalog number 80-INSMS-E10). The intra-assay and interassay coefficients of variation (CV) were 5.3 and 7.2% respectively for insulin and 4.3 and 7.8% for leptin.

Total and HMW adiponectin in serum or plasma

HMW and total adiponectin were quantified using the Adiponectin (mouse) HMW and Total ELISA manufactured by Sekisui Medical Company (Ibaraki, Japan) and distributed by ALPCO Diagnostics (Salem, NH, USA; catalog number 47-ADPMS-E01) (Ebinuma & Matsuo 2009). Both total and HMW adiponectin are quantified on the same plate alongside a single standard curve. Total adiponectin, which includes HMW (12-mer and 18-mer), MMW (hexamer), and LMW (trimer and albumin-bound trimer), was measured without any modification to their respective structures. HMW adiponectin is measured following pretreatment of samples with a specific protease that digests hexameric and trimeric adiponectin multimers. All samples were analyzed in duplicate. Tests were performed to verify that increased lipid in the serum or plasma did not interfere with the accuracy of the assay (data not shown). The intra-assay and interassay CV were 2.7 and 3.5% respectively.

Tissue adiponectin protein content

Approximately 50 mg WAT from 6-month-old *Ghr*^{-/-} and WT mice were homogenized in 150 µl PBS using a probe sonicator. Samples were centrifuged at 5000 g and the fat cake was removed from the remaining homogenate. Homogenates were stored at -80 °C until analysis with a

Table 1 Primers for mouse *Adipoq* and reference genes used for quantitative real-time PCR. All primers are listed 3'-5'

Gene	Forward primer	Reverse primer	Product length (bp)
<i>Adipoq</i>	CTCTCCTGTTCTCT-TAATCCT	ACCAAGAA-GACCTGCATCTC	218
<i>Rps3</i>	ATCAGAGAGTT-GACCGCAGTT	AATGAACCGAAG-CACACCATA	183
<i>B2m</i>	CTGGTCTTTCTA-TATCCTGGCT	CATGTCTCGATCC-CAGTAGAC	121

Quantikine Mouse Adiponectin/Acrp30 Immunoassay (R&D Systems; catalog number MRP300). Total protein content of the tissues was measured using the Bio-Rad Protein Assay, following the manufacturer's specifications (Bio-Rad; catalog number 500-0006).

RNA isolation and real-time PCR

RNA was isolated from inguinal and epididymal WAT from 2-month-old bGH, 12-month-old *Ghr*^{-/-}, and 18-month-old GHA mice and their WT littermate controls using TRIzol Reagent following the manufacturer's protocol (Life Technologies; catalog number 15596-026). Sample selection was based on mouse availability and varying lifespan of these animals. cDNA was synthesized using Maxima First-Strand cDNA Synthesis Kits and quantitative real-time PCR was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, Waltham, MA, USA). *Adipoq* expression in WAT was normalized to beta 2 microglobulin (*B2m*) and ribosomal protein S3 (*Rps3*). In our laboratory, these housekeeping genes have been determined to be the most stable among nine housekeeping genes for WAT (Xinyue Wang, unpublished results). The sequences of the primers used are shown in Table 1. Analysis of all qPCR data was performed with Biogazelle qbasePLUS (Biogazelle NV, Zwijnaarde, Belgium).

Statistical analysis

All data are represented as mean ± S.E.M. A multivariate two-way ANOVA with Tukey's honestly significant difference *post hoc* test (SPSS 17.0, IBM SPSS Statistics, Somers, NY, USA) was used to identify differences among groups. Follow-up *t*-tests and one-way ANOVAs were used to determine specific genotype or tissue differences. Pearson correlations were used to analyze the relationship between insulin, leptin, tissue weights, or body composition and circulating adiponectin levels. Differences were considered significant at *P* < 0.05.

Results

Circulating HMW and total adiponectin in GHA, *Ghr*^{-/-}, and bGH mice

Circulating concentrations of adiponectin (HMW and total) were measured at several time points for bGH, *Ghr*^{-/-}, and GHA mice and their controls, providing a life-long profile (Fig. 1A, B, C, D, E and F). For bGH animals, 2-, 6-, 9-, and 14-month-old mice were used; for GHA, 3.2-, 6-, 12-, and 16.5-month-old mice were used; and for *Ghr*^{-/-}, 6-, 12-, and 24-month-old mice were used. Sample selection was based on mouse availability and varying lifespan of these animals.

