Biochemical and Biophysical Research Communications 438 (2013) 78–83

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Oxidative damage associated with obesity is prevented by overexpression of CuZn- or Mn-superoxide dismutase

Yuhong Liu [b,1], Wenbo Qi [b,1], Arlan Richardson [a,b,c], Holly Van Remmen [a,b,c], Yuji Ikeno [a,b,d], Adam B. Salmon [a,b,e,*]

[a] The Geriatric Research Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, TX 78229, USA
[b] The Sam and Ann Barshop Institute for Longevity and Aging Studies, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78245, USA
[c] Department of Cellular & Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[d] Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[e] Department of Molecular Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 5 July 2013
Available online 18 July 2013

Keywords:
Diabetes
Mitochondria
F_2-isoprostane
Oxidative stress

ABSTRACT

The development of insulin resistance is the primary step in the etiology of type 2 diabetes mellitus. There are several risk factors associated with insulin resistance, yet the basic biological mechanisms that promote its development are still unclear. There is growing literature that suggests mitochondrial dysfunction and/or oxidative stress play prominent roles in defects in glucose metabolism. Here, we tested whether increased expression of CuZn-superoxide dismutase (Sod1) or Mn-superoxide dismutase (Sod2) prevented obesity-induced changes in oxidative stress and metabolism. Both Sod1 and Sod2 overexpressing mice were protected from high fat diet-induced glucose intolerance. Lipid oxidation (F_2-isoprostanes) was significantly increased in muscle and adipose with high fat feeding. Mice with increased expression of either Sod1 or Sod2 showed a significant reduction in this oxidative damage. Surprisingly, mitochondria from the muscle of high fat diet-fed mice showed no significant alteration in function. Together, our data suggest that targeting reduced oxidative damage in general may be a more applicable therapeutic target to prevent insulin resistance than is improving mitochondrial function.

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1. Introduction

In the United States, almost 10% of the population has been diagnosed with diabetes; more than 90% of these cases are type 2 diabetes (T2DM). T2DM is characterized largely by the presence of insulin resistance which generally describes a significant inhibition in the cellular response to insulin [1]. There are several risk factors for the development of insulin resistance, i.e., obesity, genetics, age, etc.; however, the molecular processes that regulate the onset of this pathophysiology still remain largely unclear. The growing prevalence of metabolic diseases like T2DM requires an increasing reliance on effective healthcare treatment options. Understanding the basic mechanistic processes in the etiology of these diseases will significantly aid in the development of new therapeutics.

Oxidative stress is implicated as playing a significant role in the development of many chronic diseases [2]. Several lines of evidence have suggested that oxidative stress may be a primary factor in the development of T2DM. For example, diabetics show high levels of systemic oxidative damage relative to those with normal glucose metabolism [2,3]. Similarly, oxidative damage is elevated in the obese and the severity of metabolic dysfunction among obese is directly correlated with markers of oxidative stress [4]. One of the primary sources of pro-oxidants with obesity and T2DM may be mitochondria dysfunction which promotes increased superoxide radical production. During mitochondrial respiration, superoxide radicals are produced as a byproduct when electrons passed along the electron transport chain react with molecular oxygen. Obesity, T2DM and other metabolic dysfunctions are associated with elevated glucose and fatty acids; with increased mitochondrial fuel sources, there may be a subsequent increase in number of electrons donated to ETC and thus increased likelihood of production of superoxide [5]. Obesity and metabolic dysfunction are also associated with elevated superoxide production from macrophage infiltration/activity and NADPH oxidase activity associated with chronic inflammation [6,7].

The cellular antioxidant defense system is normally sufficient to reduce, remove and repair oxidative stress/damage. However, increased pro-oxidant production, such as in metabolic dysfunction, can overwhelm this system to promote chronic oxidative stress [8,9]. There is also evidence that obesity is associated with reduced expression of several antioxidant proteins [4]. Thus, it might be predicted that increasing antioxidants will reduce reactive oxygen

* Corresponding author. Address: 15355 Lambda Drive, San Antonio, TX 78245-3207, USA. Fax: +1 (210) 562 6110.
E-mail address: salmona@uthscsa.edu (A.B. Salmon).
[1] These authors contributed equally to this manuscript.

