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Original article

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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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

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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/αFet (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 CO_2 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 × *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 $MgCl_2$, 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 × *g* for 10 min. The resulting supernatant was centrifuged at 14,000 × *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 $MgCl_2$, 0.2 mM EDTA, and 0.2 mM ATP), and were centrifuged at 7000 × *g* for 10 min. Mitochondrial pellets were then resuspended in a half volume of modified Chappell–Perry buffer and centrifuged at 3500 × *g* for 10 minutes.

The assay of mitochondrial generated ROS is based on the detection of H_2O_2 in the medium using the Amplex Red fluorescent dye (Mohanty et al., 1997). The Amplex Red reagent reacts with H_2O_2 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 H_2O_2 production with the use of a H_2O_2 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 $MgCl_2$, and 2 mM K_2HPO_4, 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