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Dietary restriction attenuates the accelerated aging phenotype of *Sod1*^{-/-} mice

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Abstract

Dietary restriction is a powerful aging intervention that extends the life span of diverse biological species ranging from yeast to invertebrates to mammals, and it has been argued that the anti-aging action of dietary restriction occurs through reduced oxidative stress/damage. Using *Sod1*^{-/-} mice, which have previously been shown to have increased levels of oxidative stress associated with a shorter life span and a high incidence of neoplasia, we were able to test directly the ability of dietary restriction to reverse an aging phenotype due to increased oxidative stress/damage. We found that dietary restriction increased the life span of *Sod1*^{-/-} mice 30%, returning it to that of wild type, control mice fed *ad libitum*. Oxidative damage in *Sod1*^{-/-} mice was markedly reduced by dietary restriction, as indicated by a reduction in liver and brain F₂-isoprostanes, a marker of lipid peroxidation. Analysis of end of life pathology showed that dietary restriction significantly reduced the overall incidence of pathological lesions in the *Sod1*^{-/-} mice fed the dietary restricted-diet compared to *Sod1*^{-/-} mice fed *ad libitum*, including the incidence of lymphoma (27 vs 5%) and overall liver pathology. In addition to reduced incidence of overall and liver specific pathology, the burden and severity of both neoplastic and non-neoplastic lesions was also significantly reduced in the *Sod1*^{-/-} mice fed the dietary restricted-diet. These data demonstrate

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that dietary restriction can significantly attenuate the accelerated aging phenotype observed in *Sod1*^{-/-} mice that arises from increased oxidative stress/damage.

Keywords

dietary restriction; aging oxidative stress; CuZnSOD

Introduction

Dietary restriction (DR) is a powerful non-genetic, anti-aging intervention that extends life span in a wide range of species from yeast and invertebrates to rodents [1]. Consistent with an anti-aging intervention, DR delays the onset or slows the progression of most age-associated diseases, including neoplasia, degenerative diseases, and immune diseases in rodents [2, 3]. Because DR increases the resistance to stresses, including oxidative stress and attenuates the age-related increase in oxidative damage [4–6], it has been hypothesized that the mechanism by which DR extends lifespan involves protection against the age-associated accumulation of oxidative damage, albeit all the data supporting this hypothesis is correlative. Although the role of oxidative stress in aging has been questioned recently [7–10], many age-associated diseases are closely associated with increased oxidative stress and have been shown to be reduced by DR [11–14].

Copper/zinc superoxide dismutase (Cu/ZnSOD) is a key antioxidant enzyme that detoxifies superoxide anions. Mice null for the *Sod1* gene (*Sod1*^{-/-} mice) have an increase in oxidative stress as measured by elevated levels of oxidative damage in lipid, DNA and protein in tissues and plasma of the mice [15–17]. The *Sod1*^{-/-} mice also show an acceleration in age-related loss of muscle mass and an increase in a number of age-related pathologies, such as hearing loss and increased cataract formation [17–20]. The *Sod1*^{-/-} mice have also been reported to have an enlarged liver as early as 3 months after birth and greater than 70% of *Sod1*^{-/-} mice have liver nodules that develop into neoplasia or hepatocellular carcinoma by 20 months of age [16], which has been correlated to changes in the expression of genes involved in neoplasia [21]. Most important, the *Sod1*^{-/-} mice have been shown to have a significantly shortened lifespan (~30%) compared to wild type control mice [16, 22].

Because the increased oxidative stress/damage arising from the absence of Cu/ZnSOD in the *Sod1*^{-/-} mice has been shown to give rise to an accelerated aging phenotype, we used this mouse model to determine if DR could reverse an aging phenotype that arises largely from oxidative stress. Our data clearly demonstrate that DR can retard aging in *Sod1*^{-/-} mice, e.g., an increased lifespan and reduced age-related pathology (e.g., tumor burden, overall liver pathology, severity of various age-related lesions, and disease burden). The increased lifespan and reduced pathology in the *Sod1*^{-/-} mice fed a DR-diet was correlated with reduced oxidative damage in several tissues of *Sod1*^{-/-} mice.

