Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile

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Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile

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Summary
Caloric restriction (CR) and down-regulation of the insulin/IGF pathway are the most robust interventions known to increase longevity in lower organisms. However, little is known about the molecular adaptations induced by CR in humans. Here, we report that long-term CR in humans inhibits the IGF-1/insulin pathway in skeletal muscle, a key metabolic tissue. We also demonstrate that CR induces dramatic changes of the skeletal muscle transcriptional profile that resemble those of younger individuals. Finally, in both rats and humans, CR evoked similar responses in the transcriptional profiles of skeletal muscle. This common signature consisted of three key pathways typically associated with longevity: IGF-1/insulin signaling, mitochondrial biogenesis, and inflammation. Furthermore, our data identify promising pathways for therapeutic targets to combat age-related diseases and promote health in humans.

Key words: caloric restriction; human; insulin/IGF-1 signaling; skeletal muscle.

Introduction
Caloric restriction (CR) without malnutrition and inhibition of the insulin/IGF-1 signaling are the most robust and reproducible interventions for extending lifespan and preventing or delaying age-related disease in a variety of species (Anderson & Weindruch, 2007; Kennedy et al., 2007; Piper & Bartke, 2008). Aging was believed to be the consequence of the inevitable wear and tear process, but in 1993, Kenyon and her associates published the first paper indicating that the inhibition of the insulin/IGF-1/FOXO pathway dramatically extends lifespan in worms (Kenyon et al., 1993). Since then, accumulating data have shown that this pathway is evolutionarily conserved and that dietary and genetic manipulations of the insulin/IGF-1/FOXO pathway extend lifespan in rodents as well (Kenyon et al., 1993; van Heemst et al., 2005; Kennedy et al., 2007; Piper & Bartke, 2008). Moreover, evidence derived from exceptionally long-lived people also supports a role for the IGF signaling pathway in human longevity (van Heemst et al., 2005; Suh et al., 2008). However, nothing is known on the molecular adaptations induced by long-term CR in humans, and in particular, on the effects of CR on the regulation of the insulin/IGF-1/FOXO pathway.

Results and discussion
As one of the primary metabolic tissues, skeletal muscle is extremely vulnerable to the effects of aging, and decreases in muscle mass and strength have been associated with increased mortality (Cesari et al., 2009). Interestingly, CR prevents or delays this age-related loss of muscle function and mass, thereby contributing to increases in both the quantity and quality of life (Aspnes et al., 1997; Mckiernan et al., 2010). Although research on CR in humans is still at an early stage, information from population and physiological studies suggest that many of the same beneficial metabolic adaptations that occur in experimental animal models are also observed in humans (Fontana & Klein, 2007; Fontana et al., 2010b). We hypothesized that the impressive metabolic changes previously described in these individuals on long-term CR would result in several molecular adaptations, including a down-regulation of the insulin/IGF-1/FOXO pathway, a key nutrient sensing pathway, shown to slow aging in several experimental animals (Kenyon et al., 1993; van Heemst et al., 2005; Kennedy et al., 2007; Piper & Bartke, 2008). We also hypothesized that the relative changes of the skeletal muscle gene expression profiles would be similar between
CR rats and humans. In this study, we studied the molecular adaptations induced by long-term CR in healthy lean men and women and compared the skeletal muscle transcriptional profiles induced by CR in rats and humans. Our results show that chronic moderate (~30%) CR in lean and weight-stable adult individuals, despite their genetic heterogeneity, results in a uniform dramatic transcriptional reprogramming of molecular pathways in skeletal muscle, which shifts cellular metabolism from growth to maintenance/repair activities. We also found that CR induces a significant down-regulation of the IGF-1/insulin/FOXO pathway both at the transcriptional and post-transcriptional level. In addition, we found that humans and long-lived CR rats, despite significant genetic, physiological, and anatomical differences, exhibit a number of key common transcriptional adaptations in a major metabolic organ (i.e., skeletal muscle), suggesting that the beneficial gains of CR on healthy longevity would be observed in humans.

