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Key Regulators of Mitochondrial Biogenesis are Increased in Kidneys of Growth Hormone Receptor Knockout (GHRKO) Mice

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Abstract

The growth hormone (GH) receptor knockout mice (GHRKO) are remarkably long-lived and highly insulin sensitive. Alterations in mitochondrial biogenesis are associated with aging and various metabolic derangements. We have previously demonstrated increased gene expression of key regulators of mitochondriogenesis in kidneys, hearts and skeletal muscles of GHRKO mice. The aim of the present study was to quantify the protein levels of the following regulators of mitochondriogenesis: peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), AMP-activated protein kinase α (AMPK α), phospho-AMPK α (p-AMPK α), sirtuin-3 (SIRT-3), endothelial nitric oxide synthase (eNOS), phospho-eNOS (p-eNOS), nuclear respiratory factor-1 (NRF-1) and mitofusin-2 (MFN-2) in skeletal muscles and kidneys of GHRKOs in comparison to normal mice. We were also interested in the effects of calorie restriction (CR) and visceral fat removal (VFR) on these parameters. Both CR and VFR improve insulin sensitivity and can extend lifespan. Results: The renal levels of PGC-1 α , AMPK α , p-AMPK α , SIRT-3, eNOS, p-eNOS and MFN-2 were increased in GHRKOs. In the GHRKO skeletal muscles, only MFN-2 was increased. Levels of the examined proteins were not affected by CR (except for PGC-1 α and p-eNOS in skeletal muscles) or VFR. Conclusion: GHRKO mice have increased renal protein levels of key regulators of mitochondriogenesis and this may contribute to increased longevity of these knockouts.

Keywords

mitochondrial biogenesis; GHRKO mice; skeletal muscles; kidneys; proteins; calorie restriction; visceral fat

Introduction

Growth hormone (GH) receptor/binding protein knockout mice (GHRKO) are homozygous for the targeted disruption of the GH receptor gene (*Ghr* gene).¹ These animals are GH-

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Conflict of Interest: None known

resistant and characterized by extended longevity, reduced weight and body size, undetectable GH receptor levels, elevated serum GH, greatly reduced plasma levels of IGF-I and insulin, low or normal glucose, enhanced insulin sensitivity, improved resistance to oxidative stress, reduced oxidative damage and lower incidence and delayed onset of fatal neoplastic diseases.¹⁻¹¹

The biogenesis of mitochondria is a process by which new mitochondria are formed. These organelles play an essential role in energy homeostasis and metabolism, contain enzymes indispensable for various biochemical processes and are crucial for apoptosis regulation and for proper cell viability.¹² Disturbances in mitochondrial biogenesis affect oxidative stress resistance, energy production and metabolism and may result in the development of age-associated degenerative diseases.

Numerous factors are involved in the regulation of mitochondrial biogenesis, including peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), AMP-activated protein kinase (AMPK), sirtuins, endothelial nitric oxide synthase (eNOS), nuclear respiratory factor-1 (NRF-1) and mitofusin-2 (MFN-2). Mitochondrial biogenesis can be enhanced, among others, by calorie restriction (CR).¹³⁻¹⁶ PGC-1 α is the key regulator (“master regulator”) of mitochondrial biogenesis, playing an important role in the activation and control of this process through regulation of physiological signals and transcription factors.^{17,18} AMPK is a cellular energy sensor, activated by an increase in intracellular adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio.¹⁹ In skeletal muscles, AMPK stimulates glucose transport and fatty acid oxidation. Activated AMPK phosphorylates PGC-1 α and enhances its activity.²⁰ Sirtuins (NAD⁺-dependent deacetylases) are enzymes involved in various biological processes, including DNA repair and the maintenance of chromosome stability. SIRT-3 is one of members of the sirtuin family. This factor regulates mitochondrial metabolism, adaptive thermogenesis, energy homeostasis, and apoptosis.^{21,22} eNOS generates nitric oxide that activates mitochondrial biogenesis through the transcriptional activation of PGC-1 α .^{23,24} NRF-1 is a nuclear DNA-binding factor which among other functions, regulates transcription of various genes and plays an important role in the development of nervous and muscle system.²⁵ Interestingly, the increased glucose transport capacity has been shown in transgenic mice overexpressing NRF-1 in skeletal muscles.²⁶ MFN-2 regulates mitochondrial biogenesis and fusion and is involved in maintenance of the mitochondrial network architecture.^{27,28} Decreased expression of MFN-2 may be linked to metabolic disturbances resulting in development of obesity.²⁷

We have previously demonstrated enhanced gene expression of certain regulators of mitochondriogenesis in kidneys of male GHRKO mice and in heart and skeletal muscles of female GHRKO vs. normal (N) mice and we suggested that these alterations may contribute to the mechanisms of longevity regulation in these mutants.²⁹

Calorie restriction (CR) is a well known experimental intervention to delay aging and increase lifespan.³⁰ Normal mice subjected to CR resemble certain phenotypic features of GHRKO mice, including reduced plasma IGF-I and insulin levels and high insulin sensitivity. Moreover, lifespan of normal mice subjected to CR resembles that of GHRKO mice fed *ad libitum*.³¹

Surgical visceral fat removal (VFR; removal of epididymal and perinephric fat depots) improves insulin signaling in N mice and rats and extends longevity in rats,³²⁻³⁴ thus mimicking the effects of CR. Unexpectedly, this intervention promoted insulin resistance in GHRKO mice in spite of the beneficial effects of VFR in genetically normal animals (Masternak *et al.*, personal comm.).