The life-long total adiponectin profile shows that circulating levels of total adiponectin were increased in *Ghr*^{-/-} and GHA mice compared with WT controls at every time point (Fig. 1B and C). In GHA mice, total adiponectin increased over life, reaching levels comparable to *Ghr*^{-/-} mice by 1 year of age. Total adiponectin was significantly decreased in bGH mice compared with WT at most ages,

but at 14 months of age, the difference between bGH and WT mice was no longer significant (Fig. 1A).

HMW adiponectin has not been previously reported at any age in bGH, GHA, or *Ghr*^{-/-} mice. Circulating total and HMW adiponectin levels were reported previously for 12- and 24-month-old WT mice (Sackmann-Sala *et al.* 2012a,b). Like total adiponectin, circulating HMW adiponectin was increased in *Ghr*^{-/-} mice compared with WT controls at all time points (Fig. 1F). HMW adiponectin was also increased in GHA mice compared with WT at 3.2, 12, and 16.5 months, but the increase was not significant at 6 months of age (Fig. 1E). In bGH mice, HMW adiponectin was significantly decreased in 2-month-old mice compared with WT, but there was no significant difference at older ages (Fig. 1D).

The ratio of HMW to total adiponectin has been suggested to be a valuable way to report HMW adiponectin findings, as it reflects the preferential change in HMW adiponectin production, rather than a change in all MW forms (Waki *et al.* 2003). The ratio of HMW to total

Figure 1

Circulating total adiponectin, HMW adiponectin, and the HMW/total adiponectin ratio in bGH, GHA, and *Ghr*^{-/-} mice and their matched WT controls. Data are presented as mean $\pm$ S.E.M. *Significant difference from WT at a given age as determined by ANOVA. (A, B, C, D, E and F) Total and

HMW adiponectin were measured side-by-side by ELISA in bGH (A and D), GHA (B and E), and *Ghr*^{-/-} (C and F) mice. (G, H and I) The ratio of HMW adiponectin to total adiponectin was calculated for bGH (G), GHA (H), and *Ghr*^{-/-} (I) mice.

Table 2 Significant Pearson correlations in bGH, GHA, and *Ghr*^{-/-} mice and their WT controls across lifespan

Factors	<i>r</i>	<i>n</i>	<i>P</i>
Total adiponectin×leptin	0.507	190	<0.001
Total adiponectin×insulin	-0.175	190	0.013
Total adiponectin×total fat	0.409	183	<0.001
Total adiponectin×inguinal	0.563	123	<0.001
HMW adiponectin×leptin	0.516	190	<0.001
HMW adiponectin×total fat	0.426	183	<0.001
HMW adiponectin×inguinal	0.490	123	<0.001
HMW/total ratio×leptin	0.239	190	0.001
HMW/total ratio×total fat	0.276	183	<0.001

adiponectin in *Ghr*^{-/-} mice was significantly increased compared with WT in 6-month-old mice but was not different in 1- and 2-year-old *Ghr*^{-/-} mice (Fig. 1I). GHA mice had significantly increased HMW to total adiponectin ratios at 12 and 16.5 months of age but not at 3.2 or 6 months (Fig. 1H). bGH mice and matched WT controls had similar HMW/total adiponectin ratios throughout life (Fig. 1G).

Correlational analysis

Correlational analysis was performed on all bGH, GHA, and *Ghr*^{-/-} mice at all time points used in this study. Significant correlations are summarized in Table 2. The analysis revealed a strong positive ($P \leq 0.001$) correlation of total, HMW, and HMW/total adiponectin to circulating leptin concentrations. Total adiponectin was negatively correlated with circulating insulin concentrations. Correlation of total fat mass and individual depot masses with adiponectin levels revealed a strong positive ($P < 0.001$) correlation of total, HMW, and HMW/total adiponectin with total fat mass. Of note, total and HMW adiponectin were only significantly correlated with the mass of the inguinal fat pad ($P \leq 0.002$) but not with the mass of the epididymal or mesenteric depots.

