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Mouse models of growth hormone action and aging: A proteomic perspective

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Abstract

Growth hormone (GH) is a protein secreted by the anterior pituitary and circulates throughout the body to exert important actions on growth and metabolism. GH stimulates the secretion of insulin-like growth factor-I (IGF-I) which mediates some of the growth promoting actions of GH. The GH/IGF-I axis has recently been recognized as important in terms of longevity in organisms ranging from *C. elegans* to mice. For example, GH transgenic mice possess short lifespans while GH receptor null (GHR−/−) mice have extended longevity. Thus, the actions of GH (or IGF-I) or lack thereof impacts the aging process. In this review, we summarize the proteomic analyses of plasma and white adipose tissue in these two mouse models of GH action, i.e., GH transgenic and GHR−/− mice. At the protein level, we wanted to establish novel plasma biomarkers of GH action as a function of age and to determine differences in adipose tissue depots. We have shown that these proteomic approaches have not only confirmed several known physiological actions of GH, but also resulted in novel protein biomarkers and targets that may be indicative of the aging process and/or new functions of GH. These results may generate new directions for GH and/or aging research.

Introduction

Growth hormone (GH) is a hormone secreted by the anterior pituitary and circulates throughout the body to exert important actions on growth and metabolism. In most organs, GH stimulates the secretion of another hormone called insulin-like growth factor-I (IGF-I), which mediates several, but not all, of the actions of GH. The GH/IGF-I axis has recently been recognized as an important regulator of aging, as attenuation of the GH/IGF-I axis results in increased lifespan [1].

We have generated two mouse lines in which GH signaling is affected. The first is a transgenic mouse that expresses the bovine (b) GH gene, whose transcription is directed by the mouse metallothionein regulatory sequences [2]. This mouse is giant due to elevated levels of GH/ IGF-I and is hyperinsulinemic in spite of a lean phenotype. The bGH mouse has a lifespan of approximately 12–18 months and dies prematurely due to heart, liver, and

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Conflict of interest
None

kidney complications. The second mouse strain is one in which the GH receptor (R) gene has been disrupted [3]. This GHR−/− mouse is dwarf with low levels of IGF-I. Since there is no expression of the GHR, the mouse is GH insensitive or resistant, similar to human Laron Syndrome (LS) patients, who have mutations in the GHR gene [4]. The GHR−/− mouse is also obese with fat accumulating primarily in the subcutaneous depot. Surprisingly, this mouse has an extended lifespan of approximately 30–36 months. As such, the GHR−/− mouse is used by many for longevity studies.

In this review, we describe proteomic parameters of plasma and white adipose tissue in bGH and GHR−/− mice. In plasma we focused on putative biomarkers of GH action and aging. In individual adipose depots we searched for proteins affected by GH action and/or aging.

Endocrine parameters and aging issues in bGH and GHR−/− mice

Some of the primary actions of GH involve the regulation of glucose and lipid metabolism, as well as insulin sensitivity, which are relevant in view of GH's role in aging. Below we review the major metabolic characteristics of GH transgenic and GHR−/− mice. For the GH transgenic mice, we have included references that use other lines (denoted as 'GH transgenic' instead of 'bGH transgenic'), which show similar metabolic characteristics to our bGH mouse line.

GH transgenic mice

Insulin, glucose and insulin sensitivity—GH is a diabetogenic hormone, i.e., it inhibits the action of insulin [5, 6]. Thus, GH transgenic mice are giant with high insulin levels and normal or low glucose [7–15] (Table 1), suggesting these animals are insulin-resistant. Interestingly, they show normal or better glucose tolerance than WT littermates [12, 13, 16], at least when young, perhaps due to a robust pancreas [17] that secretes increased insulin in response to a glucose challenge [13]. Peripheral tissues such as liver, muscle and fat all have been shown to be insulin resistant [8, 9, 18], consistent with the whole-body insulin-resistant state due to the elevated levels of GH. Plasma adiponectin, an insulin sensitizer, is decreased, whereas resistin, tumor necrosis factor (TNF)-α and interleukin (IL)-6 are increased in these mice [15], which may contribute to their insulin resistance.

Body composition and lipid profile—Adult bGH mice have increased lean mass and reduced fat mass, consistent with GH's lipolytic effect on fat and anabolic action on muscle [2, 19]. They are also resistant to diet-induced obesity [13, 14]. Interestingly, bGH mice younger than 4 months have a greater normalized fat mass than WT mice [19] suggesting a role of GH in adipocyte differentiation and proliferation early in development. However, adult bGH mice have proportionally less subcutaneous fat than other adipose depots when compared to WT mice [14], suggesting a more pronounced lipolytic action of GH in subcutaneous fat [20].

GH transgenic mice have altered lipid profiles compared to controls (Table 1). Remarkably they have increased cholesterol including high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol [10, 12, 13, 15].

Premature aging and reduced lifespan—bGH mice live on average for ~ 14 months, significantly shorter than WT littermates, which live for ~ 2 years [2, 21, 22]. The pathophysiology of these mice shares several similarities with acromegalic humans, who have excess GH levels due to pituitary adenomas. Acromegalic patients often have kidney problems [23] and cardiovascular diseases [24]. Likewise, GH transgenic mice develop progressive glomerulosclerosis [25] and cardiovascular diseases [26–28]. In addition, GH

transgenic mice show liver inflammation and liver tumors at old ages [29–31]. One of the causes of premature aging may be increased oxidative stress in GH transgenic mice in the kidney, liver and carotid artery at later ages [32–34].