The present study was undertaken to quantify the protein levels of PGC-1 α , AMPK α , phospho-AMPK α (p-AMPK α), SIRT-3, eNOS, phospho-eNOS (p-eNOS), NRF-1 and MFN-2 in skeletal muscles of female GHRKO and N mice subjected to 40% CR or fed *ad libitum* (EXPERIMENT 1) and in kidneys of male GHRKO mice subjected to VFR or sham-operated (EXPERIMENT 2). The results of the present study may indicate whether alterations in the levels of the examined proteins reflect changes in expression of the corresponding genes. Additionally, it was of interest to compare the levels of these proteins between males and females.

Materials and Methods

Animals

Normal and GHRKO mice used in the present study were produced in our breeding colony, developed using animals kindly provided by Dr. J.J. Kopchick (Ohio University). Animals were produced by mating knockout (-/-) males with heterozygous (+/-) females. Normal (+/-) and GHRKO animals (-/-) were separated by phenotypic characteristics. All animal procedures were approved by the Laboratory Animal Care and Use Committee (LACUC) at the Southern Illinois University School of Medicine (Springfield, IL). The mice were housed under temperature- and light-controlled conditions (22 $\pm$ 2°C, 12 hr light/12 hr dark cycle) and fed Lab Diet 5001 chow (PMI Nutrition International, Richmond, IN) containing 4.5 % fat and 23.4 % protein.

Experiment 1

Calorie restriction (CR)

Starting at 2 months of age, forty six (46) normal and GHRKO female mice were grouped according to average body weight within the phenotype, and divided into four experimental groups: normal-fed *ad libitum* (N-AL) (10 animals), normal-CR (N-CR) (14 animals), GHRKO *ad libitum* (KO-AL) (10 animals), and GHRKO-CR (KO-CR) (12 animals). Food-restricted animals were subjected to gradually introduced 40% CR (which corresponded to 60% of the food consumed by their AL counterparts). The gradually introduced 40% CR means that calorie-restricted mice received 90% of daily food consumption of AL animals of the same genotype for 1 week, then 80% for the second week, 70% for the third week and maintaining 60% for the rest of study. Food consumption of AL animals was monitored throughout the study, and the CR animals were fed daily, at approximately 5:00 PM, 60% of the average amount of food consumed daily by AL animals during the preceding week. Water was available at all times to all animals. At 8 months of age, the animals were fasted overnight, anesthetized using isoflurane, bled by cardiac puncture and euthanized by decapitation. Hind-limb skeletal muscles were rapidly collected, quickly frozen on dry ice and stored at -80°C until processed.

Experiment 2

Visceral fat removal (VFR)

At the age of approximately 6 months, forty three (43) normal and GHRKO male mice were grouped according to average body weight within the phenotype, and divided into four experimental groups: normal sham-operated (N-sham) (11 animals), normal subjected to visceral fat removal (N-VFR) (11 animals), GHRKO sham-operated (KO-sham) (10 animals), and GHRKO subjected to visceral fat removal (KO-VFR) (11 animals). The animals were anesthetized with ketamine/xylazine, shaved and prepared in the usual sterile fashion. Mice were supplied with ibuprofen in drinking water starting 2 days before and up to 3 days after the surgery. Tap water was available at all times to all animals. In the VFR

group, the epididymal fat pads were removed using blunt dissection through a midline incision and perinephric fat pads were removed via flank incisions. We removed as much epididymal and perinephric fat as was possible without compromising blood supply to the testes and to the adrenals. For sham operations, the abdominal cavity and both sides of the back were incised and the visceral fat was mobilized, but not removed. Two months after VFR or sham operations, the animals were fasted overnight, anesthetized using ketamine/xylazine, bled by cardiac puncture and euthanized by decapitation. Kidneys were rapidly collected, quickly frozen on dry ice and stored at -80°C until processed.

Protein extraction and Western blotting—Total proteins were obtained from tissue homogenates. Approximately 100 mg samples of skeletal muscles and kidney tissue were homogenized in 1 ml ice-cold T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL), with Protease Inhibitor Cocktail Kit (Pierce Biotechnology, Rockford, IL), Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, St. Louis, MO) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, St. Louis, MO)]. After mixing, homogenates were centrifuged at 16000 *g* for 30 minutes. Protein concentrations were assessed using Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) in accordance with manufacturer's protocol.

