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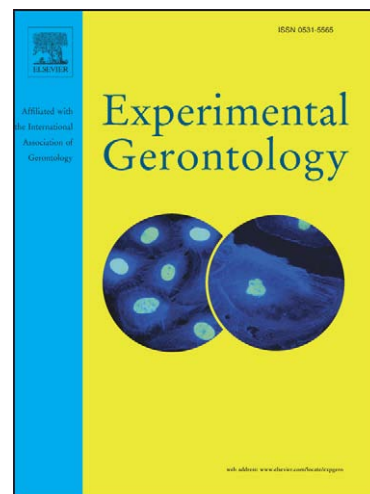
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Short-term consumption of a resveratrol-containing nutraceutical mixture mimics gene expression of long-term caloric restriction in mouse heart

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Abstract

An active area of aging research is focused on identifying compounds having the ability to mimic the effects of caloric restriction (CR). From two to five months of age, we fed male B6C3F₁ mice either a 40% CR diet, a control diet supplemented with a commercially available nutraceutical mixture (NCM) containing resveratrol, quercetin and inositol hexaphosphate, or a diet supplemented with an equivalent dose of chemical-grade resveratrol (RES; 1.25 mg resveratrol kg⁻¹ day⁻¹) from two to five months of age. Cardiac gene expression profiles were generated for the three groups of treated mice and compared to age-matched control (CO) mice. All three treatments were associated with changes in several cytoskeletal maintenance pathways, suggesting that RES and NCM are able to mimic short-term CR. CR uniquely affected several immune function pathways while RES uniquely affected multiple stress response pathways. Pathway analysis revealed that NCM (but not CR or RES) regulated multiple metabolic pathways that were also changed by long-term CR, including glucose and lipid metabolism, oxidative phosphorylation and chromatin assembly. Examination of key genes and pathways affected by NCM suggests that Foxo1 is a critical upstream mediator of its actions.

Introduction

Calorie restriction (CR) is the only dietary intervention known to increase maximum lifespan and slow multiple aspects of the aging process in diverse species (Weindruch and Walford, 1988). Although the effects of CR are robust, the difficulty of adhering to a low-calorie diet has increased interest in identifying compounds with the ability to mimic the effect of CR (Ingram et al., 2006). Many studies have reported beneficial effects of the components of red wine on cardiovascular health (Opie and Lecour, 2007), thus an active area of research has focused on the ability of these compounds to mimic CR. One compound which has received considerable attention is resveratrol, a naturally-occurring polyphenol found in red wine. Resveratrol has been suggested to extend lifespan of model organisms in a manner dependent on activation of sirtuin genes (Howitz et al., 2003; Wood et al., 2004), though some studies have failed to observe an effect of resveratrol on lifespan and sirtuin activation (Bass et al., 2007; Kaeberlein et al., 2005). Studies in mice are also conflicting: high doses of resveratrol prevent early mortality as a result of a high fat diet high fat feeding by activating the Sirt1 enzyme (Baur et al., 2006), but low doses of resveratrol do not appear to act through an increase in Sirt1 protein levels or activity (Barger et al., 2008). However, the latter study did show that resveratrol markedly mimicked the gene expression profile of CR in heart, brain and muscle and prevented age-related declines in cardiac function, suggesting that resveratrol may slow aspects of the aging process in mammals.

Because little is known about the *in vivo* effects of the consumption of resveratrol at low doses, and because other phytochemicals may enhance the bioactivity of resveratrol (De Santi et al., 2000), we studied cardiac gene expression in mice consuming either a commercially available nutraceutical mixture containing a low dose of resveratrol and other compounds thought to enhance its bioavailability and compared this to mice fed the same dose of chemical-grade resveratrol. To determine if the action of either of these treatments mimics the effect of CR, we measured gene expression in a group of age-matched mice fed a control diet or subjected to CR. Finally, we identified metabolic pathways affected by short-term treatment and compared these results to a previous microarray study (Lee et al., 2002) of mouse cardiac aging and long-term CR (LTCR).

Materials and Methods

Animals and diets. Male B6C3F₁ hybrid mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age. Upon arrival, mice were individually housed and provided with 84 kcal week⁻¹ of a control diet based on the AIN-93M formulation. This caloric intake is 10-20% less than the average ad libitum intake and prevents obesity. At two months of age, mice were either maintained on the control diet (CO) or were switched to one of three experimental diets (n=10 mice per group): CR (63 kcal week⁻¹), supplemented with resveratrol (RES, 1.25 mg kg⁻¹ body weight) from Sigma-Aldrich (St. Louis, Missouri), or supplemented with a commercially-available nutraceutical mixture

(NCM) containing a cocktail of phytochemicals (Longevinex®, Resveratrol Partners, LLC, Las Vegas, NV). The complete formulation of the NCM diet is as follows: giant knotweed extract ($2.5 \text{ mg kg}^{-1} \text{ day}^{-1}$, which provided an equivalent dose of resveratrol as in the RES diet), quercetin dihydrate ($0.31 \text{ mg kg}^{-1} \text{ day}^{-1}$), rice bran extract to provide inositol hexaphosphate ($0.94 \text{ mg kg}^{-1} \text{ day}^{-1}$), rice bran oil ($4.75 \text{ mg kg}^{-1} \text{ day}^{-1}$) and sunflower lecithin ($0.69 \text{ mg kg}^{-1} \text{ day}^{-1}$). The calorie intake of the RES and NCM diets was the same as the CO diet. All diets were provided as dustless precision pellets manufactured by Bio-Serv (Frenchtown, New Jersey). Details on the CO and CR diets are provided elsewhere (Pugh et al., 1999).

