Short report

Skeletal muscle mitochondrial DNA deletions are not increased in CuZn-superoxide dismutase deficient mice

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A B S T R A C T

Mitochondrial DNA (mtDNA) deletion mutations are proposed contributors to aging-related muscle fiber loss and atrophy, but evidence of a causal role for these mutations in muscle aging is lacking. Elucidating the etiology of in vivo mtDNA deletion mutations will help to better understand and test the possible roles of these mutations in aging. The implication of mtDNA mutations in aging is based on the susceptibility of mtDNA to oxidative damage by reactive oxygen species (ROS) due to residing in mitochondria, the primary source of endogenous ROS. Cells possess many pathways for neutralizing ROSs, including a variety of superoxide dismutases (SOD). Mice lacking CuZnSOD (Sod1−/−) mice have high levels of oxidative damage in many tissues including skeletal muscle and are a model for testing the role of oxidative damage in the formation of mtDNA deletion mutations. The increased DNA oxidative damage in Sod1−/− mice is associated with increased mtDNA deletion mutations in a variety of tissues, but skeletal muscle mtDNA mutations have not been reported. We hypothesized that a lifelong absence of mouse muscle CuZnSOD would increase mtDNA deletion mutation frequency and focal accumulation of these mutations in aging mouse skeletal muscle. Focal accumulations of mtDNA deletion mutations were detected by histochemical staining for cytochrome c oxidase (cytOX) activity and detection of cytOX-negative fibers, a marker of focal mtDNA mutation accumulation, within approximately 20,000 muscle fibers through a distance of 1000 μm. Total DNA was extracted from intervening unstained sections and mtDNA deletion mutation frequency was measured by a droplet digital PCR. Droplet digital PCR quantification of mtDNA deletion mutations showed no difference in mtDNA deletion mutation frequency in Sod1−/− mouse muscle compared to wild-type mice and we observed no significant increase in the number of cytOX-negative muscle fibers, in Sod1−/− mice compared to wild-type mice. These data demonstrate that not all changes in cellular oxidative stress are linked to mtDNA deletion mutations and shift the focus to other etiologies for these mutations that need to be clarified to better test their possible role in aging.

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1. Introduction

MtDNA deletion mutations have been implicated in aging and numerous age-related diseases (Wallace and Fan, 2009). In addition to the age-related increase in mtDNA deletion mutations in aging skeletal muscle, these mutations focally accumulate in muscle fiber segments where they result in respiratory chain deficiencies, fiber atrophy, fiber splitting and increased oxidative damage (Aiken et al., 2002; Wanagat et al., 2001). The mitochondrial and cellular dysfunction caused by mtDNA mutations is thought to result in fiber loss, thereby contributing to the loss of muscle mass and strength with aging. The cause of mtDNA mutations is not known and is attributed to replication or repair errors and oxidative damage (Larsson, 2010). While oxidative damage has long been suggested as a possible cause of mtDNA mutations, the evidence is mixed. Mice lacking the ooxoguanine DNA glycosylase (OGG1) do not display mitochondrial dysfunction or increased mtDNA mutation rates (de Souza-Pinto et al., 2001; Halsne et al., 2012). Conversely, a decrease in oxidative stress through mitochondrial targeting of catalase results in reduced mtDNA deletion mutations in transgenic mouse heart and skeletal muscle (Schriner et al., 2005; Dai et al., 2010). These models suggest that the type and source of oxidative stress determine the impact of this stress and mouse models of modulated ROS metabolism offer opportunities to further test the role of oxidative damage in mtDNA deletion mutation.

CuZn-SOD (SOD1) belongs to the superoxide dismutase family of enzymes that convert superoxide to hydrogen peroxide and is the
major superoxide scavenger in the cytoplasm, nucleus, lysosomes and mitochondrial intermembrane space (Elchuri et al., 2005). Mice lacking CuZn-SOD (Sod1−/− mice) have increased levels of oxidative damage to skeletal muscle protein, lipid and nucleic acid. The increase in oxidative damage is associated with a mean lifespan of ~21 months, about a 30% reduction from WT littermates (Elchuri et al., 2005). The decrease in lifespan is accompanied by a significantly lower muscle mass as early as 3–4 months with further reductions by 20 months to nearly 50% lower in the Sod1−/− mice than the age-matched WT mice (Muller et al., 2006). The greatest effect on muscle mass occurs in the gastrocnemius and plantaris muscles while the soleus is relatively spared, a pattern similar to that seen in usual mouse aging (Muller et al., 2006, 2007).

