Divergent Effects of Caloric Restriction on Gene Expression in Normal and Long-Lived Mice

Michal M. Masternak, Khalid Al-Regaiey, Michael S. Bonkowski, Jacob Panici, Liou Sun, Jian Wang, Grzegorz K. Przybylski, and Andrzej Bartke

1Departments of Internal Medicine and Physiology, Geriatrics Research, Southern Illinois University, School of Medicine, Springfield.
2Institute of Human Genetics in Poznan, Polish Academy of Sciences, Poland.

Long-lived Ames dwarf mice share many phenotypic characteristics with animals subjected to caloric restriction (CR) but they are not CR mimetics. CR prolongs longevity in both normal and Ames dwarf mice. Using real-time polymerase chain reaction and western blot, we have examined the expression of genes related to insulin signaling in the liver of normal and dwarf mice subjected to 30% CR. The results revealed divergent responses of dwarf and normal animals to CR raising an interesting possibility that CR may affect longevity of normal and dwarf mice by different mechanisms. Moreover, effects of dwarfism on the expression of the examined genes differed from the effects of CR, thus adding to the evidence that these long-lived mutants are not CR mimetics.

Ames dwarf (df/df) mice are homozygous for a recessive loss-of-function mutation at the Prop1 locus (Prop1). Consequently, they are deficient in growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) with secondary suppression of circulating levels of insulin-like growth factor-1 (IGF1), thyroid hormones, insulin, and glucose (1,2). Dwarf mice live approximately 50% longer than normal mice from the same colony (3). Caloric restriction (CR), an intervention known to delay aging and to increase life span, reduces body weight and plasma levels of insulin, IGF1, glucagon, and thyroid hormone. We have recently reported that CR further extends the life span of Ames dwarf mice similar to its effects on the life span of other normal and dwarf mice (4). Results obtained in Ames dwarf mice, in other long-lived mutants, and in CR animals suggest that insulin signaling may have a very important role in aging (5).

To further probe the relationships between longevity genes, CR, and insulin signaling, we have examined the effects of Ames dwarfism and CR on the hepatic expression of insulin receptor (IR), insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), peroxisome proliferator-activated receptor gamma (PPARγ), peroxisome proliferator-activated receptor alpha (PPARα), and IGF1. Expression of glucose transporter 4 (GLUT4) was also examined to generate data for future comparisons with other insulin target organs. We also analyzed the levels of IR, IRS1, IRS2, PPARγ, PPARα, and GLUT4 proteins. These genes and their products play an important role in the insulin signaling pathway, and the liver is one of major targets of insulin. Insulin action depends on its binding to the insulin receptor (IR) on the cell surface (6). Insulin binding to IR leads to phosphorylation of IRS1 and IRS2. Phosphorylated IRS1 and IRS2 bind the p85 regulatory subunit of PI 3-kinase, and this cascade plays the main role in insulin metabolic effects (6,7). GLUT4 is important in both the regulation of glucose uptake in muscle and fat and the maintenance of whole body glucose homeostasis (8–10). It resides in intracellular vesicles and is translocated to the plasma membrane upon stimulation by insulin. We have also analyzed expression of PPARγ, a gene whose relationship to insulin signaling remains to be thoroughly explored. PPARγ is a nuclear receptor involved in metabolism control. It promotes glucose tolerance and causes insulin sensitivity (11,12). PPARγ is the target receptor for thiazolidinediones, used in type 2 diabetic patients as insulin-sensitizing drugs (13). Another gene from the PPAR family, PPARα, is involved in the regulation of hepatic lipid metabolism by influencing transport and uptake of fatty acids (14,15). IGF1 involvement appears to be important in the control of longevity. Ames dwarf and Snell mice with hereditary GH deficiency and GH-resistant GH receptor knock-out (GHR-KO) mice have greatly suppressed circulating IGF1 levels and live approximately 50% longer than their normal siblings (3,16–18). IGF1 action is mediated primarily by its receptor, IGF1R. Female mice heterozygous for the IGF1R knockout were recently reported to live 33% longer (19).