Mice were fed the above diets with water provided ad libitum. Body weights were recorded approximately every other week. Body weights were analyzed by repeated measures analysis of variance. At five months of age, mice were euthanized by cervical dislocation after a 12-hour fast, blood was collected from the body cavity and tissues were collected and flash-frozen in liquid nitrogen. Serum was collected from the blood and stored with the tissues at -80°C . All experimental protocols received approval from the Animal Care and Use Committee at the William S. Middleton Memorial Veterans Hospital, which is fully compliant with the American Physiological Society's "Guiding Principles in the Care and Use of Animals" (AmericanPhysiologicalSociety, 2002).

Blood parameters. Serum glucose levels were measured with a LifeScan OneTouch glucose monitoring system. Insulin levels were measured using an

enzyme immunoassay from Alpco Diagnostics (Salem, New Hampshire). Results from treated mice were compared to CO mice with two tailed *t*-tests.

Microarray analysis. Gene expression profiles were obtained from seven mice per group using the Mouse Genome 430 2.0 Array from Affymetrix (Santa Clara, California). Sample preparation and hybridization procedures have been described in detail elsewhere (Lee et al., 2002). Probe sets on the array that did not correspond to a single Entrez Gene identifier were removed from the data set; when a gene was represented by more than one probe set, we only analyzed the probe set having the greatest signal intensity (averaged across all 28 arrays in the study). Gene expression of treated mice was compared to CO mice using two-tailed *t*-tests; genes were considered to be differentially expressed at $p < 0.01$.

To understand the functional significance of genes that were significantly changed by a particular treatment, we annotated expression data with the Biological Process terminology from the Gene Ontology (GO) Consortium. For this analysis, we only considered GO terms at Level 3 or higher to exclude ambiguous pathways (e.g. "Metabolism"); additionally, only GO terms that were represented by at least 10 genes were analyzed. We used Fisher's exact test to determine if a GO term was enriched by differentially expressed genes relative to what would be expected by chance (Al-Shahrour et al., 2004; Scheer et al., 2006; Zeeberg et al., 2003). GO terms with a p -value < 0.05 from Fisher's exact test were considered to be statistically significant. To determine if Biological

Processes changed with short-term treatment were also changed with age or long-term CR, we performed the same pathway analysis on a previously-published dataset of the effects of age and long-term CR (LTCR) in mouse heart (Lee et al., 2002).

Quantitative RT-PCR. Aliquots of total RNA used in the microarray analysis were diluted to $10 \text{ ng } \mu\text{L}^{-1}$ for confirmation of microarray data using quantitative RT-PCR. TaqMan FAM-labeled, primer-probe sets were purchased from Applied Biosciences Inc. (Foster City, CA) for the following genes: Ucp3 (Mm00494074 m1), Pdk4 (Mm00443325 m1), Pgc-1 α (Mm00447183 g1), Sirt1 (Mm0490758 m1), Foxo1 (Mm00490672 m1) and mTOR (Mm00444968 m1). Ribosomal protein L32 (Rpl32, Mm02528467 g1) was chosen as the housekeeping gene because it was unchanged in expression by any treatment according to the microarray data.

Each sample was analyzed in duplicate in a total reaction volume of 20 μL consisting of 50 ng of total RNA in a reaction mixture with EZ RT-PCR (Applied Biosystems) reagents and 1 μL of the 20x TaqMan primer-probe mix. Reactions were run on an Eppendorf Realplex s² (Eppendorf North America, Westbury, NY) using the following conditions: 50 °C for 2 minutes, 60 °C for 30 minutes, 95 °C for 5 minutes and 45 cycles at 95 °C for 15 seconds and 60 °C for 45 seconds. The threshold cycle (Ct) values were determined by the Eppendorf software and the Ct value for each sample was obtained by calculating the arithmetic mean of the duplicate values. Changes from CO values were calculated by the δ - δCt method.

Delta (δ) threshold values were computed by the difference between the Ct value of the gene under consideration and that of the housekeeping gene for that mouse sample. The mean δ Ct for CO samples provided the reference value for δ - δ Ct calculations (one CO sample was identified as an outlier—expression data for many genes was 4-20 standard deviations above the mean of the remaining six samples—and was removed from the analysis). Fold change was calculated by as $2^{-\delta\delta Ct}$.

Results

Body weight and blood parameters. Body weights of CR mice were significantly lower than CO mice at every time point after the initiation of CR (Figure 1A). Body weights of RES and NCM mice were not different from CO mice. Glucose and insulin levels were also not significantly changed by any dietary intervention (Figures 1B and 1C, respectively), although there was a trend for lower glucose levels in CR and NCM mice.

Gene expression data. For the 20,341 unique genes represented on the array, 2,829 genes were significantly changed by at least one or more treatments. Table 1 lists each treatment and the number of genes differentially expressed by that treatment or combination of treatments. Overall, NCM had the greatest effect on gene expression with 2,406 genes changed by NCM either alone or in combination with another treatment. RES affected the expression of 866 genes

overall; surprisingly, CR affected the expression of only 304 genes overall. A complete list of differentially expressed genes is shown in Supplemental Table 1.

