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Age-dependent mitochondrial energy dynamics in the mice heart: Role of superoxide dismutase-2☆

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ABSTRACT

The aging process alters cardiac physiology, decreases the number of cardiomyocytes and alters the energy metabolism. Mitochondrial dysfunction in aging is believed to cause these functional and phenotypic changes in the heart. Although precise understanding of alterations of mitochondrial respiration in aging is necessary to manage heart diseases in the elderly population conflicting data on the function of specific complex of electron transport chain of the heart mitochondria limits the intervention process. We have addressed these issues using the assay of mitochondrial coupling and electron flow to assess specific functional defects in mitochondria isolated from young or aged mice. Our results demonstrate that cardiac mitochondria from older mice utilize oxygen at a decreased rate via complex I, II or IV compared to younger mice. We further show that mitochondrial function decreases in young $Sod2^{+/-}$ mice heart compared to young wildtype mice. However, the mitochondrial function remains unchanged in older $Sod2^{+/-}$ mice heart compared to younger $Sod2^{+/-}$ mice heart. Further, the oxygen consumption remains similar in old wildtype mice and old $Sod2^{+/-}$ mice heart mitochondria. The expression and activity of Sod2 in young or old $Sod2^{+/-}$ mice heart remain unchanged. These data demonstrate that decreased oxygen utilization in older age could have resulted in decreased mitochondrial ROS-mediated oxidative damage requiring less Sod2 for protection against mitochondrial oxidative stress in older wildtype or older $Sod2^{+/-}$ mice.

1. Introduction

Elderly populations are at a greater risk of heart disease and suffer increased myocardial damage during and following an episode of heart attack. Aged hearts are more likely to fail due to ischemia–reperfusion injury compared to younger hearts (Hare, 2001; Lesnefsky and Hoppel, 2003). Additionally, aged heart suffers greater damage during reperfusion of ischemic myocardium resulting in myocardial infarction and progressive death of the heart tissue (Lesnefsky and Hoppel, 2006; Lesnefsky et al., 2001b; Ventura-Clapier et al., 2008). Mitochondria play a vital role in cardiac energy output and are critically important in energy-demanding cardiac functions (Dai and Rabinovitch, 2009). Oxidation of biological fuels is a critical source of energy required for efficient functioning of the heart (Lesnefsky et al., 2001a). The oxidation of fuels such as NADH, pyruvate or succinate is accomplished via the mitochondrial electron transport chain (ETC). During this process energy in the form of ATP is produced due to the coupling of ETC to the proton extrusion forming the proton gradient that subsequently generates ATP via ATP synthase. However, electron leak during the passage of electrons via ETC produces superoxide anions ($O_2^{\bullet-}$), which are converted to various forms of reactive oxygen species (ROS), and are detrimental to the very ETC which produces them (Brand and Nicholls, 2011). Constant burning of fuels by oxidative process over the life span of an organism is believed to oxidize the components of mitochondrial respiratory system that diminishes the function of various complexes of the mitochondrial ETC (Brand and Nicholls, 2011; Lesnefsky and Hoppel, 2006).

Superoxide dismutase-2 (Sod2) also known as manganese superoxide dismutase (MnSOD) is a mitochondrial matrix enzyme that converts $O_2^{\bullet-}$ (impermeable to mitochondrial membrane) to H_2O_2. H_2O_2 is permeable to mitochondrial membrane and is further degraded to molecular oxygen and water by catalase. Sod2 serves as a first line of defense to protect the mitochondria against deleterious $O_2^{\bullet-}$ in physiological and pathophysiological conditions. Although, free radical theory of aging (Harman, 1956) suggests that continuous generation of $O_2^{\bullet-}$ in the mitochondria throughout the life span of an organism initiates and accelerates the aging process, the supportive data to conclusively prove this theory remains inadequate. There has been no impact of 50% decrease in Sod2 on the life-span of mice (Jang and Remmen, 2009). Although partial decrease (~50%) in Sod2 expression in $Sod2^{+/-}$ mice heart decreases mitochondrial

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oxygen consumption in young mice, its effect on older mice has not been investigated. Taken together, it remains questionable whether accumulation of oxidative products decreases mitochondrial function in aging.