0006-291X/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.07.029

species (ROS) or oxidative damage and prevent metabolic defects associated with obesity. The superoxide dismutases (SOD) represent the primary cellular defense against superoxide radicals. SODs catalyze the conversion of superoxide to H_2O_2 which can then be subsequently converted to water by other antioxidants [10]. In mammals, there are two intracellular forms of SOD: CuZnSOD (Sod1) which is located primarily in the cytoplasm and MnSOD (Sod2) which is located in the mitochondria [11]. Increased expression of Sod1 (TgSOD1) or Sod2 (TgSod2) in transgenic mice has been shown to reduce oxidative stress in vivo [12–13].

We previously showed that mice with increased Sod2 expression were protected from obesity-induced insulin resistance [14]. These results support the idea that mitochondrial superoxide is a primary determinant of metabolic dysfunction. In this study, we utilized TgSOD1 and TgSod2 mice to directly test whether (1) elevated antioxidants prevent oxidative stress and damage with obesity and (2) reduction of oxidative stress can prevent metabolic dysfunction.

2. Methods

2.1. Animals

All transgenic mice utilized in this study were previously characterized [12–14]. All mice were of the C57BL/6J genetic background. In brief, CuZnSod transgenic mice (TgSOD1) were originally generated using a large fragment of human genomic DNA containing the SOD1 gene and Sod1 activity in tissues of TgSOD1 mice is 2–5-fold higher than that of wild type (WT) mice [12]. Mice overexpressing MnSod (TgSod2) were generated using a 13-kb genomic Sod2 clone isolated from C57BL/6J mice, which encompassed 2 kb of the native Sod2 promoter [13]. MnSod activity in tissues of TgSod2 mice is approximately 2-fold higher that of WT [13]. All mice used in these studies were male and were maintained under pathogen-free barrier conditions in a temperature-controlled environment at cage density of 2–4 mice/cage for the duration of studies. From birth until approximately 6 months of age, mice were maintained on a commercial mouse chow (Teklad Diet LM485) provided ad libitum. At approximately 6–8 months of age, mice were randomly assigned to either a low fat defined diet (10% kcal from fat, D12450B, Test Diets, Richmond IN) or to a high fat defined diet (45% kCal from fat, D12451, Test Diets). Diets were provided ad libitum and food consumption and body weight were monitored bi-weekly for a period of 3 months. Body composition of non-anesthetized mice was analyzed by Quantitative Magnetic Resonance imaging (QMRi) using an EchoMRI 3-in-1 composition analyzer (Echo Medical Systems, Houston, TX). For tissue collection, mice were euthanized by CO_2 inhalation followed by cervical dislocation.

2.2. Glucose tolerance tests

Fasting blood glucose was measured following 6 h fast during the light cycle (09:00–15:00) using a ONE Touch Ultra handheld glucometer. Glucose tolerance tests were performed after this 6 h fast and mice were given 1.5 g glucose (Sigma, St. Louis MO)/kg of body weight by intraperitoneal (IP) injection. Blood glucose levels were measured at 0, 30, 60, and 120 min following injection by glucometer. Area under curve (AUC) was calculated for each animal using the Trapezoid method.

2.3. Isolation of skeletal muscle mitochondria

Mitochondria were purified from freshly collected whole hindlimb skeletal muscle as described previously [15]. Hind limb skeletal muscles (gastrocnemius, tibialis anterior, and vastus lateralis) were excised, weighed, bathed in 150 mM KCl, and placed in Chappell–Perry buffer I with the protease nagarse. Muscles were minced, homogenized and centrifuged for 10 min at 600*g* with the resulting supernatant passed through cheesecloth and centrifuged at 14,000*g* for 10 min. The final pellet containing isolated mitochondria was washed once in modified Chappell–Perry buffer II with 0.5% bovine serum albumin (BSA) and twice in modified Chappell–Perry II buffer without BSA. Protein concentration was measured by the Bradford method (Bio-Rad, Richmond, CA) and mitochondria were used immediately.

