Effects of GHRKO Visceral Fat Transplant on Insulin Signaling

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ABSTRACT

Insulin sensitivity has been positively correlated with a healthy and extended lifespan, while insulin resistance, decreased insulin sensitivity, has been linked to aging and is the main indicative of type 2 diabetes. Growth Hormone Receptor/Binding Protein Knockout mice (GHRKO), although obese, are characterized by high insulin sensitivity and a prolonged lifespan. Due to the absence of growth hormone receptors (GHR), growth hormone (GH) is unable to activate its downstream pathway. Interestingly, the secretory activity of visceral fat in GHRKO mice is altered stimulating insulin sensitivity. In this study, we transplanted normal (N) mice with GHRKO visceral fat pads to determine the role of visceral fat developed with the absence of GH signaling on the insulin-signaling pathway in animals with physiologically normal GH action. We found that the visceral fat transplant (VFT) helped the normal mice gain the beneficial effects of fat developed in the absence of GH and caused improvement of their whole body insulin sensitivity when comparing with sham-operated mice and with mice that received visceral fat from N animals. In presented study, RT-PCR was used to determine the levels of hepatic mRNA expression between three experimental groups including Normal-sham mice (N-S), normal mice transplanted with visceral fat from normal animals (N-N), and normal mice receiving visceral fat from GHRKO mice (N-KO). Additionally, Western Blot and ELISA were used to determine the level of total and phosphorylated proteins.
By studying the effect of visceral fat transplant from GHRKO or N mice on the whole body insulin signaling in N male mice, and testing different genes expression and proteins quantification, we can shed light on the mechanism by which white adipose tissue (WAT) regulates whole body insulin sensitivity and longevity as well as understanding the role of WATs in development of diabetes and the process behind insulin resistance.
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INTRODUCTION

Growth Hormone Receptor growth hormone binding protein, knockout mice (GHRKO), also known as Laron dwarf mice, are characterized by high insulin sensitivity, and extended longevity [1]. GHRKO mice are growth hormone (GH) resistant due to the lack of activity of their GH receptor, therefore halting GH from activating its downstream response. They have a high level of GH in circulation, but due to the resistance to GH these mice have low levels of circulating Insulin-Like Growth factor-1 (IGF-1) [1]. They are also obese, small, and hypoinsulinemic [2]. Insulin sensitivity has been linked to increased longevity and delayed aging [3]. In addition, with the absence of growth hormone signaling, the secretory activity of visceral fat in GHRKO mice is modified and stimulates insulin sensitivity rather than causing insulin resistance [2]. In this experiment, we demonstrated the role of visceral fat transplant (VFT) developed with the absence of GH signaling on insulin signaling in normal (N) mice. Using Real-Time Polymerase Chain Reaction (RT-PCR), Western Blot, and Enzyme-Linked Immunosorbent Assay (ELISA), we determined the changes in the insulin signaling pathway of normal mice with implanted visceral fat from normal or GHRKO mice in the liver, one of the major insulin signaling organs. We concluded that the visceral fat transplant from GHRKO mice helped the N mice gain the beneficial effects of fat lacking GH and caused improvement of their whole body insulin sensitivity.
BACKGROUND

Growth hormone is one of the first therapeutic proteins in the world to be approved for human use. It is released by the pituitary gland, and regulated by the hypothalamus. Beside stimulation of growth by activating its receptor in different tissues, the GH acts on the liver and causes the production and release of IGF-1 [4]. GHRKO mice are known to live 40%-55% longer than their normal littermate and display considerable alterations of the hepatic expression of genes associated with insulin signaling indicating improved insulin action in the liver [5].

Type 2 diabetes mellitus (DM) usually caused by insulin resistance and associated with co-morbidities such as obesity or metabolic syndrome, has dramatically increased worldwide due to changes in lifestyle in children and adolescents [6] (American Diabetes Association). Moreover, the fact that 78.6 million of U.S. adults are obese is alarming since obesity is related to diabetes; which had a medical cost of $147 billion in 2008 (Center for Disease Control and Prevention). In the United States, 29.1 million Americans have diabetes, which represents 9.3% of the population. The highest percentage is among seniors, age 65 and older, with 25.9% or 11.8 million seniors having diabetes. Furthermore, about 1.7 million new cases of diabetes are diagnosed every year. Diabetes, as of 2010, is the 7th leading cause of death in the US accounting for 250,000 deaths (American Diabetes Association). The GHRKO mice, although obese, are protected from age-related diseases including diabetes and cancer [2]. In addition, studies done with members of an Ecuadorian family with Laron syndrome
showed that dwarfism protected its bearer against age-related diseases like diabetes and cancer [7].