Impact on key aging/CR genes. Previous studies in mouse heart (Barger et al., 2008; Lee et al., 2002) have identified three metabolic genes that are robustly increased in expression in mouse heart in response to long-term CR (LTCR): uncoupling protein 3 (Ucp3), pyruvate dehydrogenase kinase 4 (Pdk4) and peroxisome proliferator activated gamma coactivator 1 alpha (Pgc-1 α). In the current study, Ucp3 expression was significantly increased 1.8-fold in CR mice and 2.8-fold in NCM mice (Figure 2A). Ucp3 expression was increased 2.0-fold in RES mice but this did not reach statistical significance ($p=0.018$). Pdk4 was abundantly expressed and was significantly increased in both RES and NCM mice (2.8- and 3.3-fold, respectively; Figure 2B). Pgc-1 α expression was significantly increased in expression only in NCM mice (1.9-fold, Figure 2C). Thus, for these three transcriptional metabolic biomarkers of LTCR, the effects of a three-month treatment with NCM were far more robust than the same duration of CR.

We also examined if there was an effect of treatment on other genes thought to be important in the aging process. The mammalian sirtuin proteins are proposed to be critical mediators of the effect of CR and resveratrol; of the seven sirtuin genes identified in mice, only Sirt1 was changed by any treatment in this study (decreased -1.7-fold by NCM from the microarray; Figure 2D). The forkhead transcription factor Foxo1 is the mammalian homolog of the *C. elegans*

daf-16 and *Drosophila* dFoxo genes which have been shown to regulate longevity in these species (Hwangbo et al., 2004; Murphy, 2006); Foxo1 expression was significantly increased only by NCM (1.7-fold, Figure 2E). Finally, the mammalian target of rapamycin (mTOR), which has been shown to modulate longevity in model organisms and regulate oxidative energy metabolism (Cunningham et al., 2007; Schieke and Finkel, 2006; Schieke et al., 2006), was significantly increased in expression only by NCM (1.5-fold, Figure 2F). As shown in Figure 3, the expression pattern of these genes was confirmed by quantitative RT-PCR with the following exceptions: Ucp3 expression was significantly increased by RES, Pdk4 expression was significantly increased by CR and Sirt1 expression was significantly different in the NCM group. Expression patterns for the other three genes shown in Figure 2 were similar between the microarray and RT-PCR assays.

Insulin and insulin-like signaling (IIS) regulate aging in many species and reduced IIS has been associated with longevity (Rincon et al., 2005). Although we did not observe a decrease in circulating insulin levels, several genes involved in IIS were uniquely regulated by NCM. We observed an increase in the expression of the binding proteins Igfbp4 and Igfbp6 and an increase in insulin degrading enzyme, Ide (Supplemental Table 1). Taken together, the expression pattern of these genes would predict a lowering of IGF-1 levels (Clemmons, 2007; Hoeflich et al., 1999; Holly and Perks, 2006) and decreased local levels of insulin.

Pathway analysis. Table 1 summarizes the number of GO terms changed with each treatment and Table 2 lists selected GO terms that were significantly changed by treatment (a complete list of GO terms changed in this study is shown in Supplemental Table 2). CR affected immune function pathways including leukocyte chemotaxis and T cell differentiation. RES affected several stress response pathways, including cellular response to stress, response to DNA damage stimulus, ER overload response and positive regulation of JNK cascade. NCM was predominantly associated with changes in energy metabolism, including glucose metabolism, tricarboxylic acid metabolism, lipid metabolism, fatty acid beta-oxidation and oxidative phosphorylation. This was associated with increases in the expression of several key genes associated with these pathways including Cpt1a, Cpt1b, Srebf1 and Lipe (Supplemental Table 1). NCM also affected “chromatin assembly and disassembly” and “transcription initiation from RNA polymerase II promoter”, two GO terms that were previously found to be changed by both CR and resveratrol in multiple tissues (Barger et al., 2008). The insulin receptor signaling pathway was also significantly affected by NCM alone. Six of the 11 GO terms affected by all three treatments were associated with aspects of cytoskeletal maintenance.

Overlap between short-term NCM treatment and LTCR: Because several transcriptional biomarkers of LTCR were also changed by short-term RES or NCM treatment, we wished to determine the extent of the overlap between pathways affected by short-term treatment and LTCR using the same pathway

analysis. The most striking similarity between a short-term treatment and LTCR was for NCM, with many metabolic pathways changed by both NCM and LTCR including glucose metabolism, tricarboxylic acid metabolism and oxidative phosphorylation (Figure 4).

Discussion.

CR remains the only dietary intervention that extends maximum lifespan and retards multiple aspects of biological aging in diverse species (Guarente, 2008; Weindruch and Walford, 1988). Because of the difficulty in adhering to a low calorie diet, there is expanding interest in identifying molecules which mimic the effects of CR. A leading candidate is resveratrol, and several studies suggest that resveratrol extends lifespan in model organisms (Howitz et al., 2003; Wood et al., 2004), although contradicting data exist (Bass et al., 2007; Kaeberlein et al., 2005). We recently reported that a low dose of resveratrol ($4.9 \text{ mg kg}^{-1} \text{ day}^{-1}$) mimics the gene expression profile of long-term CR in multiple tissues of mice (Barger et al., 2008). Here we show that short-term treatment of a nutraceutical mixture containing an even lower dose of resveratrol in combination with other phytochemicals induces transcriptional shifts in heart of young mice that are similar to that seen with long-term CR. As discussed below, we also present a working model based on the gene expression data that identifies Foxo1 as an upstream regulator of the action of NCM.

