2016

Long-term calorie restriction enhances cellular quality-control processes in human skeletal muscle

Ling Yang
Harvard University

Danilo Licastro
CBM Scrl

Edda Cava
Washington University School of Medicine in St. Louis

Nicola Veronese
Washington University School of Medicine in St. Louis

Francesco Spelta
Washington University School of Medicine in St. Louis

See next page for additional authors

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
Yang, Ling; Licastro, Danilo; Cava, Edda; Veronese, Nicola; Spelta, Francesco; Rizza, Wanda; Bertozzi, Beatrice; Villareal, Dennis T.; Hotamisligil, Gokhan S.; Holloszy, John O.; and Fontana, Luigi, "Long-term calorie restriction enhances cellular quality-control processes in human skeletal muscle." Cell Reports.14,3. 422-428. (2016).
https://digitalcommons.wustl.edu/open_access_pubs/4534

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact engeszer@wustl.edu.
Long-Term Calorie Restriction Enhances Cellular Quality-Control Processes in Human Skeletal Muscle

Graphical Abstract

Highlights
- Calorie restriction increases health-span and lifespan in model organisms
- Little is known about the metabolic and molecular effects of CR in humans
- CR inhibits inflammation in part by increasing serum cortisol concentration
- CR elevates expression of genes and proteins that enhance protein quality control

Authors
Ling Yang, Danilo Licastro, Edda Cava, ..., Gökhan S. Hotamisligil, John O. Holloszy, Luigi Fontana

Correspondence
lfontana@dom.wustl.edu

In Brief
Yang et al. show that calorie restriction without malnutrition in humans inhibits inflammation, at least in part by elevating serum cortisol concentration, and increases chaperone and autophagy genes and proteins involved in protein quality control and organelle homeostasis in the removal of dysfunctional proteins and organelles from cell.
Long-Term Calorie Restriction Enhances Cellular Quality-Control Processes in Human Skeletal Muscle

Ling Yang, 1,11 Danilo Licastro, 2,11 Edda Cava, 3,4,11 Nicola Veronese, 3,5 Francesco Spelta, 3,6 Wanda Rizza, 3,7 Beatrice Bertozzi, 3 Dennis T. Villareal, 2,3 Gökhan S. Hotamisligil, 1 John O. Holloszy, 1 and Luigi Fontana 3,9,10,*

1Department of Genetics and Complex Diseases and Sabri Ulker Center, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA
2GBM Scrl—Genomics, Area Science Park, Basovizza, 34149 Trieste, Italy
3Division of Geriatrics and Nutritional Sciences and Center for Human Nutrition, Washington University School of Medicine, St. Louis, MO 63110, USA
4Department of Experimental Medicine, University of Rome “La Sapienza,” 00161 Rome, Italy
5Division of Geriatrics, Department of Medicine, University of Padova, 35128 Padova, Italy
6Department of Medicine, University of Verona, 37129 Verona, Italy
7Department of Food and Human Nutrition Science, University Campus Bio-Medico, 00128 Rome, Italy
8Baylor College of Medicine and Michael E. DeBakey VA Medical Center, Houston, TX 77030, USA
9Department of Clinical and Experimental Sciences, Brescia University, 25121 Brescia, Italy
10CEINGE Biotecnologie Avanzate, 80122 Napoli, Italy
11Co-first author
*Correspondence: lfontana@dom.wustl.edu

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

Calorie restriction (CR) retards aging, acts as a hormetic intervention, and increases serum corticosterone and HSP70 expression in rodents. However, less is known regarding the effects of CR on these factors in humans. Serum cortisol and molecular chaperones and autophagic proteins were measured in the skeletal muscle of subjects on CR diets for 3–15 years and in control volunteers. Serum cortisol was higher in the CR group than in age-matched sedentary and endurance athlete groups (15.6 ± 4.6 ng/dl versus 12.3 ± 3.9 ng/dl and 11.2 ± 2.7 ng/dl, respectively; p ≤ 0.001). HSP70, Grp78, beclin-1, and LC3 mRNA and/or protein levels were higher in the skeletal muscle of the CR group compared to controls. Our data indicate that CR in humans is associated with sustained rises in serum cortisol, reduced inflammation, and increases in key molecular chaperones and autophagic mediators involved in cellular protein quality control and removal of dysfunctional proteins and organelles.