Concomitant with muscle mass loss, muscle mitochondrial hydrogen peroxide production is increased greater than 100% in 20-month-old as compared to 5-month-old mice, resulting in elevated oxidative damage to proteins, lipids, and DNA (Muller et al., 2006, 2007). Oxidative DNA damage, as measured by levels of 8-oxo dG, increased with age, roughly two-fold between 5-month-old and 20-month-old mice (Elchuri et al., 2005). Interestingly, immunohistochemical analysis of Sod1−/− liver sections using an anti-8-oxo dG antibody to detect nucleic acid oxidative damage revealed strong cytoplasmic staining that suggests mtDNA oxidation (Elchuri et al., 2005). MtDNA deletion mutations are increased from three to 20-fold in a variety of Sod1−/− mouse tissues including the liver, kidney, brain, heart, coelac, spleen and skin, but skeletal muscle has not been examined (Zhang et al., 2002). Because CuZnSOD is present in the mitochondrial intermembrane space as well as the cytosol, we wanted to measure the impact of a lack of CuZnSOD on skeletal muscle mitochondrial DNA deletion mutations in the Sod1−/− mouse.

The Sod1−/− mouse is an ideal model to address the question of whether oxidative stress impacts mtDNA mutation or accumulation of these mutations in aging skeletal muscle. We hypothesized that increased oxidative stress in Sod1−/− mice would cause an increase in mtDNA deletion mutations and accumulation of these mutations in individual muscle fibers. We measured mtDNA deletion mutation frequency via a novel, emerging droplet digital PCR technique and focal respiratory chain defects as markers of clonal mtDNA accumulation. We found that mtDNA deletion mutations are not increased in Sod1−/− mice and focal accumulations of mtDNA deletion mutations are not affected by the absence of CuZnSOD in mouse muscle. These data suggest that oxidative DNA damage may not be the cause of mtDNA deletion mutations in mouse muscle, or that these mutations may result from other types or sources of oxidative damage.

2. Materials and methods

2.1. Animals

Sod1−/− mice were bred and maintained in the laboratory of Dr. Van Remmen at the Barshop Institute for Longevity and Aging Studies in San Antonio. Mice were maintained under conditions previously described (Jang et al., 2012). 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.

2.2. Muscle tissue preparation

The triceps surae muscle of 17-mo-old Sod1−/− mice was dissected and snap frozen in liquid nitrogen. Previously frozen muscles were embedded and cryosectioned. Serial sections were stained as described below and total DNA was isolated from intervening sections.

2.3. Muscle histochemistry

Histochemical staining for cytOX was performed on 10 micron thick cryosections of mouse muscle. CytOX stained slides were counterstained with hematoxylin and eosin to aid in the identification of cytOX-negative fibers. Staining was performed as previously described (Wanagat et al., 2001). Individual cytOX-negative fibers were counted through 100 serial histological sections and the number of cytOX-negative fibers was normalized to volume of tissue examined.

2.4. Droplet digital (3D) PCR quantitation of mtDNA deletion mutations

MtDNA deletion mutations were measured by droplet digital PCR as previously described (Taylor et al., 2014). Briefly, total DNA was isolated and purified by proteinase K digestion and phenol:chloroform extraction. Ten micrograms of total DNA was digested with TaqI (New England Biolabs) and extracted again with phenol:chloroform. The final concentration of digested total DNA was adjusted to fall within the linear range for the Poisson calculation for the expected number of droplets in the digital PCR. Reaction mixtures containing the digested DNA were prepared and droplets generated on a DG8 cartridge (Bio-Rad) before thermocycling was carried out using the following protocol: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, and 63.5 °C for 4 min. The following primer/probe sets were used with mouse total DNA for mtDNA deletion detection — control site: 5′-GAC ACA AAC TAA AAA GCT CA-3′ (forward primer), 5′-TAA GTG TCC TGC AGT AAT GT-3′ (reverse primer), and 5′-6FAM-CCA ATG GCA TTA GCA GTC CGG C-MGB-3′ (probe) and major arc: 5′-AGG CCA CCA EAC TCC TAT TG-3′ (forward primer), 5′-AAT AGT AGG CGT TTG ATT GG-3′ (reverse primer), and 5′-6FAM-AAG CAC TAC CAT ATG GTA TAA-MGB-3′ (probe). The thermally cycled droplets were then analyzed by flow cytometry on a QX100 Droplet Digital PCR system (Bio-Rad). The number of mtDNA deletion mutation genomes per droplet was calculated by QuantaSoft software (Bio-Rad).

2.5. Statistical analysis

All data with normal distribution were presented as means ± SEM. Student’s t-test was used to compare differences between groups.