We first sought to determine whether CR modifies the gene expression profile in human skeletal muscle. To this end, we recruited and studied 15 middle-aged (58.7 ± 7.4 years), weight-stable very lean (BMI = 19.2 ± 1.1 kg/m²) members of the Calorie Restriction Society who have been practicing ~30% CR with adequate nutrition (at least 100% of RDI for each nutrient) for an average of 9.6 years and a control group of 10 nonobese (BMI = 25.3 ± 2.3 kg/m²) age-matched controls eating a typical Western diet (Table 1). Principle component analysis (PCA) revealed a very distinct separation of groups based on dietary manipulation (Fig. 1A). Remarkably, we found that CR in humans induces dramatic and uniform changes to the gene expression profile within skeletal muscle that are strong enough to overcome the genetic, nutritional, and geographical heterogeneity of this human population. Furthermore, comparison of our study population expression profiles with a younger (31.2 ± 2.0 years) nonobese (BMI = 25.3 ± 2.3 kg/m²) human cohort showed that our CR was grouped closer to the younger profile by PCA (Table 1 and Fig. 1B). To further investigate the role of CR in modifying skeletal muscle gene expression in humans, we next performed parametric analysis of gene set enrichment (PAGE), a computational method that determines differences between genes

Table 1 Characteristics of the study subjects

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<th>Humans</th>
<th>Rats</th>
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<tr>
<td></td>
<td>WD-Y (n = 5)</td>
<td>WD-M (n = 10)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31.2 ± 2.0</td>
<td>58.0 ± 7.4</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>5/0</td>
<td>8/2</td>
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<tr>
<td>Body composition</td>
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<tr>
<td>Height (m)</td>
<td>1.73 ± 8.8</td>
<td>1.76 ± 0.0</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>78.4 ± 16.3</td>
<td>78.1 ± 9.7</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.0 ± 5.0</td>
<td>25.3 ± 2.3</td>
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<tr>
<td>DEXA – NMR</td>
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<tr>
<td>Fat mass (%)</td>
<td>–</td>
<td>24.0 ± 7.4</td>
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<tr>
<td>Trunk fat (%)</td>
<td>–</td>
<td>25.6 ± 6.8</td>
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<tr>
<td>Lean mass (%)</td>
<td>–</td>
<td>73.1 ± 6.0</td>
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WD-Y – younger individuals eating Western diets (WD); WD-M – middle-aged individuals on WD; AL – ad libitum laboratory diet. For humans, P-value comparison CR – WD-M; NMR, nuclear magnetic resonance.

Values are mean ± SD.
profiling also indicates a CR-induced decrease in the inflammatory response, which is implicated in a range of age-related diseases. The beneficial effects of CR may involve an elaborate crosstalk between these highly conserved inflammatory, nutrient- and energy-sensing pathways.

We then wanted to determine whether the overall physiological and molecular response of long-term CR was similar in humans and rats. Rats were subjected to either 40% CR or ad libitum (AL) diets early in life. As expected, our rats on a CR-fed diet had increased mean and maximum lifespan compared with those on an ad libitum-fed diet (Fig. S1). In humans, nevertheless, such early initiation of CR would be detrimental to growth and development. Therefore, human subjects included in this study were middle-aged men and women. However, it is well known that in rodents, CR initiated at adulthood (12–19 months of age) is as effective as CR begun early in life at decelerating mortality rate, extending remaining lifespan, and inducing dramatic changes in the gene expression profile (Dhabhi et al., 2004). As expected, CR reduced body weight, reduced fat mass, and increased the lean to fat mass ratio in both middle-aged humans and rats compared with those on Western or AL diets, respectively (Table 1). Interestingly, both species exhibited similar responses to CR in several physiological, hormonal, and biochemical markers. Previous studies on these individuals indicate that long-term CR in humans results in improved cardiometabolic profile, markedly lower blood pressure and inflammation, improved left ventricular diastolic function and heart rate variability, and lower serum concentration of insulin, leptin, sex hormones, triiodothyronine, and core body temperature (Fontana & Klein, 2007; Cangemi et al., 2010; Fontana et al., 2010a,b; Soare et al., 2011), all of which are hallmark adaptations also seen in rats in response to CR (Weindruch & Walford, 1988; de Cabo et al., 2003). Interestingly, the effect of CR on circulating IGF-1 concentrations was not significantly reduced in humans unless protein intake is also reduced (Fontana et al., 2008).