The visceral fat pad, also called intra-abdominal fat, is known to increase the risk of insulin resistance, metabolic syndrome, and type 2 diabetes, while subcutaneous fat lowers the risk of type 2 diabetes and promotes insulin sensitivity. Therefore, the site of fat accumulation seems to play a critical role in the mice’s health rather than the amount of fat. Even though, GHRKO mice are obese with the evidence of visceral obesity, they are insulin sensitive and have a healthy extended life span. Furthermore, since GHRKO mice are known to be obese, it was shown that their visceral fat regulates insulin signaling differently than the same fat pad in normal littermates [2]. In addition, visceral fat removal from normal mice improved their insulin sensitivity and increased their life span, yet the same experimental procedure showed the opposite results on insulin signaling in GHRKO mice. Therefore, the obesity in GHRKO mice is due to increase visceral fat, which indicates that the role of visceral fat in the GHRKO mice diverges from its role in normal mice in the regulation of insulin signaling [2].

Glucose intolerance, or insulin resistance, increases mostly with unhealthy weigh gain. However, it was also observed that impaired glucose tolerance correlates with age starting in the thirties and forties and continues throughout the adult life, usually resulting in the unresponsiveness of tissue to insulin [8] (American Diabetes Association). Moreover, many studies show that improved insulin sensitivity can have a beneficial effect on longevity and health span [3]. Since calorie restriction (CR) is the
most efficient intervention to increase lifespan and delay aging [9], the physiological modifications detected in GH resistance mice resemble some of its effects. However, GHRKO mice were immune to CR and did not gain any extra life extension or improvement of insulin sensitivity [10].

Overall, growth hormone triggers, via its receptors, a cascade of intracellular signals affecting different physiological aspects including fat depletion, growth, and glucose metabolism [11]. GHRKO mice, although obese, are insulin sensitive and have an increased life span [2]. Visceral fat, even though it is commonly referred to as “bad fat”, seems to have positive effects in GHRKO mice, however its removal in normal mice caused a delay onset of diabetes [12].

In this project, we studied the effect of visceral fat transplant from GHRKO or N mice on the whole body insulin signaling in N male mice by testing different genes in the liver such as Insulin Receptor (IR) and IGF-1 since they play a role in the insulin signaling pathway. This study helped shed light on the mechanism of insulin signaling in extending life span and increasing insulin sensitivity. The experiment is also beneficial to understand diabetes and the mechanism behind insulin resistance. Since GH and IGF-1 constitute one of the well-confirmed pathways involved in the regulation of lifespan and aging, the GHRKO mice give us a special opportunity to study these pathways and determine their characteristics [13]. Furthermore, other studies showed that Ames dwarf mice, characterized by absence GH in circulation and suppression of
blood IGF-1 also exhibit extended longevity and increased insulin sensitivity with similarly altered function of WAT [14, 15].
MATERIALS AND METHODS

Animals

In the present study, we used approximately 6 months old male GHRKO and normal mice. Three different experimental groups were proposed (n=10): Normal-sham mice (N-S), normal mice transplanted with visceral fat from normal animals (N-N), and normal mice receiving visceral fat from GHRKO mice (N-KO).

Surgery

Fat donor N and GHRKO mice were anesthetized with isoflurane and euthanized by cervical dislocation. Visceral fat was collected and placed in chilled sterile saline solution. At the same time the recipient mice were prepared for survival surgeries. They were anesthetized with ketamine:xylazine (130mg and 8.8mg per Kg of body weight). The mice were also given ibuprofen in their drinking water, concentration of 1mg/ml, for 2 days prior to surgery and 3 days after to ease any pain and discomfort. The mice were shaved and prepared in a sterile environment using alcohol and betadine. The abdomen of recipient mice was dissected by the midline central flank incision. Fat collected from donor mice was minced with a scalpel and then placed in to the visceral cavity of the recipient mice.

In the sham group, the abdominal cavity was surgically opened and the visceral fat located, but nothing was neither added nor removed. The mice were then monitored paying a close attention to their body weight and food intake. The transplanted fat corresponds to 3% body weight of the recipient. Eight days after surgery, the mice were
subjected to glucose tolerance test and four days later they were fasted over-night and sacrificed the next morning. Liver tissue was collected and snap frozen in liquid nitrogen.