In summary, chronic GH excess promotes insulin resistance, abnormal pathology of multiple organs with increased oxidative stress. Interestingly, many of these adverse phenotypes are observed as the mice age, indicating the importance of studying GH action in the context of lifespan rather than at a single age.

GHR−/− mice

GHR−/− mice, which were generated in our laboratory in the mid-1990s [3], show many phenotypes and endocrine parameters analogous to Laron Syndrome (LS) patients, who have disrupted GH signaling due to mutations in the GHR gene. These characteristics in both mice and men have been recently reviewed [4, 35]. Below, we will summarize several phenotypes related to aging.

Insulin, glucose and insulin sensitivity—GHR−/− mice have low-normal levels of glucose [12, 14, 20, 36–41] with very low levels of insulin throughout their lifespan compared to WT mice [14, 20, 36–43]. Glucose tolerance, however, is impaired perhaps due to a decreased size of the pancreas [39, 44], which results in reduced insulin secretion relative to WT mice upon a glucose stimulus [40]. Regarding tissue specific insulin-induced signaling, the heart and muscle of these mice are more insulin sensitive compared to control mice [43, 45–47].

Body composition and lipid profile—GHR−/− mice are obese and accumulate excess subcutaneous fat independent of sex or age [2, 14, 20, 39]. Interestingly, this ‘obese’ mouse actually shows unchanged or lower fat mass in the perigonadal depot [20], suggesting the importance of depot-specific adipose tissue in health and longevity. The fact that bGH mice have less subcutaneous fat implies that subcutaneous fat is the most GH sensitive (and perhaps insulin sensitive) among all fat pads. Also, in contrast to bGH mice, GHR−/− mice have decreased plasma triglyceride and cholesterol levels [12, 39, 42, 48], as summarized in Table 1.

Increased lifespan and reduced incidence of age-related diseases—GHR−/− mice are long-lived, in fact they are the longest lived laboratory mouse strain. The molecular mechanism(s) responsible for this extended lifespan is the object of intense research. Calorie restricted (CR) mice share many similar phenotypes with GHR−/− mice [49]. Although CR extends the lifespan in organisms as diverse as *C. elegans* and mice [50], CR does not further improve the lifespan of GHR−/− mice [43], indicating a possible overlap in the mechanisms for the increased longevity.

Consistent with their longer lifespan, GHR−/− mice suffer rarely from certain age-related diseases such as cancer [51]. GHR−/− mice also show increased insulin sensitivity compared to WT controls throughout lifespan, and are protected from nephropathy caused by experimentally-induced diabetes [52]. Importantly, these coincide with the reduced cancer and diabetes incidences found in LS individuals [53, 54]. Thus, from mouse to man, disruption of the GH receptor gene results in reduced age-related diseases such as diabetes and cancer, which may be the reason why GHR−/− mice are long-lived.

In summary, GHR−/− mice are obese, have altered lipid profiles, yet have increased insulin sensitivity and longevity. Together with GH transgenic mice, they make excellent animal models to study GH action(s) as it relates to aging. However, many issues remain unresolved such as the molecular mechanism(s) of longevity associated with GH signaling.

Genomic and Proteomic results will certainly add to the knowledge base concerning GH's physiological effects as they relate to aging.

Plasma proteomic profiles in bGH and GHR−/− mice

Although the results of many physiological studies have been reported in these GH-related mouse models, little has been done at the proteomic level. We have analyzed the proteomic profiles of these animals as a function of age at both the plasma and the adipose tissue level. In this section, we will summarize the findings on the plasma proteome.

Plasma proteins have long been used as biomarkers for diseases. Our objective was to identify biomarkers of GH action and/or aging using a proteomic approach. We have characterized the plasma proteomes in bGH and GHR−/− mice of various ages (2 months to 24 months) in two longitudinal cohorts using 2-DE and MS/MS [55, 56]. The general workflow is shown in Figure 1. We have developed a sample processing protocol where the plasma sample is diluted in a buffer containing urea, thiourea, non-ionic (e.g., Triton X-100) and zwitterionic (e.g., CHAPS) detergents, a reducing agent (tributylphosphine), carrier ampholytes (e.g., Biolytes 3–10) and protease inhibitors, as reviewed previously [57, 58]. We have discovered that the majority of plasma proteins migrate between isoelectric points (pIs) of 5 and 8, therefore we used first dimensional 17-cm immobilized pH gradient strips of pH 3–10 and subsequently sectioned the strip to obtain the portion between pH 5–8, which was used for the second dimension (SDS-PAGE). In the case of plasma samples, we found good resolution of proteins below ~50 kDa in 15% polyacrylamide gels, without the interference of albumin, which is a highly abundant plasma protein. In this system, we did not deplete albumin since we found albumin fragments to be affected by aging [59]. The final gels were stained with SYPRO Orange, a fluorescent dye that shows good linearity with protein quantity. The gel images were analyzed using PDQuest (Bio-Rad). Selected spots were excised from the gels and analyzed by MS and MS/MS using MALDI-TOF and MALDI-TOF-TOF, respectively. The MS and MS/MS data were subjected to online searches using MASCOT at Matrixsciences.com. For a detailed protocol, refer to [59]. Approximately 160 mouse plasma protein spots were resolved in this system. The identification of selected proteins that exhibit significant differences in bGH and/or GHR−/− mice is shown in Figure 2.