Materials and methods

Animals

The *Sod1*^{-/-} mice used in this study were generated by Dr. Charles Epstein and Ting-Ting Huang and are genotyped as previously described [16]. Female *Sod1*^{-/-} mice were fed a standard NIH-31 chow (19.1% protein, 5.8% fat, 62.7% carbohydrate) (Teklad Diet LM485; Harlan Teklad, Madison, WI) and were housed at 4 mice/cage under barrier conditions in micro-isolator cages on a 12-h dark/light cycle. For tissue collection, animals were sacrificed in the morning without fasting by CO₂ inhalation followed by cervical dislocation, and the tissues were immediately excised and placed on ice or in liquid nitrogen

depending on the procedures performed later. All tissues following collection were stored at -80°C and analyzed within 30 days. DR was initiated at 2 months of age by feeding the DR mice 60% of diet (by weight) consumed by the mice fed *ad libitum* (AL) as described previously [23, 24]. For the lifespan experiments, the mice were allowed to live out their lifespan, i.e., there was no censoring of mice when measuring survival. Lifespans for mice were determined by recording the ages of spontaneous death of the mice. The mean, median, 10% (the mean lifespan of longest-lived 10% animals), and maximum (the age of death for the longest-lived mouse in the cohort) lifespans were calculated from the survival data for each group.

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the Audie L. Murphy Veterans Hospital.

Measurement of lipid peroxidation

Lipid peroxidation was measured in mouse tissues by the levels of F_2 -isoprostanes as previously described [25]. Mouse tissue (200 mg) was homogenized, and whole lipid was extracted with chloroform-heptane. The levels of F_2 -isoprostanes (esterified) were determined using gas chromatography-mass spectrometry and expressed as ng F_2 -isoprostane per gram of tissue.

Western blot analysis

Mouse tissues were homogenized in ice cold RIPA buffer supplemented with a cocktail of inhibitors for proteases and phosphatases (Roche, Indianapolis, IN). Equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Target proteins were detected with the following specific antibodies against: p21, cyclin D1 and D3, Met (Cell Signaling, Danvers, MA), SOD1 (Assay Designs, Ann Arbor, MI); β -tubulin (Sigma, St. Louis, MO).

Pathological assessment

Upon death, the mice in the survival groups were necropsied for gross pathological lesions as previously described [26–28]. A list of lesions was compiled for each mouse that included both neoplastic and non-neoplastic diseases. Based on these histopathological data, tumor burden, disease burden, and severity of each lesion in each mouse were assessed. The tumor burden was calculated as the sum of the different types of tumors in each mouse. For example, a mouse that had lymphoma and adenocarcinoma had a tumor burden of 2. The disease burden was similarly calculated as the sum of the histopathological changes in a mouse. The severity of neoplastic and renal lesions was assessed using the grading system described below.

The percentage of tumor-bearing mice and overall incidence of disease were calculated for each experimental group. The percentage of tumor-bearing mice was calculated as the percentage of mice that had one or more neoplastic lesions. For this assessment, all neoplastic lesions were counted without regard to severity and thus included both incidental (not severe enough to be the cause of death) as well as those that were fatal (severe enough to be the cause of death).

The severity of neoplastic lesions and glomerulonephritis was determined using grading systems previously described [28]. The severity of these lesions was determined because the high prevalence of lesions allowed for a finer dissection of the effect of the genetic manipulation.

Glomerulonephritis was graded in the order of increasing severity: Grade 0: no lesions; Grade 1: minimal change in glomeruli (minimal glomerulosclerosis); Grade 2: minimal glomerulosclerosis with a few (less than 10) casts in renal tubules; Grade 3: minimal glomerulosclerosis with more than 10 casts in renal tubules; and Grade 4: glomerulosclerosis with interstitial fibrosis.

The determination for the severity of neoplastic lesions was based on previously reported criteria [28], that is the histopathological findings of tumor cell involvement as follows: Grade 1: primary site only; Grade 2: primary site and intra-organ or one other organ metastasis; Grade 3: metastasis to 2–3 organs; and Grade 4: metastasis to more than 4 organs or Grade 3 + additional pathology, e.g., pleural effusion, ascites, and subcutaneous edema, etc. Hydrothorax, ascites, and subcutaneous edema are common complications associated with advanced neoplastic disease.