METHODS

Animals

Ames dwarf (Prop1) heterozygous recessive (df/df) mice were produced by mating heterozygous females and homozygous mutant males in our breeding colony. In this colony, the Prop1 mutation is maintained on a heterogeneous genetic background. All animal protocols were approved by the Southern Illinois University Laboratory Animal Care Committee. Animals were maintained under temperature- and light-controlled conditions (20°C–23°C, 12-hour light/12-hour dark cycle). Animals were group-housed according to sex and genotype. Mice matched for average......
body weight (BW) within genotype at 8 weeks of age were divided into two treatment groups: CR or fed ad libitum (AL). AL animals were allowed unlimited access to food. Under these conditions, Ames dwarf mice consume more food per gram body weight than normal animals (20). Food consumption expressed per gram of “metabolic body weight” (body weight0.75) does not differ between normal and Ames dwarf mice (unpublished observations). CR animals were subjected to 30% CR starting at 2 months of age using a previously described protocol (20). Thus, we had the following four experimental groups: N-AL, N-CR, df/df-AL, and df/df-CR, consisting of six males from each normal group and seven males from df/df groups. At the age of 18 months, the animals were anesthetized using isoflurane and killed via decapitation. Tissues were rapidly collected, washed with 0.9% saline, and snap-frozen on dry ice for analysis.

**Insulin and Glucose Analysis**

The insulin and glucose in plasma were analyzed by commercial kits “Ultra insulin ELISA kit” (Crystal Chem, Inc., Downers Grove, IL) and Glucose kit (Sigma, Ronkonkoma, NY).

**Standard Curve Plasmid Preparation**

All genes in the experiment were amplified by polymerase chain reaction (PCR). The products were ligated in pGEM-T easy vector (Promega, Madison, WI). The mix included 12 ng of PCR product, 12 fmol of pGEM-T easy vector, 2x Rapid Ligation Buffer, 0.5 µl T4 DNA ligase, and H2O up to 5 µl of total reaction. The reaction ran overnight at 4°C. The ligation products were used to transform the DH5α bacteria (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. After transformation, the bacteria were grown on agar plates with antibiotic to select only the colony with the plasmid that provided them with ampicilnine resistance. A few colonies were selected and diluted in 50 µl LB medium, and then the PCR was performed. The colonies, which included the expected PCR product, were grown overnight in 5 ml of LB medium. Further, the plasmid was extracted from the bacteria using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The concentration of the plasmid was checked by spectrophotometer with the absorbance at 260 nm. On the basis of the result, the concentration was calculated; the copy number of the plasmid was then calculated. The standard curve was prepared in dilutions from 10^6, 10^5, 10^4, 10^3, 300, and down to 100 copies per 2 µl.

**The Extraction of mRNA**

Total hepatic mRNA was prepared using phenol/chloroform according to the method of Chomczynski and Sacchi (21).

**cDNA**

Five micrograms of total RNA was incubated for 30 minutes with RQ1 DNase (Promega) in 37°C. Afterwards 400 ng of Oligo dT primers (Promega) were added and incubated 10 minutes in 72°C. After cooling down the mix on ice, Superscript II buffer, DTT, dNTP mix, and Superscript II reverse transcriptase (Invitrogen) were added. The reaction mix was incubated 80 minutes at 42°C.

**Real-Time PCR**

For the PCR reaction, a “Smart Cycler” machine (Cepheid, Sunnyvale, CA) with iQ SYBR Green Surer mix (BioRad, Hercules, CA) was used. In each reaction, there were two primers, backward and forward (Table 1). Three steps of the PCR included denaturing at 94°C for 2 minutes, annealing at 62°C for 30 seconds with fluorescence reading, and extension at 72°C for 30 seconds. All sample groups were analyzed in a single reaction to avoid the false difference causes by different efficiency in separate reactions. Standard curve was used to quantify the real-time PCR results (22). The copy number of each analyzed gene was normalized to the expression of the housekeeping gene β2-microglobulin (B2M) using the formula x/y * 1000 (x–analyzed gene, y–housekeeping gene), which gave us a copy number of the gene of interest per 1000 copies of B2M.

**Western Blot**

Half of the liver homogenate for RNA extraction was taken for protein analysis. We added protease and phosphatase inhibitors and 1% of Triton-X 100 (Sigma). After mixing, homogenates were centrifuged at 1600 rpm for 30 minutes and the supernatant was removed. Protein concentrations were checked using BCA (bicinchoninic acid) assay (Pierce, Rockford, IL) according to the company’s protocol. Laemml sample buffer was added to the protein and was heated in a thermocycler in 99°C for 5 minutes and then cooled to 4°C. Forty µg of the protein was separated by electrophoresis using BioRad equipment at 120 V for 80 minutes. Proteins were transferred by wet transfer onto nitrocellulose membrane at 80 V for 60 minutes. After the transfer, membranes were washed using TBS (total serum bilirubin) (pH 7.6) and blocked with 3% BSA.
(bovine serum albumin) in TBS for 1 hour at room temperature. After blocking, membranes were washed with TBS containing 0.05% Tween-20 (TBST) 3 times for 15 minutes each time. In the next step, the membranes were incubated with the primary antibody specific for the protein of interest (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted in 3% BSA at 4°C overnight. After incubation, the blots were washed 3 times with TBST for 15 minutes each time and incubated with a secondary antibody for 1 hour at room temperature. Horseradish peroxidase activity from secondary antibody was detected using the ECL chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ). The membranes were analyzed using Gene Snap software (SynGene, Frederick, MD), and the results were quantified using Gene Tools (SynGene).

