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GROWTH HORMONE ABOLISHES BENEFICIAL EFFECTS OF CALORIE RESTRICTION IN LONG-LIVED AMES DWARF MICE

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Abstract

Disruption of the growth hormone (GH) axis promotes longevity and delays aging. In contrast, GH over-expression may lead to accelerated aging and shorter life. Calorie restriction (CR) improves insulin sensitivity and may extend lifespan. Long-lived Ames dwarf (df/df) mice have additional extension of longevity when subjected to 30% CR. The aim of the study was to assess effects of CR or GH replacement therapy separately and as a combined (CR+GH) treatment in GH-deficient df/df and normal mice, on selected metabolic parameters (e.g., insulin, glucose, cholesterol), insulin signaling components (e.g., insulin receptor [IR] β -subunit, phosphorylated form of IR [IR pY1158], protein kinase C ζ/λ [p-PKC ζ/λ] and mTOR [p-mTOR]), transcription factor p-CREB, and components of the mitogen-activated protein kinases (MAPK) signaling (p-ERK1/2, p-p38), responsible for cell proliferation, differentiation and survival. CR decreased plasma levels of insulin, glucose, cholesterol and leptin, and increased hepatic IR β -subunit and IR pY1158 levels as well as IR, IRS-1 and GLUT-2 gene expression compared to *ad libitum* feeding, showing a significant beneficial diet intervention effect. Moreover, hepatic protein levels of p-PKC ζ/λ , p-mTOR and p-p38 decreased, and p-CREB increased in CR mice. On the contrary, GH increased levels of glucose, cholesterol and leptin in plasma, and p-mTOR or p-p38 in livers, and decreased plasma adiponectin and hepatic IR β -subunit compared to saline treatment. There were

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CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

no GH effects on adiponectin in N mice. Moreover, GH replacement therapy did not affect IR, IRS-1 and GLUT-2 gene expression. GH treatment abolishes the beneficial effects of CR; it may suggest an important role of GH-IGF1 axis in mediating the CR action. Suppressed somatotrophic signaling seems to predominate over GH replacement therapy in the context of the examined parameters and signaling pathways.

Keywords

Growth hormone; calorie restriction; Ames dwarf mice; metabolism; transcription factors; MAPK signaling

1. INTRODUCTION

The Ames dwarf (df/df) mice, first described by Schaible & Gowen (1961), are homozygous for a spontaneous recessive loss-of-function mutation at the Prophet of pituitary factor-1 (*Prop-1*) gene (Sornson et al., 1996), which is required for Pituitary factor-1 (Pit-1) activation (Andersen et al., 1995). Pit-1 is known to be responsible for differentiation of three types of anterior pituitary cells – somatotrophs, lactotrophs and thyrotrophs (Sornson et al., 1996). Thus, as a result of this mutation, Ames dwarfs are deficient in circulating growth hormone (GH), prolactin and thyroid-stimulating hormone (TSH). Intriguingly, despite such very severe hormonal disturbances, df/df mice live 49%-69% longer (males and females, respectively) than their normal counterparts (Brown-Borg et al., 1996). These dwarfs are characterized by extremely low insulin-like growth factor-1 (IGF-1) and thyroid hormone levels (Bartke & Brown-Borg, 2004), reduced body core temperature (Hunter et al., 1999), enhanced insulin sensitivity (Dominici et al., 2002), increased activity of antioxidant enzymes, e.g. – catalase and Cu/Zn superoxide dismutase (Brown-Borg and Rakoczy, 2000; Hauck and Bartke, 2000), reduced hepatic levels of DNA and protein oxidation (Brown-Borg et al., 2001), as well as reduced incidence of fatal neoplastic diseases (Ikeno et al., 2003), and decreased mammalian Target of Rapamycin (mTOR) signaling (Sharp and Bartke, 2005). The Ames dwarf mice have also a beneficial adipocytokine profile, characterized by increased adiponectin and decreased proinflammatory cytokines (TNF α and interleukin-6) levels (Wang et al., 2006).

One should emphasize that lack of GH production and severely reduced IGF-1 level in Ames dwarf mice may be, as in other long-lived mice with suppressed somatotrophic signaling, considered beneficial (Bartke and Westbrook, 2012; Junnila et al., 2013), given that GH may exert, among others, insulin blocking and diabetogenic effects. Actually, in contrast to Ames dwarfs, giant mice over-expressing GH exhibit a severe reduction in lifespan, and are characterized by hyperinsulinemia and increased insulin resistance (Bartke, 2003). Consistently, elevated GH level may promote insulin resistance in humans and different laboratory animals (Kopchick et al., 1999; Bartke, 2003; Wang et al., 2007). Furthermore, GH transgenic mice demonstrate various symptoms of accelerated aging, and increased frequency of hepatocellular tumors, including cancers (Steger et al., 1993).

Calorie restriction (CR) is a well-known experimental model to delay aging and increase lifespan (Weindruch and Sohal, 1997). This diet intervention also improves insulin

sensitivity, including wild-type and long-lived Ames dwarf mice (Masternak et al., 2004; Masternak et al., 2009; reviewed in Bartke and Westbrook, 2012). Furthermore, df/df mice experience additional extension of lifespan when subjected to 30% CR, showing increase in median, average and maximal lifespan in both sexes (Bartke et al., 2001; reviewed in Bartke et al., 2008). Calorie-restricted Ames dwarf mice showed also a significantly lower incidence of fatal neoplasms (Ikeno et al., 2013).

