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Short Article

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Resveratrol Delays Age-Related Deterioration and Mimics Transcriptional Aspects of Dietary Restriction without Extending Life Span

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SUMMARY

A small molecule that safely mimics the ability of dietary restriction (DR) to delay age-related diseases in laboratory animals is greatly sought after. We and others have shown that resveratrol mimics effects of DR in lower organisms. In mice, we find that resveratrol induces gene expression patterns in multiple tissues that parallel those induced by DR and every-other-day feeding. Moreover, resveratrol-fed elderly mice show a marked reduction in signs of aging, including reduced albuminuria, decreased inflammation, and apoptosis in the vascular endothelium, increased aortic elasticity, greater motor coordination, reduced cataract formation, and preserved bone mineral density. However, mice fed a standard diet did not live longer when treated with resveratrol beginning at 12 months of age. Our findings indicate that resveratrol treatment has a range of beneficial effects in mice but does not increase the longevity of ad libitum-fed animals when started midlife.

INTRODUCTION

In developed countries, much of the population now survives to the point where chronic age-associated diseases such as cardiovascular disease, cancer, diabetes, sarcopenia, osteoporosis, stroke, and kidney disease are major determinants of morbidity and mortality (Crews, 2005). Numerous studies have shown that dietary restriction (DR) alleviates many of these conditions in mammals. Reduction of caloric intake to 30%–50% below ad libitum levels, or every-other-day feeding (EOD) of a nutritious diet, can delay the onset of age-related diseases, improve stress resistance, and decelerate functional decline (Barger et al., 2003; Goodrick et al., 1982; McCay et al., 1935). Although DR has beneficial effects in humans (Heilbronn et al.,

2006), such a diet is unlikely to be widely adopted and would pose a significant risk to the frail, critically ill, or the elderly. As such, we have focused on the development of ''DR mimetic'' compounds that provide some of the benefits of DR without a reduction in caloric intake (Ingram et al., 2004). Strategies that have been proposed include inhibition of glycolysis (2-deoxyglucose) (Lane et al., 1998), enhancing insulin action (glucophage/metformin) (Dhahbi et al., 2005), and small molecule activators of SIRT1 (e.g., 3,5,4′-trihydroxystilbene/resveratrol) (Howitz et al., 2003).

Sirtuins are a family of NAD^+-dependent deacetylases and ADP ribosyltransferases that are homologous to the *Saccharomyces cerevisiae* Sir2 protein. Extra copies of *SIR2* or its homologs extend life span in yeast (Kaeberlein et al., 1999), worms (Tissenbaum and Guarente, 2001), and flies (Rogina and Helfand, 2004), and are proposed to underlie some of the physiological effects of DR in simple organisms (Anderson et al., 2003; Lin et al., 2000) and in mammals (Boily et al., 2008; Bordone et al., 2007; Chen et al., 2005). To study sirtuins in mammals, we employed resveratrol, a small polyphenol identified in an in vitro screen for SIRT1 activators that can extend the life span of *S. cerevisiae* (Howitz et al., 2003; Jarolim et al., 2004), *Caenorhabditis elegans* (Viswanathan et al., 2005; Wood et al., 2004), *Drosophila melanogaster* (Bauer et al., 2004; Wood et al., 2004), and the vertebrate fish *Nothobranchius furzeri* (Valenzano et al., 2006), although one group has failed to detect a significant effect in worms or flies (Bass et al., 2007). In the first three species, life-span extension is dependent on *SIR2*, and this has not yet been tested for *N. furzeri*. In obese mice, resveratrol improves a number of health parameters, including glucose homeostasis, endurance, and survival (Baur et al., 2006; Lagouge et al., 2006; Sun et al., 2007), at least partly due to the increased activity of SIRT1 and AMPK (Baur et al., 2006; Lagouge et al., 2006).

Here, we test the hypothesis that resveratrol imparts health benefits by inducing DR physiology. At 1 year of age, C57BL/6NIA mice were placed on a standard control diet (SD) or DR by every-other-day feeding (EOD) with or without resveratrol. We present evidence that long-term resveratrol treatment slows age-related degeneration and functional decline and mimics the gene expression patterns induced by DR. We discuss the potential implications of these findings for human health.

RESULTS

We previously reported that resveratrol improves the health and survival of obese mice fed a high-calorie diet (Baur et al., 2006). This raised two key questions: (1) Can resveratrol improve the health of nonobese mice, and (2) if so is this due to an ability to mimic the effects of DR? To answer these questions, we examined the effects of resveratrol on mice fed SD ad libitum, subjected to EOD feeding, or fed a high-calorie diet (HC) ad libitum. Initially, each dietary group was divided into no resveratrol (negative control; SD, EOD, or HC), low resveratrol (100 mg/kg of food, SDLR, EODLR, or HCLR), or resveratrol (400 mg/kg of food, SDR, EODR, or HCR). Later, additional groups of mice were given a higher dose of resveratrol along with the standard or HC diets (2400 mg/kg of food, SDHR, or HCHR). The HC plus resveratrol (HCR) group was the subject of a previous

report, and that nomenclature is preserved herein (Baur et al., 2006).

Resveratrol Mimics Transcriptional Effects of DR

One of the most comprehensive ways to assess global changes in physiology is to compare transcriptional changes across major organs and tissues (Spindler, 2006). To test the hypothesis that resveratrol is a mimetic of DR, we compared the transcriptional profiles of resveratrol and EOD feeding in liver, skeletal muscle, adipose, and heart. Z ratios were calculated for each gene as described previously (Cheadle et al., 2003), and false discovery rates were estimated using Rankprod (Hong et al., 2006). A subset of the expression changes was verified by RT-PCR (Figure S1). Most transcriptional changes induced by resveratrol were subtle (fold change < 1.5) and tissue specific. The ten largest changes induced by resveratrol treatment in each tissue are listed in Table S1. In liver Cyp7A1, a rate-limiting enzyme in the conversion of cholesterol to bile acids (Russell and Setchell, 1992) was upregulated, while glucose-6-phosphatase, a rate-limiting enzyme in the production of glucose (Trinh et al., 1998), was repressed. In skeletal muscle and heart, numerous transcripts involved in contractility were altered, while in white adipose tissue (WAT) the most prominently affected transcripts were beta defensins, antimicrobial peptides involved in innate and adaptive immunity (Bowdish et al., 2006). The two changes that were consistent across all four tissues based on the Rankprod analysis were reductions in the expression of the anion exchanger *Slc4a1*, and the interferon-inducible transcript *Ifi27/Isg12*, which could indicate suppression of inflammatory responses. Microarray data on the effects of SIRT1 overexpression in these tissues are not available, making it currently difficult to assess whether SIRT1 is a mediator of these effects. No global correlation with previous studies of SIRT1 overexpression in cultured NIH 3T3 or β cells (Moynihan et al., 2005; Revollo et al., 2004) was observed (data not shown).