The probable cause of death was determined independently by the two pathologists based on the severity of the pathology found at necropsy. In cases with neoplastic lesions, mice with Grade 3 or 4 lesions were categorized as death by neoplastic disease.

Two pathologists separately examined all of the samples without knowledge of their genotype or age. In more than 90% of the cases, there was agreement by the two pathologists. In cases where the two pathologists did not agree or where disease did not appear severe enough, the cause of death was categorized as unknown.

Statistical analysis

In most cases, student t-test or one way ANOVA with post ad-hoc test was used to compare the difference between control mice and DR mice. Specific statistical analysis for life span and pathology were described in the figure legends where they were required [29, 30].

Results

DR rescues the life span of *Sod1*^{-/-} mice

In this study, the *Sod1*^{-/-} mice were fed a standard chow either AL or 60% of the food consumed by the mice fed AL (DR group). As shown in Figure 1 and Table 1, *Sod1*^{-/-} mice on the DR-diet had a significantly increased life span. The median life span was increased 30% for the *Sod1*^{-/-} mice on DR (880 vs. 674 days). The maximum lifespan (as measured by the 90th percentile survival) of the *Sod1*^{-/-} mice on DR was also significantly increased (991 vs. 862 days). We also compared the survival data in this study with our data previously collected from female C57BL6 wild type mice fed AL which were on the same diet and under the same housing conditions as the current study [31]. Using this comparison, the life span of the *Sod1*^{-/-} mice on the DR-diet was similar to the life span of wild type control mice fed with standard chow, indicating that the DR diet completely reversed the adverse effect incurred by the loss of the *Sod1* gene. The body weight and tissue weights of the *Sod1*^{-/-} mice fed AL and the DR-diet was determined and shown in the supplemental data composition. DR reduced the body weight significantly in the first three months on DR when compared to mice fed AL, which may be largely due to the significant reduction of lean body mass of *Sod1*^{-/-} mice on DR (supplemental Figures S1 and S2). We also observed a trend of reduction of liver size in DR *Sod1*^{-/-} mice, when compared to *Sod1*^{-/-} mice fed AL, although not reaching statistical significance (supplemental Figure S3). Due to the significant reduction of body weight, the *Sod1*^{-/-} mice on DR had a significantly higher liver to body weight ratio (supplemental Figure S3).

DR reduces the end-of-life pathology in *Sod1*^{-/-} mice

As shown in Table 2, the overall incidence of pathological lesions was significantly lowered in the *Sod1*^{-/-} mice fed DR. The major neoplastic diseases observed in the AL *Sod1*^{-/-} mice were lymphoma, hepatocellular carcinoma (HCC), and hemangioma in the liver and spleen. The neoplastic diseases were usually associated with metastasis to the other organs or other pathological lesions, e.g., pleural effusion, ascites, hemorrhage in pleural and/or abdominal cavities, or severe congestion and edema in the lung. The *Sod1*^{-/-} mice on DR showed a lower incidence of all lesions including neoplastic and non-neoplastic lesions than the *Sod1*^{-/-} mice fed AL (total lesions: 97.6% for AL groups vs. 86% for DR group; neoplastic lesions: 51.2% for AL group vs. 32.6% for DR group; non-neoplastic lesions: 97.6% for AL group vs. 86% for DR group).

We also compared the severity of several neoplastic diseases using the grading system described in Materials and Methods. We found the lymphoma was significantly less severe in the DR *Sod1*^{-/-} mice than AL group (grade score of 0.02 vs. 0.13 for DR and AL group respectively, $p = 0.05$). For other types of neoplasms, we generally found that the DR *Sod1*^{-/-} mice tend to have less severe neoplastic lesions compared to the AL group; however, the differences did not reach statistical significance. When we compared the severity of all types of tumors, the DR *Sod1*^{-/-} mice had less severe neoplastic lesions compared to the AL group (grade score of 0.03 vs. 0.12 for DR and AL group respectively, $p = 0.04$). Neoplastic lesions occurred earlier in AL mice than in DR mice (mean age when neoplastic lesions were identified: 847 vs. 620 days for DR and AL group respectively). Therefore, DR not only reduced the incidence of tumors, but also retarded the progression of tumors in the *Sod1*^{-/-} mice. However, whether DR delayed the initiation of those tumors which occurred later in *Sod1*^{-/-} mice is still not clear.