Although it is generally believed that mitochondrial function declines with age in the heart, controversies do exit due to variation in experimental procedures to assess mitochondrial function (Tatarkova et al., 2011; Van Remmen and Richardson, 2001). For example, studies have shown that aging process has no effect on cardiac mitochondrial function (Davies et al., 2001; Miro et al., 2000). In contrast, other studies have shown that mitochondrial function either declines or remains unaltered with the aging process (Kumaran et al., 2004; Rodriguez et al., 2007). Isolation procedure has been identified to be critical for functional analysis of cardiac mitochondria (Fannin et al., 1999). There are two distinct sub-populations of cardiac mitochondria; the subsarcolemmal (SSM) mitochondria that lie superficially beneath the plasma membrane, and the inter-fibrillar (IFM) mitochondria located between the myofibrils (Fannin et al., 1999; Palmer et al., 1977). Whereas only polytron homogenization releases mostly SSMs, protease digestion releases the IFMs (Lesnefsky et al., 2001a; Lesnefsky et al., 2004; Palmer et al., 1977). Studies have shown differential effect of the aging process on these two populations of mitochondria within the myocyte (Palmer et al., 1977). The assessment of integrated mitochondrial respiration by studying the maximal rate and coupling of oxidative phosphorylation uncovers age-related defects in oxidative metabolism not evident by isolated measurement of the enzyme activity of the individual electron transport chain complexes (Lesnefsky and Hoppel, 2006). Thus, the results and conclusions of experiments with aged mitochondria could be affected by the isolation procedure, specific endpoint measurement or modulation of maximal respiration, coupling and specific enzyme assays of each complex.

We sought to determine the effect of age on mouse heart mitochondrial coupling and electron flow with normal or decreased levels of Sod2 using wild-type (WT) or $Sod2^{+/-}$ mice. First, we optimized the bioenergetics assay with mouse heart mitochondria/well using the state-of-the art XF24 mitochondrial flux analyzer (Seahorse Bioscience, Billerica, MA). In this assay we integrated mitochondrial coupling and the electron flow experiments in the same assay plate and used uncoupler for maximal respiration determination and ADP for phosphorylating state 3 respirations to assess true mitochondrial capacity (Brand and Nicholls, 2011).

In this report we show that in aged mice a decline in function of complex I–IV occurs in the heart mitochondria. The decline is more pronounced in IFM mitochondria. Further, mitochondria isolated from the hearts of young $Sod2^{+/-}$ mice show diminished mitochondrial function compared to young WT mice. However, aged $Sod2^{+/-}$ mice do not show any change in cardiac mitochondrial function compared to young $Sod2^{+/-}$ mice. In addition, there was no difference in mitochondrial function between old WT and old $Sod2^{+/-}$ mice. The level of Sod2 expression and activity remains similar in young or old $Sod2^{+/-}$ mice which is about 50% of the WT mice. These studies demonstrate that whereas Sod2 levels affect mitochondrial function in young mice it does not affect mitochondrial function in older mice heart.

2. Materials and methods

2.1. Cell culture and transfections

Adenosine 5′-diphosphate sodium salt (ADP), antimycin A, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), ascorbic acid, succinate, malate, pyruvate and rotenone were purchased from Sigma Chemical Company (St. Louis, MO). Other chemicals used in the mitochondrial isolation buffer and mitochondria assay buffer (MAS) were purchased from Sigma chemical Co. Human microvascular endothelial cells (HMVEC) were purchased from Lonza CO, NJ, and were grown and propagated in endothelial basal medium with supplements (EGM-MV2; Lonza, Co., NJ). HMVEC were transfected with Sod2 siRNA (3′-GGA GCA CGC UUA CUACCUUUUdTdT-5′) or luciferase (Luc) siRNA (3′CUU ACG CUG AGU ACU UCG Att) obtained from Dharmacon, Arvada, CO, and Qiagen (Valencia, CA) using Xtremegene transfection reagent (Roche Biotech, Indianapolis, IN). After 48 h of transfection cells were sub-cultured and 25,000 cells were seeded onto v7 tissue culture plate (Seahorse Biosciences, Billerica, MA) for mitochondrial bioenergetics assay.