To test the hypothesis that resveratrol treatment induces global transcriptional changes that resemble EOD feeding, we performed principal component analysis (PCA) on the microarray data from each tissue. Each principal component (PC) can be roughly considered to represent a set of correlated changes in gene expression and to be independent of every other PC. PCs are ranked based on the contribution each makes to the total variability between samples. When the entire data set is used to generate principal components, the effects of age and diet predominate (data not shown); however, an effect of resveratrol treatment can also be observed that parallels the changes induced by EOD feeding. If the SD, SDR, and EOD groups are used for PCA (eliminating the influence of age and HC diet), the effects of resveratrol and EOD feeding correlate in the first PC (i.e., the set of correlated changes making the greatest contribution to variability between groups) in liver, muscle, and adipose. Restricting the analysis to differentially expressed genes strengthens the correlation considerably (see Figures 1A [liver versus muscle] and 1B [adipose versus heart]).

To comprehensively measure the variability between groups, we calculated pairwise distances in high-dimensional space. The position of each data point (microarray) is specified by the expression levels of many different genes, each represented mathematically as a distance in an independent spatial
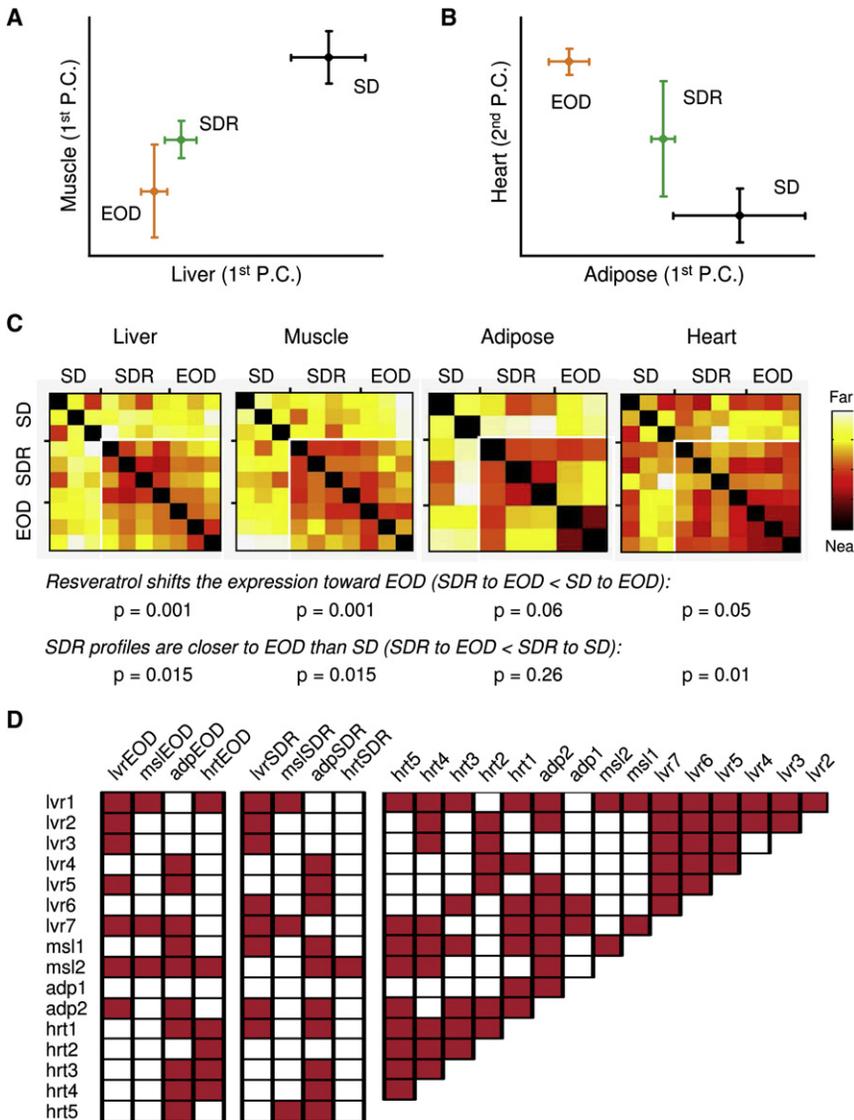
Figure 1. Resveratrol and Dietary Restriction Have Overlapping Effects on Gene Expression

(A and B) Principal component analysis (PCA) was performed on differentially expressed genes from SD, SDR, and EOD for liver, muscle, adipose, and heart. First PCs (the sets of correlated changes that capture the most variability between samples) for liver, muscle, and adipose are presented in (A) and (B). Each shows a shift of the gene-expression pattern in resveratrol-treated animals (SDR) toward that induced by every-other-day feeding (EOD). In heart, the first PC did not show this effect; however, the second PC (shown) was nearly equal and did reveal a similar shift. Error bars are 95% confidence intervals.

(C) Distances were calculated in high-dimensional space for each pair of samples. Each square shows the distance between two individual samples, ranging from zero (deep red; see comparisons to self on the diagonal) to highly divergent (white). In each tissue, distances between SDR and EOD samples (lower-right portion) tend to be smaller than those to SD samples. p values were determined by Kruskal-Wallis (nonparametric) one-way ANOVA and are similar but slightly more conservative than those obtained by standard ANOVA.

(D) To compare our transcriptional profiles of EOD and resveratrol treatment to published data on 10%–44% caloric restriction (Corton et al., 2004; Dhahbi et al., 2005, 2006; Edwards et al., 2007; Fu et al., 2006; Higami et al., 2004; Lee et al., 2002; Selman et al., 2006; Tsuchiya et al., 2004), we generated differential expression signatures (Swindell, 2008). For each pair of transcriptional profiles, the significance of the overlap in expression changes was evaluated by permutation testing and adjusted for multiple comparisons using the Benjamini-Hochberg Method (Benjamini and Hochberg, 1995). Publicly available data sets are assigned a name based on the tissue examined and described in detail in Table S2. Shaded boxes indicate significant associations (p < 0.05).

dimension; a straight line connecting any two points can always be drawn, and its length is a reflection of the similarity between the two microarrays in question. Such calculations were performed for each pair of microarrays in each tissue, and the results are presented in Figure 1C, with deep red representing a high degree of similarity, and white representing divergent samples. These calculations also allow a statistical evaluation of the hypothesis that resveratrol treatment shifted the overall pattern of gene expression toward that induced by EOD feeding. This conclusion could be reached based on spatial distances for liver, muscle, and heart. The statistical power in adipose tissue was limited by the number of samples, although the same trend was apparent. Thus, the transcriptional effects of resveratrol and EOD feeding show significant overlap in multiple tissues.