The disease burden, defined as the total number of histopathological changes in a body, can serve as a good index of age-related accumulation of tissue and cell injury [26, 28]. The data in Figure 2 show a comparison of the disease burden between the *Sod1*^{-/-} mice fed either AL or DR. The DR *Sod1*^{-/-} mice had a significantly lower disease burden than AL mice (3.254 vs. 4.407 lesions per animal for DR and AL *Sod1*^{-/-} mice, respectively). Furthermore, the DR *Sod1*^{-/-} mice showed significantly less tumor burden (defined as number of tumors per mouse) compared to the AL group (0.326 vs. 0.671 tumors per animal for DR and AL group, $p = 0.035$). These data suggest that the DR *Sod1*^{-/-} mice had a slower rate of tissue injury during aging, which possibly enabled them to maintain a better health status, and therefore increased lifespan, compared with AL group. We also found that DR improved liver function in *Sod1*^{-/-} mice indicated by lower plasma levels of alanine amino transferase (ALT) and alkaline phosphatase (ALK) (supplemental Figure S4). These data suggest less liver damage which further supports the reduction of pathology in *Sod1*^{-/-} mice by DR.

DR reduces oxidative damage in *Sod1*^{-/-} mice

Increased oxidative stress and damage has been detected in various tissues of *Sod1*^{-/-} mice, which may play a role in the increase of overall pathology and shortened lifespan of these mice. DR has been shown to increase stress resistance and reduce oxidative damage. Therefore, we determined whether DR could suppress oxidative stress-induced damage in *Sod1*^{-/-} mice by measuring the levels of F₂-isoprostanes, an indicator of lipid oxidation, in brain and liver. As shown in Figure 3A, the F₂-isoprostane levels in liver from mice of young (6–8 months) to middle (18 months) ages are significantly reduced in *Sod1*^{-/-} mice by DR when compared to the mice fed AL. However, the difference in lipid oxidation between DR and AL *Sod1*^{-/-} mice is diminished at advanced ages (24–26 months). A reduction in oxidative damage was also observed in brain tissue from dietary restricted

Sod1^{-/-} mice (Figure 3B) and in skeletal muscle [24], indicating that the reduction of oxidative stress by DR is systemic rather than limited to liver. Because our group has shown that changes of lipid oxidation parallel changes in DNA and protein oxidation in liver tissues from mice during aging and in response to DR [16, 25], we did not further measure protein and DNA oxidation in *Sod1*^{-/-} mice.

DR alters the expression pattern of liver proteins in *Sod1*^{-/-} mice

Deficiency of Cu/ZnSOD has been shown to alter the expression of several genes involved in growth signaling and cell cycle controls, which have been implicated in the progression of liver neoplasia [21, 32]. To study whether DR improves the survival of *Sod1*^{-/-} mice by improving the expression of those genes, we compared the protein expression of several important genes in the liver of *Sod1*^{-/-} mice fed DR and AL diets, and also between *Sod1*^{-/-} mice and wild type control mice.

First, we measured the expression level of p21, a cell cycle regulator under the control of p53, which has been shown to be upregulated in *Sod1*^{-/-} mice [32]. The expression of p21 has also been reported to be upregulated during aging in mouse skeletal muscle which was reduced by DR [33]. As shown in Figure 6A, DR reduced p21 protein level in the liver of wild type control mice at both young and old wild ages. In contrast, DR only significantly reduced p21 expression in the liver of *Sod1*^{-/-} mice at younger ages (6 months of age), and did not further reduce the p21 expression to a statistical significant level at advanced ages (>21 months of age). Interestingly, we found that while the level of p21 protein expression increased significantly in the liver of older wild type mice, it did not change significantly in the liver of *Sod1*^{-/-} mice with age (>21 month vs. 6 month) (Figure 4A). Noticeably, the p21 protein expression in the liver of AL *Sod1*^{-/-} mice was significantly higher than age-matched wild type AL mice at younger ages (6 month), but significantly lower than wild type AL mice at advanced ages.