We found no evidence that RES or NCM affected body weight or serum glucose or insulin levels compared to CO mice (Figure 1). As expected, CR caused a significant reduction in body weight throughout most of the study,

although there was no effect of CR on glucose or insulin levels. This is probably due to the fact that CO (and RES and NCM) mice were given ~10% less food than ad libitum intake which might obscure an effect of CR on glucose and insulin levels in young, healthy mice.

A previous microarray study in mouse heart revealed that aging is associated with a transition from fatty acid to carbohydrate metabolism and that LTCR from 14 to 30 months of age opposes the development of this metabolic shift (Lee et al., 2002). In this study, we measured gene expression in young mice of the same strain consuming the same control and CR diets used previously. A short-term (three month) CR changed the expression of relatively few genes and metabolic pathways compared to what was previously seen in old mice subjected to LTCR. An upregulation of genes involved in energy metabolism was also observed in adipose tissue of mice subjected to 10 months (but not 23 days) of CR (Higami et al., 2004), though numerous genes are differentially expressed in liver of mice subjected to short-term CR (Cao et al., 2001). This suggests that robust transcriptional regulation in response to short-term CR is tissue-specific. Nonetheless, pathway analysis indicated that CR affected pathways involved in T-cell differentiation and cytoskeletal organization (Table 2). These results are in agreement with previous findings that CR modulates the expression of genes involved in immune activation and cytoskeletal maintenance in mouse heart (Lee et al., 2002).

Resveratrol is reported to exert cardioprotective effects *in vitro*, including anti-inflammatory and anti-oxidant activity (Das and Maulik, 2006), but our

pathway analysis did not reveal a significant effect of RES on these biological processes. This is not surprising, given that we studied young, healthy mice where inflammation and oxidative damage would not be expected. However, our recent study of cardiac gene expression in old mice treated with a low dose of resveratrol from 14 to 30 months of age (Barger et al., 2008) did not reveal any effect on inflammatory or antioxidant pathways, suggesting that results from *in vitro* studies of resveratrol may not be applicable to its effect *in vivo*. In the current study, RES affected multiple stress response pathways which is in strong agreement with “xenohormesis hypothesis” for the action of resveratrol (Baur and Sinclair, 2006). This hypothesis predicts that consumption of plant secondary compounds indicative of environmental stressors can initiate a physiological response in animals that is critical for survival during times of low food availability. Although stress response pathways were not affected by CR in this study, this was observed in a re-analysis of the effect of LTCR in mouse heart (Supplemental Table 2). These data add to the increasing body of literature showing that CR is intimately linked to the stress response in mammals (Anderson et al., 2008), and that resveratrol may mimic this effect of LTCR.

Consumption of a diet supplemented with a mixture of nutraceuticals including resveratrol, quercetin and inositol hexaphosphate had the greatest impact on gene expression, affecting 85% of all differentially expressed genes and pathways (Table 1). Interestingly, several genes and metabolic pathways that are thought to be fundamental to the response to LTCR in mammals were uniquely affected by NCM. Pgc-1 α is a transcriptional coactivator regulating the

expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation (Lin et al., 2005); this gene is upregulated in multiple tissues of mice subjected to LTCR (Barger et al., 2008; Lee et al., 2002) and was increased in expression only by NCM (Figure 2C and 3C). Ucp3 and Pdk4 are two additional robust biomarkers of CR in heart (Barger et al., 2008; Lee et al., 2002), and these genes were also increased in expression in NCM mice. The expression pattern of these genes strongly suggests that NCM consumption is associated a decreased glycolysis and increased oxidative energy metabolism. Indeed, pathway analysis revealed that these GO terms were similarly changed with both LTCR (Lee et al., 2002) and with NCM consumption (Figure 4). In the previous study of LTCR in mouse heart, expression profiling revealed a pattern suggesting that fatty acid metabolism decreases with age and that LTCR markedly opposed this age-related shift (Lee et al., 2002). There was a similar expression pattern between LTCR and NCM for genes associated with fatty acid metabolism, and pathway analysis revealed that NCM significantly modulated the fatty acid metabolism pathway (Figure 4). Thus, the transcriptional data clearly show that NCM has a robust effect on metabolism similar to that seen in mice subjected to LTCR.

Although several studies propose that resveratrol's mechanism of action is by increasing the activity of the SIRT1 enzyme (Baur et al., 2006; Lagouge et al., 2006), we found that CR and a physiological dose of resveratrol (RES) did not significantly affect the expression of any of the sirtuin genes. Moreover, Sirt1 expression was significantly decreased in expression in the NCM mice from the

microarray data (Figure 2D) and Sirt1 expression was not changed by any treatment according to the RT-PCR data (Figure 3D). Taken together, these findings are in agreement with a recent study showing that SIRT1 protein levels were decreased in heart and skeletal muscle of mice fed a similar dose of resveratrol (Barger et al., 2008). Although the current data do not reveal if SIRT1 enzyme activity is altered, a growing body of evidence shows that neither CR (Chen et al., 2008) nor resveratrol at low doses increase Sirt1 expression or protein levels, suggesting that modulation of SIRT1 activity may not be essential to elicit a biological response with a low dose of resveratrol.

