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Liver specific expression of Cu/ZnSOD extends the lifespan of *Sod1* null mice

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Abstract

Genetic ablation of CuZn-superoxide dismutase (*Sod1*) in mice (*Sod1*^{-/-} mice) leads to shortened lifespan with a dramatic increase in hepatocellular carcinoma and accelerated aging phenotypes, including early onset sarcopenia. To study the tissue specific effects of oxidative stress in the *Sod1*^{-/-} mice, we generated mice that only express the human *SOD1* gene specifically in the liver of *Sod1*^{-/-} mice (*Sod1*^{-/-}/*hSOD1*^{alb} mice). Expression of hSOD1 in the liver of *Sod1*^{-/-} mice improved liver function, reduced oxidative damage in liver, and partially restored the expression of several genes involved in tumorigenesis, which are abnormally expressed in the livers of the *Sod1*^{-/-} mice. However, liver specific expression of hSOD1 did not prevent the loss of body weight and muscle mass and alterations in the structure of neuromuscular junctions. The expression of hSOD1 in the liver of *Sod1*^{-/-} mice significantly improved the lifespan of *Sod1*^{-/-} mice; however, the lifespan of the *Sod1*^{-/-}/*hSOD1*^{alb} mice was still significantly shorter than wild type mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mad.2016.01.005>.

Keywords

CuZnSOD; Oxidative stress; Lifespan; Liver-specific transgenic mice

1. Introduction

Oxidative stress induced by reactive oxygen species (ROS) is implicated in a variety of pathological conditions and diseases including ischemia–reperfusion injury, degenerative diseases, diabetes, and cancer (Barnham et al., 2004). It is also the focal point of the free radical theory of aging proposed by Harman (Harman, 1956). ROS are generated in aerobic organisms by several processes and several defense mechanisms exist to minimize the damage incurred by high levels of ROS. One of the major antioxidant enzymes is CuZn-superoxide dismutase (CuZnSOD), which is found primarily in the cytosol; however, small amounts of CuZnSOD are also found in inter-membrane space of mitochondria.

Mice with a null mutation in the *Sod1* gene have been generated and characterized (Reaume et al., 1996; Huang et al., 1997). The *Sod1*^{−/−} mice have significantly shortened lifespan (30% reduction) compared to wild type mice and many aging phenotypes are accelerated, *e.g.*, early onset of muscle atrophy, loss of hearing and hair, and thinning of skin, cataract formation (Elchuri et al., 2005; Muller et al., 2006; Ohlemiller et al., 1999; McFadden et al., 1999a; Keithley et al., 2005). However, the *Sod1*^{−/−} mice also developed enlarged liver at as early as 3 months of age with many of the mice developing hepatocellular carcinoma (HCC) (Elchuri et al., 2005). The development of HCC is correlated with an increase in DNA mutations and an up-regulation of several oncogenic genes in liver (Elchuri et al., 2005; Elchuri et al., 2007; Han et al., 2008).

The mechanism(s) by which the deletion of Cu/ZnSOD shortens lifespan and leads to accelerated aging phenotypes is poorly understood. Because Elchuri et al. (2005) initially reported that 70–80% of the *Sod1*^{−/−} mice have hepatocellular carcinoma when they die while hepatocellular carcinoma is rarely observed in the strain of wild type mice studied, it has been argued that the shortened lifespan of the *Sod1*^{−/−} mice may be related to liver cancer and as opposed to acceleration in aging arising from the increase in ROS and oxidative stress. However, our group recently showed that only 30% of the *Sod1*^{−/−} mice in our aging colony developed hepatocellular carcinoma even though the survival of all of the *Sod1*^{−/−} mice was reduced 30% compared to wild type mice. In addition, the *Sod1*^{−/−} mice still show an early onset of many phenotypes found in old mice (Zhang et al., 2013a). To gain a better insight into the effect of the null *Sod1* mutation on aging, we have generated mice that express CuZnSOD selectively in liver by crossing liver specific *hSOD1* transgenic mice (*hSOD1*^{alb}) to *Sod1*^{−/−} mice producing *Sod1*^{−/−}/*hSOD1*^{alb} mice.