Although EOD feeding and conventional caloric restriction (reducing daily energy intake by ∼40%, "CR") share key features, including extending mean and maximum life span, preventing age-related disease, and improving insulin sensitivity, they have not been shown to work through a common mechanism. There-fore, we were interested in comparing our transcriptional profiles of EOD and resveratrol treatment to previously reported effects of CR. Differences in age, strain, gender, duration of treatment, array platform, and tissue compound this analysis. Nonetheless, by testing the overlap based on differential expression signatures (Swindell, 2008), we detected significant associations between either EOD feeding or resveratrol treatment and previous studies of caloric restriction (Figure 1D). In the case of liver, the effects of EOD and resveratrol treatment overlapped significantly with those of CR for 5 of 7 published studies.

We next used parametric analysis of gene-set enrichment (PAGE) to highlight specific functional pathways within the microarray data. Gene sets were obtained from the Molecular Signatures Database (MSigDB, C2 collection) and analyzed as described previously (Subramanian et al., 2005). Pathways that were significantly altered by EOD feeding or resveratrol treatment are presented in Figure 2A. The effects of the two treatments were correlated (by direction of change) in 82% (liver), 76% (muscle), 96% (adipose), and 64% (heart) of the affected
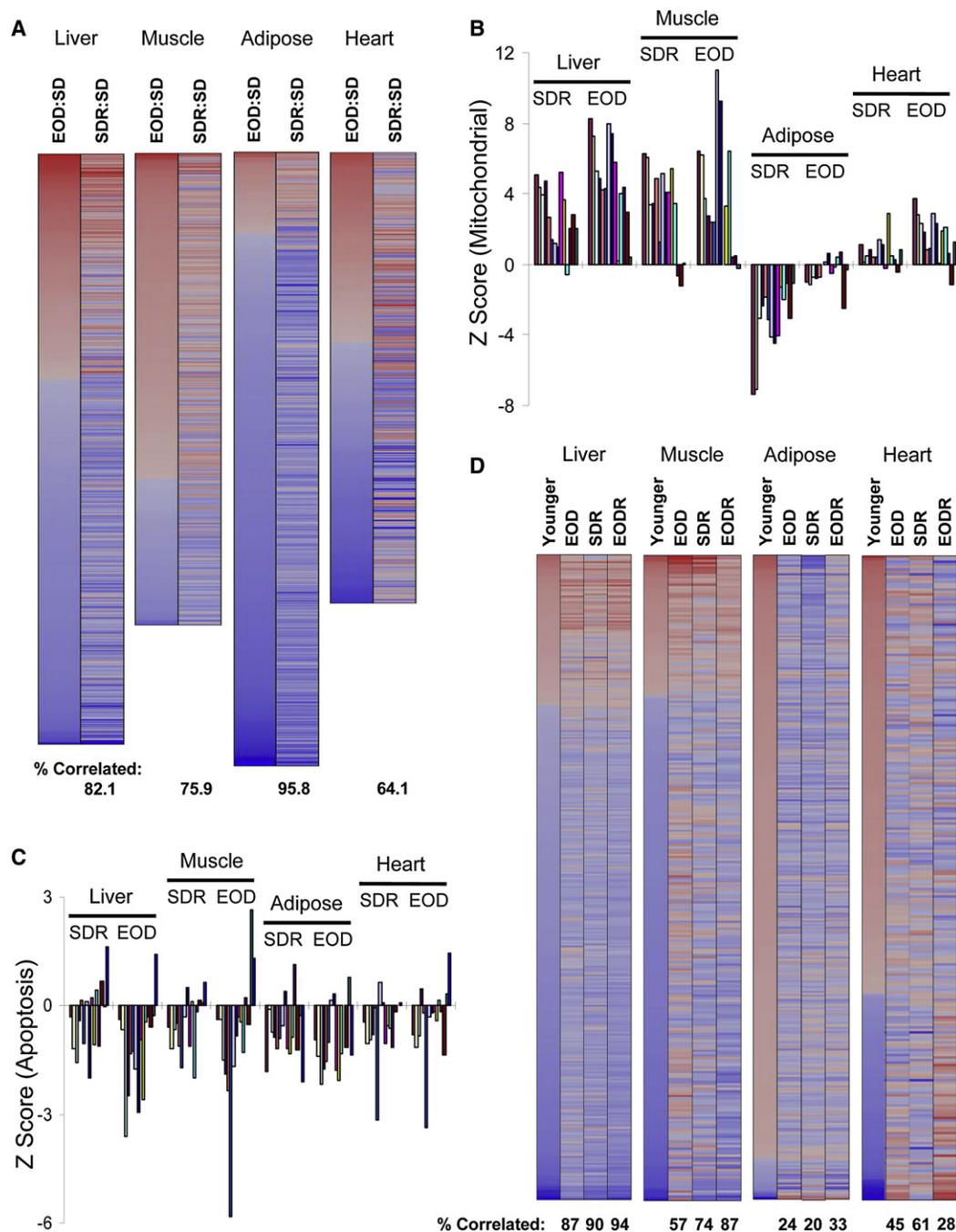
Figure 2. Resveratrol Shifts Expression Patterns in Mice on a Standard Diet toward Those of Mice on a Calorie-Restricted Diet

(A) Parametric analysis of gene-set enrichment (PAGE) was performed on microarray data from mice fed a standard diet plus resveratrol (SDR) or subjected to EOD feeding. Columns show every pathway significantly upregulated (red) or downregulated (blue) by either treatment at 27 months of age. The directions of changes induced by resveratrol and EOD feeding were highly correlated in liver (82% of 503 pathways), muscle (76% of 398 pathways), and adipose (96% of 524 pathways), and weakly correlated (64% of 375 pathways) in heart.

(B) The effect of resveratrol and EOD on mitochondria-related pathways from the PAGE analysis for liver, skeletal muscle, adipose, and heart at 27 months of age.

(C) The effect of resveratrol and EOD feeding on apoptosis-related pathways from the PAGE analysis for liver, skeletal muscle, adipose, and heart at 27 months of age. Full names and Z scores for each of the pathways represented in (B) and (C) are presented in the Supplemental Data.