Next, we examined the expression of hepatocyte growth factor receptor Met, and cell cycle regulators including cyclin D1 and D3 which have all been shown to increase in the liver of *Sod1*^{-/-} mice [16, 21]. As shown in Figure 4B, the protein levels of cyclin D1 are significantly higher in the livers of *Sod1*^{-/-} at 21 months of age. However, neither long term nor short term DR significantly altered the protein level of cyclin D1 in either *Sod1*^{-/-} or wild type mice, suggesting DR has less or no effect on the expression of cyclin D1 in liver which is similar to what has been observed in other studies [34, 35]. The effect of DR on the expression of cyclin D3 was similar to cyclin D1 (data not shown). The expression of Met increased in *Sod1*^{-/-} mice at younger ages (6 month of age, $p < 0.05$) but not in older ages (>21 months of age), comparing to age-matched wild type mice (Figure 4C). DR did not significantly affected the protein level of Met in both wild type and *Sod1*^{-/-} mice ($p > 0.05$ between AL and DR mice).

Discussion

DR has been demonstrated to be a powerful, non-genetic approach that extends the life span of a variety of animal species ranging from invertebrates to mammals [1, 2]. Although studies over the past two decades have focused on identifying the underlying mechanism(s) by which DR extends life span, the mechanism(s) responsible for the anti-aging action of DR is still poorly understood. Currently, one of the most popular hypotheses to explain how DR retards aging is through an increase in stress resistance and attenuation of stress-induced damage [3, 36]. Two approaches have been adopted to test this hypothesis. One has been the testing of whether DR directly affects oxidative damage in wild type animals during aging. This approach has provided evidence for a correlative relationship between DR and reduction of oxidative damage during aging, but with little support for a cause-effect

relationship. The second approach is to determine whether DR can reverse or attenuate a known accelerated aging phenotype arising from increased oxidative stress and damage. The underlying premise of this second approach is that if DR can attenuate or extend the shortened lifespan due to increased oxidative stress/damage by reducing such increases, it will provide direct evidence that DR does extend lifespan through the reduction of oxidative stress/damage. The *Sod1*^{-/-} mice, a model of accelerated aging, apparently is an attractive model for the second approach. The deletion of the *Sod1* gene, which encodes a major enzyme in the detoxification of superoxide, leads to an increased mitochondrial ROS generation, elevated oxidative damage in various tissues, and an accelerated aging phenotype [16, 17]. For example, the *Sod1*^{-/-} mice show a dramatically shortened life span that is associated with accelerated muscle atrophy and an increased incidence of liver cancer and other age-related pathologies. Thus, we hypothesized that if DR were able to attenuate the accelerated aging phenotype in *Sod1*^{-/-} mice, it would provide strong evidence that DR can retard aging through its action on oxidative stress/damage. Our group has reported that DR attenuated skeletal muscle atrophy in *Sod1*^{-/-} mice with reduction of ROS generation and oxidative damage [24]. In the current study, we focused on the overall impact of DR on the lifespan of *Sod1*^{-/-} mice, with respect to oxidative damage and pathology in different tissues.

DR had a dramatic effect on the lifespan of *Sod1*^{-/-} mice

DR extended the medium life span 30%, completely reversing the negative effect of deletion of the *Sod1* gene on life span, i.e., the lifespan of *Sod1*^{-/-} mice on DR was not significantly different from the life span of wild type mice. In addition, DR reduced the incidence of most pathological lesions in the *Sod1*^{-/-} mice, especially neoplastic lesions, which were shown previously to be increased in the *Sod1*^{-/-} mice. DR also significantly improved the liver function in *Sod1*^{-/-} mice, indicated by reduced serum levels of ALT and ALK. This finding is consistent with an earlier report that DR improved liver function in overweight people [37]. We have previously reported that DR attenuates the loss of muscle function and atrophy in these mice and improves mitochondrial function [24]. Thus, DR attenuates the accelerated aging-phenotype observed in *Sod1*^{-/-} mice.