The mitogen-activated protein kinases (MAPKs) comprise a group of serine/threonine kinases constituting a very complicated physiological pathways network. These enzymes are involved in the cellular adaptive responses to environmental changes, and in the signal transduction upon stimulation by numerous stimuli, including inflammatory cytokines, growth factors or stress (Pimienta and Pascual, 2007). The MAPK signaling pathways participate in the regulation of cell proliferation, differentiation, movement, survival and death (apoptosis). Several MAPK subgroups, including, among others, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and the p38 α / β are known.

The aim of the present study was to assess effects of CR or GH replacement therapy separately and a combined CR/GH treatment in GH-deficient Ames dwarf and normal mice on various metabolic parameters (insulin, glucose, triglycerides [TG], free fatty acids [FFA] and cholesterol) and adipokines (adiponectin, leptin), insulin signaling-related components (insulin receptor [IR], IR β -subunit, phosphorylated IR [IR pY1158], insulin receptor substrate-1 [IRS-1], phosphorylated form of protein kinase C ζ/λ [p-PKC ζ/λ], glucose transporter type 2 [GLUT-2], phosphorylated form of mTOR [p-mTOR]), transcription factors involved in the activation of gluconeogenesis (phospho-cyclic AMP responsive element-binding protein [p-CREB]) and in lipid metabolism (peroxisome proliferator-activated receptor α [PPAR α] and γ [PPAR- γ]), and components of the MAPK signaling pathways (phospho-p-44/42 MAPK (ERK1/2) [p-ERK1/2], phospho-p38 MAPK [p-p38]).

2. MATERIALS AND METHODS

2.1. Animals

Ames dwarf mice and their normal littermates were produced in our breeding colony by mating homozygous (df/df) males with heterozygous (N/df) females. Animals were housed under controlled temperature and light conditions (22 $\pm$ 2°C with a 12-hour light/12-hour dark cycle) and were provided *ad libitum* with a nutritionally balanced diet (Rodent Laboratory Chow 5001: 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet PMI Feeds, Inc., St Louis, MO). All animal procedures were approved by the Laboratory Animal Care and Use Committee at the Southern Illinois University School of Medicine (Springfield, IL).

2.2. Calorie restriction (CR) and growth hormone (GH) replacement therapy

Food-restricted mice were subjected to gradually introduced 30% CR with food being provided everyday at approximately 5 PM [which corresponded to 70% of the food consumed by their *ad libitum* (AL) counterparts]. Starting at the age of 5 months, calorie restriction (CR) started by receiving 90% of daily food consumption of the AL group for 1 week, then 80% for the second week, and maintaining 70% for the rest of study. Water was

available at all times to all animals. After first 2 weeks of continued 30% CR, half of the animals from each group were subjected to GH treatment.

Recombinant porcine growth hormone (GH) (Alpharma, Victoria, Australia) was dissolved in 0.1 M NaHCO₃ solution (pH~8.3) and adjusted to pH 7.8. GH was administered by subcutaneous injections (4 µg/g body weight per day). Control groups of Ames dwarf and N mice were injected with 0.9% saline daily.

The animals comprised eight (8) experimental groups (all included animals were males): normal mice fed AL and treated with 0.9% saline (N-AL-Sal; 10 animals), normal mice fed AL and treated with GH (N-AL-GH; 10 animals), normal mice calorie-restricted and treated with 0.9% saline (N-CR-Sal; 10 animals), normal mice calorie-restricted and treated with GH (N-CR-GH; 10 animals), Ames dwarf mice fed AL and treated with 0.9% saline (df/df-AL-Sal; 10 animals), Ames dwarf mice fed AL and treated with GH (df/df-ALGH; 10 animals), Ames dwarf mice calorie-restricted and treated with 0.9% saline (df/df-CR-Sal; 10 animals), Ames dwarf mice calorie-restricted and treated with GH (df/df-CR-GH; 10 animals).

At the age of approximately 7 months, after eight (8) weeks of 30% CR and six (6) weeks of GH treatment (24 hours after last injection), the animals were fasted overnight, anesthetized using isoflurane, bled by cardiac puncture, and euthanized by decapitation. Livers were rapidly collected, quickly frozen on dry ice, and stored at -80°C until processed. Whole blood was centrifuged in order to isolate the plasma supernatant, which was also stored at -80°C.

2.3. RNA extraction and complementary DNA (cDNA) transcription

RNA was extracted from liver homogenates by the method described previously (e.g., Al-Regaiey et al., 2007). The RNA concentration was measured spectrophotometrically at 260 nm. One microgram of total RNA was subjected to electrophoresis on a 1.5% agarose gel to confirm RNA integrity. Potentially contaminating residual genomic DNA was eliminated using DNase I (Promega, Madison, WI). Reverse transcription was performed, and complementary DNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction.