2. Materials and methods

2.1. Generation of transgenic mice

The cDNA of human *SOD1* gene was subcloned from a *hSOD-1* construct (kindly provided by Dr. Ting Ting Huang from Stanford University) into the plasmid pBSAlb/aFet (kindly

provided by Dr. Guntrar Shut). The transgenic mice expressing liver specific *hSOD1* gene (*hSOD1^{alb}*) were generated by the Transgenic Animal Core facility at the University of Michigan. Transgenic mice were genotyped using isolated tail DNA by polymerase chain reaction (PCR) with the following specific primers: 5'-ATGAAATGCGAGGTAAGTATGG-3' and 5'-ACATTGCCCAAGTCTCCAAC-3'. Genomic DNA was extracted from mouse tail with one-step lysis buffer (Viagen Biotech, Inc., TX) plus protease K at 55°C overnight followed by inactivation at 85°C for 1 hour. The amplification conditions are: 1 cycle of 95°C 5 minutes, 35 cycles of 95°C for 30 seconds + 54°C for 30 seconds + 72°C for 45 seconds, 1 cycle of 72°C for 5 minutes.

The *hSOD1^{alb}* mice were crossed to *Sod1^{-/-}* mice generating *Sod1^{+/-}/hSOD1^{alb}* mice, which were subsequently bred to each other to obtain the following three genotypes used in this study: wild type, *Sod1^{-/-}*, and *Sod1^{-/-}/hSOD1^{alb}* mice. Genotyping of these mice were performed using two set of primers for PCR amplification: one for *hSOD1* transgene as described above, the other for the knockout of mouse *Sod1* as described previously (Huang et al., 1997).

2.2. Animals and lifespan study

All mice were fed *ad libitum* a standard NIH-31 chow and maintained in micro-isolator cages on a 12-h dark/light cycle. For tissue collection, animals were sacrificed by CO₂ inhalation followed by cervical dislocation, and the tissues were immediately excised and placed on ice or liquid nitrogen. All procedures involving the mice were approved by the Subcommittee for Animal Studies at the Audie L. Murphy VA Medical Center and the University of Texas Health Science Center at San Antonio.

For the lifespan experiments, wild type, *Sod1^{-/-}*, *Sod1^{-/-}/hSOD1^{alb}* mice were housed four animals per cage starting at 2 months of age. Mice were assigned to survival groups at 4–8 months of age and 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 age of spontaneous death. 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 genotype.

2.3. Western blot analysis of protein expression

Tissues were homogenized with ice-cold RIPA buffer (50 mM Tris pH7.6, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, and 0.5% sodium deoxycholate) supplemented with cocktails of protease and phosphatase inhibitors (Roche Life Science). Protein concentration was determined with Pierce™ BCA Protein Assay kit. Equal amount of total protein was separated on SDS-PAGE gel and transferred onto nitrocellulose membrane. The following specific antibodies were used to detect the target proteins: cyclin D1, Met (hepatocyte growth factor), Stat3 (signal transducer and activator of transcription 3), pStat3, MIF (macrophage migration inhibitory factor) (Cell Signaling), β-tubulin (Sigma-Aldrich, MO), and CuZnSOD (Stressgen Biotechnologies Corporation, Canada).

2.4. CuZnSOD activity assay

The enzymatic activity of CuZnSOD was measured by native gel analysis as previously described (Van Remmen et al., 1999). Briefly, liver tissues were homogenized in 25 mM Tris buffer with 0.1% Triton-X 100. After centrifugation at $10,000 \times g$ for 10 min at 4°C, protein of the supernatant was quantified using the BCA method following standard protocol. Equal amount of protein was separated on native gel in cold room and CuZnSOD activity was determined by the inhibition of the xanthine plus xanthine oxidase mediated-reduction of cytochrome c.

2.5. Isolation of Skeletal Muscle Mitochondria and measures of mitochondria function

Mitochondria from skeletal muscle were isolated as previously described by Muller et al. (Muller et al., 2007). Briefly, the muscles were excised and incubated with 3 mg of nagarse/g tissue for 5 minutes in Chappell–Perry buffer (100 mM KCl, 50 mM Tris–HCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM ATP). The muscles were homogenized in pre-chilled Chappell–Perry buffer using a Potter–Elvehjem glass homogenizer with a teflon pestle. The homogenate was diluted with Chappell–Perry buffer and centrifuged at $600 \times g$ for 10 min. The resulting supernatant was centrifuged at $14,000 \times g$ for 10 minutes. The mitochondrial pellets were suspended in modified Chappell–Perry buffer (100 mM KCl, 50 mM Tris (pH 7.5), 1 mM MgCl₂, 0.2 mM EDTA, and 0.2 mM ATP), and were centrifuged at $7000 \times g$ for 10 min. Mitochondrial pellets were then resuspended in a half volume of modified Chappell–Perry buffer and centrifuged at $3500 \times g$ for 10 minutes.