3. Results

3.1. Mitochondrial DNA deletion mutation frequency in WT and Sod1−/− mouse skeletal muscles

3D PCR was performed to quantify deletion mutation frequency of mtDNA of wild type and Sod1−/− mice. The analyzed tissue was obtained from triceps surae (calf) muscle of 17-mo-old wild type and Sod1−/− mice. At an age of 17 months, the mtDNA deletion mutation frequency (per genome) was measured as 2.18 ± 0.27 × 10⁻⁷ and 2.68 ± 1.05 × 10⁻⁷ for WT and Sod1−/− mice, respectively (Fig. 1) with no
Mitochondrial DNA deletion mutation frequency in WT and Sod1−/− mice varies between mitotic and post-mitotic tissues

Because of a previous report of higher mtDNA deletion mutation rates in other tissues, we examined heart and kidney tissues from 17-mo-old Sod1−/− mice using a droplet digital PCR method. In the heart, similar to our findings in skeletal muscle, we found no increase in mtDNA deletion mutations and even a trend towards lower mutations in the Sod1−/− mice (Fig. 2). Conversely, in the kidney, a mitotic tissue, we measured a significant two-fold increase in mtDNA deletion mutations (Fig. 2). In WT 17-mo-old mice, we observed the highest mutation frequency in the kidney (1.42 × 10^−6), a 10-fold lower mutation frequency in the heart (1.30 × 10^−5) and the lowest mutation frequency in skeletal muscle (2.18 × 10^−5).

Cytochrome-c oxidase negative fibers in WT and Sod1−/− mouse skeletal muscles

As we have previously identified cytOX negative skeletal muscle fibers to harbor clonal expansions of mtDNA deletion mutations, we measured cytOX-negative fibers in the 17-mo-old WT and Sod1−/− mouse muscles to corroborate our digital PCR findings. Serial cryosections of 17-mo-old WT and Sod1−/− triceps surae stained for cytOX activity and counterstained with hematoxylin and eosin are shown in Fig. 2. Approximately 20,000 muscle fibers were examined along their length through a distance of 1000 μm with staining every 100 μm. CytOX-negative fibers (Fig. 3) were counted and normalized to the tissue volume examined. At this age, only rare cytOX-negative fibers were found and there was no significant difference in cytOX-negative fiber number between WT and Sod1−/− mouse muscles (Fig. 4, unpaired t-test, p > 0.05). The length of the cytOX-negative fiber segments was less than 100 μm in both genotypes and there was no evidence of fiber atrophy in the cytOX-negative fiber segments.

Discussion

In this study, we investigated the role of SOD1 in mtDNA deletion mutations or their focal accumulation in mouse skeletal muscle. Our results show that Sod1−/− mice, which are known to have increased DNA oxidative damage in skeletal muscle and mtDNA deletion mutations in other tissues, have no increases in muscle mtDNA deletion mutations or the occurrence of focal mtDNA deletion mutation accumulation. To quantitate mtDNA deletion mutations in a variety of mouse tissues, we used a recently developed droplet digital PCR method (Taylor et al., 2014). Currently, this droplet digital PCR is the most sensitive method for measuring mtDNA deletion mutations and is able to detect mutation frequencies as low as 1 × 10^−8 (Taylor et al., 2014). This method facilitates the measurement of mtDNA deletion mutations across a large portion of the mtDNA major arc. We validated this method in aging human brain samples and now present the first application of this method to aging mouse tissues. The mutation frequencies we found in both genotypes were quite low, similar to levels we have observed in 6-mo-old WT mice (unpublished observations), likely due to the relatively young age of the mice in the current study. Using a semiquantitative serial dilution PCR, an earlier study found increased mtDNA deletion mutation rates in many Sod1−/− mouse tissues, though they did not examine skeletal muscle (Zhang et al., 2002). While Zhang et al. found ~ten-fold increases in mtDNA deletion mutation frequency in the heart and kidney, we found no increase in the heart and only an ~two-fold increase in the kidney. The deletion frequency magnitude differs between the two approaches as well, with the semiquantitative serial dilution PCR method giving deletion mutation rates that are about 10−1000-fold higher than those we find by digital droplet PCR or as reported by other methods (Vermulst et al., 2008).