Western blot procedure was performed using the following primary antibodies: PGC-1 α (dilution: 1:1000), AMPK α (1:1000), phospho-AMPK α (Thr172) (1:1000), eNOS (1:1000) and phospho-eNOS (Ser1177) (1:1000) (all from Cell Signaling Technology, Beverly, MA), MFN-2 (1:500) (BioVision, Inc., Mountain View, CA), NRF-1 (1:1000) and SIRT-3 (1:500) (both from Abcam Inc., Cambridge, MA) and secondary goat antirabbit antibodies (1:5000) (Calbiochem, La Jolla, CA). Monoclonal anti- β -actin antibody (1:10000) (Sigma-Aldrich Corp., St. Louis, MO) was used, after stripping the membrane, as a control for protein loading.

For Western blotting, protein extracts were mixed with XT Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and heated in a thermocycler at 99°C for 5 minutes and then cooled to 4°C. Forty micrograms of the protein was separated electrophoretically using Criterion XT Precast Gel (26 wells; Bio-Rad Laboratories, Inc., Hercules, CA) for 90 minutes at 150 V. Subsequently, proteins were wet-transferred for 60 minutes at 100 V onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) at 4°C. After the transfer, membranes were rinsed briefly in Tris-buffered saline (TBS; pH 7.6) and blocked with 5% nonfat dry milk or 1% BSA (bovine serum albumin) in TBS containing 0.05% Tween 20 (TBST) for 1 h at room temperature. After blocking, membranes were washed with TBST three times for 15 minutes each time. Then, the membranes were incubated with the primary antibody specific for the protein of interest diluted in the appropriate blocking solution at 4°C overnight with shaking. After incubation, the blots were washed three times (15 min each) with TBST and incubated with an appropriate horseradish peroxidaseconjugated secondary antibody for 1 hour at room temperature. Horseradish peroxidase activity from secondary antibody was detected using the Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). Photos of blots were taken with Image Reader LAS-4000 (FujiFilm, Tokyo, Japan) and quantified for statistical analysis using Multi Gauge version 3.0 software (FujiFilm Life Science, Japan). The protein level was expressed as the Arbitrary Unit (AU)/mm². The AU is a unit to measure the emission amount of chemiluminescence material read using LAS. It represents the relative density value accumulated as linear data by a CCD (charge-coupled device) camera in the image surface.

Statistical analysis

The data are expressed as mean $\pm$ Standard Error of the Mean (SEM). Two-way analysis of variance (ANOVA) was used to evaluate the effects of the genotype and intervention (CR or VFR, respectively). To evaluate the effects of interventions within genotypes and genotypes within interventions, a *t*-test was used. A value of $p < 0.05$ was considered significant. All statistical calculations were performed using SPSS version 17.0 (SPSS, Chicago, IL) with $\alpha = 0.05$. All graphs were made using Prism 4.02 (GraphPad Software, San Diego, CA).

Results

The levels of PGC-1 α (Figure 1A), AMPK α (Figure 1B), p-AMPK α (Figure 2B), SIRT-3 (Figure 2A), eNOS (Figure 3A), p-eNOS (Figure 4A) and MFN-2 (Figure 4B) proteins were increased in kidneys of GHRKO mice (pooled KO-sham and KO-VFR) as compared to normal (N) animals (pooled N-sham and N-VFR) ($p = 0.005$, $p = 0.003$, $p = 0.049$, $p = 0.004$, $p = 0.049$, $p = 0.048$, $p < 0.001$, respectively). In comparisons based only on sham-operated mice, KO-sham mice exhibited elevated renal PGC-1 α (Figure 1A) and MFN-2 (Figure 4B) protein level in comparison to N-sham animals ($p = 0.018$, $p = 0.004$, respectively). Moreover, KO-VFR mice had increased renal protein level of AMPK α (Figure 1B) and p-eNOS (Figure 4A) as compared to N-VFR animals ($p = 0.033$, $p = 0.042$, respectively).

In skeletal muscles, only MFN-2 protein level was increased in GHRKO *vs.* N mice ($p < 0.001$) (Figure 8B). Moreover, the increased level of this protein was observed in KO-CR mice in comparison to N-CR and KO-AL animals ($p < 0.001$ both) (Figure 8B). Calorie restriction decreased skeletal muscle protein level of PGC-1 α *vs.* AL mice ($p = 0.003$) (Figure 5A) and increased protein level of p-eNOS *vs.* AL mice in this tissue ($p = 0.014$) (Figure 8A). Furthermore, KO-CR mice exhibited reduced level of PGC-1 α protein as compared to KO-AL and N-CR animals ($p < 0.001$, $p = 0.028$, respectively) (Figure 5A). Skeletal muscle p-AMPK α protein level was decreased in N-CR *vs.* N-AL mice ($p = 0.033$) (Figure 6B).