INTRODUCTION

Calorie restriction (CR) without malnutrition increases average and maximal lifespan and prevents a range of chronic disease in model organisms (Fontana et al., 2010). The mechanisms by which CR delays aging and prevents or delays chronic diseases are still unclear. Many interrelated and overlapping neuroendocrine adaptations have been proposed to play a role, including reduction of several growth factors (e.g., insulin growth factor-1 [IGF-1] and insulin) that control the insulin/IGF-1/forkhead box O (FOXO)/mammalian target of rapamycin (mTOR) pathway and an increase in serum concentrations of glucocorticoids (stress-induced hormones secreted by the adrenal cortex) (Anderson et al., 2009; Mercken et al., 2012; de Cabo et al., 2003; Omudei et al., 2013; Csiszar et al., 2013). Cortisol, the most important human glucocorticoid, regulates important metabolic functions and activates anti-stress and anti-inflammatory pathways (Sapolsky et al., 2000; Busillo and Cidlowski, 2013).

It has been hypothesized that CR works as a mild stressor to trigger a hormetic response, resulting in reduced inflammation and increased expression of stress resistance proteins, including the heat shock protein (HSP) molecular chaperones (Mattson, 2008). In particular, CR in rodents has been shown to increase the highly conserved HSP70 family, which serves crucial roles in protein homeostasis and quality control (Heydari et al., 1996; Selsby et al., 2005). HSP70 is a molecular chaperone that coordinates several key cellular functions, including the unfolding of misfolded or denatured proteins and the maintenance of these proteins in an unfolded, folding-competent state. They also protect nascently translated proteins, promote intracellular transport of proteins, and reduce proteotoxicity by stabilizing existing proteins against aggregation (Mayer and Bukau, 2005; Stricher et al., 2013).

The purpose of the present study was to evaluate some of the metabolic and molecular effects of long-term CR on stress-induced hormones and molecular pathways in healthy lean and weight-stable men and women. Serum concentrations of cortisol and aldosterone in individuals consuming a CR diet were compared with values obtained in two comparison groups: (1) age- and sex-matched sedentary individuals consuming a Western diet (WD) and (2) age-, sex-, and body fat-matched endurance runners consuming a WD. In this study, we also examined the stress-related and anti-inflammatory molecular adaptations induced by long-term CR in the skeletal muscle of healthy lean men and women.
RESULTS

CR, but Not Endurance Exercise, Induces an Increase in Serum Cortisol Levels

Participants in this study were 37 men and women (mean age 52.3 ± 11 years) consuming an ~30% CR diet for 3–15 years; 37 age-, sex-, and body fat-matched endurance athletes (EX); and 37 age-matched sedentary individuals consuming WDs. The CR individuals consumed a diet with a high nutrient-to-energy ratio, which supplied more than 100% of the recommended daily intake for all essential nutrients. All processed foods, rich in refined carbohydrates, free sugars, and partially hydrogenated oils, were strictly avoided by the CR practitioners. Energy intake in the CR group (2,779 ± 355 kcal/day; range 2,000–4,100 kcal/day) was 27% and 37% lower than in the WD group (2,811 ± 711 kcal/day; range 1,935–4,459 kcal/day), and 37% age-, sex-, and body fat-matched endurance athletes (EX); and very lean (BMI = 19.2 ± 1.1 kg/m²) volunteers of the Calorie Restriction Society and 10 age-matched nonobese control subjects (BMI = 25.3 ± 2.3 kg/m²) eating a typical WD. Heatmap analysis of the genes that are involved in the cellular stress response revealed a distinct separation of groups based on diet (Figure 2). Remarkably, we found that CR in humans induces a significant increase in several heat shock and cytosolic folding transcripts within skeletal muscle (Table 2). We found that a highly significant number of transcripts along the heat shock factor (HSF)/HSP70 pathway were altered by CR. In particular, the HSF1, HSF2, HSP70-1, and HSP70-2 transcripts were significantly upregulated 1.78-, 3.75-, 2.2-, and 12.4-fold, respectively (Table 2). Consistent with the gene expression changes, skeletal muscle of humans on long-term CR showed an ~1.8-fold increase in HSP70 protein levels relative to WD controls (p = 0.0033; Figure 3). The glucose-regulated protein 78 (GRP78) protein level also tended to be higher in the CR group than in the control group, but the difference did not reach statistical significance (p = 0.057).