The effects of DR on *Sod1*^{-/-} vs. wild type mice displayed both similarities and differences. DR extended the lifespan of *Sod1*^{-/-} mice, which was associated with a reduction of oxidative damage, overall disease incidence and tumor burden. These changes in oxidative stress and pathology are similar to what has previously been reported in wild type (C57Bl/6) mice in response to DR [23, 27]. However, DR did not extend the lifespan of *Sod1*^{-/-} mice beyond that of wild type mice, e.g., DR increases the lifespan of wild type C57BL/6 mice 20 – 30% [23, 27, 38, 39]. In addition, DR reduced disease burden to a lesser extent in *Sod1*^{-/-} mice compared to wild type mice (35% vs. 60% for *Sod1*^{-/-} and wild type mice, respectively) [27]. This difference may reflect physiological or epigenetic changes that occur in *Sod1*^{-/-} mice during development that cannot be corrected by DR.

One of the major mechanisms by which DR extends lifespan is by the attenuation of tumorigenesis [12, 14]. DR reduces cancer by reducing either tumor incidence or tumor growth rate or both. Spindler et al has previously reported that DR initiated late in life extended mouse lifespan associated with a significant reduction in the rate of tumor growth [40]. It is possible that DR delayed the tumor growth rate in *Sod1*^{-/-} mice, contributing to the longer lifespan relative to *Sod1*^{-/-} *ad libitum* fed mice. However, the reduced tumor burden in *Sod1*^{-/-} mice also suggests that DR reduces tumor incidence in *Sod1*^{-/-} mice. Finally, aside from reducing tumor growth rate and tumor incidence, DR may also delay the onset of tumorigenesis to a later age. A carefully designed cross-sectional study would be required to define the effects of DR on initiation, progression and incidence of tumors in the *Sod1*^{-/-} mice over the lifespan.

Because Cu/ZnSOD is a major antioxidant enzyme, the molecular consequence of deleting Cu/ZnSOD expression is increased oxidative stress/damage, leading to accelerated aging [16, 17]. To determine if the anti-aging phenotype of DR in the *Sod1*^{-/-} mice was correlated to reduced oxidative stress/damage, we measured the levels of oxidative damage (F₂-isoprostanes, a measure of lipid peroxidation) in livers and brains of mice. DR reduced the levels of lipid peroxidation in these tissues of *Sod1*^{-/-} mice, especially before 12 months of age, to levels that were not significantly different from wild type mice. Thus, our study with *Sod1*^{-/-} mice demonstrates that the reduction of oxidative damage by DR strongly correlates with the suppression of an accelerated aging phenotype that arises from increased oxidative stress/damage. Although we did not measure the effect of DR on the level of oxidative damage to proteins and DNA, we believe the effect should be similar as our previous study indicated that the level of lipid oxidation parallels that of protein and DNA oxidation in liver during aging and in response to DR [16, 25].

Because the *Sod1*^{-/-} mice have a null mutation in the *Sod1* gene, DR is not protecting the *Sod1*^{-/-} mice from oxidative stress/damage simply by up regulating Cu/ZnSOD. Based on the previous studies on the effect of DR on the antioxidant defense system, we envision that DR globally enhances multiple pathways leading to increased stress resistance in the *Sod1*^{-/-} mice. In a separate study, our group has reported that DR significantly improved the anti-oxidative stress mechanisms in skeletal muscle of *Sod1*^{-/-} mice, including improved MnSOD activity, increased aconitase expression, and reduced ROS production from mitochondria [24]. Therefore, DR may protect *Sod1*^{-/-} mice partially by reducing oxidative stress induced damage through these pathways.