2.4. Real-time polymerase chain reaction (RT-PCR)

The real-time polymerase chain reaction (RT-PCR) was carried out using the Smart Cycler instrument (Cepheid, Sunnyvale, CA) with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The three steps of the PCR included: denaturation at 94°C for 2 minutes, annealing at 62°C for 30 seconds with fluorescence reading, and extension at 72°C for 30 seconds. In addition, a melting curve was done for each reaction to evaluate the potential of nonspecific products. β_2 -microglobulin (B2M), which was previously validated in our laboratory as the most appropriate gene for normalizing the data (Masternak et al., 2005), was used as a housekeeping gene. Gene expression was assessed by measuring steady state levels of mRNA. Relative expression from RT-PCR was calculated using the equation $2^{A-B}/2^{C-D}$ (where A = Cycle Threshold [Ct] number for the gene of interest in the first control sample; B = Ct number for the gene of interest in the

analyzed sample; C = Ct number for the housekeeping gene in the first control sample; D = Ct number for housekeeping gene in the analyzed sample). The first control was expressed as 1.00 by this equation, and all other samples were calculated in relation to this value. Then, the results in the control group (NAL-Sal) were averaged. All other outputs were divided by the mean value of the relative expression in the control group to yield the fold change of the expression of genes of interest compared to the control group. For RT-PCR, the primers used are listed in Table 1.

2.5. Protein extraction and Western blotting

Approximately 100 mg liver samples were homogenized in 500 μ L of ice-cold homogenizing buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton 100), with protease inhibitors cocktail and phosphatase inhibitors cocktails (Sigma-Aldrich, St. Louis, MO) and were spun at 16,000g for 45 minutes. The supernatant was removed and stored at -80°C . Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) according to the manufacturer's instructions.

For Western blotting, protein extracts were mixed with XT Sample Buffer (Bio-Rad Laboratories, Inc.) 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 10% Criterion XT Precast Gel (26 wells; Bio-Rad Laboratories, Inc.) for 90 minutes at 150 V. Subsequently, proteins were wet transferred for 60 minutes at 100 V onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) 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% bovine serum albumin in TBS containing 0.05% Tween 20 (TBST) for 1 hour 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 minutes each) with TBST and incubated with an appropriate horseradish peroxidase-conjugated 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).

Western blot procedure was performed using the following primary antibodies: phospho-p44/42 MAPK (ERK1/2) (p-ERK1/2), phospho-p38 MAPK (p-p38), phospho-PKC ζ / λ (p-PKC ζ / λ), phospho-cAMP response element-binding protein (CREB) Ser133 (p-CREB), phospho-mTOR (p-mTOR) (Cell Signaling Technology, Beverly, MA), and secondary goat antirabbit and goat antimouse antibodies (Calbiochem, La Jolla, CA). Monoclonal anti- β -actin antibody (Sigma-Aldrich Corp.) was used as a control for protein loading.

Photos of blots were taken with a CCD camera (Hitachi Genetic Systems, Alameda, CA) and quantified for statistical analysis using GeneTools software (SynGene, Cambridge, U. K.). Blots of three animals per group were shown on the figures 4 & 5.

2.6. Assessment of blood chemistry

Blood glucose was measured in blood from the tail using a glucometer ONE Touch Ultra, Life Scan, Inc., by cutting the tip of the tail with a razor blade before anesthetizing of the animals. Following glucose measurement animals were anesthetized with isoflurane and blood was collected by cardiac puncture to isolate plasma for assessment of insulin, adiponectin, leptin, free fatty acids (FFA), as well as triglycerides and cholesterol. Plasma insulin levels were determined using Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL). Plasma adiponectin levels were determined using Mouse Adiponectin ELISA Kit (Linco Research, St Charles, MO). Leptin levels were evaluated using Mouse Leptin ELISA Kits (Crystal Chem Inc., Downers Grove, IL). Plasma FFAs were assayed using optimized enzymatic colorimetric assays (Roche, Penzberg, Germany). Triglyceride and cholesterol concentrations were measured using a Triglyceride (Liquid) Reagent Set or Cholesterol (Liquid) Reagent Set, respectively (Pointe Scientific, Inc., Canton, MI).

2.7. Liver tissue IR β -subunit and IR pY1158 levels assay

Liver samples were homogenized in an organic extraction solution of 2:1 chloroform/methanol in a 20:1 volume/weight ratio, and kept at 4°C overnight with gentle shaking. 0.6% NaCl was added to one-half volumes, an emulsification was formed by vortexing, then samples were spun at 800g for 20 minutes at 4°C. After extracting the organic phase, samples were evaporated to dryness under nitrogen and dried samples were reconstituted in 30–100 μ l of phosphate-buffered saline containing 1% Triton X-100.

Liver IR β -subunit level was measured using Insulin Receptor (β -subunit) ELISA kit (EMD Millipore, Billerica, MA), and liver IR pY1158 level was evaluated using IR (pY1158) ELISA Kit, Human, Mouse, Rat (Fisher Scientific UK, Ltd., Loughborough, UK).

2.8. Insulin tolerance test (ITT)

Mice were injected intraperitoneally with 0.75 IU insulin per kilogram of body weight. Blood glucose levels were measured at the time points: 0 minutes, 15 minutes, 30 minutes, and 60 minutes, using a glucometer ONE Touch Ultra (Life Scan, Inc., Milpitas, CA).

2.9. Glucose tolerance test (GTT)

After two weeks recovery from ITT, mice were injected with 2 g of glucose per 1 kilogram of body weight and the glucose levels were measured at different time points: 0 minutes, 15 minutes, 30 minutes, 60 minutes, and 120 minutes, using a glucometer ONE Touch Ultra (Life Scan, Inc., Milpitas, CA).