We have found that apolipoprotein E (APOE), mannose-binding protein C (MBP-C) and haptoglobin (HP) increased in bGH mice and decreased in GHR−/− mice, whereas RBP-4 decreased in bGH mice and increased in GHR−/− mice [55, 56] (Table 2). Several other proteins changed significantly in one but not the other genotype. For example, transthyretin (TTR) was significantly down-regulated in bGH mice, but not significantly altered in GHR −/− mice. On the other hand, apolipoprotein A-4 (APOA4) was up-regulated in GHR−/− mice but not changed in bGH mice. This indicates that in these two mouse strains, while the levels of certain plasma proteins are primarily regulated by GH (e.g., APOE, MBP-C, HP and RBP-4), others may be regulated by GH as well as other factors. The significance of the proteomic changes were discussed previously [55, 56]. Below, we focus on these novel biomarkers of GH relevant to the phenotypes of bGH and GHR−/− mice including insulin sensitivity, lipid metabolism and aging.

Insulin sensitivity

RBP-4 is secreted by the liver and adipose tissue and is the major transporter of vitamin A in the circulation. RBP-4 has been found to be associated with obesity and insulin resistance [60]. Moreover, RBP-4 has a stronger association with visceral versus subcutaneous fat [61]. The mechanism of RBP-4-induced insulin resistance is not clear, but RBP-4 is reported to attenuate the insulin-induced phosphorylation of insulin receptor substrate-1 (IRS-1) and

ERK1/2 in primary human adipocytes [62]. It has also been reported to induce proinflammatory cytokines in macrophages via c-jun N-terminal kinase and Toll-like receptor-4 [63]. In brown adipose tissue, peroxisome proliferator-activated receptors (PPAR) -α and -γ, and cAMP-mediated pathways regulate RBP-4 expression [64]. Most reports on RBP-4 are in the context of obesity and insulin resistance; very little is known about GH's effect on RBP-4. It seems paradoxical that bGH mice which are insulin resistant have lower RBP-4 and, GHR−/− mice which are insulin sensitive have higher RBP-4. However, bGH mice are lean and GHR−/− mice are obese, therefore the RBP-4 levels may correlate to obesity rather than insulin resistance in these two mouse strains. In addition, RBP-4 may represent a link that uncouples insulin resistance from obesity in the bGH and GHR−/− mice, as these two mouse strains also show seemingly counter-intuitive phenotypes of adiposity and insulin sensitivity, i.e., the lean bGH mice being insulin resistant and the fat GHR−/− mice being insulin sensitive. A role of RBP-4 in these two mouse strains deserves further study.

Lipid metabolism

Since APOE is a major apolipoprotein that carries nearly all classes of cholesterol (VLDL, LDL and HDL), higher APOE in bGH mice and lower APOE in GHR−/− mice are consistent with the higher cholesterol levels in bGH mice and lower cholesterol levels in GHR−/− mice, respectively. On the other hand, GHR−/− mice have higher APOA4 levels, which are transporter proteins of HDL, the beneficial cholesterol. Consequently, in the long-lived GHR−/− mice, the ratio of levels of APOA4 versus APOE is higher than control mice. This ratio may represent a novel marker for fitness in terms of lipid metabolism. This adds to the existing knowledge that GHR−/− mice, although obese, preferably accumulate fat in the subcutaneous depot, and have low serum cholesterol and triglyceride levels. Together these characteristics may be indicators of beneficial lipid metabolism in terms of health and longevity..

Aging and age-related diseases

Aging is associated with a low grade inflammation [65]. MBP-C and HP are both acute phase proteins indicative of inflammation; therefore their higher levels correspond to an increased inflammatory state in bGH mice, whereas their lower levels indicate that GHR−/− mice have reduced inflammation. In this regard, it has been shown that the proinflammatory cytokines TNF-α and IL-6 are increased in bGH mice [15], and monocyte chemotactic protein-1 and IL-1β are decreased in female GHR−/− mice [20]. Plasma MBP-C and HP are secreted by many tissues, but primarily by the liver. As discussed earlier, GH transgenic mice develop liver tumors at old ages, and have sustained liver inflammation and hepatocellular injury with reduced natural killer T cells [31]. Consistent with the increased inflammation, GH transgenic mice have enlarged spleen and show altered immunity, including deficiency in T helper 2 cytokine production [66] and self-antibody production leading to arthritic disorders [67]. The finding that MBP-C and HP are elevated in the plasma of GH transgenic mice further confirms their increased inflammatory phenotype. Specifically, MBP-C and HP are two plasma proteins that respond to increased GH signaling, i.e., up-regulated in bGH mouse plasma and down-regulated in GHR−/− mouse plasma. These two biomarkers of GH provide a link of GH with inflammation, an interesting observation deserving further research.