The assay of mitochondrial generated ROS is based on the detection of H₂O₂ in the medium using the Amplex Red fluorescent dye (Mohanty et al., 1997). The Amplex Red reagent reacts with H₂O₂ with a 1:1 stoichiometry producing highly fluorescent resorufin, in the presence of horseradish peroxidase. Briefly, Amplex Red reagent (1 μM) and horseradish peroxidase (5 U/2 ml) were added as previously described by Muller et al. (Muller et al., 2007). All the assays were performed at 37°C and fluorescence was followed at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a Fluoroskan Ascent Type 374 multiwell plate reader. The slope of the increase in fluorescence was converted to the rate of H₂O₂ production with the use of a H₂O₂ standard curve.

Mitochondrial respiration was measured by oxygen consumption using a Clark electrode system (Hansatech Instruments Ltd., Norfolk, UK), as previously described by Jang et al. (2010). The respiratory buffer consisted of 125 mM KCl, 10 mM HEPES, 5 mM MgCl₂, and 2 mM K₂HPO₄, pH 7.44, with 0.3% BSA. Respiration rates were measured using substrates that enter the electron transport chain selectively at the following specific complexes: for complex I, glutamate (1.7 mM) and malate (1.7 mM); for complex II, succinate (2.5 mM) with an NADH dehydrogenase inhibitor (5 mg/ml rotenone); and for complex III, duroquinol (500 mM). State 3 respiration was determined by the addition of ADP (375 nM final concentration) to the above reactions. State 4 respiration was defined as oxygen consumption in the presence of adequate substrate but without added ADP. The respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration rates. The activity of cytochrome oxidase, which represents the activity of complex IV, was measured with a Clark electrode (24) using 500 mg of mitochondrial protein in the following buffer:

50 mM potassium phosphate (pH 7.4), 40 mM cytochrome c, 12.5 mM ascorbate, 0.63 mM N,N,N₉,N₉ tetra-methyl-*p*-phenylenediamine, and 0.03% Triton X-100.

2.6. Measurement of lipid peroxidation

Lipid peroxidation measured as the levels of F₂-isoprostanes was performed as previously described (Ward et al., 2005). The tissues (200 mg) were homogenized, and whole lipid was extracted with chloroform–heptane. The levels of F₂-isoprostanes were determined with gas chromatography–mass spectrometry (GC–MS) as initially described by Morrow and Roberts (1999) and currently used in our laboratory.

2.7. Neuromuscular junction staining

Immunofluorescence images of NMJs were prepared as previously described by Jang et al. (2010, 2012). Briefly, gastrocnemius muscle was fixed for 1 h at room temperature (4% paraformaldehyde in PBS), followed by teasing into bundles of muscle fibers and incubation with 100 mM glycine. After removal of the overlying connective tissue, muscles were permeabilized and blocked for 1 h in PBS containing 2% bovine serum albumin, 4% normal goat serum, and 0.5% Triton X-100. To label axons, acetylcholine receptors (AChR), and muscle fibers, muscles were incubated with rabbit polyclonal antibodies against neurofilament (Chemicon, Temecula, CA) and synaptophysin (Zymed, San Francisco, CA), followed by incubation with fluorescein-conjugated species specific secondary antibodies, Alexa594-conjugated α -Bungarotoxin, and Alexa647-conjugated phalloidin. To quantify the number and density of AChRs, muscles were stained only with Alexa594 α -BBungarotoxin followed by fixation with 1% formaldehyde in PBS and flat-mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a Nikon Eclipse TE2000-U (Melville, NY) or Zeiss (Oberkochen, Germany) LSM 510 confocal microscope.

2.8. Statistical analysis

All data are presented as mean $\pm$ SEM, except the survival data which is explained in the following paragraph. Statistical evaluation between samples or treatments was conducted by one- or two-way ANOVA or student t-test. Difference was considered statistically significant with $p < 0.05$.

The survival data were analyzed with the help of software developed by Therneau (Therneau and Grambsch, 2000; Team, 2012). The Cox proportional hazard model was fitted to the data with the *Sod1*^{−/−} mice as the reference group being compared to the wild type and *Sod1*^{−/−}/*hSOD1*^{alb} mice. This contrast was chosen because it was the most direct way to test the hypothesis that the *Sod1*^{−/−} mice have impaired survival and expressing Cu/ZnSOD in the liver significantly reverses this impairment. Sex was also included as a covariate or a stratifying variable. Restricted means and standard errors were also calculated from Kaplan-Meier curves and are reported. Estimates for the ages of median and 90% mortality were also obtained for each group from the Kaplan-Meier curves where the sample size was sufficient. For the purposes of survival analysis, the following genotypes were grouped as wild type: *Sod1*^{+/+} or *Sod1*^{+/-} mice as well as *Sod1*^{+/+} or *Sod1*^{+/-} mice carrying the *hSOD1*-transgene.