2.10. Statistical analysis

The data are expressed as mean $\pm$ Standard Error of the Mean (SEM). To evaluate the effects of the genotype, diet intervention or pharmacological treatment, we used two-way analysis of variance (ANOVA). For analyzing differences between group means, we used a Bonferroni post-hoc test. A value of $p < 0.05$ was considered significant. All statistical

calculations were conducted using SPSS version 17.0 (SPSS, Chicago, IL) with $\alpha=0.05$. All graphs were created using Prism 4.02 (GraphPad Software, San Diego, CA).

To statistically analyze differences between normal (N) and Ames dwarf mice (df/df) (a potential genotype effect) we pooled all N mice (N-AL-Sal, N-AL-GH, N-CR-Sal and NCR-GH) and all df/df animals (df/df-AL-Sal, df/df-AL-GH, df/df-CR-Sal and df/df-CR-GH) (see the Results section). Similarly, for analyzing differences between *ad libitum* and calorie-restricted mice (a potential diet intervention effect) we pooled all AL animals (N-AL-Sal, NAL-GH, df/df-AL-Sal and df/df-AL-GH) and all CR mice (N-CR-Sal, N-CR-GH, df/df-CR-Sal and df/df-CR-GH) (see the Results section). Moreover, for analyzing differences between saline-treated (Sal) mice and animals treated with GH (a potential pharmacological treatment effect) we pooled all Sal-treated animals (N-AL-Sal, N-CR-Sal, df/df-AL-Sal and df/df-CR-Sal) and all GH-treated mice (N-AL-GH, N-CR-GH, df/df-AL-GH and df/df-CR-GH) (see the Results section).

Importantly, all statistically significant genotype, diet intervention and pharmacological treatment main effects have not been shown on the figures but they have been mentioned in the Results section. The showing of these relationships could make the figures difficult to interpret. Only the differences between particular experimental groups have been shown on the figures.

3. RESULTS

3.1. Body weights

Body weights follow up showed that both df/df and N mice had strong reduction of body weight in response to CR. As expected GH treatment caused growth of df/df mice subjected to AL or CR diet, however, the same dosage of GH did not affect the growth of N animals. (Supplemental data 1).

3.2. Effects of calorie restriction (CR)

Two-way analysis of variance (ANOVA) indicated that calorie restriction (CR) decreased plasma levels of insulin, glucose, TG, cholesterol and leptin compared to animals fed *ad libitum* ($p=0.0001$, $p=0.0001$, $p=0.002$, $p=0.0001$, $p=0.02$, respectively) (Figures 1A, 1B, 1E, 1G, 1H, respectively), showing a significant diet intervention effect. Similarly, CR decreased insulin, glucose and cholesterol in Sal-treated N mice compared to N-AL-Sal animals ($p=0.045$, $p=0.01$, $p=0.046$, respectively) (Figures 1A, 1B, 1G, respectively), and the diet also decreased glucose in Sal-treated Ames dwarf mice compared to df/df-AL-Sal mice ($p=0.032$) (Figure 1B). Moreover, a significant genotype $\times$ diet intervention interaction for insulin was detected ($p=0.0001$) (Figure 1A). The insulin resistance index (HOMA) was significantly affected by diet intervention (as well as by genotype), being reduced in CR mice compared to AL mice (and in Ames dwarf compared to normal (N) animals) ($p=0.0001$ both) (Figure 1C). Unexpectedly, FFA increased in mice subjected to CR compared to AL animals ($p=0.0001$) (Figure 1F). Moreover, lack of CR effect (i.e., pooled CR vs. AL mice) on adiponectin level has been observed ($p=0.74$) (Figure 1D), although a significant genotype $\times$ diet intervention interaction for this adipokine ($p=0.022$) was observed. Liver IR

β -subunit and IR pY1158 increased in calorie-restricted mice in comparison with AL animals ($p=0.0001$ both) (Figures 2A, 2B) (a significant diet intervention effect). Consistently, CR increased liver IR β -subunit and IR pY1158 in Sal-treated N compared to N-AL-Sal mice ($p=0.002$, $p=0.0001$, respectively) (Figures 2A, 2B, respectively) and increased IR pY1158 in Sal-treated df/df mice compared to df/df-AL-Sal animals ($p=0.0001$) (Figure 2B). Two-way ANOVA also indicated that diet intervention increased IR, IRS-1 and GLUT-2 gene expression in CR mice compared to animals fed *ad libitum* ($p=0.0001$, $p=0.002$, $p=0.013$, respectively) (Figures 3A, 3B, 3C, respectively). Consistently, IR, IRS1 and PPAR γ mRNA level increased in df/df-CR-Sal mice compared to df/df-AL-Sal animals ($p=0.012$, $p=0.024$, $p=0.002$, respectively) (Figures 3A, 3B, 3E, respectively). A significant genotype $\times$ diet intervention interaction for IR ($p=0.009$) (Figure 3A) was detected. Moreover, PPAR α and PPAR γ gene expression increased in calorie-restricted mice compared to AL animals ($p=0.0001$ both) (Figures 3D, 3E, respectively). The protein level of p-PKC ζ/λ , p-mTOR and p-p38 is decreased in CR mice ($p=0.003$, $p=0.018$, $p=0.001$, respectively) (Figures 4A, 4B, 5B, respectively). In contrast, p-CREB was significantly increased by CR ($p=0.011$) (Figure 4C). The diet intervention did not significantly affect protein levels of p-ERK1 (Figure 5A).

