Published in final edited form as:

Free Radic Biol Med. 2013 January ; 54: 116–124. doi:10.1016/j.freeradbiomed.2012.08.573.

The peroxidase activity of mitochondrial superoxide dismutase (MnSOD/SOD2)

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Abstract

Manganese superoxide dismutase (MnSOD) is an integral mitochondrial protein known as a first line antioxidant defense against superoxide radical anions produced as by-products of the electron transport chain. Recent studies have shaped the idea that by regulating the mitochondrial redox status and H₂O₂ outflow, MnSOD acts as a fundamental regulator of cellular proliferation, metabolism and apoptosis thereby assuming roles that extend far beyond its proposed antioxidant functions. Accordingly, allelic variations of MnSOD that have been shown to augment levels of MnSOD in mitochondria result in a 10-fold increase in prostate cancer risk. In addition, epidemiologic studies indicate that reduced glutathione peroxidase (GPx) activity along with increases in H₂O₂ further increase cancer risk in the face MnSOD overexpression. These facts led us to hypothesize that, like the Cu, Zn-counterpart, MnSOD may work as a peroxidase, utilizing H₂O₂ to promote mitochondrial damage, a known cancer risk factor. Here we report that MnSOD indeed possesses peroxidase activity that manifests in mitochondria when the enzyme is overexpressed.

INTRODUCTION

Manganese superoxide dismutase (MnSOD) is a homotetrameric protein that is exclusively confined to mitochondria in mammalian cells [1-3]. In the mitochondrial matrix, MnSOD rapidly scavenges and dismutates O₂^{•-} producing H₂O₂ and O₂ at a 1:1 ratio. Because O₂^{•-}

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in fairly specific contexts can act as an oxidant $E^{\circ}_{\text{OO}^-/\text{H}_2\text{O}_2} = +0.9 \text{ V}$ vs $E^{\circ}_{\text{OO}^-/\text{O}_2} = -0.33 \text{ V}$ [4], the capacity of MnSOD to act as a superoxide dismutase has been regarded as protective to mitochondria against oxidative damage. Further studies on the consequences of MnSOD downregulation to mitochondrial function confirmed that at normal levels MnSOD is a first line mitochondrial antioxidant defense against electron transport chain-derived collateral oxidative stress [5-8]. In support of this idea, studies by several authors showed that mild (2-3 fold) MnSOD overexpression effectively reduces mitochondrial $\text{O}_2^{\bullet-}$, generally correlating with improved mitochondrial function [5,9,10]

Other studies confirmed the prominent role of MnSOD in preserving the activity of Fe-S cluster containing enzymes in mitochondria (notably aconitase and NADPH oxidase complex [11]) and implicated Cu,ZnSOD in acting as a complementary defense mechanism against superoxide-dependent enzyme inactivation in mitochondria [12]. A turning point in the field took place in the late 1990's when studies by Oberley [13-17], St Clair [18,19], Melendez [20-26] and others [27-29] showed that MnSOD expression impinges significant changes on cell signaling events, strongly suggesting that MnSOD has roles in mitochondria that extend far beyond that of an antioxidant enzyme.. With the demonstrations that MnSOD directly impacts cell proliferation [13,30] and bi-directionally regulates p53 [31-36], many groups have contributed to show that MnSOD is a critical player working centrally in the control of mitochondria-dependent regulation of signaling networks. Moreover, the demonstration that the expression of mitochondrial catalase reverses many of the effects elicited by MnSOD overexpression indicated that H_2O_2 is critically involved in the mediation of MnSOD-dependent effects [37]. Along the same lines, epidemiologic studies demonstrated that MnSOD accumulation in mitochondria resulting from frequent polymorphisms encoding for the A-containing enzyme becomes an accurate prostate cancer risk factor when cellular antioxidant systems that detoxify H_2O_2 are deactivated or overwhelmed [38,39]. Together with findings that MnSOD overexpression sensitizes cells and in particular mitochondria to H_2O_2 [18, this work] these epidemiological observations led us to surmise that in addition to its well-documented superoxide dismutase, MnSOD might possess an undocumented peroxidase activity which would enable the enzyme to directly interact with its product H_2O_2 . We also hypothesized that such activity would be especially evident in conditions when MnSOD is upregulated, an intriguing possibility that would be in accordance to the observation that MnSOD overexpression can either protect or worsen [18,40,41]mitochondrial functions in a context dependent manner. Using various approaches, here we show that MnSOD analogously to inorganic Mn-complexes [42-44] possesses peroxidase activity that manifests in mitochondria when the enzyme is overexpressed. Such activity leads to mitochondrial dysfunction and increased sensitivity of the organelle to oxidative stress. Taken together, our findings suggest that the levels of MnSOD in mitochondria are likely critical in determining cellular outcomes. Our novel findings should contribute to the understanding of the multiple roles of MnSOD in cells and, importantly, in elucidating its role in signaling, oxidative stress sensitivity and cancer risk.