Of the 630 genes changed in expression by both RES and NCM, all 630 genes were changed in the same direction for both diets (Supplemental Table 1). However, NCM affected the expression of 2,406 genes altogether, suggesting that resveratrol alone is responsible for only a modest proportion of the genes changed in expression by the NCM diet. Several lines of evidence suggest that the transcriptional activity of NCM results from the additional nutraceuticals enhancing the bioactivity of resveratrol. As shown in Table 2, many of the metabolic pathways changed by the NCM diet are similar to those reported in mice fed resveratrol, however those studies required a dose of resveratrol 17-320 fold greater than that used in the present study (Baur et al., 2006; Lagouge et al., 2006). The inclusion of inositol hexaphosphate (IP6) may have contributed to the enhanced activity of resveratrol, as consumption of IP6 improves absorption of another polyphenol, blackcurrant anthocyanins, in rats and humans (Matsumoto et al., 2007). Quercetin, a polyphenol found in a variety

of plant sources has been reported to inhibit sulfation of resveratrol (De Santi et al., 2000) and therefore would be predicted to increase resveratrol absorption. It is possible that these compounds, or other compounds in the giant knotweed extract, may have independently or additively affected gene expression, though this cannot be determined from the current study.

The prevailing hypothesis explaining the mechanism by which resveratrol mimics CR at the transcriptional level is through activation of the family of sirtuin proteins (Baur et al., 2006; Lagouge et al., 2006). However, the general relevance of these findings is questionable because of the dose of resveratrol required to activate the sirtuin proteins appears to greatly exceed the amount of resveratrol that would be obtained through normal wine consumption. Moreover, others have shown that the activation of sirtuins is an assay-specific, non-physiological effect (Kaeberlein et al., 2005). We show that short-term consumption of a nutraceutical mixture containing a dose of resveratrol 17-320 fold lower than that used in previous studies in rodents affects the expression of genes involved in metabolism in a manner similar to that seen with LTCR, and that this is associated with a decrease in the expression of Sirt1.

Interestingly, expression of the forkhead transcription factor Foxo1 was upregulated only by the NCM diet (Figure 2E and 3E). Foxo1 is a homologue of the *daf-16* gene in *C. elegans* and the *dFoxo* gene in *Drosophila*, both of which have been linked with increased lifespan in response to decreased IIS (Giannakou et al., 2004; Hwangbo et al., 2004; Lin et al., 2001). Reduced IIS results in decreased phosphorylation of the FOXO1 protein, which can then enter

the nucleus to promote the expression of key longevity genes. We observed a transcriptional profile consistent with decreased IIS, and in combination with increased Foxo1 expression, our data suggest that nuclear accumulation of FOXO1 protein would be enhanced in NCM mice. A putative increase in FOXO1 nuclear abundance in NCM mice is further supported by the finding that DAF-16 nuclear translocation was enhanced and lifespan increased in *C. elegans* treated with quercetin (Kampkotter et al., 2008), one of the components of NCM.

A working model outlining how NCM induces a metabolic shift in gene expression similar to that seen with LTCR is presented in Figure 5. A central feature of this model is a postulated increase in the nuclear abundance of FOXO1 protein in NCM mice, supported by the finding of increased expression of Foxo1 (Figures 2 and 3) and a transcriptional signature of decreased insulin/insulin-like signaling (Table 2 and Supplemental Table 1). Moreover, we observed a striking overlap between NCM and metabolic pathways known to be modulated by FOXO1 including glucose, lipid and fatty acid metabolism (Furuyama et al., 2003; Kamei et al., 2003; Zhang et al., 2006). We propose that the metabolic effects of NCM are due FOXO1-dependent modulation of Pgc-1 α (Daitoku et al., 2003; Southgate et al., 2005), which is consistent with our observation of increased Foxo1 and Pgc-1 α expression in NCM mice. Furthermore, our model proposes that regulation of mitochondrial oxidative function by NCM/FOXO1 is dependent on an interaction between Pgc-1 α and mTOR; this is consistent with studies showing that the age-related decline in the mTOR pathway is prevented by LTCR (Linford et al., 2007) and that the

PGC-1 α /mTOR protein complex is required to maintain mitochondrial oxidative function (Cunningham et al., 2007). Taken together, our data suggest that FOXO1 is a major upstream regulator of metabolic regulation induced by LTCR, and that these shifts in energy metabolism are amenable to short-term dietary intervention.

In summary, we found that short-term CR has modest effects on cardiac gene expression, whereas there is a remarkable overlap in metabolic pathways changed with LTCR and short-term consumption of a mixture of nutraceuticals. This overlap was not seen with a short-term treatment of a low dose of research grade resveratrol, a proposed CR mimetic. We propose a model based on published studies and our transcriptional profiling that highlights FOXO1 as a major upstream regulator that responds to NCM treatment and induces a shift in cardiac gene expression exhibiting a striking overlap in genes and metabolic pathways affected by LTCR. Future studies will test the validity of this model and seek to reveal the extent that NCM treatment can retard the aging process based on transcriptional profiling, age-associated changes in physiological function and longevity.

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Figure and Table legends

Figure 1. Body weight, serum glucose and serum insulin levels, n=10 mice per group. Values are means \pm standard error. Body weight (A) of CR mice was significantly lower than CO mice. NCM mice tended to have a slightly lower body weight than CO mice, but this was not statistically significant. Serum glucose (B) and insulin (C) levels of treated mice were not significantly different from CO mice.

Figure 2. Expression of selected genes relevant to the effects of CR. Data represent average signal intensity (+ standard error) from the microarray. Significant ($P<0.01$) differences from CO mice are indicated with an asterisk.

Figure 3. RT-PCR confirmation of genes shown in Figure 2. Data represent fold change in expression (+ standard error) compared to CO mice. Significant differences are indicated with † ($P<0.05$) or * ($P<0.01$).