3.3. Effects of GH therapy

In contrast to mice subjected to CR, animals treated with growth hormone (GH) have shown increased plasma levels of glucose, TG, cholesterol and unexpectedly leptin, compared to saline-treated mice ($p=0.0001$, $p=0.0001$, $p=0.0001$, $p=0.022$, respectively) (Figures 1B, 1E, 1G, 1H, respectively). Thus, a significant pharmacological treatment effect was detected. Consistently, GH increased plasma levels of insulin, glucose, TG, FFA and cholesterol in df/df-AL-GH mice compared to df/df-AL-Sal animals ($p=0.045$, $p=0.0001$, $p=0.0001$, $p=0.029$, $p=0.025$, respectively) (Figures 1A, 1B, 1E, 1F, 1G, respectively). A significant genotype $\times$ pharmacological treatment interaction for glucose ($p=0.009$) (Figure 1B) was found. Growth hormone replacement therapy decreased plasma adiponectin and hepatic IR β -subunit levels compared to saline-treated mice ($p=0.0001$, $p=0.011$, respectively) (Figures 1D, 2A, respectively) (a significant pharmacological treatment effect). Consistently, GH decreased plasma adiponectin level in df/df-AL-GH mice compared to df/df-AL-Sal animals ($p=0.0001$) (Figure 1D), with a significant genotype $\times$ pharmacological treatment interaction ($p=0.0001$). Moreover, lack of GH effects on adiponectin in N mice was observed (Figure 1D). In N mice, GH treatment has changed p-mTOR protein level only, leading to the increase of p-mTOR concentration in N-AL-GH mice compared to N-AL-Sal animals ($p=0.035$; Figure 4B). Plasma insulin and FFA concentrations and liver IR pY1158 level were not significantly affected by GH treatment ($p=0.381$, $p=0.1$, $p=0.529$, respectively) (Figures 1A, 1F, 2B, respectively). Unexpectedly, GH also did not change HOMA score ($p=0.248$) (Figure 1C). Moreover, GH replacement therapy did not significantly affect IR, IRS-1 and GLUT-2 gene expression (Figures 3A, 3B, 3C, respectively). In contrast, animals treated with GH had increased p-mTOR, p-CREB and p-p38 protein levels compared to saline-treated mice ($p=0.0001$, $p=0.001$, $p=0.005$, respectively) (Figures 4B, 4C, 5B, respectively). Lack of GH effect has been observed for p-PKC ζ/λ protein level (Figure 4A).

3.4. ITT and GTT after CR and/or GH therapy

Insulin tolerance test (ITT) and glucose tolerance test (GTT) showed improved insulin sensitivity and glucose handling after CR in Ames dwarf (Figures 6A and 6B, respectively) and N mice, respectively (Figures 6E and 6F, respectively). However, GH replacement therapy (4ug/g/day) decreased insulin sensitivity of these mutants with no changes of glucose handling (Figures 6C and 6D, respectively). GH did not alter insulin signaling and improved glucose tolerance in normal mice (Figures 6E and 6F, respectively). Results of combined treatment with GH and CR showed that GH abolishes the effects of CR on insulin sensitivity and glucose handling in Ames dwarfs (Figures 6C and 6D, respectively).

3.5. Effects of genotype

Plasma levels of insulin, glucose, TG, FFA and cholesterol were decreased in Ames dwarf (df/df) mice compared to normal (N) mice ($p=0.0001$, $p=0.002$, $p=0.0001$, $p=0.002$, $p=0.0001$, respectively) (Figures 1A, 1B, 1E, 1F, 1G, respectively), showing a significant genotype effect. Moreover, plasma adiponectin, IR β -subunit and IR pY1158 levels were increased in df/df animals ($p=0.0001$, $p=0.0001$, $p=0.012$, respectively) (Figures 1D, 2A, 2B, respectively). Consistently, gene expression of IR was increased in Ames dwarf mice ($p=0.001$) (Figure 3A), and IRS-1 showed a tendency for elevated mRNA level in these mice compared to N animals ($p=0.07$) (Figure 3B). Moreover, PPAR γ mRNA level was increased in Ames dwarfs compared to N mice ($p=0.006$) (Figure 3E). Consistently, plasma levels of insulin, glucose, TG, cholesterol as well as HOMA score were decreased, and plasma adiponectin and liver IR β -subunit level were increased in df/df-AI-Sal compared to N-ALS mice ($p=0.0001$, $p=0.021$, $p=0.0001$, $p=0.023$, $p=0.0001$, $p=0.0001$, $p=0.0001$, respectively) (Figures 1A, 1B, 1E, 1G, 1C, 1D, 2A, respectively). Protein levels of p-CREB, p-ERK1/2 and p-p38 were decreased in df/df mice ($p=0.006$, $p=0.0001$, $p=0.003$ respectively) (Figures 4C, 5A, 5B, respectively) (a significant effect of genotype).