**Methodology**

The experiment was performed using the liver tissue samples collected as described above. RNA and proteins were isolated and tested using RT-Polymerase Chain Reaction, Western Blot, and ELISA. RT-PCR was used to determine the relative levels of mRNA expression in the tissue and compare it between the different groups. Tested genes include Insulin Receptor (IR), Peroxisome proliferator-activated receptors family (PPAR), Insulin Receptor Substrate-1 and 2 (IRS-1, IRS-2), Insulin like Growth Factor-1 (IGF-1), RAC-beta serine/threonine-protein kinase (AKT2), Forkhead box protein O1 (FOXO1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a), Phosphoinositide 3-kinase (PI3K), Signal transducer and activator of transcription 1, and 3 (STAT3, STAT1), Glycogen synthase kinase 3 beta (GSK3b), and Glucose transporter 2 (GLUT2). Western Blot and ELISA were used to determine the level of total and phosphorylated proteins. Proteins tested included IR Beta-subunit, IRS-1, GSK3b, AKT2, STAT3, PGC1-a, PPARg, IR pY1158, GSK-3b pS9, and AKT pS473. This analysis helped us determine the up- or down-regulation of a specific gene and protein in the different groups, and the way it affects insulin signaling.

*Glucose Tolerance Test* (GTT): Animals were fasted over-night for 12-16 hours, and the body weight checked in the morning. The tail was pinched along with a
razor to initiate bleeding causing a cut of about 1-2 mm. Using standard glucometers and about 0.5-1μl of blood, the basal glucose level was checked. After the initial reading, the mice were injected with 2g of glucose per 1 Kg of body weight, then the blood glucose was measured at 15, 30, 60, 90, and 120 min after the first reading. The GTT test, which measures the ability of the body to clear glucose, starts with a sharp incline of glucose levels after injection. Mice exhibiting a rapid drop of blood glucose to basal levels have a greater glucose tolerance. This proves the mice’s ability to respond to changes in blood glucose and regulate it by secreting adequate amount of insulin.

**Insulin Tolerance Test (ITT):** Animals had access to the food over-night, then removed one hour before the test, and the body weight was checked. The tail was pinched with a razor blade and the cut was about 1-2 mm. Using glucometers and 0.5-1μl of blood, the blood glucose was measured. After the initial measurement, the mice were injected with 1IU of insulin per 1 KG of body weight. After that, the blood glucose was measured at 15, 30, and 60 min after insulin stimulation. ITT test was used to measure the whole body insulin sensitivity. A rapid drop in blood glucose levels (% of baseline) indicates insulin responsiveness of the animals. This indicates that the injected insulin produced a more significant effect on the body’s blood glucose levels; which in turn suggests an increase in insulin sensitivity.

**Insulin stimulation:** Approximately half the mice from each group were injected with either a high dose of insulin (10 IU per Kg body weight), while the other half were injected with saline as controls to stimulate insulin signaling in target organs.
Two minutes after insulin stimulation, the mice were sacrificed and the liver was collected and snap frozen in liquid nitrogen. After that, it was stored at -80\(^\circ\) C until further analysis [15].

Statistics: GraphPad Prism5 software and Microsoft Excel were used to calculate the statistical difference between the mice groups. On Prism 5, the statistics were calculated using one-tailed P values for unpaired t tests. A P value less than 0.05 was considered statistically significant.

Real-Time Polymerase Chain Reaction: About 75mg of the liver tissue sample was homogenized by placing it in a 1.5mL Eppendorf safe-lock tube with 0.5mm Zirconium Oxide beads and 700μL of Qiazol extraction buffer. The tube was then placed in a bullet blender homogenizer at speed 9 for 3min then incubated for 5min at room temperature (RT) to lyse the cells and separate the soluble from the insoluble, this step can be repeated until the sample is well homogenized. The mRNA was then isolated using the Qiagen mRNA purification kit following the Quick-start Protocol included in the kit, which resulted in a purified mRNA in nuclease free water (NF H2O). RNA concentration was then determined by the Nanodrop procedure with the Epoch Gen5 Plate reader (see appendix for procedure details), and then converted into cDNA using the Bio-Rad iScript reverse transcription kit. A master mix containing 5X iScript reaction mix and iScript reverse transcriptase was prepared then added to the mRNA template (previously extracted) in a PCR tube following the protocol. The mixture was then placed into a Bio-Rad MJ Mini Personal Thermal Cycler, with conditions set at 25\(^\circ\) C for
5 min, 42°C for 30 min, 85°C for 5 min, then held at 4°C. The step yielded 40 ul of cDNA in NF H2O, then 60 ul of NF H2O was added to each sample to narrow the cycle threshold (Ct) range of B2M in RT-PCR (see below). The cDNA samples were then stored at 4°C if used after a short period of time or frozen at -20°C if kept for an extended period [16].