Tissue adiponectin content

Owing to the dramatic increase in circulating adiponectin and unique WAT distribution in *Ghr*^{-/-} mice with a preferential enlargement of the inguinal (subcutaneous) depot, we measured the adiponectin protein content of three adipose depots (mesenteric, inguinal, and epididymal) in 6-month-old *Ghr*^{-/-} mice to determine the source of the increased circulating adiponectin (Berryman *et al.* 2010). Several methods of normalization of these data were considered. When normalized to total protein content, there were no differences

between genotypes in adiponectin content, although there was a depot difference in *Ghr*^{-/-} mice. Specifically, mesenteric WAT had significantly decreased adiponectin content (0.462 ± 0.014 ng adiponectin/ μ g protein) when compared with epididymal and inguinal WAT (0.879 ± 0.009 and 0.956 ± 0.130 ng adiponectin/ μ g protein respectively) in *Ghr*^{-/-} but not in WT mice. However, normalization to total protein content does not take into account the dramatic differences in depot size and body size (therefore blood volume). To account for these differences, we normalized tissue adiponectin content to depot weight/body weight. With this method of normalization, there was a significant decrease in adiponectin in the epididymal depot and a significant increase in the inguinal depot of *Ghr*^{-/-} mice when compared with WT littermate controls (Fig. 2).

Adiponectin mRNA expression

To further elucidate the potential source of changes in circulating adiponectin, adiponectin (*Adipoq*) expression was measured at a single time point for three models: 12-month-old *Ghr*^{-/-} mice, 18-month-old GHA mice, and 2-month-old bGH mice and each group's WT controls. It is important to note that due to the age differences between the genotypes used in this series of experiments, expression data of a given mouse strain may be compared only to its corresponding WT controls and not between transgenic lines. Adiponectin expression did not differ from WT in epididymal or inguinal WAT in 12-month-old *Ghr*^{-/-}, 18-month-old GHA, or 2-month-old bGH mice (data not shown). No significant effect of

Figure 2

Tissue adiponectin content. Total adiponectin protein in mesenteric (Mes), epididymal (Epi), and inguinal (Ing) adipose tissue from 6-month-old *Ghr*^{-/-} and WT adipose tissue homogenates was measured by ELISA. Data are presented as mean $\pm$ S.E.M. Within a genotype or depot, bars without a common letter are statistically different as determined by ANOVA. $n = 9-10$.

Figure 3

Expression of adiponectin in the epididymal (Epi) and inguinal (Ing) adipose depots of 2-month-old bGH mice and their WT littermate controls. Data are presented as mean $\pm$ S.E.M. Within a genotype or depot, bars without a common letter are statistically different as determined by ANOVA and *post hoc* tests.

depot was found in GHA or *Ghr*^{-/-} mice at the ages used. However, in bGH mice but in WT mice, inguinal WAT had significantly lower adiponectin expression than epididymal WAT (Fig. 3).

Circulating HMW and total adiponectin in Ames, HiGH, and AOiGHD mice

To confirm the results found in bGH, GHA, and *Ghr*^{-/-} mice, circulating total and HMW adiponectin was also measured at about 6 months of age in three additional models of modified GH signaling (Table 3): HiGH mice, which have a more modest elevation in GH/IGF1 axis than bGH mice; AOiGHD mice, which have an adult onset GH deficiency; and Ames dwarf mice, which lack GH as well as prolactin and TSH. HiGH mice on a HF diet had a significant decrease in total and HMW adiponectin in circulation when compared with controls. However, there was no significant difference between AOiGHD mice and

their controls. Ames dwarf mice showed a significant increase in total and HMW adiponectin when compared with phenotypically normal heterozygous controls. No difference in the HMW/total adiponectin ratio was observed for HiGH, AOiGHD, or Ames dwarf mice compared with their respective controls.

Circulating HMW and total adiponectin in GH- and IGF1-injected mice

To determine the differential effects of GH and IGF1 and to examine the effects of acute GH administration, total and HMW adiponectin was measured in HF-fed mice injected with GH and/or IGF1 (Fig. 4). Mice injected with GH or IGF1 twice daily experienced a significant decrease in total adiponectin (Fig. 4A). Mice injected with both IGF1 and GH had circulating total adiponectin levels lower than those injected with either hormone alone, indicating an additive effect of GH and IGF1 (Fig. 4A). HMW adiponectin was significantly decreased in mice injected with GH compared with controls, but was not changed in mice injected with IGF1. Mice injected with both IGF1 and GH showed a decrease in HMW adiponectin similar to those injected with only GH. Regarding the ratio of HMW to total adiponectin, mice injected with GH or GH and IGF1 had significantly decreased ratios when compared with controls (Fig. 4B).