4. DISCUSSION

It is known that calorie restriction (CR) improves insulin sensitivity and increases lifespan in most strains of wild-type mice (Masternak et al., 2004; Masternak et al., 2009). Moreover, CR further increases sensitivity to insulin and extends longevity in long-lived Ames dwarf mice (Masternak et al., 2004; Masternak et al., 2009). Therefore, these beneficial effects of CR seem to be fully consistent with our findings in calorie-restricted mice (compared to *ad libitum* animals; with detected significant diet intervention effect), showing decreased plasma insulin and glucose levels, decreased HOMA score, increased hepatic concentrations of IR β -subunit and IR pY1158, and increased IR, IRS-1, GLUT-2, PPAR α and PPAR γ gene expression – the parameters which may be involved in insulin sensitivity regulation. In another long-lived mouse strain, characterized by reduced somatotrophic signaling, namely – growth hormone receptor (GHR) knockout (GHRKO) mice (or “Laron dwarfs”), 30% CR failed to enhance insulin sensitivity and further increase overall, median, or average lifespan (Bonkowski et al., 2006). This diet regimen only increased maximal lifespan in female GHRKO mice, although it also increased insulin sensitivity and extended lifespan in normal animals (Bonkowski et al., 2006). Similarly, every other day feeding, resulted in a 10-15% reduction in the average daily food intake, increased median lifespan in normal mice but did

not extend this parameter in GHRKO dwarfs (Bonkowski et al., 2009). Therefore, it seems that the mechanisms involved in mediating the effects of CR and GH receptor deletion, but not lack of GH production (as seen in Ames dwarfs), on lifespan are similar and, presumably, overlap. In contrast to the lack of effects on lifespan, CR can change levels of metabolic parameters in GHR-disrupted mice. For example, CR decreased plasma glucose, insulin and leptin levels in GHRKO mice (as well as in normal animals) compared to *ad libitum* mice (Al-Regaiey et al., 2005). In our study, CR also showed a significant diet intervention effect for these three metabolic parameters, leading to the decrease of their levels in mice subjected to the diet. In contrast, CR did not change plasma level of adiponectin. Similarly, Al-Regaiey et al. (2005) showed that plasma level of this “good” adipokine was not significantly affected by CR in normal and GHRKO mice. Presumably, effects of adiponectin and CR may somehow overlap. Increased liver IR β -subunit in N-CR-Sal mice compared to N-AL-Sal animals and lack of changes of this parameter in df/df-CR-Sal mice compared to df/df-AL-Sal animals are consistent with the data on liver total IR obtained in long-lived GHRKO dwarfs (Bonkowski et al., 2009). However, in contrast to Laron dwarfs, where there were no effects of CR on liver IR pY1158 (Bonkowski et al., 2009), in the current study, the active form of IR increased in df/df-CR-Sal mice compared to df/df-AL-Sal animals.

It is well accepted that CR represents the most powerful intervention to improve lipid metabolism. Introduction of restricted diet provides quick down-regulation or normalization of cholesterol and triglycerides as well as improvement in glucose and insulin metabolism which was also indicated in our study. However, in contrast to CR regimen, our study showed that GH replacement therapy abolished or even reversed beneficial effects of this diet intervention on the examined metabolic parameters. In the present study, GH increased plasma levels of glucose, TG, cholesterol and leptin, and decreased adiponectin level, demonstrating a significant treatment effect. Consistently, GH led to the increase of plasma insulin, glucose, TG, FFA and cholesterol levels, and to the decrease of adiponectin in Ames dwarf mice compared to saline-treated df/df animals. Interestingly, GH did not change these parameters in N mice. This divergent response to GH treatment by N and df/df mice is most likely regulated by different sensitivity to GH. Ames dwarf mice are GH deficient with fully functioning GH receptor. Supplying GH to this dwarf animals with selected established dosage efficiently activates the GH signal and growth of the animals, however the same dosage does not stimulate growth in N mice which produce normal physiological GH. Importantly, supplying the same dosage as was established for df/df mice may suppress pituitary production and disrupt pulsatile production of GH in N mice. Due to this complication we could partially suppress rather than activate GH signal in N mice. This could be one of the causes of differential effect on insulin and glucose metabolism in N when comparing with df/df mice. More studies with higher dosages in N mice would need to be completed, however increasing the same dosage in GH-sensitive df/df mice could cause some pathological abnormalities. However, over-all blood lipids and glucose regulation findings show important negative regulation of blood lipids by GH and more importantly it indicated that this hormonal therapy neutralizes beneficial effects of diet. GH replacement therapy also decreased insulin sensitivity of Ames dwarfs during ITT test. The mentioned detrimental effects of GH seem to be consistent with the findings observed in short-lived

transgenic mice with GH over-expression (GH-Tg). Wang et al. (2007) have shown increased plasma levels of insulin, cholesterol and resistin, and decreased adiponectin level in GH-Tg mice compared to N mice. Consistently, in the study on pioglitazone effects, increased plasma insulin and decreased adiponectin levels in GH-Tg mice were detected (Gesing et al., 2013). Therefore unexpectedly, GH replacement therapy does not improve the beneficial metabolic “status” of GH-deficient and long-lived Ames dwarf mice. It was also previously shown that twice daily injection of GH decreased IR β -subunit and IR pY1158 in the liver of Ames dwarf mice (Masternak et al., 2010). It corroborates well-known disadvantageous characteristic of transgenic mice with GH over-expression (Bartke, 2003). Consistently, GH and thyroxine replacement therapy in GH- and TSH-deficient Ames dwarfs increased plasma insulin and decreased hepatic IR total levels (Louis et al., 2010). These findings may confirm a crucial role of suppressed somatotrophic signaling in metabolism regulation. Moreover, one could conclude that the effects of Ames dwarfism, CR and GH therapy on whole animal insulin sensitivity presumably reflect alterations in insulin signaling not only in the liver but also in other important insulin target organs, the skeletal muscle and the adipose tissue.