By adding SYBR Green Master Mix, forward and reverse primers specific to 2μL of cDNA of our target gene for a total volume of 20μL, we amplified our gene of interest using the Applied Biosystems 7900 HT Fast RT-PCR system. In addition, a melt curve was generated with each run to determine the specificity of the primers and eliminate false positive results. For prime sequences used (table 1) and the PCR protocol, see appendix. After that, the data was analyzed and its statistical significance was determined using the GraphPad “Prism” software [15, 17].

After running the RT-PCR, the data was normalized using Beta 2 Microglobulin (B2M) as a housekeeping gene. A maximum Ct values range of 3 between the samples indicated normalized samples. To calculate the relative expression of the genes, the equation $2^{A-B}/2^{C-D}$ was used, where A is the threshold cycle number of the first control sample of the gene of interest, B is the threshold cycle number in each gene of interest sample, C is the threshold cycle value of the first B2M in the control sample, and D is the threshold cycle number of B2M in each sample. The formula resulted in a relative expression of 1 for the first control sample, and then all the other samples were calculated in relation to the first sample. After that, the average of
the N-Sham group was calculated and used as a denominator for the other groups average to calculate the fold change in gene of expression compared to the control group [18].

*Protein Extraction:* Total proteins were extracted from about 100mg liver tissue using Thermo Scientific’s Tissue Protein Extraction Reagent (T-PER) protocol, and protease and phosphotase inhibitors. Using the bullet blender, the samples were homogenized in the extraction buffer as mentioned in the RNA extraction. After centrifugation, at 15,000 rpm for 15 min at 4°C, the aqueous layer below the fat was transferred into a new tube and centrifuged again. The relatively fat-free protein extract was first diluted 1:10, then stored at -80°C [15].

Thermo Scientific’s Pierce BCA Protein Assay, a copper (II) colorimetric protein assay, Kit was used to measure the total protein concentration in every sample following the protocol. For start, 10μL per well of the diluted sample and bovine serum albumin (BSA) standards were pipetted into a 96 well flat bottom plate in duplicate, then 200μL of BCA working reagent was added to each well. After that, the plate was incubated for 30min at 37°C, and then cooled to RT. The absorbance, at 562nm, was read in the Epoch Gen5 reader. A standard curve was also generated from the BSA standards, allowing the extrapolation of the protein’s concentrations. Using Microsoft Excel, we calculated the volume of each protein sample needed, to be used for western blot, to allow loading 64μg of protein within 16μL volume per well. A 4X sample buffer
mixed with 2-Mercaptoethanol in a 19:1 ration was added in a 1:3 dilution (sample buffer : sample protein and T-PER).

**Western Blot:** This method was used to determine the levels of a target total and phosphorylated proteins. This approach uses antibodies against the target proteins; therefore a high level of specificity is observed. First, the sample, with the protein of interest, is separated by size using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). After that, the proteins are transferred from the gel to a polyvinylidene fluoride (PVDF) membrane, which has a high affinity for proteins. This transfer allows the proteins to remain in relatively the same locations as on the gel. Since only a small area of the protein will cover the membrane, a blocking buffer is added to inhibit unspecific binding of the primary antibody used in the next step. The primary antibody, monoclonal antibody against the target protein, will bind to the protein of interest. Then the membrane is washed to remove any unbound antibodies. After that, a secondary antibody is added targeting the constant region of the primary antibody. It also has a substrate linked to its constant region that will fluoresce at a specific wavelength. A second wash is done to remove any unbound secondary antibodies, and then the bands on the membrane are detected using the Odyssey instrument since the substrate on the secondary antibodies will emit fluorescence (Al-Regaiey et al., 2004). The PVDF membrane was imaged with the Li-Cor Odyssey Infrared Imager and ImageStudio software. The signal values were measured and used in GraphPad Prism for statistical analysis.
Enzyme-Linked Immunosorbent Assay (ELISA): Although similar to western blot, this technique allows for the detection of a specific antigen in the sample with greater sensitivity using monoclonal antibodies without the need to run the samples in an SDS-PAGE. The samples are instead placed in a 96 wells plate and assayed. The protein is first sandwiched between the bound and the detection antibodies, then a secondary antibody is added targeting the Fc region of the detection antibody; which is also bound to horse radish peroxidase (HRP). The addition of stabilized chromogen causes fluorescence when it interacts with HRP. Using the BioTek Plate Washer, ELx50, the plate is washed between every step. A color change indicates a positive result, which implies the presence of the target protein; the fluorescence is then measured using a plate reader. Fluorescence is proportional to concentration; the brighter the fluorescence, the more proteins of interest are present.
RESULTS

Effect of VFT on insulin sensitivity and glucose tolerance

The Glucose tolerance test indicated that transplanting GHRKO visceral fat into N animals improved glucose handling in N-KO mice when comparing with N-Sham animals, while there was no effect in N-N when comparing with N-Sham group (Fig. 1).