Figure 4. Ability of short-term NCM treatment to affect metabolic pathways previously reported to be changed by age and/or long-term CR (LTGR). For each metabolic pathway, treatments causing a significant change in that pathway are indicated in parentheses. Genes significantly changed by treatment are shaded to indicate down- or up-regulation (genes not significantly changed by any treatment are not shown).

Figure 5. Proposed model to explain mechanism by which NCM mimics the expression of genes and metabolic pathways affected by long-term CR. NCM uniquely increases the expression of Foxo1 which subsequently induces a shift in gene expression reminiscent of LTCR. See Discussion for detailed explanation.

Supplemental Table 1. Complete list of genes changed by at least one treatment ($P < 0.01$). Fold change values of genes significantly increased in expression are shaded in yellow, fold change values for genes significantly decreased in expression are shaded in blue.

Supplemental Table 2. Complete list of Gene Ontology (GO) Biological Process terms that were changed ($P < 0.05$) by at least one treatment in the CR, RES and NCM groups. Re-analysis of a previous dataset reporting the effect of LTCR revealed an overlap between the effects of LTCR and the short-term treatments in the current study. Where statistically significant, the effect of LTCR is indicated with the appropriate p-value.

Table 1. Summary of treatment effects on the 2,829 genes and 220 Gene Ontology Biological Process terms significantly changed by at least one treatment. Genes were considered significantly different at $P < 0.01$; GO Biological Process terms were considered significantly different at $P < 0.05$ (see Methods for details).

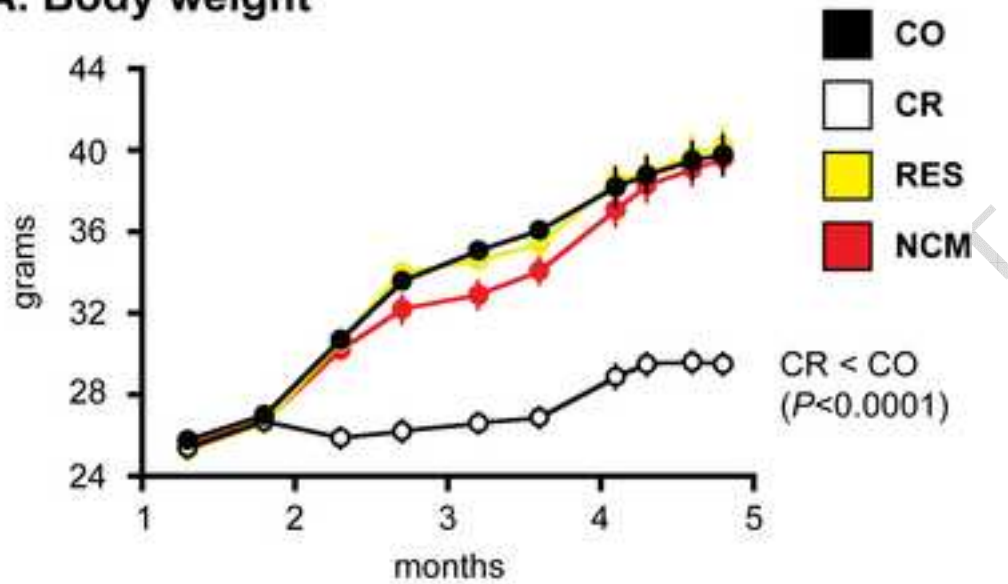
Treatment effect	# genes	# GO Terms
Unique to CR	187	11
Unique to RES	224	22
Unique to NCM	1711	131
CR & RES	12	1
CR & NCM	65	17
RES & NCM	590	27
All	40	11
	2829	220

Table 2. Selected Gene Ontology Biological Processes affected by treatment (a complete list is shown in Supplemental Table 2). * indicates pathways affected by LTCR in a previous study (Lee et al., 2002); ** indicates pathways previously shown to be affected by CR and resveratrol in multiple tissues (Barger et al., 2008).

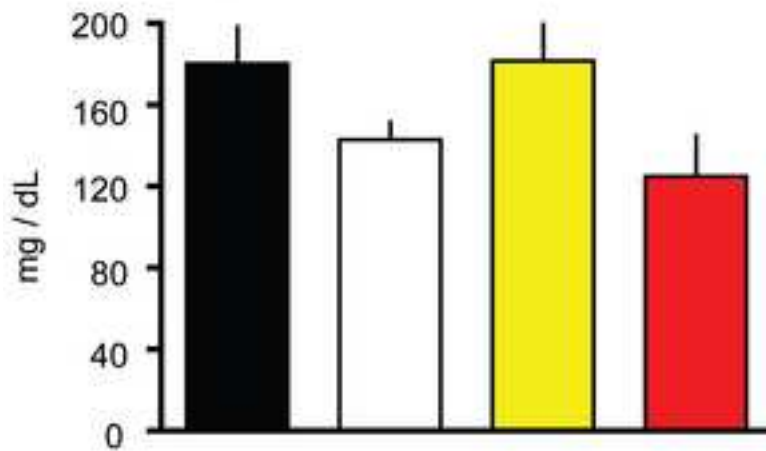
Gene Ontology Biological Process Term	GO Identifier
CR	
T cell differentiation	GO:0030217
leukocyte chemotaxis	GO:0030595
actin polymerization and/or depolymerization	GO:0008154 *
cytoskeleton organization and biogenesis	GO:0007010
RES	
cellular response to stress	GO:0033554 *
response to DNA damage stimulus	GO:0006974
ER overload response	GO:0006983
positive regulation of JNK cascade	GO:0046330
ubiquitin cycle	GO:0006512 **
actin polymerization and/or depolymerization	GO:0008154 *
cytoskeleton organization and biogenesis	GO:0007010
NCM	
glucose metabolic process	GO:0006006 *
tricarboxylic acid cycle	GO:0006099 *
lipid metabolic process	GO:0006629 *
fatty acid metabolic process	GO:0006631
oxidative phosphorylation	GO:0006119 *
ATP synthesis coupled electron transport	GO:0042773 *
insulin receptor signaling pathway	GO:0008286
chromatin assembly or disassembly	GO:0006333 **
transcription initiation from RNA polymerase II promoter	GO:0006367 **
ubiquitin cycle	GO:0006512 **
actin polymerization and/or depolymerization	GO:0008154 *
cytoskeleton organization and biogenesis	GO:0007010