EXPERIMENTAL PROCEDURES

Chemicals

Recombinant MnSOD from human mitochondria was produced by the protein expression core facility at the National Institute of Environmental Health Sciences, MitoTracker Red CMXRos and Amplex Red were obtained from Molecular Probes/Invitrogen, (Carlsbad, CA), the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Alexis, (Plymouth Meeting, PA) and purified by distillation under vacuum. All antibodies against mitochondrial electron transport chain complex components were purchased from Invitrogen, (Carlsbad, CA). Anti-MnSOD was obtained from Santa Cruz Biotechnologies, (Santa Cruz, CA). Sodium phosphate was purchased from Mallinckrodt Baker Inc. (Paris, KY). Chelex 100 resin was purchased from Bio-Rad Laboratories (Hercules, CA). Buffers used in the experiments were treated with Chelex 100 resin to remove traces of transition metal ions. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were analytical grade or better.

Reconstitution of MnSOD with MnCl_2

Recombinant human MnSOD (10 mg/mL) was mixed with PBS and 10 mM MnCl_2 . After 30 min, the protein was desalted using polyacrylamide spin desalting columns from Pierce Thermo Scientific (Rockford, IL) according to the manufacturer instructions. The desalted MnSOD was diluted 10 times with Chelex-treated 100 mM phosphate buffer pH 7.4 and transferred to a Protein Concentrator (Thermo Scientific Pierce, Rockford, IL). The ultrafiltration device was centrifuged at 4°C to a minimal protein solution volume (approximately 50 μL). The dilution followed by the ultrafiltration was repeated three times for the total buffer exchange and protein wash from remaining MnCl_2 .

Cell Cultures

MCF-7 cells stably expressing an empty vector (neo) or MnSOD (Mn11) were a generous gift from Dr. Larry Oberley, University of Iowa. The cells were cultured in RPMI 1640 media supplemented with 10% FBS, penicillin (30 mg/L)/ streptomycin (50 mg/L) and neomycin (50 mg/L). The cells were grown under a 5% CO_2 atmosphere at 37°C. Treatments with glucose oxidase or exogenous H_2O_2 were performed in serum-free media for 15 minutes prior to replenishment with pre-conditioned media.

Confocal Microscopy

Cells were plated onto MatTek glass bottom culture dishes (1.5 mm thickness) and allowed to adhere overnight. After treatments were performed, cells were washed with PBS and fixated with 4% paraformaldehyde. Cells were permeabilized using methanol (−20°C). Images were recorded using a Zeiss LSM510UV microscope.

Electron Paramagnetic Resonance Experiments

EPR spectra were recorded on a Bruker EMX EPR spectrometer (Billerica, MA) operating at 9.81 GHz with a modulation frequency of 100 kHz and equipped with an ER 4122 SHQ

cavity. All experiments were performed at room temperature with a 10-mm quartz flat cell. CAT1H was purchased from Alexis and used as supplied

Visible Spectrometry studies

All optical measurements were carried out with a Varian Cary 100 Bio spectrophotometer.

Gel electrophoresis and Western Blot Analysis

Protein derivatives were analyzed by separating the protein fractions by their molecular weight on 4-12 % Bis-Tris gels under reducing and denaturing conditions (NuPAGE system, Invitrogen, Carlsbad, CA) followed by electroblotting on nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-T. After blocking, membranes were washed once with TBS-T (TBS pH 7.4 with 0.05% Tween) and incubated with primary antibody rabbit anti-DMPO serum 1:5,000 (Dojindo, Rockville, MD), rabbit anti-SOD2 1:1,000 (Abcam), After 3 washes with TBS-T, secondary antibody anti-rabbit IgG-alkaline phosphatase 1:5,000 (Pierce Chemical Co., Rockford, IL), in washing buffer, was added and incubated for 60 min. After 3 more washes with TBS-T, the antigen-antibody complexes were analyzed with a chemiluminescence system (CDP-Star, Roche Molecular Biochemicals, IN). Gels were stained with the Coomassie-based stain SimplyBlue™ (Invitrogen, Carlsbad, CA) according to the manufacturer instructions.

Electron Microscopy

Samples from control and Mn11 cells treated and untreated with H₂O₂ were evaluated using EM to define mitochondrial structural changes. Sections (approximately 1 mm cubes) were rapidly fixed in diluted Karnovsky's fixative and processed for EM. Embedded sections (0.5 μ) were cut with a glass knife and stained with toluidine blue for orientation. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and viewed on a Philips Morgagni electron microscope (Philips, Amsterdam NL). Structurally damaged mitochondria were operationally defined as having loss or dissolution of 25% of cristae; alteration of size and number of mitochondria per cell and vacuolization were also considered.