Figure 1- Glucose Tolerance Test (GTT) measured by blood glucose levels in GHRKO and N-mice following VFT or sham surgery.
Similarly insulin tolerance test showed that N-KO mice are characterized by higher insulin sensitive than N-normal and N-sham animals. However, implanting visceral fat from N animals to N recipient did not change insulin sensitivity (Fig. 2).

Figure 2- Insulin Tolerance Test (ITT) measured by blood glucose levels in GHRKO and N-mice following VFT or sham surgery.
The overall sensitivity of the mice can be measured using the Homeostatic Model of Assessment (HOMA), which is the relationship between insulin levels and peripheral glucose and it is calculated by the formula (insulin x glucose)/22.5. A relatively lower HOMA score represents improved insulin sensitivity. Normal mice transplanted with GHRKO visceral fat show a reductions HOMA score, therefore an improved insulin sensitivity compared to the sham group. However, N-N mice had the same HOMA score compared to the sham group (Fig. 3). Moreover, insulin plasma analysis revealed a significant reduction of insulin levels in N-KO group when compared to N-N (P= 0.0033) and N-sham groups (P= 0.028) (Fig. 4). In addition, the analysis of plasma leptin showed a decreased of leptin in N-KO mice compared to the N-N group (P=0.0361) (Fig 5).

Figure 3- Insulin Sensitivity measured by HOMA analysis. Different letters represent statistical significance (P < 0.05).
Figure 4- Effect of VFT on plasma insulin levels. Different letters represent statistical significance (P < 0.05).

Figure 5- Effects of VFT on Plasma Leptin levels. Different letters represent statistical significance (P < 0.05).
Effect of VFT on insulin signaling in liver

Analysis of the hepatic mRNA expression of different genes related to insulin signaling-pathway (Table 2) using RT-PCR revealed different gene expression between the three groups. The expression of IGF-1, IRS1, and AKT2 mRNA in N-N groups was decreased when compared to N-S mice (P= 0.0201, P= 0.0318, and P= 0.0301, respectively), but no significant change was observed in the N-KO mice when comparing with N-S animals (Fig 6A, B, E). The mRNA expression of PPARa and PPARg was increased in N-KO mice compared to the N-N mice (P= 0.0115 and P=0.0155, respectively) and to the N-Sham groups (P= 0.0276 and P= 0.0119, respectively) (Fig. 6C-D). However, FOXO1 gene expression was increased in N-N mice in comparison to N-S group and to N-KO mice (P= 0.0022 and P= 0.0139 respectively) (Fig 6F). In addition, the gene expression of IR, GLUT2, GSK3b, PGC1a, and STAT showed no significant difference between the three groups (Fig 7).
Figure 6: Relative gene expression of IGF-1, IRS1, PPARa, PPARg, AKT2, and FOXO1 in the liver.

Different letters represent statistical significance (P < 0.05).
Figure 7- Relative hepatic gene expression of PGC1a, IR, PI3K, STAT3, STAT1, and GSK3b. Different letters represent statistical significance (P < 0.05).
Effects of VFT on protein expression in liver

The hepatic protein expression of total IR obtained using ELISA showed that the N-KO mice had increase protein levels compared to N-N group (P= 0.0303), while no significance was observed with the N-S group (Fig 8).

Figure 8- Protein expression of total IR in the liver. Different letters represent statistical significance (P < 0.05).
Before stimulation, the level of phosphorylated IR did not differ between all three experimental groups. However, after challenging the mice with insulin before sacrifice the phosphorylation of IR was decreased in N-N animals when comparing with N-S and N-KO mice (P=0.0401 and P= 0.0006 respectively) (Fig 9).

Figure 9- Protein expression of phosphorylated IR in the liver. Different letters represent statistical significance (P < 0.05).
Using ELISA, we determined the protein expression of GSK3b in the liver, which showed a decrease of pGSK3b in N-N mice compared to N-S group and to N-KO mice (P = 0.007 and P = 0.0352, respectively) (Fig 10).