In our study, decreased levels of p-PKC ζ/λ and p-mTOR in CR mice compared to AL animals were detected. The atypical PKC (aPKC) isoforms ζ and ι (ι also known as λ in rodents) are downstream of phosphatidylinositol 3-kinase (PI3K). PKC ι is a human oncogene and is frequently amplified in human cancers (see review – Parker et al., 2013); also it is known that aPKC ι/λ overexpression is involved in the progression of several types of cancer (e.g., Ishiguro et al., 2009; Kato et al., 2013). Importantly, p-PKC ζ/λ protein level was decreased in GHRKO mice (Al-Regaiey et al., 2007), the long-lived animals which are characterized by lower incidence and delayed onset of fatal neoplastic diseases (Ikeno et al., 2009). PI3K, AKT and mTOR constitute important signaling pathway (PI3K/AKT/mTOR), which is involved in the regulation of neogenesis. The inhibition of this signaling is considered to be a potential therapy in cancer (Polivka & Janku, 2013). Moreover, rapamycin – the mTOR pathway inhibitor can produce lifespan extension (Harrison et al., 2009) in mice apparently reflect in slower aging (Wilkinson et al., 2012). Similarly to our results, decreased levels of p-PKC ζ/λ (Al-Regaiey et al., 2007), mTOR and p-mTOR (Bonkowski et al., 2009) were reported in long-lived GHRKO mice. Therefore in the context of the above mentioned observations, the CR-induced reduction of p-PKC ζ/λ and p-mTOR in the present study, could be considered fully beneficial.

Our findings show increased liver level of phosphorylated form of cAMP response element binding protein (p-CREB) in pooled CR mice compared to pooled AL animals. One should emphasize that this transcription factor is involved in the regulation of the hepatic gluconeogenesis (Oh et al., 2013) and is a downstream target of ERK pathway. Moreover, cAMP/PKA/CREB pathway is known to play an important role in maintaining fasting glucose homeostasis. In another study, p-CREB was increased in GHRKO mice vs. N animals (Al-Regaiey et al., 2005). Therefore, one could hypothesize that increased p-CREB level may be considered beneficial, given that CR and GHR disruption are two experimental procedures leading to extended longevity. However, in contrast, we have also demonstrated significantly decreased p-CREB protein level in insulin-sensitive Ames dwarf mice compared to N animals. It is also known that knockdown of hepatic CREB expression in

rodents can enhance insulin sensitivity and reduce lipid content in the liver (Erion et al., 2009). Consistently, the liver p-CREB protein expression was decreased in insulin-sensitive TSH receptor knockout mice (Wang et al., 2013). Thus, alterations in p-CREB levels, observed in our study are quite difficult to interpretate, and a full role of CREB undoubtedly requires further studies.

As mentioned earlier, the MAPK signaling pathways play an important role in the regulation of cell proliferation, differentiation and survival. In the present study, CR decreased p-p38 hepatic protein levels and did not affect p-ERK1/2 compared to AL mice. Moreover, p-ERK1/2 and p-p38 were decreased in long-lived df/df mice. Thus, one could hypothesize that reduced MAPK signaling pathways may play potentially beneficial role in lifespan regulation. This hypothesis may be, in some part, supported by the study by Al-Regaiey et al. (2007). In this study we have demonstrated decreased skeletal muscles protein level of another MAPK signaling pathway component – p-JNK1 in GHRKO mice. However, in contrast to the present findings, hepatic protein level of MAPK signaling-related factor pp38 was increased in GHRKO mice (Al-Regaiey et al., 2005). Interestingly, Okuyama et al. (2010) have concluded that the ERK-MAPK cascade may promote longevity of metazoans. Therefore, a physiological role of the MAPK signaling pathways, similarly to ambiguous roles of the above mentioned transcription factors, is still not fully understood and further studies are required to clarify these complicated networks.

5. CONCLUSION

Summing up, one could conclude that GH abolishes numerous beneficial effects of CR. These findings tempt us to speculate that GH-IGF1 axis seems to be an important regulatory pathway in mediating the action of CR. Moreover, GH treatment does not benefit GH-deficient Ames dwarf mice, although GH replacement therapy plays a very important role in treatment of pathological GH deficiencies in humans. Apparently, severe congenital suppression of somatotrophic signaling constitutes a strongly beneficial feature of these mutants and overshadows the potentially beneficial effects of GH replacement during adult life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- GH treatment abolishes beneficial effects of CR in GH-deficient Ames dwarf mice
- GH-IGF1 axis may be an important regulatory pathway in mediating the action of CR
- Congenital GH deficiency constitutes a strongly beneficial feature of Ames mutants

Blood chemistry

Fig. 1. Plasma levels of metabolic parameters and HOMA score

Plasma level of insulin (A) and glucose (B), HOMA score (C), plasma level of adiponectin (D), triglycerides (E), free fatty acids; FFA (F), cholesterol (G) and leptin (H) in normal mice (N) fed *ad libitum* (AL) and treated with 0.9% saline (N-AL-Sal), N mice fed AL and treated with growth hormone (GH) (N-AL-GH), N mice calorie-restricted (CR) and treated with 0.9% saline (NCR-Sal), N CR mice treated with GH (N-CR-GH), Ames dwarf (df/df) mice fed AL and treated with 0.9% saline (df/df-AL-Sal), df/df mice fed AL and treated with GH (df/df-ALGH), df/df CR mice treated with 0.9% saline (df/df-CR-Sal), df/df CR

mice treated with GH (df/df-CR-GH). Values are means $\pm$ SEM. a, b, c, d, e – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$).