Several proteins including APOE, A2M, CLU and MBP-C are involved in Alzheimer's disease (AD), which is an age-related disease. APOE has been well recognized as a player in AD. In humans, the *APOE* allele 4 is associated with a higher risk of AD [68]. One allele of *A2M* confers increased risk of AD [69], CLU variants have also been found to be associated with AD risks [70, 71], and higher total levels are found in AD patients [72]. MBP-C binds

to amyloid β and may play a role in tissue homeostasis [73]. It has been found that the cerebral spinal fluid levels of MBP-C are lower in AD patients compared to the controls, although serum levels do not differ [74]. Even though there is no direct evidence linking plasma levels of APOE, A2M and MBP-C with AD, the fact that GH regulates these proteins involved in AD is intriguing.

In summary, plasma proteomic studies have revealed that GH alters several proteins involved in lipid metabolism, insulin sensitivity, and inflammation and may also play a role in AD

White adipose tissue proteomic profiles in GHR−/− mice

White adipose tissue (WAT) is classified into two main groups, subcutaneous (under the skin) and intra-abdominal or visceral (lining the internal organs, such as the stomach, kidneys, intestine and reproductive organs). Intra-abdominal WAT is more closely associated with classical obesity-related conditions (type-2 diabetes, insulin resistance, cardiovascular disease, etc.), whereas subcutaneous WAT is considered “less unhealthy” [75–78]. As described above, GHR−/− mice are dwarf, but exhibit a marked accumulation of WAT, localized mainly in the subcutaneous region. The fact that GHR−/− mice exhibit enlarged subcutaneous WAT depots while other depots remain unchanged highlights the depot-specific actions of GH on adipose tissue [20]. Also, given that GHR−/− mice are very insulin sensitive and show extended lifespan, understanding the physiology of WAT in different depots of these mice could be applicable to current human health issues. For these reasons, we studied the proteomic profiles of WAT from different depots from GHR−/− mice and WT littermates, including one subcutaneous depot (inguinal) and three intra-abdominal ones (retroperitoneal, mesenteric and epididymal). To better investigate the mechanisms of extended longevity in GHR−/− mice, we included two time points: one where mice are adult (12 months) and one representing old age (24 months). The main focus of our analysis was proteins that showed differences among WAT depots, between age groups, and genotypes. For 2DE, we used the procedures described above for plasma, following homogenization of the tissue and separation of lipids, which if present in a sample can generate problems in protein resolution. For a detailed protocol, refer to [79, 80]. A representative image of a 2D-gel of WAT is shown in Figure 2.

We observed that except for spot intensity differences, the resulting protein profiles of different WAT depots displayed similar spot patterns. Only a few protein spots (myosin regulatory polypeptide 9 and transgelin) were unique to mesenteric WAT and not detected in the other depots (Figure 2). These spots contained smooth muscle proteins which we believe to originate from large blood vessels more densely present in mesenteric fat [80].

Out of 166 well resolved spots, 70 showed significant differences among the groups evaluated. Some of the main observations resulting from the analysis of the differences among WAT depots and between age groups are summarized in Table 3. Regarding differences among the four WAT depots studied, protein profiles of inguinal (subcutaneous) WAT suggested a lower metabolic rate in this depot than in intra-abdominal (mesenteric and epididymal) fat pads. For example, subcutaneous WAT displayed lower levels of glycolytic enzymes and antioxidant proteins, among others [80]. A lower metabolic rate paralleled by a diminished production of ROS might result in decreased cellular stress in subcutaneous WAT which could contribute to its overall “healthiness” when compared to intra-abdominal fat pads (see above). When the effects of age were analyzed, similar changes were observed in all WAT depots, where enzymes involved in glycolysis, the Kreb’s cycle, and antioxidant proteins showed higher levels in aged mice than in adult animals [79]. Seemingly, advancing age leads to increased metabolic rate and ROS production in WAT, which are consistent

with a decrease in insulin sensitivity in all depots. Considering the basal depot-differences just described, it was surprising to find similar changes in all WAT depots in terms of metabolic changes with aging. However, even at old age, the differences among WAT depots in metabolic enzyme levels seem to persist, suggesting that subcutaneous WAT remains the "less unhealthy" depot regardless of age. For instance, given that subcutaneous fat is progressively lost in humans with advancing age, the close link between aging and metabolic disease could partly depend on the abundance of the subcutaneous WAT depot [81].

Finally, differences between WAT depots in GHR−/− and wild-type mice involved several proteins that have been associated with aging processes or aging diseases [Apolipoprotein A-1 (APOA1) and TTR, among others]. This analysis is the subject of a recently submitted manuscript [Sackmann et al, submitted] and therefore will not be detailed here. Overall, the effects of GH on WAT depot proteomes were not as striking in terms of the number of proteins affected (12 out of 166 spots analyzed, Figure 2) as the effects observed in depot masses and adipocyte sizes, where differences between genotypes are impressive [20]. However, the extended lifespan of GHR−/− mice might result from a combination of effects of GH on the morphology, architecture and cellular biology of WAT depots (as well as other tissues).