Figure 10- Protein concentration of GSK3b using ELISA in the liver. Different letters represent statistical significance (P < 0.05).
DISCUSSION

The key novel conclusion in the present study is that the VFT of GHRKO fat into N mice improved their whole body insulin sensitivity. Growth hormone receptor knockout mice are characterized by insulin sensitivity and extended life span, with delayed symptoms of age related diseases such as cancer and diabetes. Although they have high levels of GH in circulation, IGF-1 levels are low due to the absence of GHR; thus the GH is unable to activate its downstream pathway [1, 2]. These results were also observed in other long-living animals such as dwarf mice since their GH pathway was disrupted. Hence, alteration in IGF-1 signaling pathway appeared to be the link between GH and aging [19]. Surgical removal of visceral fat from GHRKO mice decreased insulin sensitivity, while caused the opposite effects in normal mice. Therefore, VF in GHRKO mice appeared to have an altered secretory activity thus promotes insulin sensitivity [2]. The absence of GH signaling seemed to play a significant role in longevity; which allows these mice models to provide, in comparison to normal controls, an overview of the insulin-signaling pathway [11].

In the present study, VFT from GHRKO mice into normal mice gave us the opportunity to better understand the role of VF in insulin signaling. As predicted, the N-KO mice had an increase in their insulin sensitivity as indicated by the GTT and ITT tests compared to the N-S group. However, the N-N mice didn’t exhibit any change in their insulin sensitivity as observed in the GTT and ITT tests. In addition, the HOMA score showed that N-KO mice had an improved insulin sensitivity compared to the
control group. In conjunction with ITT and GTT tests, HOMA score affirmed that VFT from GHRKO mice into N animals helped the mice becoming more insulin sensitive compared to the control groups. It also showed that N-N mice insulin sensitivity was not worsened due to VFT.

Insulin plasma test shows low plasma levels of insulin in N-KO mice indicating a decrease in insulin secretion and increase in insulin sensitivity compared to the N-S mice. In addition, plasma leptin test confirmed the previous finding since low levels of plasma leptin were observed in the N-KO mice. High plasma leptin concentration is an indication of obesity and corresponds to adipose tissue mass [20]. Moreover, no significance in either of insulin or leptin plasma is observed between N-N and N-S groups.

As expected, the N-KO mice improved their glucose tolerance and insulin sensitivity, as observed in the results above, when compared N-S groups. However, no significance was observed between N-N and N-S mice; which indicate that the increase of VFT in N mice didn’t not decrease their insulin sensitivity. These results demonstrate that the VF from GHRKO helped the N mice gain the beneficial effects of fats lacking the GH and helped improve their whole body insulin sensitivity; which support the previous data stating that the VF from GHRKO mice is considered as “good” fat [2]. In addition, the VFT of normal fat into normal animals did not seem to worsen the situation compared to the sham group, this could be explained by the possibility that the mice
might have a threshold in which they can still manage the increase of VF without any consequences on their insulin sensitivity.

The VFT led to an up- and down-regulation of several genes involved in the insulin and growth hormone-signaling pathway in the liver, which is one of the major insulin organs. The two significant differences between N-KO mice and the N-S were observed in the gene analysis of PPARa and PPARg. An increase in these gene expressions in N-KO mice signifies an increase in lipogenesis. Increase of PPARg indicates an increase in insulin sensitivity, while an increase in PPARa indicates an increase in the usage of fatty acids [9]. These findings correlates with the previous results stated above, that the N-KO mice had an increase in insulin sensitivity.

From the gene analysis of IGF1, IRS1, AKT2, and FOXO1 we observe a trend toward a decrease of these genes in N-N mice compared to N-S. Although, such trend can indicate a start of a decrease in insulin sensitivity, the ITT and GTT tests showed no such results. These results shows that the N-N mice might have needed more time for their insulin sensitivity to decrease, or more VF was need to be transplanted. In the other hand, N-KO mice had no significant difference in their gene expression compared to the N-S group, which could indicate that the GHRKO VF targets the whole body insulin sensitivity.

The protein expression of total and phosphorylated IR was measured using ELISA. Since IR requires stimulation and phosphorylation to activate its downstream pathway, N-N mice showed a decrease in phosphorylated IR compared to N-S group,
indicating that transplanting N mice with normal VF inhibited the activation of IR. However, N-KO mice had no significant differences with the N-Sham group. This data indicates that transplanting normal VF inhibited insulin signaling, while transplanting GHRKO VF had no effects.

ELISA was also used to determine the protein expression of phosphorylated GSK3b at Serine 9. In response to insulin stimulation, GSK3b is phosphorylated at Serine 9 and deactivated via the PI3K/AKT pathway. This results in a decrease in phosphorylation and activation of glycogen synthase, which in turn causes an increase in glycogen synthesis. In the present data, N-N mice show a decrease in pGSK3b, compared to N-S group, indicating a decrease in glycogen homeostasis, therefore promoting the inhibition of insulin signaling. N-KO mice show no significant difference compared to the N-S groups. This result indicates that N-N mice show a decrease in their insulin signaling, however not enough to be considered significant and imply insulin resistance.