Fig. 2. Levels of insulin receptor (IR) β -subunit and phosphorylated IR in the liver
 Level of IR β -subunit (**A**) and phosphorylated IR (IR-pY1158) (**B**) in N-AL-Sal, N-AL-GH, N-CR-Sal, N-CR-GH, df/df-AL-Sal, df/df-AL-GH, df/df-CR-Sal, df/df-CR-GH (the group descriptions as in Fig. 1). Values are means $\pm$ SEM. a, b, c, d, e, f, g – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$).

Hepatic mRNA expression

Fig. 3. mRNA levels in the liver

Hepatic mRNA expression of insulin receptor (IR) (A), IR substrate 1 (IRS-1) (B), glucose transporter 2 (GLUT-2) (C), peroxisome proliferator-activated receptors α (PPAR α) (D), and peroxisome proliferator-activated receptors γ (PPAR γ) (E) in N-AL-Sal, N-AL-GH, N-CR-Sal, N-CR-GH, df/df-AL-Sal, df/df-AL-GH, df/df-CR-Sal, df/df-CR-GH (the group descriptions as in Fig. 1). The data from real-time PCR were normalized by the housekeeping gene β 2-microglobulin (*B2M*) and expressed as the relative expression.

Values are means $\pm$ SEM. a, b, c – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$).

Fig. 4. Protein levels in the liver - 1

Hepatic protein level of phosphorylated form of protein kinase C ζ/λ (p-PKC ζ/λ) (A), phosphorylated form of mTOR (p-mTOR) (B), and phospho-cyclic AMP responsive element-binding protein (p-CREB) (C) in normal mice (N) fed *ad libitum* (AL) and treated with 0.9% saline (N-AL-Sal), N mice fed AL and treated with growth hormone (GH) (N-AL-GH), Ames dwarf (df/df) mice fed AL and treated with 0.9% saline (df/df-AL-Sal), df/df mice fed AL and treated with GH (df/df-AL-GH), N mice calorie-restricted (CR) and treated with 0.9% saline (N-CR-Sal), N CR mice treated with GH (N-CR-GH), df/df CR mice treated with 0.9% saline (df/df-CR-Sal), df/df CR mice treated with GH (df/df-CR-GH). β -actin for protein loading control (D). Values are means $\pm$ SEM. a, b, c – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$).

Fig. 5. Protein levels in the liver - 2

Hepatic protein level of phospho-p-44/42 MAPK (ERK1/2) (p-ERK1/2) (A) and phospho-p38 MAPK (p-p38) (B) in N-AL-Sal, N-AL-GH, df/df-AL-Sal, df/df-AL-GH, N-CR-Sal, N-CR-GH, df/df-CR-Sal, df/df-CR-GH (the group descriptions as in Fig. 4). Values are means $\pm$ SEM. a, b, c – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$).

Fig. 6. Insulin Tolerance Test (ITT) and Glucose Tolerance Test (GTT)

ITT (A) and GTT (B) in normal mice (N) fed *ad libitum* (AL) and treated with 0.9% saline (N-AL-Saline), N mice calorie-restricted (CR) and treated with 0.9% saline (N-CR-Saline), Ames dwarf (df/df) mice fed AL and treated with 0.9% saline (df/df-AL-Saline), and df/df CR mice treated with 0.9% saline (df/df-CR-Saline). ITT (C) and GTT (D) in Ames dwarf (df/df) mice fed AL and treated with 0.9% saline (df/df-AL-Saline), df/df mice fed AL and treated with GH (df/df-ALGH), df/df CR mice treated with 0.9% saline (df/df-CR-Saline), and df/df CR mice treated with GH (df/df-CR-GH). ITT (E) and GTT (F) in N-AL-Saline, N

mice fed AL and treated with GH (N-AL-GH), N-CR-Saline, and N CR mice treated with GH (N-CR-GH). Values are means $\pm$ SEM. a, b, c – values that do not share the same letter in the superscript are statistically significant ($p < 0.05$). Figure (A) previously reported by Masternak et al. (2009): Insulin sensitivity as a key mediator of growth hormone actions on longevity. *J Gerontol A Biol Sci Med Sci.* 64, 516-521 (Reference 30 in our current submission).

Table 1

Primers used for gene expression analyses.

Gene	GenBank accession no.	Forward (5'-3')	Reverse (5'-3')
<i>β₂-microglobulin</i>	NM_009735	aagtatactcacgccacca	aagaccagtccttgctgaag
<i>IR</i>	NM_010568	atgaggccaaccttctggaa	acgggacattctccatgtct
<i>IRS-1</i>	L24563	agcccaaaagcccaggagaata	ttccgagccagtctctctcta
<i>GLUT-2</i>	NM_012879	ggtcagaactacctctcttg	gtgtgtgtggaattgtcctc
<i>PPARα</i>	NM_011144	cgaagcctacctaagaactt	atctggccacaagcgtctt
<i>PPARγ</i>	NM_011146	caaagtagaacctgcctctcc	ccttcacaagcatgaactcc