2.4. Measurement of mitochondrial H_2O_2 release

The rate of mitochondrial H_2O_2 production was measured using the Amplex red (Molecular Probes, Eugene OR)–horseradish peroxidase method as described in [16]. Amplex red reagent was made in ROS buffer (125 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, 2 mM K_2HPO_4, pH 7.44) with 77.8 μM Amplex-Red, 1 unit/ml of HRP and 37.5 units/ml of SOD (to convert all $O_2^{\cdot-}$ into H_2O_2). All assays were performed in black 96-well plates 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 (Labsystems, Helsinki, Finland). For each assay, one reaction well contained buffer only and another contained buffer with mitochondria to estimate the mitochondria H_2O_2 release without substrate. Mitochondria complex substrates/inhibitors were added at following concentrations: glultamate/malate (5 mM), succinate (10 mM), rotenone (1 μM). The slope of the increase in fluorescence was converted to the rate of H_2O_2 production with the use of a known H_2O_2 standard curve. Data are expressed in pmol H_2O_2/min/mg mitochondrial protein.

2.5. Measurement of mitochondrial membrane potential

Membrane potential was monitored by fluorescence quenching of the positively charged dye Safranin O as previously described [17]. Safranin O fluorescence was measured by following emission wavelength of 590 nm (excitation wavelength of 485 nm) using a Fluoroskan Ascent type 374 multiwell plate reader (Labsystems). For all assays, samples of 5 μg of mitochondrial protein in 100 μl of ROS buffer (described in Section 2.4) with 5 μM of Safranin O were distributed in 96-well black plates. Mitochondria complex substrates were added to reactions at concentrations given for measurement of H_2O_2 production as in Section 2.4. Safranin O fluorescence quenching was determined in the presence of respiratory chain substrates and inhibitors. The relative decrease in Safranin O fluorescence was taken as a measure of the mitochondrial membrane potential.

2.6. Measurement of ATP production

ATP production of isolated mitochondria was measured using a luminometric assay (ATP Bioluminescence Assay CLS II; Roche, Indianapolis, IN) that follows the change in luminescence at 560 nm. Equivalent amounts of 4 μg of mitochondrial proteins in 100 μl of ROS buffer were mixed with glutamate and malate as Complex I substrates at concentrations given for measurement of H_2O_2 production in Section 2.4 were added to a 96-well white plate. Luciferase (Roche) and 0.3 mM ADP were then added to start the reaction, and the rates of ATP production were determined at 560 nm using Fluoroskan Ascent type 374 multiwell plate reader (Labsystems).The slope of the increase in luminescence is converted to the rate of ATP production with a standard curve.

2.7. Measurement of lipid peroxidation

F_2-isoprostanes were measured in tissues using the gas chromatography–mass spectrometry method of Roberts and Morrow as previously described [18]. Tissue levels of F_2-isoprostanes are expressed as nanograms F_2-isoprostanes per gram tissue.

2.8. Statistical analysis

For body composition, glucose metabolism and mitochondrial function experiments, data were analyzed by one-way ANOVA for each diet independently with the target comparison among genotypes fed the indicated diet followed by Newman–Keul's post hoc analysis. Isoprostane data were not normally distributed and were analyzed by ANOVA on ranks followed by Dunn's post hoc analysis. Isoprostane data were also analyzed post hoc by *T*-test comparing each levels in each high fat-fed group to that of low fat-fed WT.

3. Results

3.1. Glucose metabolism

After consumption of high fat diets for 3 months, body weight and fat mass were significantly increased (Fig. 1A). Low fat fed mice weighed 33.2 ± 0.8, 30.1 ± 2.3, and 32.0 ± 0.5 g and high fat fed mice weighed 39.2 ± 1.8, 38.8 ± 2.3, 43.3 ± 2.9 g (WT, TgSOD1, TgSod2 respectively). Body fat percentage also significantly increased with high fat feeding from 25.0% ± 1.7 (WT), 21.8% ± 2.9 (TgSOD1) and 25.2% ± 2.3 (TgSod2) to 38.8% ± 3.1, 35.2% ± 3.2, and 42.8% ± 3.0 respectively. Among genotypes, however, we found no significant difference in body weight, fat mass, or percentage body fat for each individual diet. We also found no significant difference in food consumption among the three genotypes of mice tested (data not shown).