Table 1. Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>CR Group (n = 37)</th>
<th>EX Group (n = 37)</th>
<th>WD Group (n = 37)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52.3 ± 11.4</td>
<td>53.7 ± 11.0</td>
<td>54.1 ± 9.4</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>32/5</td>
<td>32/5</td>
<td>32/5</td>
<td>NS</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.73 ± 0.3</td>
<td>1.74 ± 0.3</td>
<td>1.76 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.5 ± 6.3</td>
<td>62.8 ± 8.3</td>
<td>80.1 ± 14.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.3 ± 1.4</td>
<td>22.4 ± 2.2</td>
<td>25.2 ± 3.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>10.6 ± 5.8</td>
<td>13.4 ± 5.7</td>
<td>23.7 ± 6.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>50.6 ± 7.4</td>
<td>56.4 ± 8.4</td>
<td>56.5 ± 11.6</td>
<td>0.009</td>
</tr>
<tr>
<td>Cortisol (µg/dl)</td>
<td>15.6 ± 4.6</td>
<td>27.2 ± 2.7</td>
<td>12.3 ± 3.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>63.2 ± 67</td>
<td>67.1 ± 60</td>
<td>61.0 ± 45</td>
<td>NS</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.6 ± 0.8</td>
<td>2.2 ± 1.2</td>
<td>6.9 ± 5.7</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

NS, nonsignificant. Values are mean ± SD.

*Significantly different from the WD group: p < 0.0001.

**Significantly different from the EX group: p < 0.001.

CR-Induced Molecular Adaptation in Human Skeletal Muscle

To investigate the effects of long-term CR on stress-related molecular pathways, we performed skeletal muscle biopsies in a subset of 15 middle-aged (58.7 ± 7.4 years), weight-stable and very lean (BMI = 19.2 ± 1.1 kg/m²) volunteers of the Calorie Restriction Society and 10 age-matched nonobese control subjects (BMI = 25.3 ± 2.3 kg/m²) eating a typical WD. Heatmap analysis of the genes that are involved in the cellular stress response revealed a distinct separation of groups based on diet (Figure 2). Remarkably, we found that CR in humans induces a significant increase in several heat shock and cytosolic folding transcripts within skeletal muscle (Table 2). We found that a highly significant number of transcripts along the heat shock factor (HSF)/HSP70 pathway were altered by CR. In particular, the HSF1, HSF2, HSP70-1, and HSP70-2 transcripts were significantly upregulated 1.78-, 3.75-, 2.2-, and 12.4-fold, respectively (Table 2). Consistent with the gene expression changes, skeletal muscle of humans on long-term CR showed an ~1.8-fold increase in HSP70 protein levels relative to WD controls (p = 0.0033; Figure 3). The glucose-regulated protein 78 (GRP78) protein level also tended to be higher in the CR group than in the control group, but the difference did not reach statistical significance (p = 0.057).

Figure 1. Long-Term Effects of CR on Serum Cortisol Concentration and Inflammation

(A) Serum cortisol concentration from the cross-sectional comparison of individuals on a CR diet (n = 37), EX individuals (n = 37), or sedentary individuals on a typical WD (n = 37). *p < 0.0001. Data are mean ± SE.