3. Results

3.1. Generation of *Sod1*^{-/-} mice that express hSOD1 specifically in the liver

Liver-specific *hSOD1* transgenic mice were generated as described in the Methods section using the human cDNA for *SOD1* fused to the albumin promoter. As can be seen in Fig. 1A and B, the expression of *hSOD1* gene was detected only in the liver of the transgenic mice as measured by *hSOD1* mRNA and Cu/ZnSOD protein levels. No *hSOD1* expression was detected in skeletal muscle, heart, kidney and brain. The liver specific male *hSOD1* transgenic mice were bred with female *Sod1*^{+/-} mice to generate *Sod1*^{-/-} mice that are null for *Sod1* but express *hSOD1* specifically in the liver, i.e., *Sod1*^{-/-}/*hSOD1*^{alb} mice. As shown in the Western blots in Fig. 1C, *Sod1*^{-/-}/*hSOD1*^{alb} mice expressed the human CuZnSOD in the liver at a level approximately 20% of the level found in wild type mice. The human CuZnSOD protein expressed in the mouse liver was enzymatically active as measured by native gel activity assay (Fig. 1D), and the activity of CuZnSOD was also approximately 20% of the CuZnSOD found in wild type mice.

One of the major phenotypes observed in *Sod1*^{-/-} mice is a dramatic increase in oxidative damage in all tissues (Elchuri et al., 2005; Muller et al., 2006). Therefore, we first determined if re-introducing *hSOD1* into the liver of *Sod1*^{-/-} mice could significantly reduce oxidative damage by measuring the levels of F₂-isoprostanes, which are a marker of lipid oxidation (Roberts and Morrow, 2002). As shown in Fig. 2, the F₂-isoprostane levels were ~2.5-fold higher in the *Sod1*^{-/-} mice compared to the wild type mice, and the levels of F₂-isoprostanes were significantly reduced (40%) in the livers of the *Sod1*^{-/-}/*hSOD1*^{alb} mice. However, the *Sod1*^{-/-}/*hSOD1*^{alb} mice still showed a significantly higher level of oxidative damage than the wild type mice, which is not unexpected because the level of *Sod1* expression in the *Sod1*^{-/-}/*hSOD1*^{alb} mice was only ~20% of wild type. The data in Fig. 2 also show that while the levels of F₂-isoprostanes were significantly increased in brain, heart, and skeletal muscle of the *Sod1*^{-/-} mice, the levels of F₂-isoprostanes were not reduced in these tissues of *Sod1*^{-/-}/*hSOD1*^{alb} mice. The data in Fig. 2 showing that the level of oxidative damage is only reduced in the liver of *Sod1*^{-/-}/*hSOD1*^{alb} mice confirms the tissue specific expression of *Sod1* in the *Sod1*^{-/-}/*hSOD1*^{alb} mice.

3.2. Liver specific expression of hSOD1 reduces liver damage and improves liver function in *Sod1*^{-/-} mice

First, we determined whether liver-specific expression of *hSOD1* improved the physiological parameters of the liver previously reported to be altered in *Sod1*^{-/-} mice, including body weight and liver size (Muller et al., 2006). As shown in Fig. 3A, the liver-specific expression of *hSOD1* in *Sod1*^{-/-} mice did not significantly increase the body mass of *Sod1*^{-/-} mice to that of wild type mice. The supplementary data show that this lower body weight in the *Sod1*^{-/-} and *Sod1*^{-/-}/*hSOD1*^{alb} mice was maintained throughout the lifespan of the mice. However, the liver-specific expression of *hSOD1* attenuated the liver hypertrophy seen in the *Sod1*^{-/-} mice. The liver weight of the *Sod1*^{-/-}/*hSOD1*^{alb} mice was comparable to the wild type mice, which was 30% smaller than that of *Sod1*^{-/-} mice.

Because liver damage or disease can lead to increased plasma free fatty acids and lactic acid (Kalhan et al., 2011; Ritz and Heidland, 1977). we measured the plasma levels of free fatty acids and lactic acid in the livers of the mice (Fig. 3B). Plasma levels of free fatty acids and lactic acid were significantly higher in *Sod1^{-/-}* mice compared to wild type mice but was restored to wild type levels in the *Sod1^{-/-}/hSOD1^{alb}* mice, demonstrating an improvement of liver function in *Sod1^{-/-}/hSOD1^{alb}* mice that is comparable to wild type mice.