Protein Isoforms in Mouse Plasma

In the 2-DE proteomic analysis, we routinely discover multiple isoforms identified to be the same protein, most likely due to post-translational modifications (PTMs). For example, in plasma, seven protein spots at approximately 35 kDa are identified as alpha-2 macroglobulin have been identified (A2M, Figure 1). In most situations, the isoforms of the same protein show similar expression changes between genotypes. For example, five plasma APOE isoforms are increased in bGH mice and decreased in GHR−/− mice, indicating that APOE is altered at the total protein level, but the proportions of different isoforms remain the same. However, for certain proteins, different isoforms behave differently from each other. For example, the isoforms 1 and 2 of A2M in plasma are up-regulated in bGH mice but isoforms 5 and 6 are down-regulated, with the total expression of A2M unaltered between bGH and control mice. In this case, GH does not change total A2M levels, but rather, regulates specific PTMs that result in selective enrichment of certain isoforms and loss of others. By conventional methods such as immunoblotting, A2M cannot be detected to be a target of GH action. PTMs can be easily detected by 2-DE, provided that the PTM results in changes in the charge of the protein, even if the molecular weight (MW) is only minimally altered.

We are interested in identifying the PTMs that cause these pI shifts of proteins in gels. For example, as a major target tissue of GH, liver is also a main source of plasma proteins, therefore it is natural to ask whether it is possible that GH induces a specific kinase that posttranlationally modifies certain liver proteins that are secreted into the blood, and whether these secreted proteins are affected by aging. One way of predicting or identifying PTMs is to theoretically calculate the pIs of the target protein for a given PTM (e.g., phosphorylation) and see whether they match the observed MWs and pIs on the 2-D gel. The mouse A2M is composed of two subunits- 165 kDa and 35 kDa, and the 35 kDa subunit is detected in our 2-DE system [55] shown in Figure 1. The 35kDa subunit of A2M has a theoretical pI of 7.09 and the seven observed isoforms had pI ranging from 5.65 to 7.08 (Table 4), which means isoform 7 (Figure 1) is most likely the un-modified form and isoforms 1–6 have been modified by PTM(s). A2M is known to be phosphorylated [82] and glycosylated on multiple Asn residues [83]. These PTMs are likely the reason for A2M to exist as multiple isoforms with similar sizes but different pIs on the 2D gel. Table 4 shows the theoretical and observed MWs and pIs of the seven A2M isoforms. Using an online tool

to calculate the MW and pI of a protein with varying number of phosphorylated residues (http://scansite.mit.edu/calc_mw_pi.html), it was revealed that isoform 2 (observed pI 5.85) was consistent with the addition of three phosphate groups (predicted pI 5.81) and isoform 3 (observed pI 5.08) was consistent with the addition of two phosphate groups (predicted pI 5.08). The pIs of other isoforms cannot be explained by phosphorylation alone, but may be results of other PTM(s) (e.g., glycosylation) and/or a combination of phosphorylation and other PTM(s). It will be useful to develop tools to predict MW and pI with PTMs other than phosphorylation, as well as combinations of different PTMs.

In addition to the theoretical calculation and prediction based on known PTMs, it is ultimately the experimental detection that demonstrates whether a PTM is present or not. Traditional MS and MS/MS can achieve this, but peptides with certain modifications such as phosphorylation are difficult to identify, resulting in under-representation of peptides with PTMs in the MS or MS/MS spectrum, leading to low detection rates of PTMs. Further, computer algorithms and programs that accurately and efficiently detect PTMs in the MS or MS/MS spectrum of a protein are needed given the large number of potential PTMs. Currently, the identification of PTMs, which are important elements to understand the regulation of novel biological processes by GH remains a bottle neck. For example, if A2M isoform 2 (which is up-regulated in bGH mouse plasma) proves to have two phosphorylation groups, then GH may regulate a kinase (either in liver cells or in plasma) that phosphorylates A2M, and it would be of interest to study that kinase for a physiological function regulated by GH.

Protein isoforms in WAT

With respect to WAT, there are at least two interesting cases of PTMs in our proteomic analysis: APOA1 and carbonic anhydrase 3 (CA-III). The case of APOA1 is commonly observed in 2D-gels of many different tissues, appearing as a characteristic array of spots aligned at ~25 kDa between pIs of ~5.5 and ~6.5. In our analysis of WAT, six isoforms of APOA1 were found to vary significantly between age groups, and two showed differences among depots (Figure 3). When reviewing the literature, one can easily find associations of APOA1 with atherosclerosis, inflammation, and other age-related diseases [84, 85]. However, the PTMs giving origin to the detected isoforms of APOA1 remain uncharacterized. The theoretical MW and pI of secreted APOA1 are 27.9 and 5.42 respectively. The Uniprot database includes methionine sulfoxides at residues 110 and 136, a phosphoserine at 191 and a N-linked glycation at residue 263 as reported modifications of the human protein [86]. Among these, only serine-phosphorylation generates variations in pI and could therefore be responsible for the detection of a second isoform with similar MW but different pI. Nevertheless, the change in charge would result in a more negative pI, which is opposite to the increased pI detected in APOA1 spots. Given the important role played by APOA1 in lipid transport in the body and its consequences for human health, it is of great interest to unveil the nature of the PTMs present in its various isoforms and how they affect the protein's activity.