Fasting blood glucose was also significantly affected by diet with the average glucose levels from high fat fed mice significantly higher than that of mice fed low fat diets (Fig. 1B). However, increased expression of either Sod1 or Sod2 did not affect fasting blood glucose levels in mice fed either diet. Glucose tolerance was not different among mice fed low fat diets; i.e., area under curve (AUC) analysis of GTT showed no difference among low fat WT, TgSOD1 and TgSod2 (Fig. 1C). Diet had a significant impact upon glucose tolerance with high fat-fed mice significantly more glucose intolerant than low fat fed mice. We had previously shown that female TgSod2 mice were protected from high fat diet-induced insulin resistance [14]. In this study, we confirm that overexpression of Sod2 in male mice reduces glucose intolerance caused by high fat feeding (Fig. 1C). Interestingly, we also found that TgSOD1 mice were similarly protected from glucose intolerance caused by high fat feeding (Fig. 1C). Thus, overexpression of

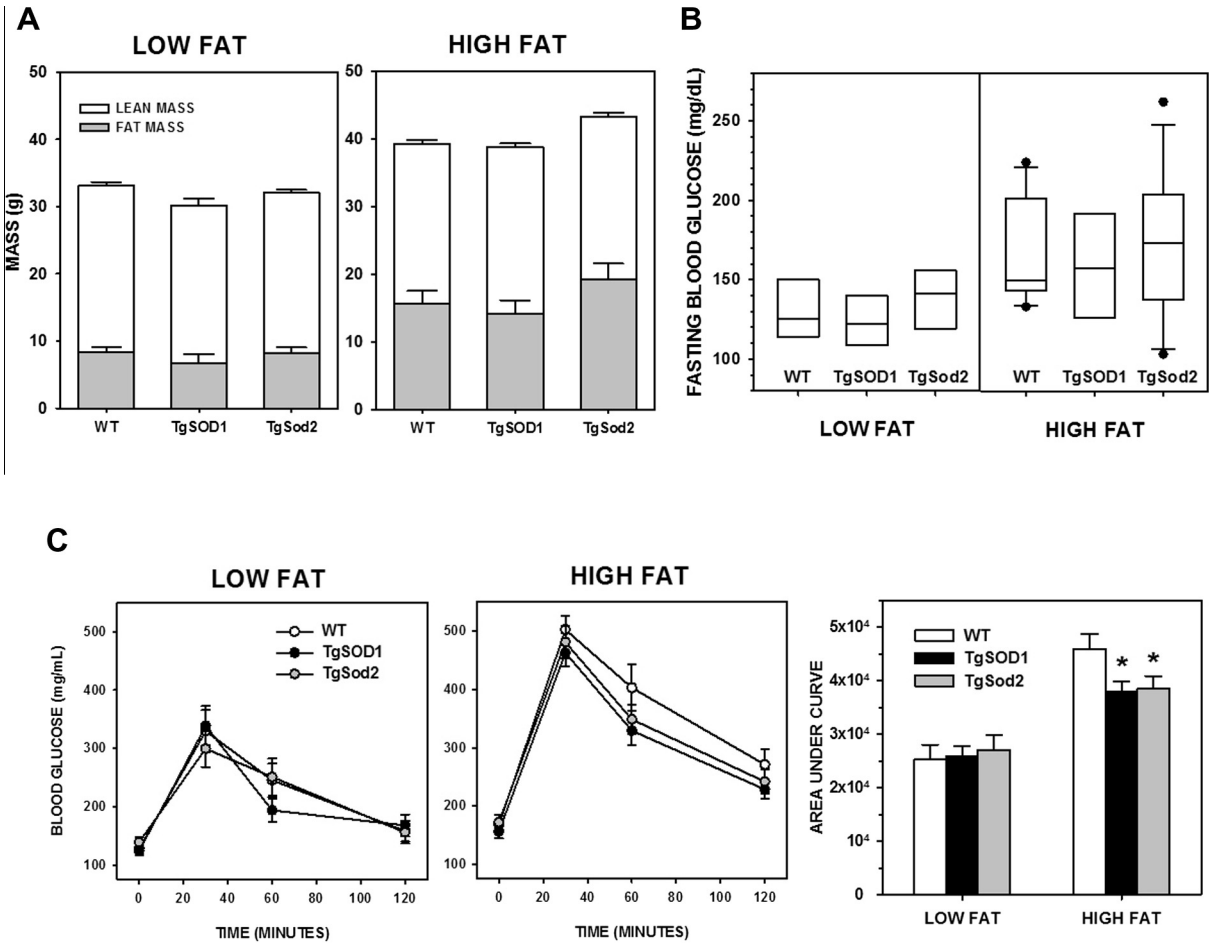

Fig. 1. Increased expression of either Sod1 or Sod2 prevents glucose intolerance with high fat diet. (A) Mean fat mass and lean mass of indicated mice as measured by qMRI. (B) Fasting (6 h) blood glucose for indicated groups presented as box plots with horizontal line indicating median value, whiskers indicating 95% CI, and dots representing outliers within sample group. (C) Glucose tolerance test (left) and area under curves (AUC) (right). Each dot or bar represent mean (±SEM) for indicated groups. Asterisks represent significant difference ($p < 0.05$) by ANOVA. For all, means were calculated from 4 to 9 animals per group.

SOD in either primarily cytosol (Sod1) or primarily mitochondria (Sod2) is beneficial in preserving glucose metabolism when mice are fed high fat diet but do not prevent obesity.

3.2. Tissue oxidative damage

To assess whether superoxide dismutase expression prevented oxidative damage associated with obesity, we measured tissue levels of F_2-isoprostanes in muscle and adipose. F_2-isoprostanes are stable markers of lipid peroxidation formed by free radical oxidation of fatty acids, primarily arachidonic acid, and are one of the most reliable indices of in vivo oxidant stress [18]. In WT mice, high fat feeding significantly