Our group has previously shown that skeletal muscle mitochondria isolated from *Sod1^{-/-}* mice show dramatic alterations in mitochondria function, *e.g.*, mitochondria from *Sod1^{-/-}* mice generate more ROS and produced less ATP with reduced membrane potential (Muller et al., 2007; Jang et al., 2010). As shown in Fig. 3C, the function of liver mitochondria isolated from *Sod1^{-/-}/hSOD1^{alb}* mice is comparable to that of wild type mice; no significant differences were observed in ATP production, oxygen consumption, membrane potential, and ROS generation.

To further characterize the liver of the *Sod1^{-/-}/hSOD1* mice, we measured the expression of several genes that have been previously shown altered in the liver of *Sod1^{-/-}* mice that could play a role in promoting tumorigenesis or tumor growth (Elchuri et al., 2005, 2007; Han et al., 2008). We focused on Met and cyclin D1 because they have been associated with liver tumorigenesis/progression (Goyal et al., 2013; Matsuda et al., 2013). As shown in Fig. 4, the levels of cyclin D1 were increased significantly in the liver of adult (8–10 months) *Sod1^{-/-}* mice and the levels of cyclin D1 were significantly reduced in the *Sod1^{-/-}/hSOD1^{alb}* mice to levels that are comparable to wild type mice. The difference in the expression level of cyclin D1 diminished in old animals (20–24 months). The levels of Met on the other hand were similar in the three groups of mice in adult mice; however, Met levels were significantly increased in the old *Sod1^{-/-}* mice compared to the *Sod1^{-/-}/hSOD1^{alb}* or wild type mice. We also measured the phosphorylation of Stat3, an indicator of the activation of Stat3 signaling, which regulates a number of pathways important in tumorigenesis. The phosphorylation of Stat3 was increased significantly in the liver of adult and old *Sod1^{-/-}* mice. The introduction of *hSOD1* attenuated the increase in Stat3 phosphorylation in the liver of *Sod1^{-/-}* mice. Thus, the reduction in liver weight, circulating fatty acids and lactate, and reduction in the expression of several genes upregulated in hepatocarcinoma, demonstrate that overexpressing *hSOD1* in the livers of mice null for *Sod1* is able to rescue the liver dysfunction observed in the *Sod1^{-/-}* mice.

3.3. Effect of liver-specific expression of hSOD1 on skeletal muscle in *Sod1^{-/-}* mice

Because the accelerated age-related loss of muscle mass is one of the most dramatic phenotypes observed in the *Sod1^{-/-}* mice (Muller et al., 2006), we determined whether the liver-specific reintroduction of *hSOD1* would rescue the muscle loss in *Sod1^{-/-}* mice. It has been argued that the accelerated muscle loss may be caused by liver cancer, a condition known as cachexia (Argiles et al., 2014). However, indirect evidence from our group suggested that the sarcopenia phenotype in the *Sod1^{-/-}* mice was not due to cachexia (Muller et al., 2006). As shown in Fig. 5A, the muscle mass of the hind leg of *Sod1^{-/-}/hSOD1^{alb}* mice was significantly smaller than wild type control mice and similar to the *Sod1^{-/-}* mice for the *Sod1^{-/-}/hSOD1^{alb}* mice. The same was true for the mass of specific

muscle types, *e.g.*, quadriceps, gastrocnemius, and tibia anterior (Fig. 5A). Correlated with the failure to prevent muscle loss, liver-specific expression of *hSOD1* did not reduce the level of oxidative damage in skeletal muscle, *e.g.*, the level of lipid oxidation was similar to that found in the muscle of *Sod1^{-/-}* mice (Fig. 2). In addition, we found that the alterations in the function of mitochondria isolated from gastrocnemius muscle of *Sod1^{-/-}/hSOD1^{alb}* mice were similar to that of *Sod1^{-/-}* mice (Fig. 5B), *i.e.* mitochondria produced more ROS, generated less ATP, and consumed less oxygen than age-matched wild type littermates.

Our group has also shown that one of the characteristics of the sarcopenia phenotype observed in the *Sod1^{-/-}* mice is the disaggregation of neuromuscular junctions (Jang et al., 2010). As shown in Fig. 5C, the neuromuscular junctions in *Sod1^{-/-}/hSOD1^{alb}* mice exhibited similar structural abnormalities as those found in the *Sod1^{-/-}* mice, further indicating that the muscle loss in *Sod1^{-/-}* mice is independent of liver dysfunction. These data indicate that the accelerated sarcopenia phenotype observed in *Sod1^{-/-}* mice is largely due to oxidative damage in the muscle and neuronal tissue and is not due to liver dysfunction/cancer.