Discussion

Total adiponectin levels have been previously reported to be elevated in a few strains of mice with decreased GH action, while circulating total adiponectin has been reported to decrease in mice with increased GH action (Berryman *et al.* 2010). Our results confirm this. All mouse models of increased GH action, bGH, HiGH, and GH-injected mice have significantly decreased total serum adiponectin levels when compared with their respective

Table 3 Total and HMW adiponectin in additional genotypes with altered GH action. *n* = 6 for AOiGHD and HiGH mice, while *n* = 20 for Ames dwarfs

Model	Age (months)	Diet	Genotype	Total (ng/ml)	HMW (ng/ml)	Ratio
Ames dwarf	6.0	Chow	Control	8526 $\pm$ 625	3150 $\pm$ 530	0.35 $\pm$ 0.04
			Dwarf	20 206 $\pm$ 886 ^a	8446 $\pm$ 520 ^a	0.42 $\pm$ 0.02
AOiGHD	6.5	HF	Control	32 068 $\pm$ 4383	7906 $\pm$ 638	0.27 $\pm$ 0.04
			AOiGHD	24 535 $\pm$ 2858	6538 $\pm$ 720	0.30 $\pm$ 0.07
HiGH	6.5	HF	Control	26 153 $\pm$ 2286	4495 $\pm$ 143	0.18 $\pm$ 0.01
			HiGH	16 686 $\pm$ 578 ^a	3259 $\pm$ 405 ^a	0.24 $\pm$ 0.03

^aSignificant difference from controls determined via ANOVA.

Figure 4

Circulating total adiponectin, HMW adiponectin, and the HMW/total adiponectin ratio in HF-fed mice injected with GH, IGF1, or a combination of GH and IGF1 (Combo). Data are presented as mean $\pm$ S.E.M. Within a parameter, bars without a common letter are statistically different as determined by ANOVA. Circulating total adiponectin was measured by ELISA in mice fed a HF diet and injected with GH, IGF1, or a combination of the two (A). The ratio of HMW adiponectin to total adiponectin in mice fed a HF diet and injected with GH, IGF1, or both (B).

controls. By contrast, most mouse lines with decreased GH action (GHA, *Ghr*^{-/-}, and Ames dwarf mice) have increased circulating total and HMW adiponectin. These findings fit nicely with data reported for individuals with acromegaly who have reduced serum adiponectin and Laron syndrome patients who have increased total and HMW adiponectin (Lam *et al.* 2004, Kanety *et al.* 2009). In our study, the only exception to this trend was with AOiGHD mice, which had no significant difference in total and HMW adiponectin levels despite reduced GH signaling. This exception may, in part, be explained by the late onset and relatively small reduction in circulating GH and/or by an effect of the HF diet. AOiGHD mice have a 60% reduction in GH signaling that occurs only after maturity is reached. By contrast, *Ghr*^{-/-}, Ames dwarf, and GHA mice have greater reductions in GH signaling throughout life. Perhaps adiponectin in HF-fed AOiGHD remains unchanged due to the opposing effects of diet-induced obesity (DIO), which tends to reduce adiponectin, and reduced GH action, which tends to increase adiponectin (Louer *et al.* 2012). Thus, the partial reduction in GH might not be sufficient to relieve the inhibitory effect of DIO on adiponectin.

The bGH, GHA, and *Ghr*^{-/-} mice have been previously shown to have significant differences in lifespan and adiposity (Coschigano *et al.* 2000, 2003, Bartke 2003, Magon 2009, Palmer *et al.* 2009, Berryman *et al.* 2010). Additionally, factors that affect and are affected by adipokine concentrations (e.g. adiposity and glucose tolerance) often vary with age (Arai *et al.* 2011, Schautz *et al.* 2012). Considering the variation in lifespan of these mice, comparing adipokine levels at a single time point is not appropriate, as a given chronological age may represent a different biological age in each genotype. For example, 1 year is nearing the end of a bGH mouse's lifespan, while it is only a quarter of a *Ghr*^{-/-} mouse's lifespan and about half way through the lifespan of a WT mouse. Therefore, it might be expected that total and HMW adiponectin would vary with advancing age. Though it has been reported that adiposity increases throughout life in *Ghr*^{-/-} and WT mice (Berryman *et al.* 2004, 2010), our data show that total adiponectin levels remain relatively constant in adult mice on a chow diet. Unlike total adiponectin, circulating levels of HMW adiponectin and the HMW/total adiponectin ratio varied with age. These data highlight the importance of careful age selection when designing an experiment investigating HMW adiponectin.