2.2. Animals and isolation of mitochondria

Wildtype c57BL/6 or $Sod2^{+/-}$ mice were purchased from Jackson laboratory (Bar Harbor, ME) and were bred and maintained in the animal facility of Texas Tech University Health Sciences Center (TTUHSC), Lubbock, TX. The protocol was approved by the institutional animal care and use committee (IACUC) of the TTUHSC. Hearts of mice (young, 2–4 months or old, 22–28 months of age) were surgically removed from anesthetized animals. Mitochondria from hearts of mice were isolated following the protocol for rats published by Rogers et al. (2011) with following modifications. One mouse heart was minced and homogenized at 4 °C using polytron homogenizer (Mini Genie, Fisher Biotech) in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM HEPES, pH 7.2, 1 mM EGTA and 0.5% fatty acid free BSA). The homogenate was centrifuged at 27,000 ×*g* for 10 min. The pellet was resuspended in the same buffer and was centrifuged at 500 *g* for 5 min. The supernatant was passed once through 70 μ filters and once through 40 μ filters (BD Biosciences, CA) and then centrifuged for 5 min at 10,000 ×*g*. The mitochondrial pellet was suspended in mitochondrial isolation buffer without BSA and protein was estimated with bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Mitochondria were suspended at 1–2 μg/50 μl in 1× mitochondrial assay buffer (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, and 0.2% fatty acid free BSA; pH 7.2 at room temperature) and plated into each well of the v7 assay plate of XF24 analyzer.

2.3. Isolation of subsarcolemmal (SSM) and interfibrilar (IFM) mitochondria

SSM and IFM were isolated from mice heart following the methods developed by Palmer et al. for Fisher 344 rats with modifications (Palmer et al., 1977). Briefly, mice hearts were washed and minced in ice-cold buffer A (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4, 2 mM EGTA and 0.2% fat free BSA). The minced tissue was homogenized in a polytron homogenizer (Mini Genie, Fisher Biotech.) for 3–4 s at a setting 6. The polytron homogenate was centrifuged at 500 ×*g* for 10 min at 4 °C using ss34 rotor (Sorval) using a Sorval RC5 centrifuge. Buffer A was added to polytron pellet in a Potter–Elvehjem homogenizer and centrifuged at 500 ×*g* for 5 min. The original supernatant from the polytron homogenizing and Potter–Elvehjem supernatant were pooled and centrifuged at 3000 ×*g* for 10 min to obtain subsarcolemmal mitochondria (SSM). The Potter–Elvehjem pellet was resuspended in buffer B (100 mM KCl, 50 mM MOPS, pH 7.4, 2 mM EGTA, 0.2%BSA) and Peptidase (Sigma Chemical Co, St. Louis, MO) at a concentration of 5 mg/g of heart tissue and resuspended using Potter–Elvehjem homogenizer. The homogenate was diluted 2-fold with buffer B and centrifuged 5000 ×*g* for 5 min. The pellet was resuspended in original volume of buffer B without peptidase and centrifuged 300 *g* to pellet the nuclei. The resulting supernatant was centrifuged at 3000 ×*g* for 10 min to obtain the IFM mitochondria. Protein was quantified using BCA (Pierce, Rockford, IL) protein assay.

2.4. XF24 instrument setup and analysis

2.4.1. Isolated mitochondria

XF24 instrument was equilibrated at 37 °C overnight. 1 μg of mouse heart mitochondria was plated in each well of the XF24 v7 plate in a