Except for reported above skeletal muscle PGC-1 α (Figure 5A) and p-eNOS (Figure 8A), CR (pooled N-CR and KO-CR mice) or VFR (pooled N-VFR and KO-VFR mice) did not change the levels of the examined proteins in kidneys or skeletal muscles as compared to AL (pooled N-AL and KO-AL) or sham-operated (pooled N-sham and KO-sham) animals, respectively (Figures 1-8).

Discussion

We have previously demonstrated increased expression of genes coding for certain key regulators of mitochondrial biogenesis in kidneys (PGC-1 α , AMPK, SIRT-3, eNOS and MFN-2) and skeletal muscles (PGC-1 α) in GHRKO mice.²⁹ In the present study, the renal protein levels of PGC-1 α , AMPK α , p-AMPK α , SIRT-3, eNOS, p-eNOS and MFN-2 were also increased in long-lived GHRKO mice as compared to normal (N) animals. Therefore, the increased renal expression of genes for the regulators of mitochondriogenesis in GHRKO mice is associated with the corresponding changes of their protein products. It could be hypothesized that this increase in protein levels as well as in gene expression may contribute to the extended lifespan of these mutants.

In the present study, the protein level of PGC-1 α – the “master regulator” of mitochondrial biogenesis was shown to be increased in kidneys of GHRKO mice. This alteration corresponds to the results obtained by Al-Regaiey *et al.*³⁵ in livers of male GHRKO mice and may constitute a potential beneficial effect in these knockouts. In contrast, decreased PGC-1 α expression may lead to lipid accumulation in skeletal muscles, and finally to

insulin resistance, obesity and diabetes.³⁶ Moreover, PGC-1 α -null mice demonstrate reduced mitochondrial function and reduced thermogenic capacity.³⁷

The targeted disruption of the *Ghr* gene was associated with an increase in renal level of AMPK α and its phosphorylated form – p-AMPK α vs. N mice. The phosphorylation of a threonine residue (Thr172) within the kinase domain of the α -catalytic subunit is required for AMPK activation. Certain apolipoprotein A1 mimetic peptides may exhibit anti-obesity effects in obese diabetic B6v-Lep ob/J mice, through a decrease in visceral fat and an increase of renal pAMPK.³⁸ Interestingly, AMPK is considered to be a new target for antidiabetic drugs.³⁹ Therefore, the elevated p-AMPK α (and also AMPK α) level, as seen in the present study, may be considered beneficial for GHRKO mice.

The increased SIRT-3 protein level in the kidneys of GHRKO mice, demonstrated in the present study, may also be assumed to be beneficial for these knockouts. High levels of SIRT-3 expression were detected in various metabolically active tissues including kidney, brown fat, liver and brain.⁴⁰ Moreover, SIRT-3 may contribute to the regulation of longevity in mice.⁴¹

Our findings included increased renal protein level of eNOS and its phosphorylated active form – p-eNOS in GHRKOs in comparison to N mice. One could speculate that these alterations may be beneficial for GHRKO mice. Some metabolic disturbances, including insulin resistance, can be associated with reduced eNOS expression⁴² and eNOS(-/-) knockout mice are characterized by hypertension, impaired angiogenesis and insulin resistance⁴³ with fasting hyperinsulinemia and hyperlipidemia.⁴⁴ Moreover, lack of eNOS may accelerate glomerular and tubulointerstitial injury,⁴⁵ leading to tubular disruption and tubular cell death.⁴⁶ In contrast, eNOS overexpression prevented the development of renovascular hypertension in C57BL/6 mice.⁴⁷

It is known that PGC-1 α stimulates MFN-2 mRNA and protein expression in muscle cells.⁴⁸ In the present study, mice with targeted disruption of the *Ghr* gene had increased levels of the MFN-2 protein (and also PGC-1 α , as reported above) in the kidneys. Similarly, the increased MFN-2 protein level was demonstrated in skeletal muscles in GHRKO mice, even though no changes in PGC-1 α protein between GHRKO and N mice were detected in this tissue. However, Adhihetty *et al.*⁴⁹ demonstrated up-regulated MFN-2 protein in heart muscle of PGC-1 α knockout mice, showing that MFN-2 level does not have to depend on the levels of PGC-1 α . A change in the opposite direction, namely a decrease of MFN-2 expression was observed in patients with obesity and type 2 diabetes.^{27,50} Numerous mitochondrial defects were observed in experimental models of mitofusin-deficient muscles.⁵¹ Moreover, Chen *et al.*⁵² showed that MFN-2-deficient mice die at midgestation. Therefore, the elevated level of MFN-2 protein, as seen in the present study, may be – by analogy - regarded as beneficial for GHRKO mice.

PGC-1 α induces transcription of nuclear respiratory factor-1 (NRF-1).⁵³ However, in the present study, the NRF-1 protein level was unchanged in the kidneys of GHRKO mice as compared to N animals, even though the PGC-1 α expression was increased in this tissue in GHRKOs. Therefore, one could speculate that PGC-1 α may potentially act in GHRKO animals *via* other transcription factors.