DR has profound effect on the expression of genes involved in cellular function, metabolism and stress response, including those involved in cell cycle and tumorigenesis [33, 41, 42]. Edwards et al reported that p53-mediated transcriptional program is elevated with age and is reduced by DR in rodent models [33]. As reported previously by our group, p53 mediated gene expression is elevated earlier in life in *Sod1*^{-/-} mice [32], which correlates with the increase of oxidative stress and accelerated aging phenotype of *Sod1*^{-/-} mice. In this study, we observed a significant increase of p21 expression, a gene involved in p53 mediated responses, in the liver of *Sod1*^{-/-} mice at young ages when compared to age-matched wild type mice. While the expression of p21 increased with age in wild type mice, it did not increase significantly in *Sod1*^{-/-} mice. DR reduced p21 expression in wild type mice at all ages, but only had significant effect on p21 expression at younger *Sod1*^{-/-} mice. Why the expression of p21 and its response to DR with age differs between *Sod1*^{-/-} mice and wild type mice remains to be determined. The fact that DR did not significantly reduce p21 expression in the liver of old *Sod1*^{-/-} mice correlates with the less reduction of F₂-isoprostanes in the liver of old *Sod1*^{-/-} mice by DR (Figure 3). It is interesting that the level of p21 in the liver of older *Sod1*^{-/-} mice is lower than age-matched wild type mice, although there is more oxidative stress in older *Sod1*^{-/-} mice which is supposed to increase p53/p21 response. This may be due to the possibility that the loss of *Sod1* gene leads to molecular changes that alter the response of p21 during aging. It has been reported that *Sod1*^{-/-} mice have profound molecular changes in different tissues, especially in the liver, which may affect the expression of p21 during aging in a manner different from that of wild type mice [32, 43]. It may be also due to the fact that with age the liver of *Sod1*^{-/-} mice become more susceptible to neoplastic transformation due to persistent oxidative damage, leading to a regenerative phenotype as demonstrated in the study by Busuttil et al [43]. Liver transformation may require the down regulation of p21 to escape cell cycle arrest [44]. Our data (Figure S3) further support this notion in that old *Sod1*^{-/-} mice had significant increase in liver size, comparing to age-matched wild type mice.

DR has also been reported to improve liver function in obese people [37]. Our study also found that DR improved liver function of *Sod1^{-/-}* mice, indicated by reduction of ALT and ALP in the plasma. In the current study, we also investigated the effect of DR on the expression of several genes which have been reported previously to play a positive role in liver pathology of *Sod1^{-/-}* mice [21]. Generally, our data did not found a significant effect of DR on cyclin D1, cyclin D3 and Met in the liver of *Sod1^{-/-}* mice as well as wild type mice, suggesting that DR has little or no effect on these genes. Nonetheless, our study using a genetic mouse model provides further support for the beneficial effect of DR on liver health.

In summary, our study with *Sod1^{-/-}* mice provides the first direct evidence that the anti-aging action of DR can occur through a mechanism that involves reduced oxidative stress/damage. However, one must be careful in extrapolating our data in *Sod1^{-/-}* mice, which show an accelerated aging phenotype with elevated oxidative stress/damage, to normal animals with aging that may occur through mechanisms other than oxidative damage/stress. In other words, in animal models without elevated oxidative stress/damage, DR may alter other pathways than oxidative stress/damage that might have an anti-aging phenotype in these animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Life span of female *Sod1*^{-/-} mice. Survival curves are shown for female *Sod1*^{-/-} mice fed AL (43 mice, open triangle) or a DR-diet (44 mice, solid triangle). The survival curve shown for wild type mice fed AL (65 mice, open square) is derived from the data collected from a previous survival study for female C57BL6 mice conducted by our laboratory [45]. The curve of wild type C57BL6 male mice fed DR was a summary of the data collected in our institute which have been published or unpublished [23, 27]. The curve was superimposed onto the figure.

Figure 2.

Tumor and disease burden of *Sod1*^{-/-} mice. Tumor or disease burden was expressed as the average number of distinct tumors or lesions observed in a single animal as described by Ikeno et al [26, 28]. *p<0.05 indicates statistical significance between *Sod1*^{-/-} mice fed AL (grey bar) and DR diet (solid bar).

Figure 3.

Effect of DR on lipid peroxidation (n =6). The levels of F₂-isoprostan (ng per gram of tissue) in the liver and brain from *Sod1*^{-/-} mice fed AL (grey bars) or a DR-diet (solid bar) and wild type mice fed AL (open bars) is shown. Each value represents the mean and SEM for 6 animals per group. *p<0.05 indicates statistical significance between *Sod1*^{-/-} fed AL and wild type mice; #p<0.05 between *Sod1*^{-/-} mice on DR and fed AL; &p<0.05 between *Sod1*^{-/-} on DR and wild type mice fed AL.