increased the levels of F_2-isoprostanes in skeletal muscle and adipose tissue (Fig. 2). Under normal chow–fed conditions, young TgSOD1 and TgSod2 mice show little to no reduction in F_2-isoprostanes compared to WT mice [12,13]. However, we found here that overexpression of Sod1 or Sod2 significantly reduced the accumulation of lipid peroxidation caused by high fat feeding. In muscle, F_2-isoprostanes in high fat TgSOD1 and high fat TgSod2 muscle were not statistically different from low fat WT suggesting significant reduction in oxidative damage. Similarly, F_2-isoprostane levels in either high fat TgSOD1 or TgSod2 adipose were not significantly different than levels in low fat fed WT mice. Among adipose samples from high fat fed mice, F_2-isoprostanes were significantly lower in TgSod2 adipose than those of both other groups of high fat fed mice (ANOVA ON RANKS $p = 0.022$, Dunn's post hoc). These findings suggest Sod1 or Sod2 expression prevents elevated oxidative damage associated with obesity.

3.3. Mitochondria function

Insulin resistance in several models has been associated with mitochondria dysfunction including defects in respiration and increased ROS production. In this study, we measured H_2O_2 release (a surrogate for superoxide production), membrane potential, and ATP production from mitochondria isolated from the hindlimb skeletal muscle of mice from all groups. In WT samples, H_2O_2 release from mitochondria isolated from skeletal muscle was not affected by obesity in this study (Fig. 3A). We found no effect of diet whether H_2O_2 release was measured in the absence of respiratory substrates (not shown), in the presence of Complex I-linked substrates glutamate and malate (not shown) or in the presence of Complex II-linked substrates succinate and rotenone (Fig. 3A). Diet also had no significant effect within genotypes; i.e., H_2O_2 release was the same in mitochondria from low fat TgSOD1 or TgSod2 mice compared to their respective high fat fed counterparts. Similarly, there was no significant difference in membrane potential or ATP production between mitochondria isolated from low or high fat fed mice (Fig. 3B and C). Taken together, these results suggest that feeding mice high fat diet in this study did not cause mitochondria dysfunction as measured by these assays.