(B) Inverse relation between serum cortisol concentration and serum TNF-α concentration in the CR group. Pearson correlation was used to assess associations between continuous variables.
Next, we investigated whether long-term CR in human skeletal muscle also exerts favorable effects on genes and proteins that regulate the cellular homeostatic mechanism, autophagy, which is essential for removing damaged cell organelles, as well as dysfunctional proteins, and for maintaining metabolic health (Yang et al., 2010). Accumulating data show that aging is associated with a decline in autophagy, and enhancing autophagy promotes longevity in both model organisms and rodents (Morimoto and Cuervo, 2014; Madeo et al., 2015). In addition, induction of autophagy by glucocorticoid has been evidenced in several cell types (Braun and Marks, 2015; Wang et al., 2015). We found that CR significantly upregulated many autophagy genes, including ULK1, ATG101, beclin-1, APG12, microtubule-associated protein 1 light chain 3 (LC3), GAPRAP/GATE-16, and autophagin-1 (Table 2). Consistent with some of these gene expression changes, we found that beclin-1 and LC3 protein expression levels were significantly higher in the skeletal muscle of the CR volunteers than in the WD control subjects (Figure 3).

Finally, we examined the effects of CR on molecular effectors downstream of the glucocorticoid receptor to which cortisol and other glucocorticoids bind. We found that CR significantly downregulated the transcript factors necrosis factor κB (NF-κB), signal transducer and activator of transcription 5 (STAT5), and c-FOS and, downstream, the mRNA levels of multiple inflammatory cytokines, including TNF-α, interleukin-6 (IL-6), interleukin-8 (IL-8), and inducible nitric oxide synthases (iNOS; Figure 4). Consistent with the transcriptional downregulation of these inflammatory pathways, we found that the serum concentration of TNF-α was significantly lower in the CR group than in the WD group (0.8 ± 0.5 pg/ml versus 1.6 ± 0.9 pg/ml, \( p \leq 0.0002 \)) demonstrating that CR in humans dampens the detrimental chronic inflammation (Hota-misligil, 2006).

**DISCUSSION**

It has been hypothesized that CR exerts its beneficial effects via a hormetic response that results in activation of the protein chaperones (e.g., HSP70 and GRP78) and autophagy, as well as in the inhibition of inflammation in rodents (Morimoto and Cuervo, 2014; Yu and Mattson, 1999; Arumugam et al., 2010; Lee and Notterpek, 2013). However, the hormonal and molecular effects of long-term CR with adequate nutrition on stress-related factors have not been carefully evaluated in humans on long-term severe CR. In this study, we found that serum cortisol concentration, a major stress hormone, was significantly higher in the CR group than in sedentary or exercising subjects eating a WD and was notably inversely correlated with serum TNF-α levels. We also found that key stress-induced cytosolic chaperones and autophagic transcript and protein levels were significantly higher and inflammatory factors were lower in the skeletal muscle of CR individuals than in age-matched controls, providing evidence for a CR-induced enhancement of protein quality control and of the ability of cells to eliminate damaged proteins and organelles.