(D) PAGE analysis was reexamined to identify changes in transcriptional patterns between control (SD) mice at 18 and 27 months of age for liver, muscle, adipose, and heart. For each of the pathways found to be significantly different in the 18-month-old ("younger") mice compared to 27-month-old mice (359, 270, 365, and 256 pathways in liver, muscle, adipose, and heart, respectively), the corresponding Z scores are also presented for EOD, SDR, and EODR mice at 27 months of age (compared to the same 27-month-old controls).

pathways, supporting the idea that resveratrol can mimic many effects of DR in vivo. Among the notable changes were an increase in mitochondrial gene expression in liver and muscle (Figure 2B) and a decrease in apoptosis across the four tissues (Figure 2C). Full names and Z scores for the gene sets in Figures 2 and S2 are presented in Table S5.

We also sought to identify changes in gene-expression patterns that occur during normal aging and to test whether resveratrol treatment or DR resulted in more youthful gene-expression patterns in the elderly mice. Based on a comparison between SD fed mice at 18 and 27 months of age, gene-expression patterns induced by either resveratrol treatment or EOD feeding in liver resembled patterns from mice 9 months younger (Figure 2D), while in muscle this was only true for resveratrol treatment. In adipose both EOD feeding and resveratrol enhanced changes that occurred with aging, while in heart, neither treatment had a significant effect. These results indicate that both resveratrol and EOD feeding can slow the transcriptional changes that occur with aging in some but not all tissues.

We previously showed that resveratrol opposes the majority of the transcriptional changes in liver induced by an HC diet. Using newly isolated RNA, a different array platform, and the current version of the MSigDB pathways (1687 pathways versus 522 analyzed previously), we have confirmed this result and extended our analysis to three additional tissues: skeletal muscle, adipose, and heart (Figure S2). These results from additional tissues support the conclusion that resveratrol treatment induces gene-expression profiles that resemble mice on a lower-calorie diet.

Resveratrol Delays Functional Decline

Osteoporosis is a major age-associated disease in humans (Gass and Dawson-Hughes, 2006). Resveratrol increases the osteogenic response of osteoblasts (Su et al., 2007) and bone density in ovariectomized rats (Liu et al., 2005), but the effect of resveratrol on age-induced bone loss in normal mice has not previously been tested. Following their natural deaths, femurs were removed from mice (ages 30–33 months) and analyzed by microcomputed tomography (microCT) and mechanical measurements. In the distal femur, resveratrol significantly improved the tissue mineral density (TMD) in SDLR and SDR compared to SD control bones (Figure 3A), and tended to increase trabecular thickness (p = 0.13). Cortical TMD (Figure 3B) trended higher in resveratrol-treated groups (p = 0.14), and the effect was statistically significant when the two doses were pooled. In addition, resveratrol significantly increased the bone volume to total volume ratio over the entire femur in the SD fed mice (Figure 3C). Bone strength was determined as the load that is endured by a bone prior to failing in the 3 point bend test. Resveratrol caused a trend toward increased maximum load (p = 0.18, Figure 3D); that approached statistical significance when the two doses were pooled (p = 0.058). Overall, resveratrol improved the structure and strength of the femurs tested, suggesting that it may improve bone health.

The development of age-related cataracts involves mismigration of lens epithelial cells and the accumulation of reactive oxygen species (Wolf et al., 2005). A pathologist trained in cataract assessment in aging mice, and blinded to the groups, rated lens opacity in live mice from 0 to 4 by half steps of 0.5, with 4 representing the complete lens opacity of a mature cataract. Consis-

tent with previous studies (Wolf et al., 2000), the extent of cataract formation significantly increased with age in ad libitum-fed mice (Figure 3E). Strikingly, this increase was attenuated by resveratrol treatment, which was more effective than EOD at 30 months of age.

Decreased locomotor function, resulting in the loss of balance and coordination occurs with increasing age in humans and rodents. To test the effect of resveratrol on locomoter function, we measured the time to fall from an accelerating rotarod every 3 months. Rotarod is a task that contains a learning component (Welsh et al., 2005), so it is normal to observe improved performance over time. The SDR group, however, showed a pronounced and statistically significant improvement at 21 and 24 months (Figure 3F), indicating that resveratrol improves balance and motor coordination in aged animals.

Improved Vascular Function

Increased albuminuria is a marker of vascular dysfunction in mice and a clinical marker of overall increased cardiovascular risk in humans (Guzik and Harrison, 2007; Scalia et al., 2007). Urine albumin/creatinine ratios were assessed in the SD, HC, HCLR, and HCR groups at 21 and 26 months of age. The HC mice, but not the HCLR or HCR mice, had significantly increased albumin/creatinine ratios compared to SD controls at both time points (Figure S3A), indicating that resveratrol affords protection against vascular or kidney dysfunction.

Because the original cohort had reached an advanced age and had few members remaining, we assessed vascular function in an additional cohort of mice placed on a diet containing 2400 mg/kg resveratrol at 12 months of age. Total plasma cholesterol was significantly reduced in 22-month-old nonobese mice (SDHR) following 10 months of resveratrol treatment (Figure S3B), while plasma triglycerides showed a slight trend toward a decrease (Figure S3C). Fractionating pooled plasma samples revealed that resveratrol reduced the amount of cholesterol carried in all lipoprotein fractions (Figure S3D). One potential explanation for the decrease in circulating cholesterol is diversion to bile acid synthesis via Cyp7A1, which was highly upregulated in the livers of resveratrol-treated animals (Table S1). However, changes in bile acid pool sizes were not detected (data not shown).