In conclusion, this experiment showed that the VFT from GHRKO mice into normal mice helped them enhance their insulin sensitivity and glucose tolerance, which resulted in an increase in their whole body insulin sensitivity. In other words, the VFT from GHRKO mice helped the normal mice acquire the beneficial effects of fats lacking GH and lead to an improvement of the whole body insulin sensitivity. This in turn can help us understand the mechanism that alter the secretory function of fat in these long
lived animals and translate the results to manipulate fat tissue to prevent diabetes and other age related diseases.

Figure 11- Proposed mechanism of extended longevity in GHRKO mice.
Nano-drop Protocol

To determine the RNA concentration, 2μL of RNA sample was pipetted into the Epoch Gen5 Plate Reader along with two blanks containing RNase free water. A wavelength of 260nm was used to read the absorbance and determine the relative RNA concentration. An absorbance ratio of 260/280 greater than 1.9 signified a purified sample, which can be used to make cDNA.

RT-PCR Protocol

The Applied Biosystems 7500 Fast RT-PCR system was used to quantify different genes present in the tissue. Into a 96 wells plate, SYBR Green (used for detection), forward and reverse primers, and cDNA were added. The system is a quantitative method since it compares the Ct values. It was also set up for 45 cycles and in fast mode (~40min). The temperatures for the RT-PCR were as follow:

- Enzyme Activation: 45°C for 20 sec
- Denature: 95°C for 30 sec
- Annealing and extend: 62°C for 30 sec
**Table 1 List of primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (forward and reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B2M</strong></td>
<td>F: 5'-AAGTATACTCAGCCACCCA-3'&lt;br&gt;R: 5'-CAG CGCTATGTATCAGTCTC-3'</td>
</tr>
<tr>
<td><strong>IR</strong></td>
<td>F: 5'-GTTCTTTTCTGCGTGCATTTCCA-3'&lt;br&gt;R: 5'-ATCAGGGTGCCAGTGTGCTTCTTA-3'</td>
</tr>
<tr>
<td><strong>IRS1</strong></td>
<td>F: 5'- AGCCCCAAAAGCCCAGGAGAATA-3'&lt;br&gt;R: 5'-TTCCGAGCCAGTCTTCTCTTA-3'</td>
</tr>
<tr>
<td><strong>IRS2</strong></td>
<td>F: 5'- AGTAACCGAGGTGGCTACA-3'&lt;br&gt;R: 5'-AAGCTGAGAAGTCAAGGT-3'</td>
</tr>
<tr>
<td><strong>PI3K</strong></td>
<td>F: 5'- TAGCTGCATTGGAGCTCCTT-3'&lt;br&gt;R: 5'-TACGAACTGTGGGAGCAGAT-3'</td>
</tr>
<tr>
<td><strong>AKT2</strong></td>
<td>F: 5'- GAGGACCTTCCATGTAGACT-3'&lt;br&gt;R: 5'-CTCAGATGTGGGAAGAGTGC-3'</td>
</tr>
<tr>
<td><strong>PPARg</strong></td>
<td>F: 5'- GTCAGTACTGTCGTTTCAG-3'&lt;br&gt;R: 5'-CAGATCAGCAGACTCTGGGT-3'</td>
</tr>
<tr>
<td><strong>PPARa</strong></td>
<td>F: 5'-GGGAAAGACCAGCAACC-3'&lt;br&gt;R: 5'-TGGAAGAATCGGCCCTGC-3'</td>
</tr>
<tr>
<td><strong>PGC1a</strong></td>
<td>F: 5'-TACGCAGGGTCAACGAAAAT-3'&lt;br&gt;R: 5'-TGCTCTTTGGTGGAGCA-3'</td>
</tr>
<tr>
<td><strong>IGF-1</strong></td>
<td>F: 5'-CTGAGCTGGATGCTTCTT-3'&lt;br&gt;R: 5'-CACTCATCCACACCTTG-3'</td>
</tr>
<tr>
<td><strong>GLUT2</strong></td>
<td>F: 5'-GGTCAGAATCTACCTTCTTTG-3'&lt;br&gt;R: 5'-GTGTGTGTGGAATTTGCTC-3'</td>
</tr>
<tr>
<td><strong>STAT3</strong></td>
<td>F: 5'-AGAAAGTGTCACAAAGGCGAC-3'&lt;br&gt;R: 5'-CACGAAGGCACTTCTCCATTAAGGT-3'</td>
</tr>
<tr>
<td><strong>STAT1</strong></td>
<td>F: 5'-CGACCAGTACAGCGCTTTT-3'&lt;br&gt;R: 5'-CGGGGTCTTCTTGGGAAGTATC-3'</td>
</tr>
</tbody>
</table>
Table 2 Analyzed genes related to insulin signaling