CA-III is one of the most abundant proteins in white adipose tissue although its function is not well understood. This protein is thought to provide bicarbonate ions for reactions that involve carbon fixation (production of oxaloacetate from pyruvate for synthesis of triglycerides via glyceroneogenesis, and transformation of acetyl-CoA into malonyl-CoA for fatty acid synthesis). Apart from its putative role in anabolic reactions, CA-III might be an important regulator of intracellular pH when fatty acids are released during lipolysis [87, 88]. Therefore, the function of CA-III in WAT seems intrinsically related to the physiology of this tissue. In our proteomic analysis we found at least five spots containing CA-III that were different among WAT depots and/or age groups. These isoforms had MWs that varied

from ~27 to ~70kDa and pIs from ~7.2 to ~8. The theoretical MW and pI for CA-III are 29.2 kDa and 6.97 respectively, indicating that various PTMs could be present in the spots detected. The Uniprot database reports the possible presence of two S-glutathionyl-cysteine residues at positions 182 and 187 [86]. However, more research is needed to find the PTMs that originate the shifts in MW and pI observed in our gels. Information on this matter could provide new mechanisms of regulation of CA-III activity, with possible influence in lipid metabolism in WAT.

Conclusion

The use of transgenic and knockout mouse strains to study GH activity has further established the actions of GH in terms of growth, metabolism and aging. However, systematic analysis of tissue proteomes in these mice has only recently emerged. The application of proteomics to study these mice will uncover novel aspects of GH action and represents one of the future research directions. Our recent proteomic studies have confirmed several phenotypes of GH-related mouse strains, e.g., the proteomic studies in mouse plasma indicate that chronic overexpression of GH or complete lack of GH regulate proteins that are involved in insulin sensitivity, lipid metabolism, inflammation and aging. Furthermore, novel biomarkers of GH have been discovered revealing new physiological targets (RBP-4, HP and MBP-C) in relation to aging and age-related diseases. Additionally, proteomic studies in mouse WAT provide interesting information on the physiology of subcutaneous and intra-abdominal depots, and their behavior during aging. The impairment of GH signaling in WAT leads to clear differences among depots and affects the levels of proteins associated to aging. However, the prolonged longevity observed in GHR−/− mice could be the result of GH's combined action on several aspects of tissue morphology, architecture and cellular biology in WAT depots (and in other tissues). We anxiously await future results that will generate data on biomarkers of GH as it relates to normal physiology and the aging process.

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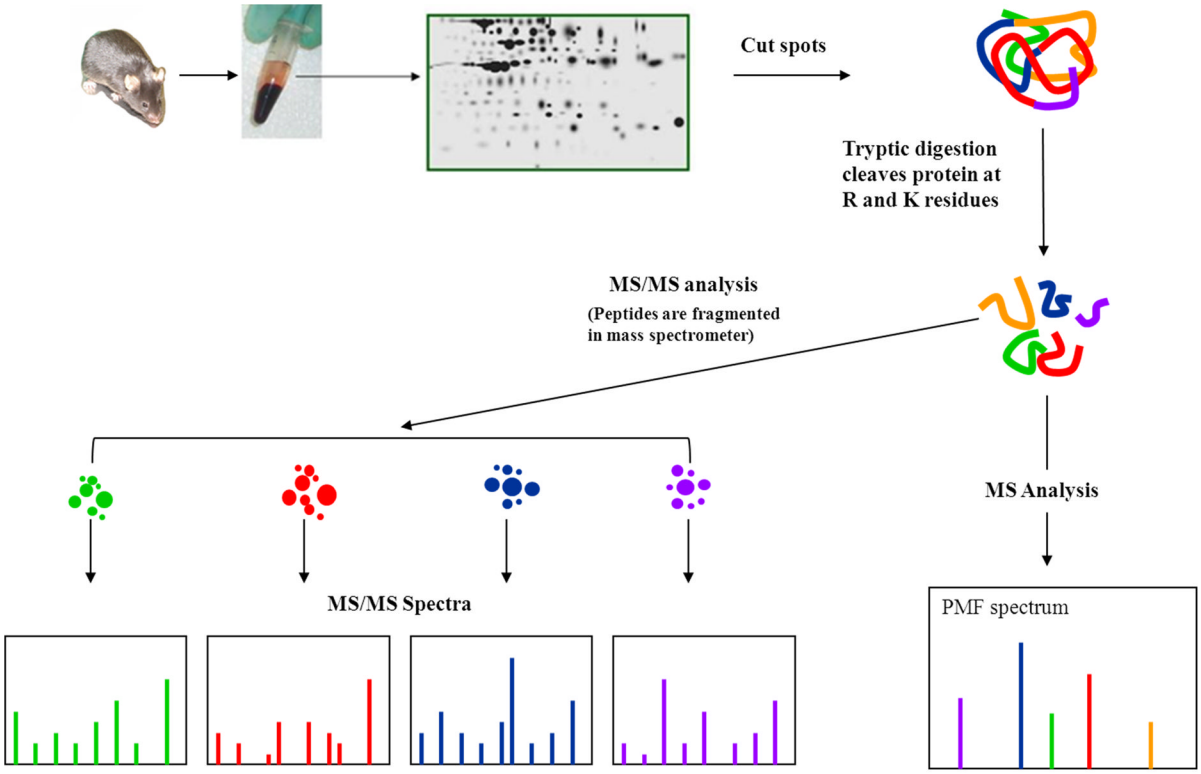

Figure 1.

A schematic flow chart of protein isolation by 2-DE, then identification by MS and MS/MS. 2-DE: two-dimensional electrophoresis, MS: mass spectrometry, PMF: peptide mass fingerprint, R: arginine; K: lysine. This figure was adapted from that found on the Michigan Proteome Consortium web page.