Aortic dysfunction and stiffening occur with increased age in humans (Lakatta and Levy, 2003; Vaitkevicius et al., 1993), and it has been suggested that resveratrol might be protective against these effects (Labinskyy et al., 2006). Therefore, we investigated the effects of resveratrol (2400 mg/kg/food) on vascular function in mice fed a SD or HC diet. Aortas of 3- (SD only) or 18-month-old animals were dissected and tested for responsiveness to the endothelium-dependent vasodilator acetylcholine (ACh). Both age-related and obesity-related functional decline were prevented by resveratrol treatment (Figure 3G). The responsiveness of aortas from SDHR mice was significantly better than that of age-matched controls and comparable to that of younger (SD, 3-month-old) controls. The HC diet significantly worsened responsiveness, and the HCHR mice were protected such that there was no difference between HCHR and age-matched SD controls. The loss of ACh-induced relaxation with aging and obesity was most likely due to increased superoxide
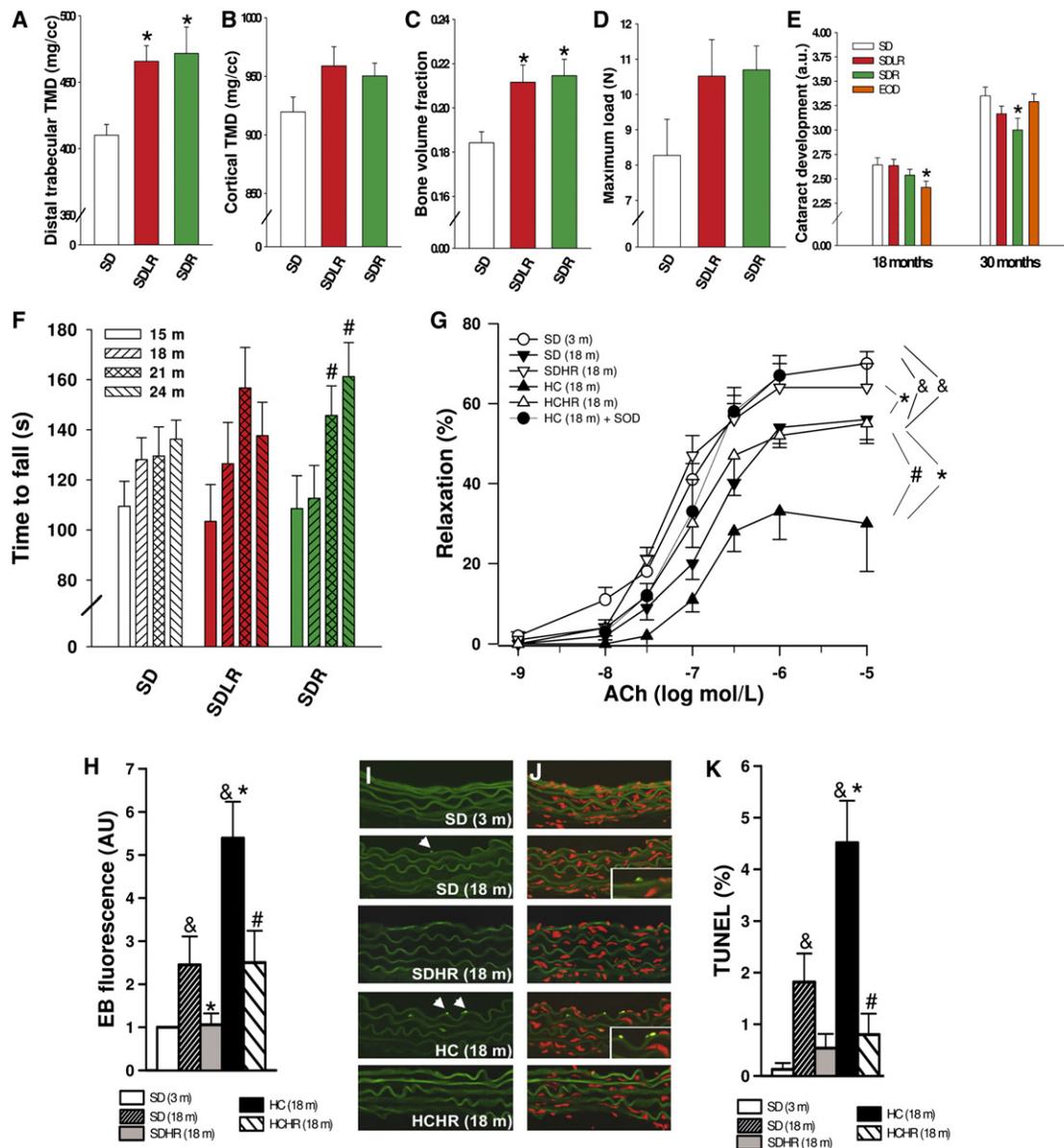
Figure 3. Resveratrol Improves the Health of Mice Fed a Standard Diet

(A) Femurs were removed following natural deaths between the ages of 30–33 months and analyzed by microcomputed tomography (microCT). In (A), distal trabecular tissue mineral density (TMD) was improved in SDLR and SDR compared to SD control bones ($p < 0.05$ for SDLR and $p < 0.01$ SDR versus SD control, *). Error bars indicate SEM.

(B) Cortical TMD tended to increase with resveratrol treatment ($p = 0.14$). Error bars indicate SEM.

(C) Resveratrol significantly increased bone volume to total volume ratio over the entire femur in SD-fed mice ($p < 0.05$ for SDLR and $p < 0.01$ SDR versus SD control, *); n = 5 for all groups in (A), (B), and (C). Error bars indicate SEM.

(D) Bone strength was tested in the postmortem femurs. Resveratrol treatment caused a trend toward increased maximum load (the load that is endured by a bone prior to it failing in the three-point bending to failure test) ($p = 0.18$), n = 5 for SD and SDLR, and n = 4 for SDR. Error bars indicate SEM.

(E) Resveratrol treatment delayed the onset of age-related cataracts. Lens opacity was scored in living mice on a scale from 0 to 4 by half steps of 0.5, with 4 representing the complete lens opacity of a mature cataract. Age-related cataract development was significantly decreased at 30 months of age in SDR mice compared to the SD control, whereas EOD feeding caused an early protective effect that was lost ($p < 0.05$, *). Error bars indicate SEM.

(F) Time to fall from an accelerating rotarod was measured every 3 months for all survivors from a predesignated subset of each group; n = 15 (SD), 11 (SDLR), and 16 (SDR). The SDR group improved significantly at 21 and 24 months versus 15 months ($p < 0.05$, #), showing increased motor coordination over time. Error bars indicate SEM.

(G) Acetylcholine-induced relaxation in aortic ring preparations. Both age-related (SD, 18 months versus SD, 3 months) and obesity-related (HC, 18 months, versus SD, 18 months) declines in endothelial function were prevented by resveratrol treatment. Preincubation with SOD restored ACh-induced relaxation in the HC 18 month rings to youthful levels; n = 6 for each group. (&$p < 0.05$ versus SD [3 months], *$p < 0.05$ versus SD [18 months], and #$p < 0.05$ versus HC [18 months]). Error bars indicate SEM.

production, since preincubation of the HC control vessels with superoxide dismutase restored function (Figure 3G).

To directly measure levels of oxidative stress aortic rings were incubated with dihydroethidine, which reacts with superoxide to generate the fluorescent molecule ethidium bromide (EB). Oxidative stress, as measured by EB fluorescence, increased with age and was attenuated by resveratrol-treatment in aortas from SD-fed mice (Figure S6P). Separately, mean EB fluorescence intensities were quantified in cross-sections of aortas from the SD 3-month-old, SD 18-month-old, HC, and HCHR mice (Figure S6Q). HC diet increased, and resveratrol attenuated, oxidative stress, to the point where HCHR aortas were not different from those of age-matched SD controls. These experiments are summarized in Figure 3H.