Figure 1

A. Body weight



B. Serum glucose



C. Serum insulin

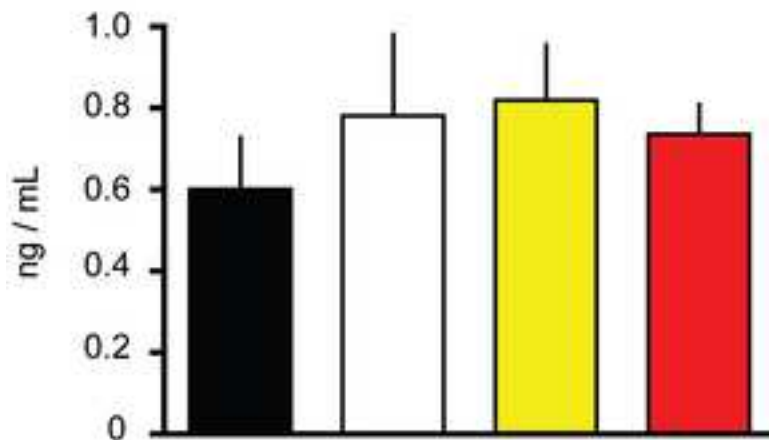


Figure 2

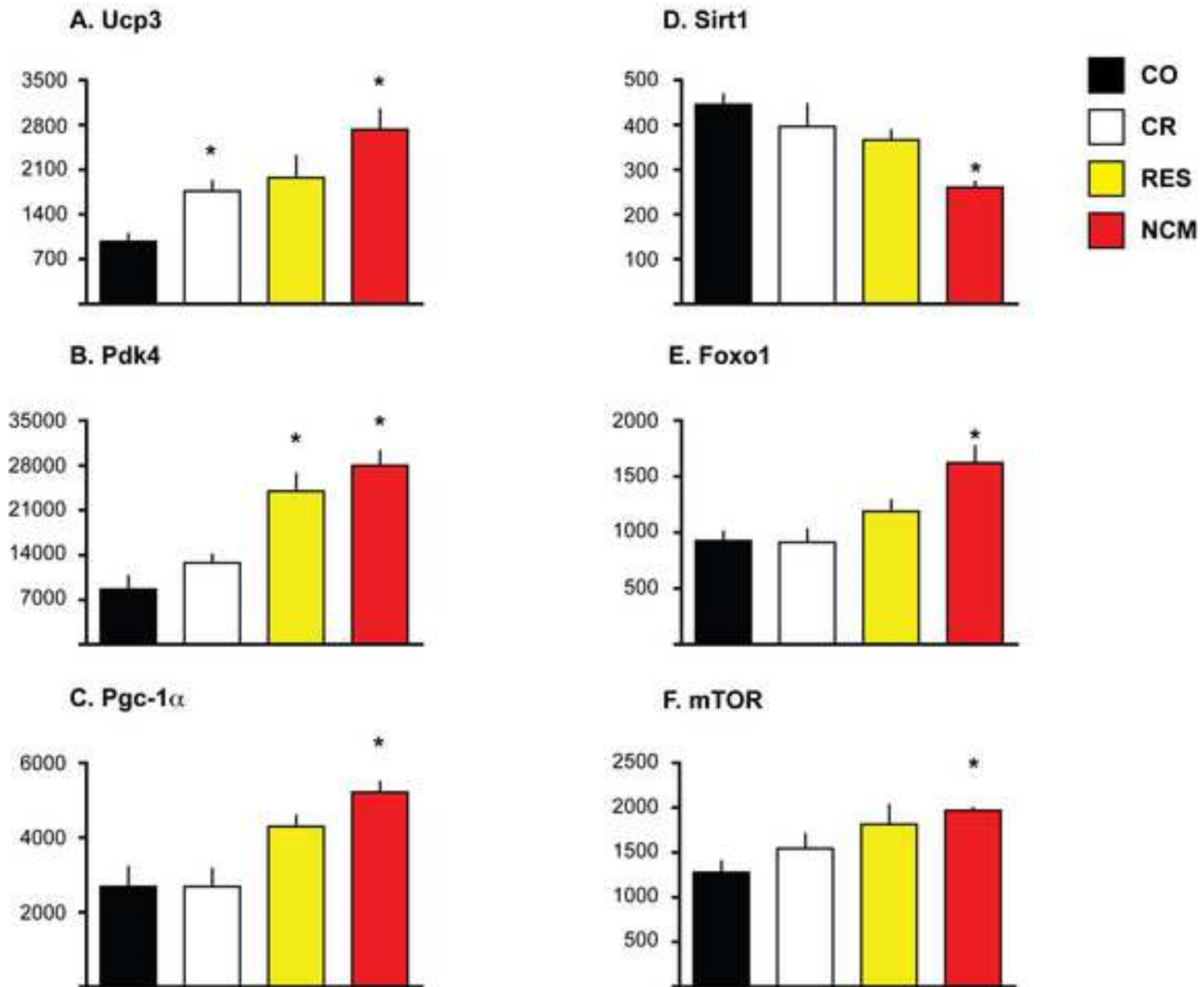


Figure 3

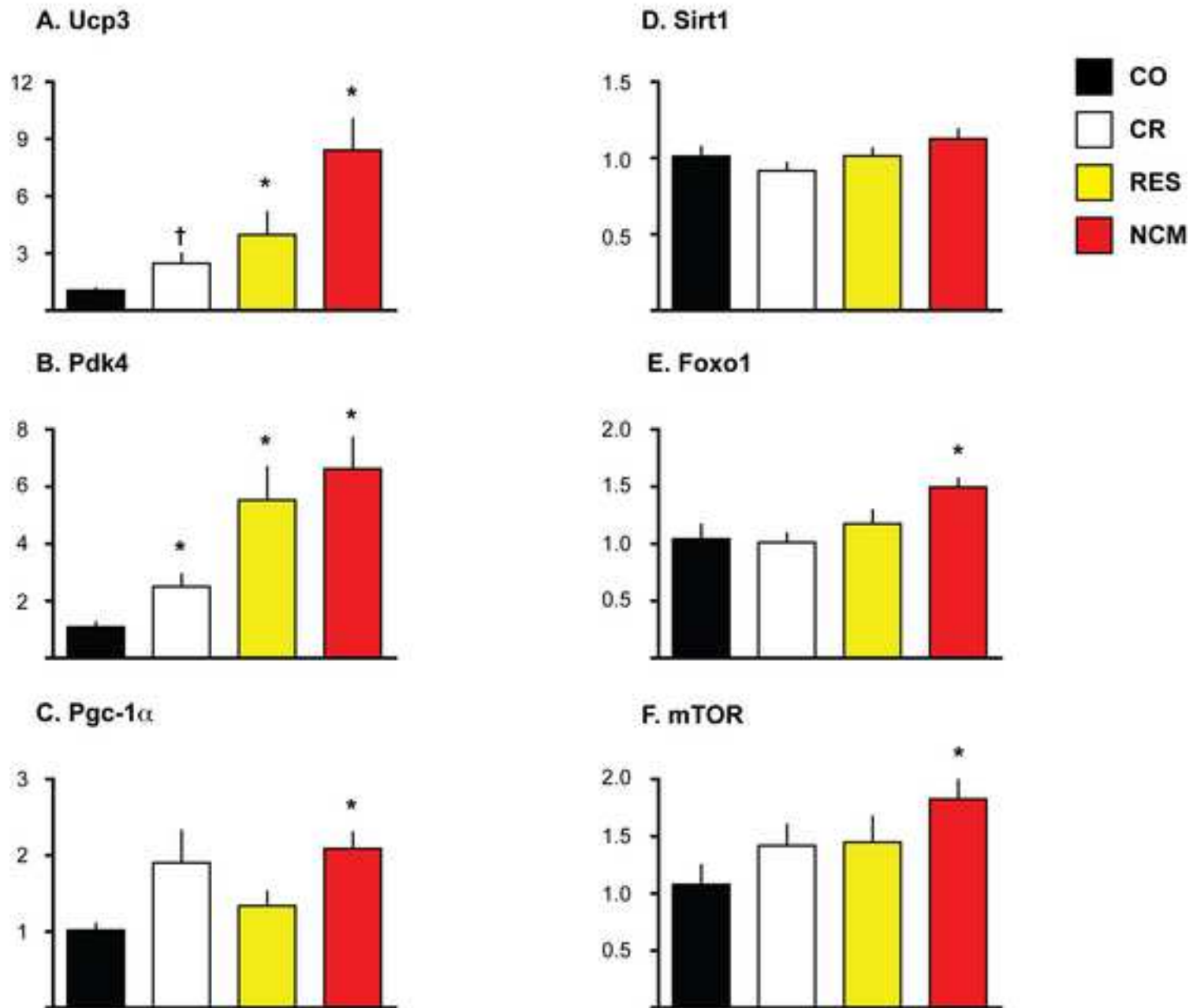
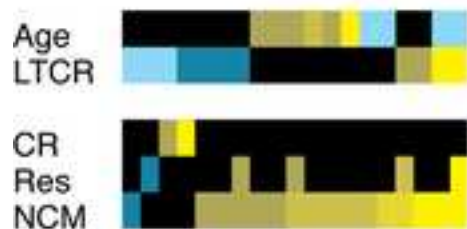
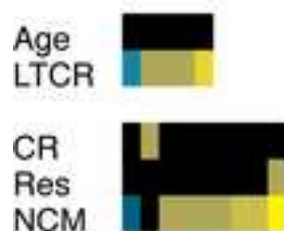


Figure 4

GO:0006006: Glucose metabolism (Age, LTCR, NCM)



GO:0006099: Tricarboxylic acid metabolism (LTCR, NCM)



GO:0006631: Fatty acid metabolism (NCM)



GO:0006119: Oxidative phosphorylation (LTCR, NCM)



Fold change



Figure 5