No previous studies on mice with altered GH action have differentiated between total and HMW forms of adiponectin. As different forms of adiponectin may influence its bioactivity and because these mice exhibit extreme differences in longevity, insulin sensitivity, and body composition, evaluating HMW adiponectin may contribute to defining the mechanisms responsible for the differences in longevity and insulin sensitivity in these mice (Coschigano *et al.* 2000, 2003, Berryman *et al.* 2004, 2011, Liu *et al.* 2004, Olsson *et al.* 2005). The HMW form of adiponectin is thought to be the most bioactive in terms of insulin sensitivity as decreases in HMW adiponectin are more closely correlated with HOMA-IR, obesity, metabolic syndrome, and insulin resistance than total adiponectin (Waki *et al.* 2003, Hara *et al.* 2006). The HMW/total adiponectin ratio has been suggested to be an appropriate way to report adiponectin complex distribution (O'Leary *et al.* 2007). Additionally, *db/db* mice, which have severe insulin resistance, have similar total adiponectin levels to *db/+* littermates, yet have a dramatic decrease in specifically the HMW form, resulting in decreases in HMW/total adiponectin ratio. The current study shows that circulating HMW adiponectin follows nearly the same pattern as total adiponectin at most time points and does not provide additional insight into insulin sensitivity

or longevity. These data suggest that, at least in the context of altered GH action, HMW adiponectin and HMW/total adiponectin ratio may not be strongly linked to insulin sensitivity.

As IGF1 levels are generally reflective of GH levels and many of the effects of GH act via IGF1, it is often difficult to differentiate between the effects of GH and IGF1. To shed light on this matter, we measured circulating total and HMW adiponectin in mice injected with GH, IGF1, or both. Injection with GH significantly reduced circulating total adiponectin. Interestingly, this decrease in adiponectin is associated with an improvement in many metabolic parameters, as injection of the same dose of GH has previously been shown to decrease fat mass and liver triglycerides and improve glucose tolerance (List *et al.* 2009). Injection with IGF1 also significantly reduced the circulating total adiponectin, though not to the extent of GH injection. Thus, we hypothesize that the actions of GH on total adiponectin levels are at least partially dependent on IGF1 action, as IGF1 injection alone lowers circulating adiponectin. Injection with both GH and IGF1 induced a greater reduction in total adiponectin than either hormone alone, implying an additive effect of GH and IGF1 on total adiponectin. Injection with IGF1 did not change the circulating levels of HMW adiponectin or the HMW/total adiponectin ratio. These data show that while both GH and IGF1 affect total adiponectin, only GH modulates HMW adiponectin. Of note, all other mouse lines used in this study were transgenic or had natural mutations resulting in chronic alterations to GH activity. GH injections into WT mice show that an acute increase in GH action also negatively regulates circulating adiponectin levels.

The strong positive correlations between adiponectin and total fat mass suggest that these changes may be due to modulation of WAT mass by GH. As GH is lipolytic, mice with increased GH action tend to have lower WAT mass, while mice with decreased GH action have increased adipose mass (Berryman *et al.* 2011). Mice with low GH action show a healthy obese phenotype, escaping the negative health consequences of obesity. We hypothesize that this is through the healthy expansion of WAT without the inflammation that typically accompanies fat expansion. To support this, increased GH signaling has been associated with increased inflammation, while *Ghr*^{-/-} mice have been shown to have lower levels of circulating inflammatory cytokines (Hattori 2009, Masternak *et al.* 2012). Likewise, inflammatory markers such as MCP1, IL6, and IL10 have been reported to increase with increased duration of

HF feeding and progression of DIO (Stanton *et al.* 2011). We propose that the often-reported reduction of adiponectin in WT HF-fed DIO mice may be due to the inflammation associated with DIO rather than simply the increase in adipose mass. This hypothesis could also explain some of the differential effects of a HF diet on adiponectin production, as the circulating adiponectin levels would depend on both the level of inflammation and the increase in adipose mass, which could vary greatly between studies.