Based on the broad phenotypical similarities observed in both species following long-term CR, we next examined whether similarities between humans and rats occurred in gene expression profiles of skeletal muscle as well. PCA revealed a very distinct separation of groups based on dietary manipulation regardless of species (Fig. 1C). PAGE analysis indicated that out of the shared changes, CR evoked a number of common genes and pathways in both species (83 genes and 93 pathways), with the majority of genes being down-regulated by CR (Fig. 3A,B). A subset of gene expression changes were verified by quantitative real-time PCR or QuantiGene-based assays (Table S2). Notably, the 5 most highly elevated transcripts were contractile proteins (Fig. 3C). The list of the 5 most highly down-regulated transcripts include proteins involved in antioxidant responses (NQO1), cholesterol metabolism (NR1H3), fibrosis (PLOD2), and cell growth (IMPDH1). The complete dataset is available at http://www.ncbi.nlm.nih.gov/geo/. We identified 93 pathways (22 up-regulated, 93 pathways), with the majority of genes being down-regulated by CR (Fig. 3A,B). A subset of gene expression changes were verified by quantitative real-time PCR or QuantiGene-based assays (Table S2). Notably, the 5 most highly elevated transcripts were contractile proteins (Fig. 3C). The list of the 5 most highly down-regulated transcripts include proteins involved in antioxidant responses (NQO1), cholesterol metabolism (NR1H3), fibrosis (PLOD2), and cell growth (IMPDH1). The complete dataset is available at http://www.ncbi.nlm.nih.gov/geo/.
Interestingly, the effects observed on these pathways by CR oppose those normally occurring during the aging process (Park & Prolla, 2005). These results indicate that humans share with other species, such as rats, similar transcriptional responses to CR that are typically associated with improved health and survival. However, this association does not imply causation for increased longevity. In summary, transcriptional patterns of CR humans suggest that CR may retard the aging process by shifting cellular metabolism from growth to maintenance and repair activities. A molecular trigger for this shift may be the genes associated with mitochondrial biogenesis and antioxidant response.

Fig. 2  Transcriptional and post-transcriptional modifications of the PI3K/AKT/FOXO pathway in human skeletal muscle by caloric restriction (CR). (A) Transcriptional down-regulation of the PI3K/AKT/FOXO signaling pathway by CR. GH, growth hormone; IGF, insulin-like growth factor; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B (PKB); FOXO-3A, forkhead box O3; FOXO-4, forkhead box O4; mTOR, mammalian target of rapamycin; LC3, microtubule-associated protein 1 light chain 3; AMPK, adenosine monophosphate-activated protein kinase; NFκB, nuclear factor-kappaB; SOD2, superoxide dismutase 2; DDB1, damage-specific DNA binding protein 1; FASL, fas ligand. Western blot of human skeletal muscle from individuals on a Western diet (WD) or caloric restricted (CR) diet. Immunoblot (B) and quantification (C) of Western blots in panel a for p-T308 AKT and p-S473 AKT was performed using NIH ImageJ and normalized to total AKT expression. (*P < 0.01, **P < 0.00003 (n = 10 WD, 15 CR samples)). Bars indicate mean ± SEM.
down-regulation of the IGF/insulin/FOXO signaling pathway, a result that connects our findings to those obtained through genetic manipulation of aging in worms, fruit flies, and rodents (Kenyon et al., 1993; van Heemst et al., 2005; Kennedy et al., 2007; Piper & Bartke, 2008).

**Experimental procedures**

**Study subjects**

**Rats**

Male Fisher 344 rats (n = 54) were randomly assigned to two groups at 2 months of age. One group was kept *ad libitum* (AL) fed throughout their lifespan, while the calorie restriction (CR) group was progressively brought down to a 40% CR. All animals were fed a NIH-31 standard chow (Harlan Teklad, Indianapolis, IN, USA). Rats were singly housed in an environmentally controlled vivarium with unlimited access to water and a controlled photoperiod (12 h light; 12 h dark). Body weights and food intake were recorded biweekly. All rats were maintained between 68–72 °F according to animal protocols and NIH guidelines. All animal procedures for this study were reviewed and approved by the Animal Care and Use Committee (ACUC) at the Biomedical Research Center (NIA/NIH).