Figure 4.

Effect of DR on gene expression in the liver (n = 4). (A) Expression of p21 protein. Statistical significance ($p < 0.05$): a = between AL and DR group; b = between *Sod1^{-/-}* mice AL or DR and wild type AL group at >21 month; c = between *Sod1^{-/-}* and wild type AL mice at 6 month; d = between AL wild type mice at 6 month and >21 month. (B) Expression of cyclin D1. * $p < 0.05$ denotes statistical significance between *Sod1^{-/-}* and wild type mice. (C) Expression of Met. Protein levels were determined by Western blot analysis (inlet picture) and quantified by normalizing to the levels of β -tubulin.

Table 1

Lifespan Characteristics of female wild type fed AL, *Sod1*^{-/-} mice fed AL, and *Sod1*^{-/-} mice fed a DR-diet.

Group (all female)	WT AL (n=65)	<i>Sod1</i> ^{-/-} AL (n=41)	<i>Sod1</i> ^{-/-} DR (n=44)	p value (With Holm Correction)
Median, days (lower and upper 95% confidence bounds)	913 (855,943)	674 (613,733)	880 (850,919)	WT AL vs <i>Sod1</i> ^{-/-} AL: $< 1 \times 10^{-16}$ WT AL vs. <i>Sod1</i> ^{-/-} DR: 0.21 <i>Sod1</i> ^{-/-} AL vs. <i>Sod1</i> ^{-/-} DR: 0.0001
Mean, days $\pm$ standard deviation	870 $\pm$ 182	654 $\pm$ 191	819 $\pm$ 217	
90%, days (lower and upper 95% confidence bounds)	1054 (1016,1189)	862 (856,918)	991 (971,1029)	WT AL vs. <i>Sod1</i> ^{-/-} AL: 2.07×10^{-2} WT AL vs. <i>Sod1</i> ^{-/-} DR: 0.21 <i>Sod1</i> ^{-/-} AL vs. <i>Sod1</i> ^{-/-} DR: 0.21
Maximum, days	1189	918	1029	
Log-Rank				WT AL vs. <i>Sod1</i>^{-/-} AL: 6.66×10^{-16} WT AL vs. <i>Sod1</i> ^{-/-} DR: 0.04 <i>Sod1</i> ^{-/-} AL vs. <i>Sod1</i> ^{-/-} DR: 0.05

The data for the *Sod1*^{-/-} mice was obtained from the survival data described in Figure 1 and the data for the wild type (WT) mice fed AL was obtained from a previous lifespan study [31]. For medians and 90th percentiles the parenthesized values are the lower and upper bounds of the 95% confidence interval. The means are shown with $\pm$ SD. The medians and 90th percentiles were compared using censored quantile regression described by Fitzenberger and Winker and implemented by Koenker [29]. These comparisons were done using custom scripts written in the R statistical language [30].

Table 2

Age-associated pathology of *Sod1*^{-/-} mice fed AL or a DR-diet.

Type of Lesions	AL			DR			Statistics (p value)
	N	Positive		n	Positive		
		#	%		#	%	
All lesions							
Total	41	40	97.6	43	37	86.0	0.004
Liver	41	25	61.0	37	15	40.5	0.029
Neoplastic Lesions							
Total	41	21	51.2	43	14	32.6	0.077
Lymphoma							
Total	41	11	26.8	43	2	4.7	0.004
Liver							
Total	41	13	31.7	37	12	32.4	ns
HCC ^a	41	12	29.3	37	11	29.7	ns
HG ^b	41	3	7.3	37	5	13.5	ns
HA ^c	41	2	4.9	37	4	10.8	ns
Non neoplastic							
Total	41	40	97.6	43	37	86.0	0.004
Liver	41	18	43.9	37	4	10.8	0.007
Kidney	41	35	85.4	40	27	67.5	ns
Lung	36	18	50.0	37	25	67.6	ns

^a hepatocellular carcinoma;

^b hemangioma;

^c hepatocellular adenoma.