While it is often reported that mice with low levels of GH signaling have increased circulating adiponectin, the adipose depot responsible for the increased adiponectin is not known. As the *Ghr*^{-/-} and GHA mice used in this study have a unique adipose distribution with a preferential enlargement of subcutaneous WAT and this depot is thought to have metabolically beneficial effects, it is possible that the increased adiponectin is simply due to the increased mass of the subcutaneous (inguinal) depot (Berryman *et al.* 2004). Additionally, the correlation of total and HMW adiponectin levels with inguinal adipose depot weights ($P \leq 0.001$) imply that this depot may be contributing more to the differences in circulating adiponectin levels. One previous study has attempted to determine the origin of elevated adiponectin in mice with decreased GH signaling. Masternak *et al.* (2012) performed visceral fat removal surgeries on *Ghr*^{-/-} and WT mice and found that circulating adiponectin was decreased after removal of epididymal and perinephric WAT in *Ghr*^{-/-} but not in WT mice, implying that visceral WAT is a primary contributor to elevated circulating adiponectin in *Ghr*^{-/-} mice. This study also found that tissue adiponectin protein content normalized to total protein content is higher in epididymal WAT than in inguinal WAT. However, we found no significant genotype or depot differences in tissue adiponectin content when normalized in this manner. As mentioned previously, normalization to total protein content does not take into consideration the differences in depot and body size. In order to compensate for these differences, the current study normalized data to depot/body weight. When normalized in this manner, adiponectin content was increased in the inguinal depot of *Ghr*^{-/-} mice. While these results appear to be contradictory, the *Ghr*^{-/-} mice used in the visceral fat removal study have a different genetic background (129Ola/BALB/c, C3H/C57) and, on this background, all depots, not just the inguinal fat pad, are enlarged (Panici *et al.* 2009). Thus, the observed difference in tissue adiponectin content could be due to different background strains. Of note, adiponectin

protein content was not assessed in all fat depots in either study. It is possible that other fat pads, such as subscapular subcutaneous fat or brown fat, could contribute substantially to the adiponectin in circulation. Thus, it is difficult to make a strong conclusion without a more comprehensive assessment. Interestingly, measurement of mRNA expression of the adiponectin gene, *Adipoq*, revealed no genotype or depot differences in bGH, GHA, or *Ghr*^{-/-}, except that bGH mice had significantly lower expression of *Adipoq* in inguinal WAT when compared with epididymal. These data suggest that adiponectin is not regulated at the level of mRNA, but rather another level of regulation (translational/posttranslational/degradation) is likely responsible for the variation in circulating adiponectin levels.

While adiponectin has often been associated with increased longevity, this paper questions its importance in influencing longevity. Several models of extended longevity via alterations in the GH/IGF1 pathway have increased circulating adiponectin, including Snell dwarf, Ames dwarf, Dwarf Lit/Lit, *Sma1*, and *Ghr*^{-/-} mice (Flurkey *et al.* 2001, Combs *et al.* 2003, Berryman *et al.* 2004, Wang *et al.* 2006, 2007, Arumugam *et al.* 2007, del Rincon *et al.* 2007, Alderman *et al.* 2009). Calorically restricted mice, which are also long-lived, show increased circulating adiponectin levels (McKee Alderman *et al.* 2010, Qiao *et al.* 2011). Additionally, mice expressing high levels of human adiponectin show increased longevity (Otabe *et al.* 2007). Links between high adiponectin levels and healthy human aging have also been reported (Arai *et al.* 2006, Bik *et al.* 2006, Atzmon *et al.* 2008). However, data from this study question this, as GHA mice have a normal lifespan (Coschigano *et al.* 2003) yet extremely high levels of circulating total and HMW adiponectin (Fig. 1B and E). These data in GHA mice demonstrate that elevated adiponectin alone is not sufficient to extend longevity in mice with reduced GH signaling. However, it should also be noted that GHA mice develop severe obesity and do not have any decrease in lifespan, indicating that the high circulating adiponectin in these mice may offer some protection from age- and obesity-associated pathologies that would normally affect obese mice.

In conclusion, the current study provides greater insight into the interplay of GH and adiponectin. The association of low GH with high adiponectin and vice versa is strengthened in this study by the measurement of adiponectin in an array of mouse strains and at varied ages. Interestingly, GH appears to modulate both total and HMW adiponectin, while IGF1 modulates only total

adiponectin. The lack of consistent association of HMW adiponectin or HMW/total adiponectin ratio with insulin sensitivity sheds doubt on the relevance of these parameters as indicators of insulin sensitivity in the context of increased or decreased GH signaling. Additionally, data showing GHA mice have dramatically increased total and HMW adiponectin throughout life without extended longevity implies a need for further investigation of adiponectin's link with aging. Using this unique system in which obesity is dissociated from insulin resistance, the current study confirms a strong negative relationship between GH signaling and adiponectin levels but questions the role of HMW adiponectin in controlling insulin sensitivity or longevity in mice.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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