**Humans**

Fifteen individuals had been on CR for an average of 9.6 years (4–20 years). Subjects were instructed by an experienced research dietician to record all food and beverages consumed, preparation methods, and approximate portion sizes for seven consecutive days. Food records were analyzed using the NDS-R program (version 4.03_31), which is the Nutrition Data System for research from the Nutrition Coordinating Center at the University of Minnesota. CR subjects consumed a variety of nutrient-dense unprocessed foods (i.e., vegetables, fruits, nuts, egg whites, fish, poultry, low-fat dairy products, whole grains, and beans) which supplied >100% of the recommended daily intake for all essential nutrients. Refined foods rich in empty calories and trans fatty acids were avoided. Energy intake was 30% lower in the CR group (1773 ± 239 kcal/day) than in the Western diet (WD) group (n = 10) (2483 ± 378 kcal/day) (P ≤ 0.0001). The percentage of total energy intake derived from protein, carbohydrate, fat, and alcohol was 22%, 50%, 28%, and 0.2%, respectively, in the CR group and 17%, 47%, 35%, and 1% in the WD group. The human study was approved by the Human Studies Committee of Washington University School of Medicine, and all participants gave informed consent before their participation. Moreover, a younger cohort of humans (n = 5) who underwent surgery for hip dysplasia was included in this study. Written informed consent was obtained from those participants, and the study was approved by the medical ethics committee of the Rizzoli Hospital.

**Body composition**

**Rats**

Measurements of lean and fat mass in whole, live rats were acquired by nuclear magnetic resonance (NMR) using the Minispec LF90 (Bruker Optics, Billerica, MA, USA).

**Humans**

Total body fat mass and fat-free mass were determined by dual-energy X-ray absorptiometry (DXA) (QDR 1000w, Hologic, Waltham, MA, USA).

**Microarray analysis**

Five rats per group at 27 months of age were sacrificed and vastus lateralis muscle was flash-frozen. Human percutaneous biopsy
Objectives: To determine if calorie restriction (CR) induces similar adaptations in rats and humans.

Methods: Vastus lateralis muscles were lysed in 1 mL of cold RIPA buffer (50 mM HEPES pH 7.4, 40 mM NaCl, 2 mM EDTA, 1.5 mM sodium orthovanadate (Na3VO4), 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium beta glycerophosphate, 0.1% SDS, 1% sodium deoxycholate, 1% Triton) supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets, using a FastPrep 24 (MP Biomedical, Santa Ana, CA, USA) instrument. Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA, USA). 20 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8%, 10%, or 16% Tris-glycine gels (Invitrogen) and transferred to Immobilon P 0.45 μM PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk/TBST, and antibodies were incubated overnight at 4 °C in 5% BSA/TBST (phospho-antibodies) or 5% milk/TBST (all other antibodies). Quantification was performed by densitometry using ImageJ software, and loading was verified by blotting for actin. Antibodies to p-T308 AKT (CST 2965), p-S473 AKT (CST 4060), and total AKT (CST 4691) were from Cell Signaling Technology. The actin (sc-7210) antibody was from Santa Cruz Biotechnology.

Statistical analysis

Subject characteristics were analyzed using Student’s t-test (two-sample equal variance; two-tailed distribution). Data are expressed as mean ± SD unless indicated otherwise. Survival curves were plotted using the Kaplan–Meier method, which included 27 rats for both CR and AL. Statistical analysis was performed using SigmaStat Program for Windows, version 3.5 (Systat Software, Inc., Chicago, IL, USA). A difference with P-values < 0.05 were considered statistically significant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. 51 Kaplan–Meier survival analyses of ad libitum (AL) and 40% caloric restricted (CR) rats.

Table 51 Z-scores of the top 100 pathways significantly changed by either WD or CR.

Table 52 RT-qPCR/QuantiGene based assay validation of micro-array data.

Table 53 Z-scores of common pathways between humans and rats.

Table 54 Z-scores of 3 central pathways altered by CR between humans and rats.

Table 55 Primer sequences and probe set region used for quantitative PCR analysis or QuantiGene based assays.