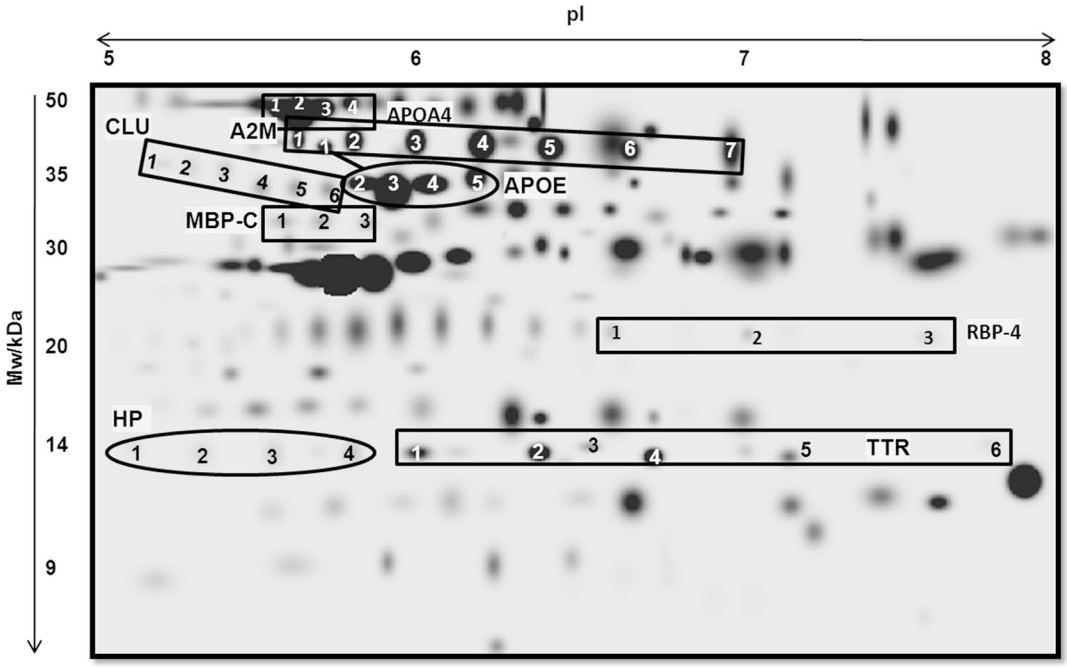

Figure 2.

2-DE gel map of mouse plasma proteome with annotations of proteins that are significantly different in bGH and/or GHR−/− mice. Mw = molecular weight; pI = isoelectric point; A2M = alpha-2 macroglobulin; APOA4 = apolipoprotein A-4; APOA1 = apolipoprotein A-1; APOE = apolipoprotein E; CLU= clusterin; HP = haptoglobin; MBP-C = mannose-binding protein-C; RBP-4 = retinol-binding protein-4; TTR = transthyretin.

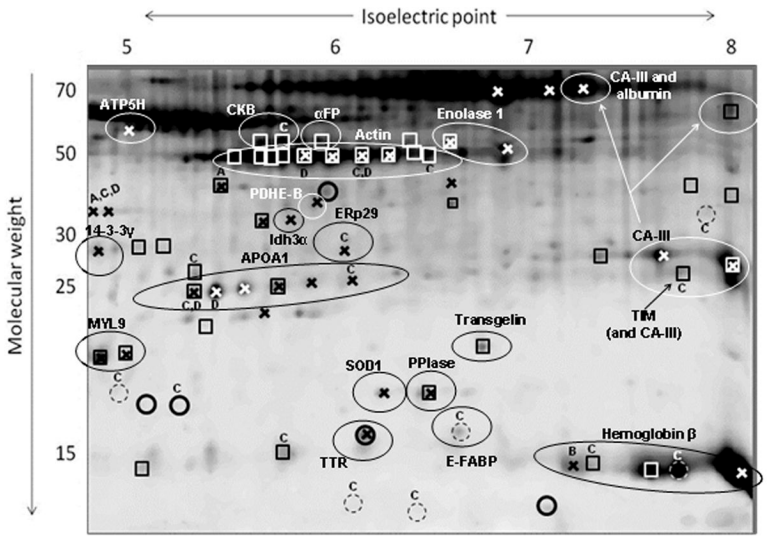

Figure 3.

2-DE gel map of mouse white adipose tissue with annotations of proteins that are significantly different between GHR−/− mice and wild-type littermates (P<0.01). Main effects are labeled using (○) for genotype; (□) for depot; and (x) for age. Significant interactions are labeled with capital letters: genotype × depot (A); genotype × age (B); depot × age (C); and genotype × depot × age (D). Dashed circles mark spots that only show significant interaction effects. The identities of certain spots are annotated; αFP = α fetoprotein; APOA1 = apolipoprotein A-1; ATP5H = ATP synthase subunit β, mitochondrial; CA-III = carbonic anhydrase 3; CKB = creatine kinase B; E-FABP = Fatty acid-binding protein, epidermal; ERp29 = Endoplasmic reticulum resident protein 29; Idh3α = isocitrate dehydrogenase [NAD+] α; MYL9 = myosin regulatory light polypeptide 9; PDHE-B = Pyruvate dehydrogenase E1 β, mitochondrial; PPIase = Peptidyl-prolyl cis-trans isomerase A; SOD1 = superoxide dismutase [Cu–Zn]; TIM = triosephosphate isomerase; TTR = transthyretin.