Chronic CR has consistently been shown to cause a dose-dependent moderate elevation (i.e., 30%–50% above baseline) of circulating corticosterone levels in both rats and mice (Yaktine et al., 1998; Levay et al., 2010). Data from a recent randomized clinical trial of 2-year mild CR in nonobese humans have shown a small, 7% transient increase of serum cortisol levels (Ravussin et al., 2015; Fontana et al., 2015). Here, we show that serum cortisol concentration is ~30% higher in humans practicing long-term severe CR than in age-matched control subjects. Our data suggest that the mechanism responsible for the sustained increase in serum cortisol concentrations induced by CR is likely related to CR itself, rather than changes in body composition, because the equally low body fat and leptin levels of the exercisers were not associated with high cortisol in the
Elevation of glucocorticoid levels is an essential adaptation required to cope with a variety of stressors (Munck et al., 1984), and in CR animals, high corticosterone level has been shown to play a role in inhibiting inflammation and cancer progression. Adrenalectomy abrogates the CR-induced cancer inhibition, and glucocorticoid supplementation partially restores cancer inhibition in CR adrenalectomized rodents (Pashko and Schwartz, 1996; Stewart et al., 2005). Whether the increased level of cortisol plays a direct role in upregulating HSPs is unclear. However, it is well known that CR increases HSF1 and HSP70 levels in rodents (Heydari et al., 1996; Selsby et al., 2005). Here, we show that long-term CR significantly upregulates transcripts along the HSF/HSP70 pathway and increases HSP70 and GRP78 protein levels in the human skeletal muscle. Because aging is associated with reduced protein folding capacity and ability to maintain homeostasis in response to stress (Ben-Zvi et al., 2009; Kayani et al., 2008), these data suggest that CR in humans prevents this decrease and may be involved in the slowing of age-dependent accumulation of damaged and dysfunctional proteins. These changes would also contribute to overall functional capacity and health of organelles, such as endoplasmic reticulum, that are integral to inflammatory and metabolic regulation (Hotamisligil, 2010). Overexpression of HSF and HSP70 has been shown to extend lifespan by 50%–100% in C. elegans (Hsu et al., 2003; Yokoyama et al., 2002). Finally, suppression of inflammation may contribute to proteostasis, because endoplasmic reticulum function is also compromised in the presence of inflammation through nitrosylation and inhibition of key adaptive molecules such as IRE1 (Yang et al., 2015).

### Table 2. Effects of CR on Stress-Inducible Chaperones and Autophagy Genes in Muscle

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Function</th>
<th>LogFC</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HSP Family of Molecular Chaperones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSF1</td>
<td>+1.78</td>
<td>6.6e-05</td>
<td></td>
</tr>
<tr>
<td>HSF2</td>
<td>+3.75</td>
<td>2.6e-12</td>
<td></td>
</tr>
<tr>
<td>HSPA70-1A and B</td>
<td>+2.16</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>HSPA70-1 like</td>
<td>+2.88</td>
<td>2e-6</td>
<td></td>
</tr>
<tr>
<td>HSPA70-2</td>
<td>+12.45</td>
<td>0.000001</td>
<td></td>
</tr>
<tr>
<td>HSPA70-4</td>
<td>+2.54</td>
<td>1e-6</td>
<td></td>
</tr>
<tr>
<td>HSPA70-5</td>
<td>–2.86</td>
<td>0.000001</td>
<td></td>
</tr>
<tr>
<td>HSPA70-6</td>
<td>–3.39</td>
<td>9.3e-5</td>
<td></td>
</tr>
<tr>
<td>HSPA70-8</td>
<td>+1.7</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>HSPA70-9</td>
<td>+2.19</td>
<td>1e-6</td>
<td></td>
</tr>
<tr>
<td>HSPA70-13</td>
<td>+1.95</td>
<td>8.0e-6</td>
<td></td>
</tr>
<tr>
<td>HSPA70-14</td>
<td>+2.35</td>
<td>0.00001</td>
<td></td>
</tr>
<tr>
<td>HSP90-AA1 (α)</td>
<td>stress inducible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP90-AB1 (β)</td>
<td>constitutive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Autophagy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ULK1</td>
<td>serine/threonine protein kinase is involved in autophagy in response to starvation</td>
<td>+1.53</td>
<td>3.8e-05</td>
</tr>
<tr>
<td>ATG101</td>
<td>cytosol protects ATG13 from proteasomal degradation, therefore stabilizing levels of ATG13 found in cells and regulating levels of macroautophagy</td>
<td>+1.45</td>
<td>8.3e-05</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>Beclin-1 plays a central role in autophagy and, with its binding partner class III phosphoinositide 3-kinase, is required for the initiation of the formation of the autophagosome in autophagy</td>
<td>+1.51</td>
<td>1.7e-7</td>
</tr>
<tr>
<td>APG12</td>
<td>Apg12 conjugation of Apg5 is required for elongation of the isolation membrane to form a complete spherical autophagosome</td>
<td>–2.07</td>
<td>1e-10</td>
</tr>
<tr>
<td>APG16L1</td>
<td>the protein encoded by this gene is part of a large protein complex that is necessary for autophagy</td>
<td>+1.59</td>
<td>4.5e-10</td>
</tr>
<tr>
<td>LC3</td>
<td>LC3 is involved in elongation of the phagophore membrane and is an important marker and effector of starvation-induced autophagy</td>
<td>+1.68</td>
<td>4.1e-7</td>
</tr>
<tr>
<td>GAPRAP</td>
<td>GAPRAP is essential for a later stage in autophagosome maturation</td>
<td>+1.47</td>
<td>3.5e-06</td>
</tr>
<tr>
<td>GATE-16</td>
<td>GATE-16 is essential for a later stage in autophagosome maturation</td>
<td>+1.27</td>
<td>0.00013</td>
</tr>
<tr>
<td>Autophagin-1</td>
<td>Autophagin-1 cleaves the carboxyl termini of the LC3, GABARAP, and GATE-16, a reaction essential for its lipidation during autophagy</td>
<td>+1.44</td>
<td>0.05</td>
</tr>
</tbody>
</table>