We next addressed whether elevated levels of Sod1 or Sod2 alone altered mitochondrial function on either diet. In both low fat and high fat diet groups, mitochondria from TgSOD1 mice showed reduced H_2O_2 production compared to either WT or TgSod2 mice when incubated in the presence of succinate and rotenone (Fig. 3A). Overexpression of Sod1 had no effect on membrane potential, but mitochondria from TgSOD1 mice fed either diet did show elevated ATP production relative to WT or TgSod2 mice when mitochondria were incubated with glutamate and malate to support mitochondrial respiration (Fig. 3B and C). A previous report showed that mitochondria isolated from skeletal muscle of young TgSod2 mice fed standard rodent chow differed little from WT mitochondria [15]. We found similar results here; in either the low fat or high fat groups, mitochondria from TgSod2 mice did not differ from those of WT mice in H_2O_2 production, membrane potential, or ATP production (Fig. 3).

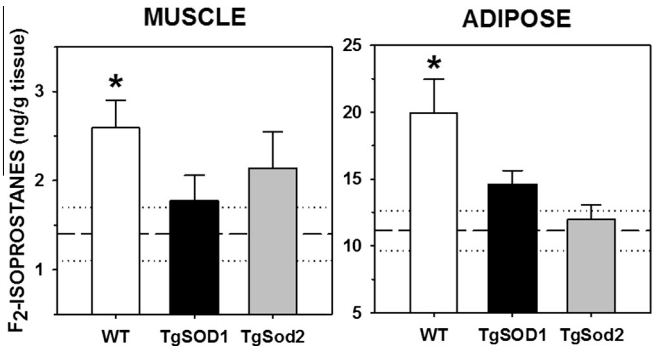

Fig. 2. Increased oxidation caused by high fat feeding is reduced by overexpression of Sod1 or Sod2. Each bar represents mean (±SEM) F_2-isoprostane levels for indicated tissue from high fat fed mice of indicated genotype. Dashed (mean) and dotted (upper and lower SEM) lines represent values of F_2-isoprostanes in indicated tissue from low fat WT mice. Asterisks represent high fat group indicated significantly different from low fat WT group ($p < 0.05$). Means are calculated from tissues of 4–9 individual animals per group.

4. Discussion

A role for oxidative stress in the development of T2DM has been largely supported by correlative evidence; i.e., increased pro-oxidant production and reduced antioxidant defense are associated with the disease [4,7–9]. Recently, a small number of studies have addressed whether direct modification of oxidative stress can alter glucose metabolism in vivo. For example, we previously reported that insulin-resistance caused by high fat feeding is reduced in female TgSod2 mice [14]. Similarly, Anderson et al. showed that targeted overexpression of catalase in the mitochondria reduced mitochondrial H_2O_2 production and improves insulin sensitivity in high fat-fed mice [8]. In this study, we show novel results that overexpression of Sod1 (TgSOD1) preserves glucose metabolism with high fat feeding and also confirm that male TgSod2 mice are similarly protected. Delineating the protective mechanisms utilized by TgSOD1 and TgSod2 mice could thus provide important insight into prevention of T2DM.

Insulin resistance and diabetes are associated with reduced mitochondrial ATP production, changes in membrane potential, increased ROS production and/or changes in mitochondrial dynamics [19]. It has been suggested that mitochondrial deficits may be the critical mechanistic link between obesity and T2DM [8]. However, there is some discrepancy in the literature on whether obesity actually negatively affects mitochondria function or whether diabetes itself is associated with mitochondrial dysfunction [20]. Furthermore, mitochondria dysfunction may actually promote insulin resistance as a mechanism to protect the cell from energy excess [14]. This current study supports the notion that mitochondrial function is not directly linked to changes in glucose metabolism associated with high fat feeding. We found that high fat feeding had little effect on ATP production, membrane potential, or ROS production of mitochondria isolated from muscle. In addition, glucose tolerance of low fat TgSOD1 mice was not different from low fat WT mice despite our finding that TgSOD1 mitochondria had significantly reduced ROS production and increased ATP production. However, isolated mitochondria and the particular complex substrates utilized here may not fully recapitulate mitochondrial function in vivo [21]. Thus, alternative approaches to monitor mitochondria will be beneficial to further address this question.