3.4. Liver specific hSOD1 expression extends the lifespan of *Sod1^{-/-}* mice

Because previous studies have shown that the *Sod1^{-/-}* mice have a significantly shortened lifespan (Elchuri et al., 2005; Zhang et al., 2013a; Jang et al., 2012), we compared the lifespan of the *Sod1^{-/-}/hSOD1^{alb}* mice to *Sod1^{-/-}* and wild type mice to determine the role of liver damage in the shortened lifespan of the *Sod1^{-/-}* mice. Both male and female *Sod1^{-/-}/hSOD1^{alb}* mice lived longer than *Sod1^{-/-}* mice. However, the lifespan was only partially restored by the liver specific expression of Cu/ZnSOD because the *Sod1^{-/-}/hSOD1^{alb}* mice lived significantly shorter than wild type mice. Fig. 6 shows the survival curves and data (Table 1) we obtained from the limited number of mice we studied. Combining data from both sexes, mean survival was lower for the *Sod1^{-/-}* mice than for wild type or *Sod1^{-/-}/hSOD1^{alb}* mice. In females, the age at median and 90% mortality followed the same pattern. In males the sample size of the *Sod1^{-/-}/hSOD1^{alb}* mice was insufficient to obtain valid median and 90% mortality ages; however, the male *Sod1^{-/-}/hSOD1^{alb}* attained median and 90% mortality at earlier ages than the control wild type males. The same pattern as observed with the females. These age differences are strictly qualitative because the sample size was not sufficient to obtain bootstrap confidence intervals that are required for hypothesis testing.

We further analyzed the mortality rate of the three groups of mice using the method developed by Therneau as described in the method section and shown in Table 2. After adjusting for sex, the mortality risk of the wild type mice was 28% that of the *Sod1^{-/-}* mice ($p = 0.0004$) and the mortality risk of the *Sod1^{-/-}/hSOD1^{alb}* mice was 49% that of the *Sod1^{-/-}* mice ($p = 0.0174$). Thus, the mortality risk of *Sod1^{-/-}* mice was 3.7-fold higher than that of wild type mice and 2.3-fold higher than that of *Sod1^{-/-}/hSOD1^{alb}* mice. Analysis of deviance was performed to determine whether it was necessary to also include an interaction term in this model, and it was found not to be necessary ($p = 0.65$). Similar hazard ratios were obtained if the data were stratified by sex or if the male and female animals were treated as one group. In all cases, the *Sod1^{-/-}* mice had significantly greater

mortality risk than either the wild type or *Sod1^{-/-}/hSOD1^{alb}* mice, indicating that the survival of *Sod1^{-/-}/hSOD1^{alb}* mice was significantly improved over *Sod1^{-/-}* mice.

4. Discussion

The *Sod1^{-/-}* mouse is of interest to the aging community because it is the only mouse model with a loss of function in a gene coding for an antioxidant protein that is not embryonically/prenatally lethal and has a shortened lifespan with accelerated aging phenotypes (Muller et al., 2006; Ohlemiller et al., 1999; Olofsson et al., 2005; McFadden et al., 1999b). For example, mice lacking *Sod2* (MnSOD) die within days after birth (Li et al., 1995), mice lacking *Gpx4* (glutathione peroxidase 4) are embryonically lethal (Ran et al., 2003) and mice lacking either *Gpx1* or *MsrA* (methionine sulfoxide reductase A) show no change in lifespan and no major phenotype (Zhang et al., 2009; Salmon et al., 2009). The *Sod1^{-/-}* mouse is also a valuable model to study the role of oxidative stress/damage on physiological processes because the level of oxidative stress/damage in tissues of the *Sod1^{-/-}* mouse is higher than any other viable mouse model with a reduction in an antioxidant enzyme (Perez et al., 2009). In addition, the ability to alter the expression of Sod1 in specific tissues makes the *Sod1^{-/-}* mouse valuable for studying the biological importance of oxidative stress in specific tissues or cell types. Using genetic approaches, one can study how increased expression of Cu/ZnSOD in a tissue alters the phenotypes observed in the *Sod1^{-/-}* mouse or one can determine the effect of knocking out the *Sod1* gene in a specific tissue or cell type. In other words, the *Sod1^{-/-}* mouse allows investigators to determine how changes in oxidative stress/damage in a specific tissue affect the global physiological status of the mouse. For example, our group has studied the effect of neuronal and muscle specific expression of *Sod1* on the accelerated sarcopenia phenotype that is a hallmark of the *Sod1^{-/-}* mouse. Sakellariou et al. (2014) showed that expressing the *hSOD1* transgene in neuronal tissue of *Sod1^{-/-}* mice prevented the loss of muscle mass and neuromuscular junction disaggregation (Sakellariou et al., 2014). On the other hand, knocking out the *Sod1* gene in muscle (Zhang et al., 2013b) or neurons (Sataranatarajan et al., 2015) alone did not recapitulate the accelerated sarcopenia phenotype observed in *Sod1^{-/-}* mice. These data demonstrate the importance of the interplay of between motor neurons and muscle in the mechanism underlying the loss of muscle arising from increased oxidative stress (Sataranatarajan et al., 2015).