LogFC, log fold change.
remains that these molecular events are downstream of other metabolic changes that are the result of CR.

Another cellular process activated by nutrient deprivation and energy stress is autophagy (i.e., cellular self-eating), which is essential to sustain cellular homeostasis by providing substrates for energy production during starvation. CR has been shown to ameliorate the age-dependent decline in autophagy in multiple organisms (Cuervo, 2008). Here, we demonstrate that long-term CR upregulates the transcription of several key autophagy genes and increases beclin-1 and LC3 protein levels in the human skeletal muscle. Unfortunately, in this experiment we could not obtain a direct measurement of autophagic flux. Nonetheless, our data, viewed in the context of data obtained on CR model organisms, strongly suggest that CR in humans activates autophagy, a vital cellular process for removal of dysfunctional organelles and damaged proteins from the cell, reduced inflammation, and improved metabolic homeostasis.

In conclusion, the results of this study demonstrate that long-term CR, with adequate intake of micronutrients, in healthy lean and weight-stable subjects is associated with sustained higher serum cortisol concentration, similar to that found in CR rodents. We also found that chronic CR in humans is associated with lower inflammation and is strongly associated with corrective changes in the cellular protein folding and autophagic apparatus. These CR-induced hormetic responses may play a key role in preserving protein quality control, preventing age-associated proteotoxicity, and increasing the capacity for degrading dysfunctional proteins and organelles, thereby preserving cell functionality and the capacity to adjust to a changing environment. These vital housekeeping homeostatic processes have been shown to protect against age-associated disease and may be involved in slowing the rate of aging in humans.

**EXPERIMENTAL PROCEDURES**

**Study Subjects**

Three groups (37 participants/group) were studied. One group (CR group) had been consuming a CR diet with adequate nutrients for 6 ± 3 years (range 3–15 years) and was recruited by contacting the Calorie Restriction Society. The second group (EX group) consisted of endurance runners who had been running an average of 48 miles/week (range 20–90 miles/week) for 21 ± 11 years (range 5–35 years) and were recruited from the St. Louis area. The EX group was matched on age, sex, and percent body fat with the CR group. The third group (WD group) comprised sedentary (regular exercise < 1 hr/week) subjects, recruited from the St. Louis area, who were eating a WD. The WD group was matched on age and sex with the CR and EX groups. The characteristics of the study participants are shown in Table 1. None of the participants had evidence of chronic disease, smoked cigarettes, or were taking medications that could affect the outcome variables. All participants reported weight stability, defined as less than a 2-kg change in body weight in the preceding 6 months. Participants recorded all food and beverage intake for 7 consecutive days. Food records were analyzed by using the NDS-R program (v.4.03, 31). The present study was approved by the Human Studies Committee of Washington University School of Medicine, and all subjects gave informed consent before their participation.