Since nitric oxide (NO) is a key mediator of ACh-induced vasorelaxation that is sensitive to oxidative stress, we measured endothelial nitric oxide synthase (eNOS) expression. The resveratrol-treated mice displayed increased levels of eNOS mRNA, suggesting an enhanced capacity for NO production (Figures S6C and S6D), which could potentially help offset inactivation by superoxide. NADPH oxidase is the primary source of $O_2^{\cdot-}$ in vascular tissue, and gp91^{phox}, its catalytic subunit, is upregulated during aging in endothelial cells (Csiszar et al., 2007b). Expression of gp91^{phox} was upregulated in response to aging and HC diet, and in both cases, the effects were reversed by resveratrol treatment (Figures S6A and S6B). Consistent with these data, vascular NADPH oxidase activity was increased in mice fed a HC diet, and this was prevented by resveratrol treatment (Figure S6O). Thus, resveratrol may enhance vasorelaxation by both increasing NO and decreasing $O_2^{\cdot-}$ production.

Apoptotic death of endothelial cells is thought to contribute to vascular pathophysiology in aging and is attenuated by DR (Csiszar et al., 2004). Since our PAGE analysis suggested that resveratrol can suppress apoptosis (Figure 2C), we performed TUNEL staining of aortas from SD 3-month-old, SD 18-month-old, SDHR 18-month-old, HC 18-month-old, and HCHR 18-month-old mice (Figures 3I and 3J). Apoptotic index (TUNEL-positive nuclei as a percentage of total propidium iodide stained endothelial cell nuclei) was increased by aging and further increased by the HC diet, but these increases did not occur in the presence of resveratrol (Figure 3K).

Inflammatory processes contribute to endothelial dysfunction in aging (Csiszar et al., 2007a) and obesity (Roberts et al., 2006) and are suppressed by DR. In agreement with in vitro studies suggesting that resveratrol can reduce the production of inflammatory cytokines and chemokines through inhibition of NF-κB (Mayo et al., 2003), we found that age- and diet-related increases in the expression of the inflammatory markers TNFα,

IL-6, IL-1β, ICAM-1, and iNOS were attenuated by resveratrol treatment (Figures S6E–S6N). Consistent with these observations, we found that a number of pathways containing NF-κB-responsive genes were suppressed in multiple tissues during the PAGE analysis (see Table S5).

Survival

We have reported that resveratrol treatment increased the survival of mice fed a HC diet to 114 weeks of age (Baur et al., 2006). Here, we provide the complete Kaplan-Meier survival analysis (Figures 4B–4D) and maximum life span (final 20% surviving) for the HC groups, as well as mice fed a standard diet or placed on an EOD feeding regimen (Figure 4E). In the context of the HC diet, resveratrol increased remaining life span of 1-year-old mice by an average of 26% for the HCLR group (p = 0.005) and 25% for HCR (p = 0.001) to the point where survival was not significantly different from that of nonobese SD controls. In the lower-dose (HCLR) group, maximum life span was also increased, while this effect did not reach significance for the higher dose (HCR) (Figure 4E). The major factor contributing to life span extension in the resveratrol-treated HC groups was a reduction in the number of deaths attributed to cardiopulmonary distress (specifically, fatty changes in the liver combined with severe congestion and edema in the lungs; Table S3).

Interestingly, the increases in longevity could be completely uncoupled from changes in body weight. While the HCR group had a very slight decrease in body weight that was not statistically significant, the HCLR group displayed a significant increase in body weight, despite consuming a similar amount of food (Figures 4A, S4, and S5). Thus, the beneficial effects of resveratrol on health and life span are not dependent on weight loss.

In the context of the standard diet, resveratrol did not increase overall survival or maximum life span (Figures 4B and 4E). Importantly, the SD control group had a life span similar to that of a much larger cohort of C57BL/6NIA mice (Turturro et al., 1999). EOD feeding produced a trend toward increased longevity compared to the SD control group, but the effect did not reach statistical significance. Our results are consistent with the previous observation that the effect of EOD on longevity is diminished in older C57BL/6 mice (Goodrick et al., 1990), which is also true of DR by 40% restriction (Weindruch and Walford, 1982). Notably, EOD feeding in combination with the lower dose of resveratrol did extend both mean and maximal life span by 15% compared to SD controls (Figures 4C and 4E). We have also tested the effect of a higher dose of resveratrol beginning at 12 months of age (SDHR) on life span, and again found that longevity was not significantly affected. (Figure 4F).

(H) Quantification of total nuclear ethidium bromide fluorescence as a marker of increased oxidative stress; n = 6 aortas for each group. This panel combines data from two experiments shown separately in Figures S6P and S6Q. Both age-related (SD [18 months] versus SD [3 months]) and obesity-related (HC [18 months] versus SD [18 months]) increases in oxidative stress were prevented by resveratrol treatment. (&p < 0.05 versus SD [3 months], *p < 0.05 versus SD [18 months], and #p < 0.05 versus HC [18 months]). Error bars indicate SEM.

(I and J) Representative TUNEL staining of aortas from mice of the indicated ages and diets. Nuclei from apoptotic endothelial cells (intense green) in aortas of SD [18 months] and HC [18 months] mice are highlighted in insets. Autofluorescence of elastic laminae (faint green) and nuclear counterstaining (propidium iodide, red) are shown for orientation purposes.