Statistical Analysis

To evaluate the differences between groups, we analyzed the data using analysis of variance (ANOVA) followed by Fisher’s PLSD (protected least significant difference) test to compare individual means.

RESULTS AND DISCUSSION

Consistent with GH deficiency, body weight was considerably lower in Ames dwarf than in normal animals (p < .0001). CR leads to the expected reduction of BW in both normal and dwarf mice (Figure 1). Plasma insulin levels were significantly reduced by CR in normal but not in dwarf mice. Plasma glucose levels were significantly reduced in dwarf as compared to normal animals regardless of caloric intake (data not shown).

IR mRNA expression in df/df-AL did not differ from values measured in N-AL animals, but the level of IR protein was significantly increased in df/df-AL (p < .0008). However, in df/df-CR mice, IR mRNA expression was reduced (p < .0007) in comparison to the N-CR group, although there was no difference in the protein level. The levels of IR mRNA and protein were significantly elevated in N-CR compared to N-AL mice (p < .0057 and p < .0333, respectively) (Figures 2A and 3A). Increase of IR after CR likely represents a response to reduced insulin release and may contribute to enhanced insulin sensitivity in CR animals. In dwarf mice, CR did not change the level of IR mRNA expression; however, it significantly decreased the IR protein level (p < .0282), suggesting that, in these highly insulin-sensitive mutants, CR may reduce rather than enhance responsiveness to insulin. This could serve as a mechanism for preventing hypoglycemia under the condition of limited availability of nutrients. Comparing both genotypes without CR treatment appears to elevate the mRNA expression of both insulin receptor substrates in Ames dwarf mice (p < .5 for IRS1 and p < .2 for IRS2). IRS1 and IRS2 protein levels were also increased in dwarf versus N (p < .0127, p < .0499, respectively) in agreement with our previous findings in 4–5-month-old female Ames dwarf mice (23). This indicates that the increase in the levels of IRS-1 and IRS-2 in the liver of Ames dwarf mice may be due to increased transcription of the corresponding genes. The mRNA expression of IRS1 and IRS2 also appears to be elevated in N-CR compared to N-AL mice (p < .1 and p < .2, respectively), but reduced in df/df-CR compared with df/df-AL (p < .2 and p < .1, respectively). The level of IRS1 protein appeared elevated in N-CR compared to N-AL, but this apparent difference was not significant (p < .0720), while IRS2 levels were not altered. However, in df/df mice, the levels of IRS2 were elevated after CR (p < .015).
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Figure 3. The level of the proteins related to insulin signaling in liver tissue of normal (N) and Ames dwarf (df/dt) mice fed ad libitum (AL) or subjected to 30% caloric restriction (CR). Equal loading of protein for western blots was verified using β-actin. Means ± SEM (standard error of mean). a,b,c = values that do not share the same letter in the superscript are statistically significant (p < .05). IR = insulin receptor; IRS1 = insulin receptor substrate 1; IRS2 = insulin receptor substrate 2; GLUT4 = glucose transporter 4; PPARγ = peroxisome proliferator-activated receptor gamma; PPARα = peroxisome proliferator-activated receptor alpha.

and the levels of IRS1 were numerically (although not significantly) higher (p < .2). The mRNA expression of IRS1 in df/dt-CR was lower than in N-CR animals (p < .0497), and expression of IRS2 mRNA appeared to be similarly affected (p < .5) (Figure 2B and 2C). The effects of genotype on the levels of the corresponding proteins were opposite, with significant increases in the levels of IRS1 and IRS2 in df/dt-CR as compared to N-CR mice (p < .0461 and p < .0008, respectively) (Figure 3B and 3C).

In comparison to normal animals, hepatic expression of the PPARγ mRNA and protein in Ames dwarfs was significantly increased (p < .05 and p < .0072, respectively). Increased levels of PPARγ could account for, or more likely contribute to, increased insulin sensitivity of these animals (23). Interestingly, this effect of the Ames dwarf gene is age dependent, with young dwarfs having reduced rather than elevated PPARγ mRNA expression (unpublished observations). Caloric restriction had no significant effect on the levels of PPARγ mRNA or protein in either genotype, although levels of PPARγ mRNA measured in df/dt-CR were less than 25% of the levels measured in df/dt-AL animals (p < .0527). This may have been due to considerable individual variation in the df/dt-AL group.