In contrast to the reported alterations in renal protein levels of several key regulators of mitochondrial biogenesis in GHRKO mice, no differences in these proteins (except for MFN-2) in skeletal muscles between knockouts and N mice were detected. These results are consistent with the unaltered expression of almost all of the corresponding genes in GHRKO vs. N mice in this tissue.²⁹ These data could be interpreted as an indication that the role of skeletal muscles in the regulation of mitochondrial biogenesis is insignificant. However, we

have recently demonstrated the increased cytochrome c oxidase (COX) activity (regarded as a measure of mitochondrial content) only in skeletal muscles without changes in the heart and kidney of GHRKO mice.²⁹ Importantly, expression of several pro-apoptotic genes and/or proteins was also changed (decreased) in skeletal muscles of GHRKO mice.⁵⁴ Yin et al. reported the increased COXIV level and, at the same time, unchanged PGC-1 gene expression and protein level in rat model of hypoxic/ischemic brain injury.⁵⁵ Thus, by analogy, the unchanged protein levels of key mitochondrial biogenesis regulators, as seen in the present study, and increased COX activity, as reported previously,²⁹ both in skeletal muscles, may not exclude an important role of this tissue in mitochondrial biogenesis regulation, and as result of that, potentially in prolonged longevity of GHRKO mice.

Calorie restriction (CR) is considered to affect numerous factors involved in mitochondrial biogenesis (including AMPK, eNOS, sirtuins and PGC-1 α), leading to the increase of activity of this process.⁵⁶ However, in the present study, levels of almost all of the examined proteins were not affected by CR. This dietary intervention affected only the level of PGC-1 α and p-eNOS proteins in skeletal muscles. Similarly, VFR did not change the level of the examined proteins as compared to sham-operated mice. It should be emphasized that these observations are not unexpected and are consistent with the results of our previous study showing no effects of CR or VFR on the gene expression of key regulators of mitochondrial biogenesis.²⁹ Importantly, in contrast to the studies showing stimulatory effects of CR on mitochondrial biogenesis, there are also other findings which cast new light on these earlier observations. Hancock *et al.*⁵⁷ have recently demonstrated that 30% CR did not induce an increase in mitochondria in heart, brain, liver, adipose tissue or skeletal muscle in male Wistar rats, as assessed by measurements of several key mitochondrial proteins, including cytochrome c, citrate synthase and cytochrome c oxidase subunit IV (COXIV), among others. Also, Sreekumar *et al.*⁵⁸ showed no effects of 40% CR on content of mitochondria in skeletal muscles in rats. Furthermore, in contrast to extended longevity and improved insulin sensitivity in normal mice subjected to CR, this intervention failed to enhance insulin sensitivity or increase average or mean lifespan of GHRKO mice.³¹ CR failed to further modify the alterations in insulin signaling in GHRKO's livers as compared to normal mice.⁵⁹ Moreover, CR did not change the insulin signaling in the heart of GHRKO mice.⁶⁰

In summary, long-lived GHRKO mice have increased renal levels of key protein regulators of mitochondrial biogenesis and this may constitute a potential benefit in terms of metabolic regulation and survival. It could be hypothesized that the alterations in the regulation of mitochondrial biogenesis in the kidneys may represent one of the mechanisms leading to extended longevity in GHRKO mice. CR or VFR either did not further improve these potentially beneficial effects or did not induce them. Since the stimulatory effect of Ghr gene deletion on key regulators of mitochondrial biogenesis occurs at both, gene and protein levels, one can speculate that this genetic intervention may have important effects on the regulation of mitochondrial biogenesis in the context of increased longevity in GHRKO mice.

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References

1. Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigano K, Wagner TE, Baumann G, Kopchick JJ. A mammalian model for Laron syndrome produced by

- targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci USA*. 1997; 94:13215–13220. [PubMed: 9371826]
2. Kopchick JJ, Laron Z. Is the Laron mouse an accurate model of Laron syndrome? *Mol Genet Metab*. 1999; 68:232–236.10.1006/mgme.1999.2890 [PubMed: 10527674]
3. Coschigano KT, Clemmons D, Bellush LL, Kopchick JJ. Assessment of growth parameters and life span of GHR/BP gene disrupted mice. *Endocrinology*. 2000; 141:2608–2613. [PubMed: 10875265]
4. Bartke A, Chandrashekar V, Bailey B, Zaczek D, Turyn D. Consequences of growth hormone (GH) overexpression and GH resistance. *Neuropeptides*. 2002; 36:201–208.10.1054/npep.2002.0889 [PubMed: 12359510]
5. Coschigano KT, Holland AN, Riders ME, List EO, Flyvbjerg A, Kopchick JJ. Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased lifespan. *Endocrinology*. 2003; 144:3799–3810.10.1210/en.2003-0374 [PubMed: 12933651]
6. Bartke A, Brown-Borg H. Life extension in the dwarf mouse. *Curr Top Dev Biol*. 2004; 63:189–225.10.1016/S0070-2153(04)63006-7 [PubMed: 15536017]
7. Coschigano KT. Aging-related characteristics of growth hormone receptor/binding protein gene-disrupted mice. *AGE*. 2006; 28:191–200.10.1007/s11357-006-9004-x [PubMed: 19943140]
8. Liu JL, Coschigano KT, Robertson K, Lipsett M, Guo Y, Kopchick JJ, Kumar U, Liu YL. Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. *Am J Physiol Endocrinol Metab*. 2004; 287:E405–E413.10.1152/ajpendo.00423.2003 [PubMed: 15138153]
9. Salmon AB, Murakami S, Bartke A, Kopchick J, Yasumura K, Miller RA. Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. *Am J Physiol Endocrinol Metab*. 2005; 289:E23–E29.10.1152/ajpendo.00575.2004 [PubMed: 15701676]
10. Sun LY, Steinbaugh MJ, Masternak MM, Bartke A, Miller RA. Fibroblasts from long-lived mutant mice show diminished ERK1/2 phosphorylation but exaggerated induction of immediate early genes. *Free Radic Biol Med*. 2009; 47:1753–1761.10.1016/j.freeradbiomed.2009.09.021 [PubMed: 19786089]
11. Ikeno Y, Hubbard GB, Lee S, Cortez LA, Lew CM, Webb CR, Berryman DE, List EO, Kopchick JJ, Bartke A. Reduced incidence and delayed occurrence of fatal neoplastic diseases in growth hormone receptor/binding protein knockout mice. *J Gerontol A Biol Sci Med Sci*. 2009; 64:522–529.10.1093/gerona/glp017 [PubMed: 19228785]
12. Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. *Annu Rev Physiol*. 2009; 71:177–203. [PubMed: 19575678]
13. Lambert AJ, Wang B, Yardley J, Edwards J, Merry BJ. The effect of aging and caloric restriction on mitochondrial protein density and oxygen consumption. *Exp Gerontol*. 2004; 39:289–295.10.1016/j.exger.2003.12.009 [PubMed: 15036388]
14. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science*. 2005; 310:314–317.10.1126/science.1117728 [PubMed: 16224023]
15. Lopez-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, Cascajo MV, Allard J, Ingram DK, Navas P, de Cabo R. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc Natl Acad Sci USA*. 2006; 103:1768–1773.10.1073/pnas.0510452103 [PubMed: 16446459]
16. Civitarese AE, Carling S, Heilbronn LK, Hulver MH, Ukropcova B, Deutsch WA, Smith SR, Ravussin E, CALERIE Pennington Team. Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Med*. 2007; 4:e76.10.1371/journal.pmed.0040076 [PubMed: 17341128]
17. Handschin C, Spiegelman BM. Peroxisome proliferator-activated receptor γ coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr Rev*. 2006; 27:728–735.10.1210/er.2006-0037 [PubMed: 17018837]

18. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*. 1998; 92:829–839. [PubMed: 9529258]
19. Reznick RM, Zong H, Li J, Morino K, Moore IK, Yu HJ, Liu ZX, Dong J, Mustard KJ, Hawley SA, Befroy D, Pypaert M, Hardie DG, Young LH, Shulman GI. Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis. *Cell Metab*. 2007; 5:151–156. [PubMed: 17276357]
20. Jager S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 α . *Proc Natl Acad Sci USA*. 2007; 104:12017–12022. [PubMed: 17609368]
21. Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem*. 2005; 280:13560–13567. [PubMed: 15653680]
22. Allison SJ, Milner J. SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways. *Cell Cycle*. 2007; 6:2669–2677. [PubMed: 17957139]
23. Leary SC, Shoubridge EA. Mitochondrial biogenesis: which part of “NO” do we understand? *Bioessays*. 2003; 25:538–541. [PubMed: 12766943]
24. Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, Carruba MO. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science*. 2003; 299:896–899. [PubMed: 12574632]
25. Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev*. 2008; 88:611–638. [PubMed: 18391175]
26. Baar K, Song Z, Semenkovich CF, Jones TE, Han DH, Nolte LA, Ojuka EO, Chen M, Holloszy JO. Skeletal muscle overexpression of nuclear respiratory factor 1 increases glucose transport capacity. *FASEB J*. 2003; 17:1666–1673. [PubMed: 12958173]
27. Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Dugaard JR, Lloberas J, Camps M, Zierath JR, Rabasa-Lhoret R, Wallberg-Henriksson H, Laville M, Palacin M, Vidal H, Rivera F, Brand M, Zorzano A. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. *J Biol Chem*. 2003; 278:17190–17197. [PubMed: 12598526]
28. Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC. Structural basis of mitochondrial tethering by mitofusin complexes. *Science*. 2004; 305:858–862. [PubMed: 15297672]
29. Gesing A, Masternak MM, Wang F, Joseph AM, Leeuwenburgh C, Westbrook R, Lewinski A, Karbownik-Lewinska M, Bartke A. Expression of key regulators of mitochondrial biogenesis in growth hormone receptor knockout (GHRKO) mice is enhanced but is not further improved by other potential life extending interventions. *J Gerontol A Biol Sci Med Sci*. 2011 in press.
30. Weindruch R, Sohal RS. Seminars in medicine of the Beth Israel Deaconess medical center. Caloric intake and aging. *N Engl J Med*. 1997; 337:986–994. [PubMed: 9309105]
31. Bonkowski MS, Rocha JS, Masternak MM, Al Regaiey KA, Bartke A. Targeted disruption of growth hormone receptor interferes with the beneficial actions of calorie restriction. *Proc Natl Acad Sci USA*. 2006; 103:7901–7905. [PubMed: 16682650]
32. Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J, Rossetti L. Surgical removal of visceral fat reverses hepatic insulin resistance. *Diabetes*. 1999; 48:94–98.10.2337/diabetes.48.1.94 [PubMed: 9892227]
33. Muzumdar R, Allison DB, Huffman DM, Ma X, Atzmon G, Einstein FH, Fishman S, Poduval AD, McVei T, Keith SW, Barzilai N. Visceral adipose tissue modulates mammalian longevity. *Aging Cell*. 2008; 7:438–440.10.1111/j.1474-9726.2008.00391.x [PubMed: 18363902]
34. Shi H, Strader AD, Woods SC, Seeley RJ. The effect of fat removal on glucose tolerance is depot specific in male and female mice. *Am J Physiol Endocrinol Metab*. 2007; 293:E1012–E1020.10.1152/ajpendo.00649.2006 [PubMed: 17652151]
35. Al-Regaiey KA, Masternak MM, Bonkowski M, Sun L, Bartke A. Long-lived growth hormone receptor knockout mice: interaction of reduced insulin-like growth factor I/insulin signaling and caloric restriction. *Endocrinology*. 2005; 146:851–860. [PubMed: 15498882]