<table>
<thead>
<tr>
<th>Genes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin receptor (IR)</td>
<td>Transmembrane receptor activated by insulin</td>
</tr>
<tr>
<td>Insulin receptor substrate 1 and 2 (IRS1 and IRS2)</td>
<td>Proteins in insulin pathway phosphorylated by IR</td>
</tr>
<tr>
<td>Glucose transporter 2 (GLUT2)</td>
<td>Mediates the transport of glucose</td>
</tr>
<tr>
<td>Insulin-like growth factor 1 (IGF-1)</td>
<td>Similar in molecular structure to insulin, can activate IR. Important role in childhood growth and has anabolic effects in adults</td>
</tr>
<tr>
<td>Protein kinase B (AKT2)</td>
<td>Plays a key role in signal transduction downstream of the insulin receptor</td>
</tr>
<tr>
<td>Forkhead box O1 (FOXO1)</td>
<td>Transcription factor, downstream pathway of insulin signaling</td>
</tr>
<tr>
<td>Peroxisome proliferator–activated receptor gamma coactivator 1 alpha (PGC-1a)</td>
<td>Transcriptional coactivator that regulates the genes involved in energy metabolism</td>
</tr>
<tr>
<td>Peroxisome proliferator–activated receptor-gamma (PPARg)</td>
<td>Nuclear receptor with a key role in adipogenesis; target receptor for thiazolidinediones (insulin sensitizers)</td>
</tr>
<tr>
<td>Peroxisome proliferator–activated receptor-alpha (PPARα)</td>
<td>Nuclear receptor regulating fatty acids b- and w- oxidation</td>
</tr>
<tr>
<td>Phosphoinositide 3-kinase (PI3K)</td>
<td>Activates the protein kinase AKT</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 1, and 3 (STAT3, STAT1)</td>
<td>Transcription factor, and regulates down stream genes in the insulin pathway.</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3 beta (GSK3b)</td>
<td>Regulatory kinase of glycogen homeostasis</td>
</tr>
</tbody>
</table>
Western Blot Protocol

*SDS-PAGE*: Due to time constraints, the Bio-Rad transfer (1X TG) and Running (1X TGS) buffers were made two days before the experiment following the direction on the package and kept at 4°C. The procedure was completed using precast 26 wells 10% Criterion TGX gels. The molecular marker, 10μL, was always loaded into the first well, followed by 16μL of the samples, and the last empty wells were loaded with 16μL of loading dye. The voltage was set at 80V for 30min while in the stacking gel, and then increased to 100V for 60min (or until the sample reaches the bottom of the gel) while in the resolving gel.

*Western Transfer*: After cutting the PVDF membrane to match the Polyacrylamide gel’s size, it was activated in methanol (MeOH) for 1 min, and then soaked in transfer buffer for 10 min. The membrane was then placed on top of the gel and sandwiched between filter paper and sponge, with the gel being on the negative electrode side and the membrane on the positive side. The complex was then placed in the tank and submerged with cooled transfer buffer. A stirring rod and ice pack were also added, and the transfer was run for 1 hour at 80V.

Once the transfer was over, the membrane was first rinsed in phosphate buffered saline (PBS) and blocked in Pierce Protein-Free (PBS) Blocking Buffer for 1 hour with shaking. After that, the membrane was washed with PBS, then incubated with the primary antibody for 1 hour with shaking. Before adding the secondary antibody, the membrane was washed with phosphate buffered saline with tween (PBS-T) four times,
5 min each, with shaking. After 1-hour incubation with the secondary antibody while protected from light, another 4 washes with PBS-T were performed, and the membrane was covered in PBS for storage.

**Antibodies:** The primary antibodies, from mouse or rabbit source, were added to a solution containing PBS-T and the blocking buffer in a 1:1 ratio. After use, they were stored at -20°C, but they were never used more than twice. The secondary antibodies, anti-mouse or anti-rabbit, were added to a mixture of PBS and the blocking buffer (1:1 ratio) and 10% SDS making a 1:20,000 dilution. The final mixture was protected from light by aluminum foil paper, and stored at 4°C after use, it was use for a maximum of two runs.
Figure 12- Flow chart of Insulin and Glucose pathways, indicating some of the proteins studied in this experiment.
REFERENCES