Caloric restriction increased PPARα mRNA expression in normal mice (p < .0082), but not in the dwarfs. As a result, PPARα mRNA expression in the df/dt-CR group was much lower than in the N-CR group (p < .0001). The observations that the mRNA expression of PPARα was affected by CR but not by dwarfism, and that CR altered PPARα mRNA expression only in normal animals, provide interesting contrast with many phenotypic similarities between Ames dwarf mice and normal animals subjected to CR (Figure 2F). These observations add to the evidence that Ames dwarfs are not CR mimetics (4,20). However, the interpretations of these findings are complicated by the lack of significant effect of the genotype or caloric intake on PPARα protein levels (Figure 3F).

GLUT4 mRNA expression was low but measurable in liver homogenates and tended to be greater in N-CR as compared to N-AL counterparts, and was increased by CR in Ames dwarf mice (p < .0167) (Figure 2D). The product of the GLUT4 gene showed similar trends (Figure 3D). We have no evidence that the detected mRNA expression of GLUT4 and its product originates in hepatocytes. The low level of GLUT4 mRNA expression could be derived from smooth muscle cells in blood vessels or other cells present in the liver.

As expected from GH deficiency of Ames dwarf mice, IGF1 mRNA expression in their livers was dramatically suppressed (p < .004). In normal animals, CR caused an unexpected increase in IGF1 mRNA expression (p < .0001), while IGF1 mRNA expression in the dwarfs was not altered by CR (Figure 1G). This is difficult to reconcile with the evidence that CR reduces peripheral IGF1 levels (24) and with our demonstration that CR prolongs life in Ames dwarf mice as it does in normal animals (4). However, it is known that reduced food intake decreases plasma IGF1 level in young mice but not in older animals (25) and, in addition, the present findings may reflect the effect of overnight fast on IGF1 mRNA expression in AL mice. Perhaps reduction in plasma IGF1 levels in mice subjected to CR does not result from reduced transcriptional activity of the hepatic IGF1 mRNA. Relating alterations in IGF1 mRNA expression to aging and life expectancy is complicated by a limited amount of information on the relative importance of systemic versus local effects of IGF1, and on IGF1 levels versus sensitivity to the IGF1 signal. In dwarf and GHR-KO mice, profound suppression of IGF1 levels is associated with a major increase in life span (3,16–18). In transgenic mice expressing a GH antagonist, plasma IGF1 levels are modestly reduced and longevity is not affected (18), while in female but not male IGF1R−/− mice, partial resistance to IGF1 prolongs life (19). In rats, recent studies of interactions of CR with GH deficiency suggest that, in this species, a modest suppression of the somatotropic axis increases longevity, while more complete suppression does not and indeed may reduce life expectancy by compromising immune function and increasing incidence of leukemia (26).

Summary

Our results, based on quantitative analysis of gene expression by real time PCR and the proteins level analyzed by western blot, lead to several novel conclusions. (a) Chronic restriction of caloric intake alters hepatic expression of genes related to the actions of insulin and IGF1. Genes affected by CR include PPARα, a gene recently suggested to play an important role in metabolic control. (b) Effects of CR on the expression of insulin-related genes and their
products differ from the effects of a loss-of-function mutation at the Prop1 locus (Ames dwarfism) in spite of generally comparable alterations in insulin and glucose levels, body core temperature, growth, and longevity. Although altered, insulin signaling is believed to be important in mediating the effects of both CR and dwarfism on longevity, the nature of these alterations is clearly different. (c) Unexpectedly, the effects of CR on gene expression in normal (wild type) and in Ames dwarf mice are very different. We have previously shown that CR effectively extends life span in both normal and dwarf mice, and thus the present results suggest an intriguing and unexpected conclusion that different mechanisms may be responsible for this effect of CR in wild-type and in long-lived mutant mice. We also have reported differences between profiles of hepatic gene expression in long-lived GHR-KO mice versus normal mice subjected to CR (27). We believe that these differences can be explored to identify alterations in gene expression, their products, and consequent differences in physiological function, which are responsible for the effects of CR and “longevity genes” on aging and life span. This will require additional studies of gene expression and protein levels at different stages of life and in different insulin target organs to further clarify the mechanisms by which insulin signaling may influence aging, and to interpret the differences in its action in normal versus long-lived mutant mice.

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Address correspondence to Michal M. Masternak, PhD, SIU School of Medicine, Department of Internal Medicine, Division of Geriatric Medicine, 801 N. Rutledge, Springfield, IL 62794-9628. E-mail: mmasternak@siu.edu

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