(K) Apoptotic index (percentage of TUNEL-positive endothelial cell nuclei) was increased in the aortas of obese mice, and this change was prevented by resveratrol treatment; n = 6 aortas for each group; 10–15 images per aorta were analyzed. (&p < 0.05 versus SD [3 months], *p < 0.05 versus SD [18 months], #p < 0.05 versus HC [18 months]). Error bars indicate SEM.
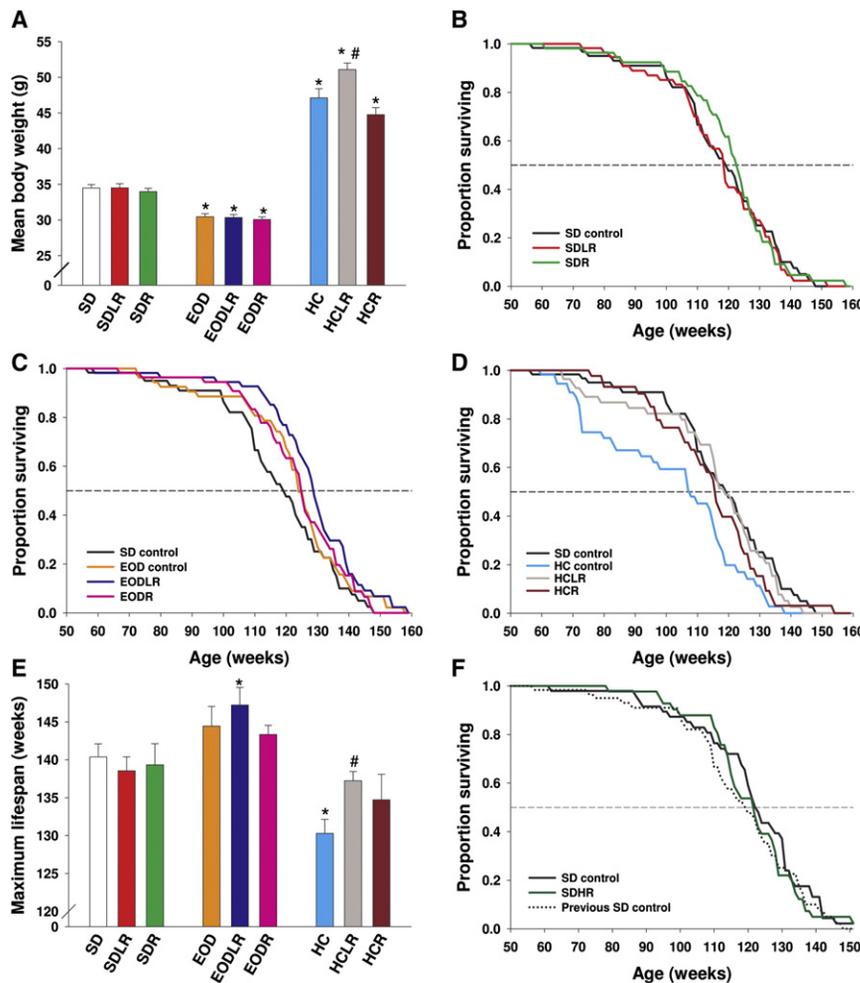
Figure 4. Effects of Resveratrol Treatment on Longevity

(A) Mean body weights over the entire life span. EOD feeding lowered body weight, and HC diet increased it ($p < 0.001$). Resveratrol did not affect body weight in mice fed the SD or EOD diets. The HCLR group was significantly heavier than the HC control group ($p < 0.01$), while the HCR group showed a slight trend toward decreased body weight that did not reach statistical significance. Error bars indicate SEM.

(B) Kaplan-Meier Survival Analyses were performed on the SD, SDLR, and SDR groups, and the curves were not significantly different by the logrank or Wilcoxon tests; n = 60 (SD), 55 (SDLR), and 54 (SDR) at the beginning of the experiment.

(C) Kaplan-Meier Survival Analyses were performed on the SD, EOD, EODLR, and EODR groups. There were no significant differences between the three EOD diet groups; however, the EODLR had increased survival compared to the SD control group as determined by both logrank ($x^2 = 7.46$, $p = 0.006$) and Wilcoxon ($p = 0.0016$) tests; n = 60 (SD) and 55 (EOD, EODLR, and EODR) at the beginning of the experiment.

(D) Kaplan-Meier survival analyses were performed on the SD, HC, HCLR, and HCR groups. The HC control group had decreased survival compared to the SD control group (logrank: $x^2 = 11.65$, $p = 0.0006$, Wilcoxon: $p = 0.0003$), whereas, survival in the HCLR and HCR groups did not differ significantly from that of SD controls. When compared to HC controls, survival was significantly increased in both the HCLR group (logrank: $x^2 = 8.31$, $p = 0.004$, Wilcoxon: $p = 0.005$) and the HCR group (logrank: $x^2 = 4.83$, $p = 0.03$, Wilcoxon: $p = 0.001$); n = 60 (SD) and 55 (HC, HCLR, and HCR) at the beginning of the experiment.

(E) Maximum life span was calculated as the mean of the final 20% of mice in each group as determined by Kaplan-Meier Analysis. Compared to the SD control group, the maximum life span was significantly increased in the EODLR ($p = 0.03$) and significantly decreased in the HC control ($p = 0.003$) groups. In addition, HCLR had significantly increased maximum life span compared to the HC control group ($p = 0.04$), and there was a trend toward increased life span in HCR mice compared to HC controls. *, $p < 0.05$ versus SD control; #, $p < 0.05$ versus HC control. Error bars indicate SEM.

(F) Kaplan-Meier Survival Analyses were performed on the SD and SDHR groups, and the curves were not significantly different. The earlier SD survival curve (broken line) is shown for reference; n = 48 (SD, SDHR), and 60 (previous SD control) at the beginning of the experiment.

Histopathology

Blinded postmortem histopathology for disease or predisease states was performed on visceral organs including the heart, kidneys, liver, spleen, lungs, and pancreas (Table S3). Resveratrol treatment did not significantly alter the distribution of pathologies in SD groups. This included neoplasias, despite the potency of resveratrol against implanted or chemically induced tumors, recently reviewed elsewhere (Baur and Sinclair, 2006). This may be related to the fact that the vast majority of these cases were lymphomas, a tumor type for which the efficacy of resveratrol has not been thoroughly assessed, and that is thought to be triggered mainly by endogenous retroviruses in mice (Kaplan, 1967; Risser et al., 1983).

DISCUSSION

Here we present a long-term evaluation of resveratrol as a DR mimetic in mice. In agreement with a concurrent study (Barger et al., 2008), we show that resveratrol induces changes in the transcriptional profiles of key metabolic tissues that closely resemble those induced by DR. In liver and muscle, these changes can also be correlated to the gene-expression patterns in younger animals, while in adipose tissue, the trend is reversed. Overall, health was improved under all dietary conditions, as reflected by the reduction of osteoporosis, cataracts, vascular dysfunction, and declines in motor coordination; however, longevity was increased only in the context of a HC diet, as reported previously (Baur et al., 2006).

The effects of DR on longevity are diminished when the regimen is initiated at increasing ages (Goodrick et al., 1990; Weindruch and Walford, 1982), although many of the characteristic transcriptional changes can be induced rapidly regardless of age (Cao et al., 2001). Indeed, our EOD feeding regimen was initiated at 12 months of age, and while average life span was increased, the effect did not reach statistical significance, except in combination with resveratrol (EODLR versus SD). Thus, it is

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possible that the age of our animals at the start of the experiment might have diminished the potential effects of resveratrol on lifespan in nonobese mice. To address this question, we are following up with life-span studies in which resveratrol treatment is begun at weaning. It may also be that resveratrol slows the general age-related decline but does not impact the specific causes of death in these mice. Indeed, resveratrol did not suppress lymphoma, a major cause of mortality in C57BL/6 mice.