**Body Composition and Hormone Measurement**

Total body fat mass and fat-free mass was determined by dual-energy X-ray absorptiometry (DXA; QDR 1000/iv; Hologic). A venous blood sample was taken in the morning after subjects fasted for 12 hr. Radioimmunoassay kits were used to measure cortisol (DSL-2100; Diagnostic Systems Laboratories) and leptin (Leptin HL-81K; Linco Research). Commercially prepared ELISA kits were used to measure serum aldosterone concentration (ALPCO) and TNF-α (Quantakine High Sensitive, R&D Systems). The coefficients of variation of all assays were less than 10%.

**Gene Expression Analysis**

All microarray data discussed in this publication were obtained from GEO: GSE38012. As previously described (Mercken et al., 2013), human percutaneous biopsy specimens of vastus lateralis muscle of 15 individuals practicing CR and 10 age-matched control eating WDs were obtained in the morning after an overnight fast. RNA was extracted from skeletal muscle samples using Trizol Reagent (Invitrogen) following the manufacturer’s instructions. The signals on each sample are normalized by log z transformation to obtain Z scores and tests for distributions as previously described (Mercken et al., 2013). Correlation analysis, sample clustering analysis, and principal-component analysis including all probes were performed to identify or exclude any possible outliers. The resulting dataset was analyzed with DIANE 6.0, a spreadsheet-based microarray analysis program. Gene expression levels were quantitated using the Quantigene Plex 2.0 assay according to the manufacturer’s protocols (Panomics/Attymetric). To determine the fold change of the genes of interest, we used Partek Genomic Suite to conduct an ANOVA on log2 transformed background-subtracted Quantigene data, generating a fold change of the CR RNA samples over the WD RNA samples.

**Immunoblotting**

Vastus lateralis muscles were homogenized 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 phophatase inhibitor and protease inhibitor cocktail tablets, using a FastPrep 24 instrument (MP Biomedicals). Protein concentration was determined by BCA assay kit (Thermo Scientific). Then, 30 μg of protein was separated by SDS-PAGE on 10% or 16% Tris-glycine gels and transferred to 0.45 μM polyvinylidene fluoride membrane (Millipore). Membranes were blocked in 3% BSA/TBST, and antibodies were incubated overnight at 4°C in 1% BSA/TBST. The membranes were incubated with antibodies to HSP70 (Enzo, SPA-811), GRP78 (Cell Signaling Technology, 3177), beclin-1 (Cell Signaling Technology, 3738), and LC3 (Novus Biologicals, NB100-2220), and loading was verified by blotting for tubulin-horseradish peroxidase (Abcam, ab21058). This was followed by incubating with the secondary antibody
Figure 4. Transcriptional Modifications of the AKT/FOXO, HSF1/HSP70, and the Glucocorticoid Receptor Pathways in Human Skeletal Muscle by CR

AKT, protein kinase B; SOD2, superoxide dismutase 2; DDB1, damage-specific DNA-binding protein 1; GCR-α, glucocorticoid receptor alpha; CBP, CREB-binding protein.

conjugated with horseradish peroxidase and visualized using the enhanced chemiluminescence system (Roche Diagnostics). Densitometric analyses of western blot images were performed by using Quantity One Software (Bio-Rad).

Statistical Analysis
One-way ANOVA was used to compare group variables, followed by Tukey post hoc testing when indicated. One-way ANOVA with Games-Howell was performed for distributions where equal variances could not be assumed. Statistical significance was set at p < 0.05 for all tests. All data were analyzed using SPSS software v.13.0. All values are expressed as mean ± SD.

ACKNOWLEDGMENTS
This work was supported by grants from the Bakewell Foundation, AFAR (American Federation for Aging Research), the Longer Life Foundation (an RGA/Washington University Partnership), and the National Center for Research Resources (UL1 RR024992), as well as, to G.S.H., in part from RGA/Washington University Partnership, and the National Center for (American Federation for Aging Research), the Longer Life Foundation (an

REFERENCES