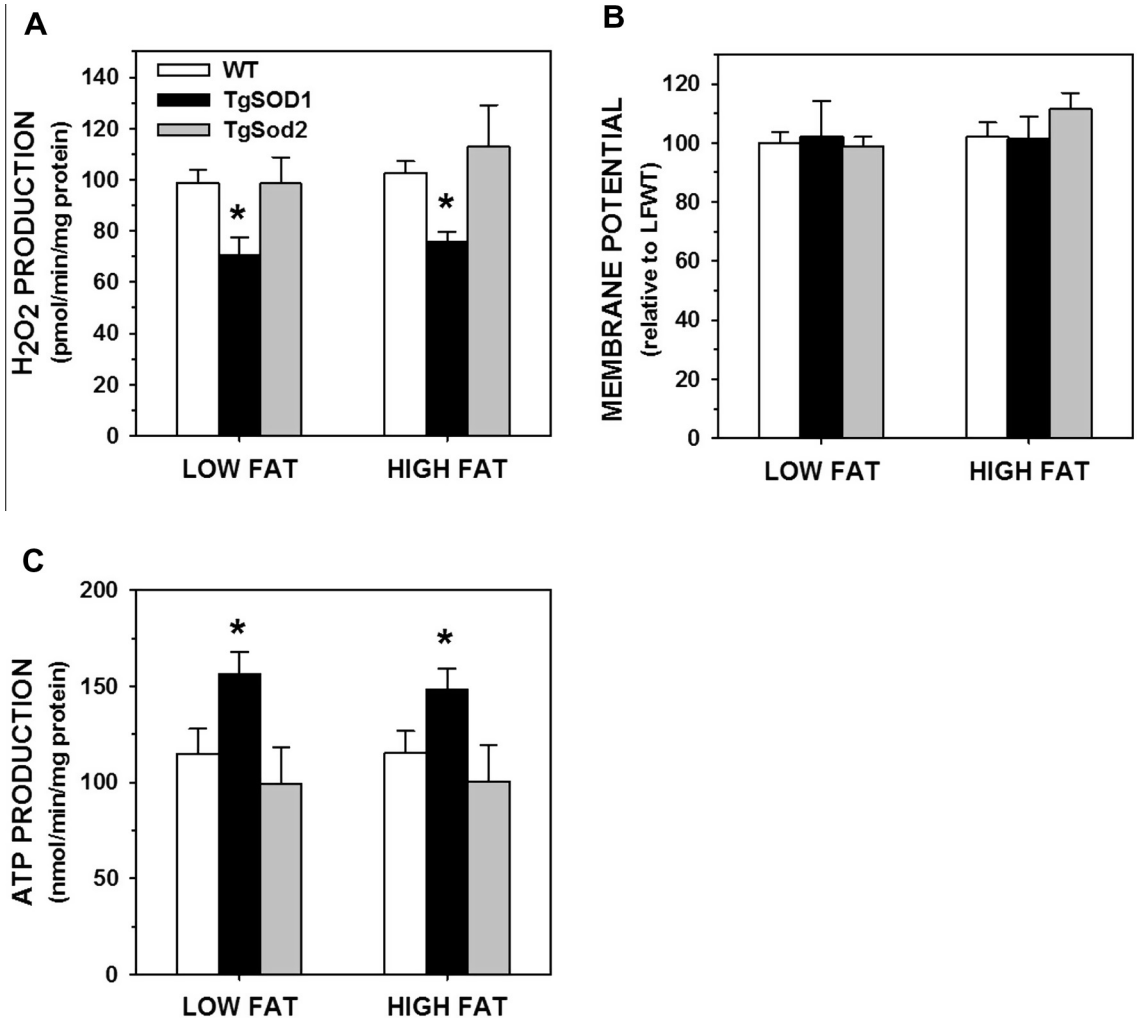

Fig. 3. Skeletal muscle mitochondria function is altered by Sod1, unaffected by Sod2 and high fat diet. (A) Mitochondrial H_2O_2 production with succinate and rotenone as substrates/inhibitors. (B) Mitochondrial membrane potential with succinate and rotenone as substrates/inhibitors. Results are given as signal relative to that of low fat wild type mitochondria. (C) Mitochondrial ATP production measured by with glutamate and malate as substrates. For all, bars represent mean values (±SEM) for $n = 8$ (WT each diet), 3 (TgSOD1 each diet), or 4 (TgSod2 each diet). Asterisks represent ANOVA among group $p < 0.05$.

Our mitochondrial ROS data are also surprising given the subcellular localization of Sod1 and Sod2. Despite mitochondrial localization, overexpression of Sod2 has previously been shown to have no effect on H_2O_2 production in isolated skeletal muscle mitochondria [13]. As previously reported, these mitochondria do however show preserved aconitase activity (indicative of reduced superoxide within mitochondria) suggesting the lack of difference in H_2O_2 release may be due to the assay utilized rather than for biological reasons. This current report is the first to note that overexpression of Sod1 reduces mitochondrial ROS release and increases ATP production. While generally described as cytosolic, Sod1 can also be found in the mitochondrial intermembrane space (IMS) [11]. Previous data have suggested that increased expression of Sod1 targeted to the IMS can reduce mitochondrial oxidative stress [22]. Thus, it seems reasonable to interpret that mitochondrial oxidative stress is reduced by overexpression of either Sod1 (as measured by H_2O_2 release) or Sod2 (as measured by aconitase activity in [13]). However, neither seems to be directly linked to glucose metabolism as (1) mitochondrial H_2O_2 release differs in low fat fed mice but glucose tolerance does not and (2) mitochondrial H_2O_2 release is not affected by diet.