The advantages of the droplet digital PCR method likely lead to the differences in mtDNA deletion mutation frequencies between the two studies. The droplet digital method is quantitative, does not require multiple rounds of PCR with nested primers, and is independent of PCR efficiency. Conversely, the serial dilution PCR method employed in the earlier study is semiquantitative as it relies on digital scanning of bands from electrophoretic gels. Semiquantitative PCR approaches rely on avoiding saturation of the PCR reaction, which would be difficult with the 65 total cycles of nested PCR in the serial dilution method (Marone et al., 2001). The serial dilution PCR method relies on the PCR efficiency of each primer pair to be equivalent. This was not shown in the earlier study and could substantially increase or decrease the apparent mutation frequency. The droplet digital PCR avoids many of the difficulties faced by other mutation detection assays and will be useful for examining mtDNA deletion mutations in many aging and disease models (Taylor et al., 2014).

The tissue differences in mtDNA deletion mutation frequencies are seen in both studies. In tissues with high metabolic demand, higher mutation frequencies are seen in mitotic tissues such as the liver and kidney while post-mitotic tissues have smaller or no increases in mtDNA deletion mutations in Sod1−/− mice. Metabolic demand or oxygen consumption does not appear to be the primary driver, as the three tissues we examined, i.e., the heart, skeletal muscle and kidney have some of the highest oxygen consumption rates. This observation would seem to support a more complicated relationship between oxygen consumption and ROS generation in the formation of mtDNA deletion mutations. Differences in mtDNA deletion mutation rates between mitotic and post-mitotic tissues with high metabolic demand may be related to the clonal expansion of cells harboring mtDNA deletions in

**Fig. 2.** Mitochondrial DNA deletion mutation frequency in heart or kidney tissue of WT or Sod1−/− mice. mtDNA deletion mutations from 17-mo-old WT and Sod1−/− mice quantified by droplet digital PCR. Columns show mean ± SEM for four mice per group. Different letters denote significance at p < 0.05.
mitotic tissues. In the aging rat kidney, we found clonally expanding groups of abnormal tubular epithelial cells, a phenomenon that we have not observed in aging skeletal muscle where mutations are confined to individual muscle fibers (McKiernan et al., 2007). These observations suggest interplay between cell metabolism and cell division in the natural history of mtDNA deletion mutations.

Our second finding, that a life-long absence of CuZnSOD does not affect focal accumulation of mtDNA deletion mutations, is demonstrated by our histological studies. Increases in mutation frequency would be reflected in greater numbers of cytOX-negative fibers in the Sod1−/− mice as each fiber contains only a single deletion mutation event, while increases in the clonal expansion of deletion mutations within fibers would be apparent as increases in the length of muscle fiber involved by the cytOX-negative defect. At 17 months of age, we found very few cytOX-negative fibers in each animal and no difference between WT and Sod1−/− mice. This is consistent with our droplet digital PCR findings and suggests that droplet digital PCR measurement of mtDNA deletion mutations may serve as a high-throughput method for screening tissues that may be of interest at the histological level. We did not observe changes in the fiber atrophy or length of the cytOX-negative fiber segments suggesting that a lack of CuZnSOD and increased in oxidative damage does not play a role in these processes.

Our observations that a systemic loss of CuZnSOD does not cause increased mtDNA deletion mutation rates or focal accumulation in aging mouse skeletal muscle contribute to the evidence against a causal role of oxidative damage in mtDNA deletion mutations in this tissue. Because mtDNA deletion mutations are not altered in Sod1−/− mouse muscle, the model does not address the possible role of mtDNA deletion mutations in muscle aging. Furthermore, the age-related muscle changes in the Sod1−/− mouse are due to changes in motor neuron redox homeostasis in this model, rather than direct effects on muscle fibers (Sakellariou et al., 2014). These data suggest that the impact of ROS depends on the type and localization of ROS production and models implicating oxidative damage in aging and age-related disease will need to focus on these details. The numerous other mouse models of modulated oxidative damage will aid in isolating the important characteristics of ROSs and oxidative damage that contribute to these processes.

One limitation of our study is the relatively young ages of the mice and the comorbidities present in this model. The mouse ages are limited by the availability of aged Sod1−/− mice and the development of hepatocellular carcinoma that develops in Sod1−/− mice by about 19 months of age (Wanagat et al., 2001). The hepatocellular carcinoma is unlikely to have confounded the mtDNA deletion mutation results as we studied 17-mo-old mice, before the formation of the carcinomas and their impact on mouse health. Conversely, in the earlier study, ages extended up to 19 months, which may have also contributed to difference from our study in mtDNA deletion mutation rates (Zhang et al., 2002).

5. Conclusions

In summary, our study shows that general increases in oxidative damage do not cause mtDNA deletion mutations in mouse skeletal muscle and do not affect the formation or progression of focal accumulations of mtDNA deletion mutations. Interventions to test the role of mtDNA mutations in aging and age-related disease should focus on specific types of oxidative damage or the impact of cell metabolism and cell division on mtDNA deletion mutations.

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