36. Liang H, Ward WF. PGC-1 α : a key regulator of energy metabolism. *Adv Physiol Educ.* 2006; 30:145–151. [PubMed: 17108241]
37. Lin J, Wu PH, Tarr PT, Lindenberg KS, St Pierre J, Zhang CY, Mootha VK, Jager S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, Wu Z, Cooper MP, Fan MC, Rohas LM, Zavacki AM, Cinti S, Shulman GI, Lowell BB, Krainc D, Spiegelman BM. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1 α null mice. *Cell.* 2004; 119:121–135. [PubMed: 15454086]
38. Peterson SJ, Kim DH, Li M, Positano V, Vanella L, Rodella LF, Piccolomini F, Puri N, Gastaldelli A, Kusmic C, L'Abbate A, Abraham NG. The L-4F mimetic peptide prevents insulin resistance through increased levels of HO-1, pAMPK, and pAKT in obese mice. *J Lipid Res.* 2009; 50:1293–1304. [PubMed: 19224872]
39. Gruzman A, Babai G, Sasson S. Adenosine monophosphate-activated protein kinase (AMPK) as a new target for antidiabetic drugs: a review on metabolic, pharmacological and chemical considerations. *Rev Diabet Stud.* 2009; 6:13–36. [PubMed: 19557293]
40. Palacios OM, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward JL III, Goodyear LJ, Tong Q. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1 α in skeletal muscle. *Aging.* 2009; 1:771–783. [PubMed: 20157566]
41. Benigni A, Corna D, Zoja C, Sonzogni A, Latini R, Salio M, Conti S, Rottoli D, Longaretti L, Cassis P, Morigi M, Coffman TM, Remuzzi G. Disruption of the Ang II type 1 receptor promotes longevity in mice. *J Clin Invest.* 2009; 119:524–530. [PubMed: 19197138]
42. Lee-Young RS, Ayala JE, Hunley CF, James FD, Bracy DP, Kang L, Wasserman DH. Endothelial nitric oxide synthase is central to skeletal muscle metabolic regulation and enzymatic signaling during exercise in vivo. *Am J Physiol Regul Integr Comp Physiol.* 2010; 298:R1399–R1408. [PubMed: 20200137]
43. Mungrue IN, Bredt DS, Stewart DJ, Husain M. From molecules to mammals: what's NOS got to do with it? *Acta Physiol Scand.* 2003; 179:123–135. [PubMed: 14510775]
44. Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, Vollenweider P, Pedrazzini T, Nicod P, Thorens B, Scherrer U. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation.* 2001; 104:342–345. [PubMed: 11457755]
45. Nakayama T, Sato W, Kosugi T, Zhang L, Campbell-Thompson M, Yoshimura A, Croker BP, Johnson RJ, Nakagawa T. Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse. *Am J Physiol Renal Physiol.* 2009; 296:F317–F327. [PubMed: 19036847]
46. Forbes MS, Thornhill BA, Park MH, Chevalier RL. Lack of endothelial nitric-oxide synthase leads to progressive focal renal injury. *Am J Pathol.* 2007; 170:87–99.10.2353/ajpath.2007.060610 [PubMed: 17200185]
47. Gava AL, Peotta VA, Cabral AM, Vasquez EC, Meyrelles SS. Overexpression of eNOS prevents the development of renovascular hypertension in mice. *Can J Physiol Pharmacol.* 2008; 86:458–464. [PubMed: 18641695]
48. Soriano FX, Liesa M, Bach D, Chan DC, Palacin M, Zorzano A. Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor- γ coactivator-1 α , estrogen-related receptor- α , and mitofusin 2. *Diabetes.* 2006; 55:1783–1791. [PubMed: 16731843]
49. Adhietty PJ, Ugucioni G, Leick L, Hidalgo J, Pilegaard H, Hood DA. The role of PGC-1 α on mitochondrial function and apoptotic susceptibility in muscle. *Am J Physiol Cell Physiol.* 2009; 297:C217–C225.10.1152/ajpcell.00070.2009 [PubMed: 19439529]
50. Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, Laville M. Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. *Diabetes.* 2005; 54:2685–2693. [PubMed: 16123358]
51. Chen H, Vermulst M, Wang YE, Chomyn A, Prolla TA, McCaffery JM, Chan DC. Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell.* 2010; 141:280–289.10.1016/j.cell.2010.02.026 [PubMed: 20403324]

52. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol.* 2003; 160:189–200. [PubMed: 12527753]
53. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell.* 1999; 98:115–124. [PubMed: 10412986]
54. Gesing A, Masternak MM, Wang F, Lewinski A, Karbownik-Lewinska M, Bartke A. Decreased expression level of apoptosis-related genes and/or proteins in skeletal muscles, but not in hearts, of growth hormone receptor knockout mice. *Exp Biol Med.* 2011; 236:156–168.10.1258/ebm.2010.010202
55. Yin W, Signore AP, Iwai M, Cao G, Gao Y, Chen J. Rapidly increased neuronal mitochondrial biogenesis after hypoxic-ischemic brain injury. *Stroke.* 2008; 39:3057–3063. [PubMed: 18723421]
56. Lopez-Lluch G, Irusta PM, Navas P, de Cabo R. Mitochondrial biogenesis and healthy aging. *Exp Gerontol.* 2008; 43:813–819. [PubMed: 18662766]
57. Hancock CR, Han DH, Higashida K, Kim SH, Holloszy JO. Does calorie restriction induce mitochondrial biogenesis? A reevaluation. *FASEB J.* 2011; 25:785–791.10.1096/fj.10-170415 [PubMed: 21048043]
58. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, Nair KS. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. *Am J Physiol.* 2002; 283:E38–E43.
59. Bonkowski MS, Dominici FP, Arum O, Rocha JS, Al Regaiey KA, Westbrook R, Spong A, Panici J, Masternak MM, Kopchick JJ, Bartke A. Disruption of growth hormone receptor prevents calorie restriction from improving insulin action and longevity. *PLoS ONE.* 2009; 4:e4567.10.1371/journal.pone.0004567 [PubMed: 19234595]
60. Giani JF, Bonkowski MS, Muñoz MC, Masternak MM, Turyn D, Bartke A, Dominici FP. Insulin signaling cascade in the hearts of long-lived growth hormone receptor knockout mice: effects of calorie restriction. *J Gerontol A Biol Sci Med Sci.* 2008; 63:788–97. [PubMed: 18772466]

Figure 1.

PGC-1 α (A) and AMPK α (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.005$ vs. N mice (the significance for genotype), ** – $p = 0.003$ vs. N mice (the significance for genotype)

Figure 2.

SIRT-3 (A) and p-AMPK α (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.004$ vs. N mice (the significance for genotype), ** – $p = 0.049$ vs. N mice (the significance for genotype)

Figure 3.

eNOS (A) and NRF-1 (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a – values that share the same letter in the superscript are not statistically significant. * – $p=0.049$ vs. N mice (the significance for genotype)

Figure 4.

p-eNOS (A) and MFN-2 (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.048$ vs. N mice (the significance for genotype), ** – $p < 0.001$ vs. N mice (the significance for genotype)

Figure 5.

PGC-1 α (A) and AMPK α (B) protein level in the skeletal muscles of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice fed *ad libitum* (AL) or subjected to 40% calorie restriction (CR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.003$ vs. AL mice (the significance for diet intervention)

Figure 6.

SIRT-3 (A) and p-AMPK α (B) protein level in the skeletal muscles of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice fed *ad libitum* (AL) or subjected to 40% calorie restriction (CR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$).

Figure 7.

eNOS (A) and NRF-1 (B) protein level in the skeletal muscles of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice fed *ad libitum* (AL) or subjected to 40% calorie restriction (CR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a – values that share the same letter in the superscript are not statistically significant.

Figure 8.

p-eNOS (A) and MFN-2 (B) protein level in the skeletal muscles of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice fed *ad libitum* (AL) or subjected to 40% calorie restriction (CR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a-b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.014$ vs. AL mice (the significance for diet intervention), ** – $p < 0.001$ vs. N mice (the significance for genotype)