If not mitochondria function, then perhaps oxidative damage itself could be a better predictor of metabolic dysfunction associated with obesity. Several studies, including this one, have reported that increased levels of lipid, protein, and DNA oxidative damage are associated with both obesity and T2DM [2–4,7–9]. It might be predicted that increased antioxidant defense should prevent oxidative damage; however, this report is one of the first to directly test this hypothesis utilizing a model of diet-induced obesity. Uptake of glucose in skeletal muscle and adipose tissue is generally considered as the significant regulator of mammalian glucose levels. In general, increased Sod1 or Sod2 did reduce F_2-isoprostanes in muscle and adipose suggesting that glucose metabolism could be directly correlated with damage in these tissues.

In this study, our data show high fat diet increases oxidative damage but does not alter ROS production from muscle mitochondria, so the question remains on what exactly is promoting oxidative stress in obese mice? One particularly attractive candidate may be elevated chronic inflammation associated with fat accumulation. Increasing adipose tissue, particularly visceral adipose, alters intra-adipose T-cell subsets and stimulates macrophage infiltration and activation within adipose and other

tissues which can stimulate production of pro-inflammatory cytokines and generate a pro-oxidative environment in the adipose [5,23]. Certainly, examining whether Sod1 and Sod2 reduce these inflammatory processes in the context of obesity and disease models should be a significant focus of future studies.

Acknowledgments

Mitochondria and F2-isoprostane measurements were performed by the Mitochondrial Function and Oxidative Damage Core Facility of the San Antonio Nathan Shock Center of Excellence in the Basic Biology of Aging. This work was supported by the Geriatric Research Education and Clinical Center of the South Texas Veterans Health Care System, and grants from the Biomedical Laboratory Research & Development Service of the Veteran's Affairs Office of Research and Development by [VA Merit Review Grants 1I01BX001023 (Y.I.) and 1I01BX000547 (A.R.)], the American Federation for Aging Research (Y.I.) and the Glenn Foundation (Y.I.).

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