In this study, we were interested in determining what role increase liver damage/pathology played in the shortened lifespan of the *Sod1^{-/-}* mice. Hepatocellular carcinoma is rare in the strain of mice used in these studies (Elchuri et al., 2005; Muller et al., 2006), and it is often argued that the decrease in lifespan, and even some of the accelerated aging phenotypes, are secondary to the dramatic increase in hepatocellular cancer. In this study, we first generated transgenic mice that expressed Cu/ZnSOD specific in liver using the albumin promoter fused to the *hSOD1* cDNA, and then generated mice that expressed Cu/ZnSOD specifically in the liver of *Sod1^{-/-}* mice by crossing the liver specific *hSOD1* transgenic mice to *Sod1^{-/-}* mice. The *Sod1^{-/-}/hSOD1^{alb}* mice displayed a reduction in oxidative damage in liver, improved liver function, and reduced markers of hepatocellular carcinoma. The liver-specific expression of *hSOD1* increased significantly the lifespan of *Sod1^{-/-}* mice; however, the lifespan of the *Sod1^{-/-}/hSOD1^{alb}* mice was still significantly lower than wild type mice, and

the *Sod1^{-/-}/hSOD1^{alb}* mice still showed the accelerated aging phenotype of sarcopenia, *e.g.*, the early onset of muscle atrophy, mitochondrial dysfunction, and the disaggregation of neuromuscular junctions. Thus, while expressing SOD1 in liver can partially rescue lifespan of *Sod1^{-/-}* mice, these mice still show the accelerated aging phenotype in muscle. Thus, the *Sod1^{-/-}/hSOD1^{alb}* mice allows one to study the effect of increased oxidative stress on aging without the confound of the dramatic increase in liver pathology in the *Sod1^{-/-}* mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The generation of liver specific transgenic mice crossed to *Sod1*^{-/-} mice. Panel A: The expression of *hSOD1* transcripts in various tissues of the *hSOD1*-transgenic (TG) and wild type (WT) mice are shown. Panel B: Western blots are shown for the levels of Cu/ZnSOD in tissues of *hSOD1*-transgenic (TG) and wild type (WT) mice. The human Cu/ZnSOD protein runs slower than mouse Cu/ZnSOD protein on SDS-PAGE gel electrophoresis (e.g., 23 vs. 19 kDa). Panel C: Western blots are shown for the levels of *hSOD1* and *mSod1* in the liver and muscle of *Sod1*^{-/-} (KO) and wild type (WT) mice (1 and 2 represents two different mice) compared to *Sod1*^{-/-}/*hSOD1*^{alb} (ALB) that are hemizygous (+/-) and homozygous (+/+) for the *hSOD1*-transgene. Panel D: Cu/ZnSOD enzymatic activity is compared in the liver of *Sod1*^{-/-} (KO) and *Sod1*^{-/-}/*hSOD1*^{alb} (ALB) mice at two different levels of protein added to

the gels. Abbreviations for tissues: K—kidney, M—skeletal muscle, L—liver, H—heart, B—whole brain.

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Fig. 2.

Effect of liver-specific expression of *hSOD1* on oxidative damage. The levels F₂-isoprostanes in liver, skeletal muscle, brain, and heart from wild type (white bar), *Sod1*^{-/-}/*hSOD1*^{alb} (black bar) and *Sod1*^{-/-} (gray bar) mice are shown for 12- to 14-month-old mice (*n* = 6 mice per group). The data are expressed as the mean ± SEM and the statistical significance shown as follows: icance: **p < 0.01 between wild type and *Sod1*^{-/-} mice; *p < 0.05 between *Sod1*^{-/-} or *Sod1*^{-/-}/*hSOD1*^{alb} mice and wild type mice. There is no significant difference between *Sod1*^{-/-} and *Sod1*^{-/-}/*hSOD1*^{alb} mice in all the tissues, except the liver.

Fig. 3.