Table 1

Characteristics of GH transgenic mice and GHR−/− mice

Phenotype	GH transgenic mouse vs. control	GHR−/− mouse vs. control
Body weight and length (nose to anus)	Higher	Lower
IGF-I level	Higher	Lower
Lifespan	Shorter	Longer
Age-related diseases	Increased incidence of liver inflammation, liver tumors, glomerulosclerosis and oxidative stress [25, 29–34]	Lower incidence of cancer [51]
Glucose level	Normal or lower [7–15]	Lower (normal in older animals) [12, 14, 20, 36–41]
Insulin level	Higher [7–11, 13–15]	Lower [14, 20, 36–43]
Insulin sensitivity	Lower [8, 9, 18]	Higher in muscle and heart [43, 45– 47], mixed results for liver [36, 41, 43, 44, 89]
Glucose tolerance	Higher [12, 16]	Lower [12, 40]
Body composition	Less fat mass [2, 19], especially in the subcutaneous fat depot [14]	More fat mass, primarily in the subcutaneous depot [2, 14, 20]
Plasma free fatty acids	Lower or no change [10, 11, 15]	No change [48]
Plasma triglycerides	Mixed results [10, 12, 13, 15]	Normal or lower [12, 39, 42, 48]
Plasma cholesterol	Higher [10, 12, 13, 15]	Lower [12, 39, 42, 48]

Table 2

Summary of protein changes in bGH transgenic mice and GHR−/− mice

Plasma protein	Change in bGH vs. WT [55]	Change in GHR−/− vs. WT [56]	Main Function
A2M	Isoforms 1 and 2 ↑; 5 and 6 ↓	↔	Protease inhibitor
APOA4	↔	Isoforms 3, 4 and total ↑	Transports HDL cholesterol
APOE	Isoforms 1–5 and total ↑	Isoforms 1,3,4 and total ↓	Transports HDL, LDL and VLDL
CLU	Isoforms 1,2 and total ↓ in bGH mice at young ages but ↑ in bGH at old ages	↔	Clearance of cellular debris and apoptosis
HP	Isoforms 1–4 and total ↑; 1–4 and total ↑ in bGH aging	Isoforms 1–3 ↓ ($p<0.05$)	Acute phase protein
MBP-C	Isoform 1 ↑	Isoform 1 ↓ ($p<0.05$)	Acute phase protein
RBP-4	Isoform 2 and total ↓ [90]	Isoforms 1–3 and total ↑	Transports retinol; involved in insulin resistance
TTR	Isoforms 1–6 and total ↓	↔	Transports RBP-4 and thyroxin T4

Numbers represent protein isoform# as shown in Figure 1. ‘Total’ is the sum of intensity of all protein isoforms. ↑ increase, ↓ decrease, ↔ unchanged. All changes are at a significance level of $p<0.01$ unless otherwise indicated ($p<0.05$ means $0.01<p<0.05$).

Table 3

Summary of main WAT proteome differences among depots and age groups in GHR−/− and WT mice

Protein function	Differences among WAT depots	Effects of Age on WAT
ATP generation	↑ in mesenteric WAT (CKB)	↑ in aged animals (ATP5H)
Glucose/lipid metabolism	↑ in epididymal WAT; ↓ in inguinal WAT (TIM; CA-III)	↑ in aged animals (ENO1; PDHE-B; Idh3 α; CA-III)
Lipid transport	↑ in mesenteric WAT (APOA1)	↑ in aged animals (APOA1)
Stress resistance	↑ in epididymal and mesenteric WAT; ↓ in inguinal WAT (HSPβ1; PRDX2)	↑ in aged animals (ERp29; SOD1; PPIase)
Smooth muscle markers	↑ in mesenteric WAT (MYL9; transgelin)	No change detected
Other	↑ hemoglobin β in mesenteric WAT	↓ TTR in aged animals

Values marked "↓" (low) or "↑" (high). Examples of proteins identified for a corresponding biological function are shown in parentheses.

Table 4

The molecular weight and isoelectric point of A2M (35 kDa subunit) isoforms

Isoform #	Theoretical MW (kDa) [a]	Observed MW (kDa) [b]	Theoretical pI [a]	Observed pI [b]	Theoretical pI with possible phosphorylation (P) [c]
1	28.2	38.3	7.09	5.65	5.57 (4P)
2	28.2	38.7	7.09	**5.85**	**5.81 (3P)**
3	28.2	38.5	7.09	**6.08**	**6.08 (2P)**
4	28.2	38	7.09	6.3	6.43 (1P)
5	28.2	37.7	7.09	6.54	
6	28.2	37.5	7.09	6.80	
7	28.2	37.6	7.09	**7.08**	**7.09 (0P)**

[a] MW and pI were calculated at http://scansite.mit.edu/calc_mw_pi.html.

[b] Observed MW and pI were estimated from the 2-D gel

[c] Theoretical pIs assuming phosphorylation were calculated at http://scansite.mit.edu/calc_mw_pi.html. 1P means one phosphorylation and 2P means two phosphorylations, etc.