There is increasing evidence that the antiaging action of DR, at least in part, stems from the attenuation of the age-associated increase in oxidative stress (Sohal and Weindruch, 1996). In particular, DR attenuates oxidative stress in the aortas of aged rodents, and this may contribute to its vasoprotective effects (Guo et al., 2002). Many of the changes we observed in resveratrol-treated animals involved a generalized reduction in oxidative stress and inflammation, consistent with known effects of DR. This was particularly apparent in the aorta, where a decrease in superoxide production was detected directly (Figure 3H), and a variety of transcripts related to inflammatory processes were repressed (Figures S6E–S6N). These changes likely had direct functional consequences, since ACh-induced responsiveness was restored by resveratrol treatment (Figure 3G). In addition, the reduction in cataract formation in resveratrol-treated animals suggests that oxidative stress was reduced in the eye (Figure 3E). One clear difference between EOD and resveratrol was that EOD strongly upregulated glutathione metabolism, whereas resveratrol had no effect. Thus, there may be differences in mechanisms by which EOD and resveratrol reduce oxidative stress. Another difference was that while both increased expression of ribosomal proteins in liver, heart, and adipose, only EOD had this effect in skeletal muscle. Since increased protein synthesis in skeletal muscle has previously been implicated as a major effect of DR (Lee et al., 1999), this result highlights a potentially important difference in the resveratrol-treated animals.

Whether it is possible to find a DR mimetic that is also safe for long-term consumption is of considerable debate in the field. Throughout the experiment, mice were subjected to behavioral tests and examined for signs of distress or disease. Postmortem histopathological assessments were performed on mice from all three dietary regimens (SD, EOD, and HC), with or without resveratrol, and no obvious detrimental change was seen. Although we did not find evidence for detrimental effects of long-term resveratrol treatment at modest doses, we cannot rule out the possibility that resveratrol exerted harmful effects that limited its ability to extend life span. In a small pilot study using 7.5 times our highest dose (18,000 mg/kg resveratrol in the food), five out of six mice died within 3–4 months, consistent with an earlier study of extremely high doses in rats (Crowell et al., 2004).

In conclusion, long-term resveratrol treatment of mice can mimic transcriptional changes induced by dietary restriction and allow them to live healthier, more vigorous lives. In addition to improving insulin sensitivity and increasing survival in HC mice, we show that resveratrol improves cardiovascular function, bone density, and motor coordination, and delays cataracts, even in nonobese rodents. Together, these findings confirm the feasibility of finding an orally available DR mimetic. Since cardiovascular disease is a major cause of age-related morbidity and mortality in humans but not mice, it is possible

that DR mimetics such as resveratrol could have a greater impact on humans. However, resveratrol does not seem to mimic all of the salutary effects of DR in that its introduction into the diet of normal 1-year-old mice did not increase longevity.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male C57BL/6NIA were purchased from the National Institute on Aging Aged Rodent Colony (Harlan Sprague-Dawley, Indianapolis, IN). Beginning at 1 year of age, mice were fed a standard AIN-93G diet (SD and EOD) or AIN-93G modified to provide 60% of calories from fat (HC) plus 0%, 0.01%, or 0.04% resveratrol. Average daily doses over the course of the study (mg/kg/day) were: 7.9 ± 0.2 (SDLR), 30.9 ± 0.6 (SDR), 7.6 ± 0.2 (EODLR), 30.4 ± 0.6 (EODR), 5.4 ± 0.2 (HCLR), 24.2 ± 0.8 (HCR), 204 ± 4 (SDHR), and 167 ± 16 (HCHR, ages 12–18 months). Additional details are provided in the supplemental material.

MicroCT and Bone Strength

Femurs were removed following natural deaths between the ages of 30–33 months, and their architecture was analyzed on an EVS microCT MS-8 system (GE Healthcare, London, ON). Mechanical behavior of the femurs was measured on an ELF 3200 test instrument (Bose Corp, Eden Prairie, MN). Additional details are provided in the Supplemental Data.

Cataracts Assessment

Age-related lens opacity was scored in all living mice by an experienced pathologist using a slit lamp, who was blinded as to the experimental groups.

Rotarod

Time to fall from an accelerating rotarod was measured for all survivors from a predesignated subset of each group; n = 15 (SD), 11 (SDLR), and 16 (SDR). The mice were tested at 15, 18, 21, and 24 months of age. At each time point the mice were given a habituation trial at a constant speed of 4 rpm. The following day, each mouse was given three trials, separated by 30 min rest periods, during which the rotarod accelerated from 4 to 40 rpm over a period of 5 min.

Vessel Isolation and Functional Studies

Endothelial function was assessed as described previously (Csiszar et al., 2007a, 2007b). In brief, aortas (n = 6 per group) were cut into ring segments 1.5 mm in length and mounted in myographs chambers (Danish Myo Technology A/S, Inc., Denmark) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose at 37°C and gassed with 95% air and 5% CO_2). After an equilibration period of 1 hr during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), they were precontracted with 10^{-6} M phenylephrine and relaxation in response to acetylcholine (ACh) over the range of 10^{-9} to 10^{-6} M was measured. In some experiments rings were preincubated for 30 min with 200 U/mL superoxide dismutase to test the involvement of oxidative stress.

Apoptosis Assays

TUNEL assays were performed on aorta sections using the Apop Tag Fluorescein in Situ Apoptosis Detection Kit (Chemicon/Millipore, Billerica, MA), according to manufacturer's instructions, as previously described (Csiszar et al., 2007b). Endothelial cells were identified by their anatomical location (Ungvari et al., 2007).

Microarrays

RNA was extracted from liver, muscle, adipose, and heart at 18 or 27 months of age and hybridized to Illumina Mouseref 8 whole genome microarrays (version 1.1). Raw data were subjected to Z normalization as described previously (Cheadle et al., 2003) and are available at http://www.ncbi.nlm.nih.gov/geo. Gene set enrichment was tested using the PAGE method as previously described (Kim and Volsky, 2005) and pathway definitions from MSigDB (Subramanian et al., 2005). Pathways involving mitochondria and apoptosis

(Figures 2B and 2C) were selected based on the names and descriptions provided by MSigDB while blinded to the data. Differentially expressed genes were converted into differential expression signatures and used to evaluate overlap with previously reported effects of 10%–44% CR as described previously (Swindell, 2008). Characteristics of the animals in 10%–44% restriction studies are presented in Table S2. Additional details, including calculation of principal components and pairwise distances, are provided in the Supplemental Data.

ACCESSION NUMBERS

The GEO accession number reported in this paper is GSE11845.

SUPPLEMENTAL DATA

Supplemental Data include six figures, five tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/8/2/■ ■ ■/DC1/.

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