The effect of liver specific expression of *hSOD1* on liver of *Sod1*^{-/-} mice. These data were obtained from 12- to 14-month-old *Sod1*^{-/-} (gray bar), wild type (white bar), and *Sod1*^{-/-}/*hSOD1*^{alb} (black bar) mice. Panel A: Body weight and liver wet weight ($n = 14, 9$, and 13 for wild type, *Sod1*^{-/-} and *Sod1*^{-/-}/*hSOD1*^{alb} mice, respectively). The data shown combine both sexes; however, the differences observed are significant for both males and females. Panel B: Plasma levels of free fatty acids (FFA) and lactic acid ($n = 4$ mice per group). Panel C: Liver mitochondrial function ($n = 4$ mice per group). The data are expressed as the mean $\pm$ SEM and the statistical significance shown as follows: * $p < 0.05$ and ** $p < 0.01$ indicates statistical significance between *Sod1*^{-/-} mice and wild type or *Sod1*^{-/-}/*hSOD1*^{alb} mice.

Fig. 4.

Expression of genes in liver associated with tumorigenesis. The levels of Met and cyclin D1 and the phosphorylation of Stat3 (pStat3/Stat3 ratio) were measured in liver tissue isolated from of 8- to 10- or 20- to 24-month-old wild type (white bars), *Sod1*^{-/-}/*hSOD1*^{alb} (gray bars), and *Sod1*^{-/-} (black bars) mice with $n = 6$ mice for each group. The data are expressed as the mean $\pm$ SEM and the symbols denote statistical significance at $p < 0.05$: between *Sod1*^{-/-} and wild type or *Sod1*^{-/-}/*hSOD1*^{alb} mice (*); between *Sod1*^{-/-} mice and wild type mice (&); between *Sod1*^{-/-} mice and *Sod1*^{-/-}/*hSOD1*^{alb} mice (#).

Fig. 5.

Effect of liver specific expression of *hSOD1* on skeletal muscle. Panel A: Wet weight of different types of skeletal muscle from wild type ($n = 10$, white bars), *Sod1*^{-/-} ($n = 6$, black bars), and *Sod1*^{-/-}/*hSOD1alb* mice, ($n = 8$, gray bars). Panel B: Skeletal muscle mitochondrial function as measured by ROS generation, oxygen consumption, and ATP generation ($n = 4$ mice for each group). Panel C: Structure of neuromuscular junctions visualized through fluorescence immunostaining and confocal microscopy for wild type (WT), *Sod1*^{-/-} (KO), and *Sod1*^{-/-}/*hSOD1alb* (ALB) mice. The data are expressed as the

mean $\pm$ SEM and the asterisk denotes statistical significance at (*) $p < 0.05$ and (**) $p < 0.001$ either between wild type mice and *Sod1*^{-/-} mice or between wild type and *Sod1*^{-/-}/*hSOD1*^{alb} mice.

Fig. 6.

Survival of *Sod1^{-/-}/hSOD1^{alb}* mice compared to *Sod1^{-/-}* and wild type mice. The Kaplan–Meier survival curves are shown for male and female mice combined. The curves represent *Sod1^{-/-}/hSOD1^{alb}* (Alb-red line, $n = 31$), *Sod1^{-/-}* (KO-blue line, $n = 25$), and wild type (WT-green line, $n = 34$) mice. Censored events are represented by the symbol 'X'. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Survival characteristics of *Sod1^{-/-}* (KO), wild type (WT) and *Sod1^{-/-}/hSod1^{Alb}* (Alb) mice.

Sex	M		F		M		F	
	KO	WT	KO	WT	Alb	WT	Alb	WT
Records	13	12	16	18	8	23		
Censored	3	3	6	7	3	7		
Events	10	9	10	11	5	16		
^a _t mean survival (days)	705	769	851	853	831	825		
^a _t SEM for rmean (days)	46	28	47	53	65	32		
Lower Bound 50% mortality	527	780	789	820	-	813		
Estimated 50% mortality	730	797	922	980	-	848		
Upper Bound 50% mortality	-	-	-	-	-	-		
Lower Bound 90% mortality	-	830	981	1042	-	901		
Estimated 90% mortality	914	876	1026	1095	-	988		

^aRestricted mean.

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Table 2

Mortality risk analysis of survival.

	β	Hazard ratio	SEM	Z	P
Stratified by sex					
Control vs KO	-1.3200	0.2671	0.3758	-3.5128	0.0004
ALB vs KO	-0.8382	0.4325	0.3523	-2.3791	0.0174
Adjusting for sex					
Control vs KO	-1.2815	0.2776	0.3681	-3.4818	0.0005
ALB vs KO	-0.7130	0.4902	0.3411	-2.0901	0.0366
F vs M	-0.1959	0.8221	0.2721	-0.7201	0.4715
Pooling M and F					
Control vs KO	-1.2979	0.2731	0.3694	-3.5134	0.0004
ALB vs KO	-0.7588	0.4683	0.3349	-2.2657	0.0235