Fluorescent Immunocytochemistry

Cells were plated on glass coverslips in 6-well plates and grown to appropriate confluence overnight. Cells were washed with PBS, and then fixated with 4% paraformaldehyde. Cells were permeabilized with 100% ethanol, and then incubated in primary antibody overnight at 4°C. Cells were washed and incubated in secondary antibody for one hour at room temperature the next day. Coverslips were then mounted on SuperFrost slides (VWR) with Vectashield Hard Set Mounting Media with DAPI (Vector Laboratories). Slides were then examined on a Nikon ECLIPSE E400 microscope and were documented using SPOT Advanced version 4.0.1 software.

Gene-specific Quantitative PCR (QPC)

QPCR was used to assay mitochondrial DNA (mtDNA) integrity in neo and Mn11-expressing cells as previously described [45,46]. Briefly, total genomic DNA was isolated in

mtDNA integrity analyzed by quantitatively amplifying a 8.9kb and a 221 bp fragments of the mitochondrial genome. Amplification of glucose oxidase (GO)-treated samples were compared to non-treated controls and relative amplifications calculated. These measurements were used to estimate the lesion frequency present on the DNA based on a Poisson distribution. MtDNA copy number was monitored and used to normalize the data obtained with the large fragment. For basal levels of lesions, non-treated Mn11 cells were compared to non-treated neo. Experiments were repeated at least 3 independent times, statistical significance was evaluated using unpaired Student's *t*-test.

RESULTS

MnSOD possesses peroxidase activity

The intrinsic peroxidase activity of MnSOD was measured using Amplex Red as the peroxidase substrate. Resulting resorufin, the oxidation product of Amplex Red, has a high extinction coefficient ($\epsilon_{571\text{nm}} = 54,000 \text{ M}^{-1}\text{cm}^{-1}$) which permitted the assessment of the peroxidase activity of small quantities of human mitochondrial MnSOD (Fig. 1). It is important to note that the resorufin generation results from a 2-electron oxidation of Amplex Red which allows the specific determination of peroxidase activities. Incubation of MnSOD with Amplex Red in the presence of H_2O_2 led to a marked increase of visible absorption at 571 nm that was found to be H_2O_2 (Fig. 1A) and MnSOD (Fig. 1B) dose-dependent.

Purified recombinant human MnSOD had a specific dismutase activity of 1130 U/mg of protein. Incubation with 10 mM MnCl_2 for 30 min in PBS, followed by the removal of the excess metal (see Experimental Procedures), increased the enzyme specific activity as superoxide dismutase by 1.9-fold (2179 U/mg of protein) (supplemental material Fig. 1). This protein preparation in the presence of H_2O_2 and Amplex Red showed a 1.8-fold higher peroxidase activity (0.94 and 1.69 mU/mg of protein) (Fig. 1C) confirming that both the superoxide dismutase and the peroxidase activities depend on the enzyme-bound manganese atoms.

Fig. 1D shows complementary immuno-spin trapping experiments. Results showed that in the absence of sacrificial substrates and in the presence of H_2O_2 , MnSOD oxidizes its own amino acid side chains, as demonstrated by 5,5,-dimethyl-pirroline-*N*-oxide (DMPO) labeling in a typical peroxidase reaction.

MnSOD peroxidase activity leads to mitochondrial protein oxidation

Next we interrogated whether MnSOD possesses the capacity to act as a peroxidase in mitochondria of cultured cells. Based on numerous studies that showed the protective effects of mild MnSOD overexpression, it seemed unlikely to us that at basal to slight overexpression levels MnSOD would act as a detrimental peroxidase negatively impacting mitochondria homeostasis. It is known, nevertheless, that in certain advanced cancers MnSOD is markedly overexpressed. Although commonly reported, the biological significance of this observation is unknown. Therefore, we examined in MCF-7 cells, a breast-cancer derived cell line, whether MnSOD exhibits peroxidase activity when markedly overexpressed (8 – 10 fold). To this end, we performed experiments in MCF-7 cells

expressing empty vector (heretofore called neo), and in a derivative where MnSOD is ectopically expressed (referred to as Mn11). These cell lines were derived by Dr. Larry Oberley (University of Iowa) and have been extensively studied [47,48]. The levels of MnSOD overexpression in Mn11 are about 8-10 fold above control as quantified by us by immunofluorescence (Fig. 2B). Experiments shown in Fig. 2A and C indicate that at this level of overexpression, MnSOD can produce evident protein oxidation in mitochondria when in the presence of H₂O₂. Fig. 2A shows using immuno-spin trapping (40-49) DMPO-trapped adducts on MnSOD itself in the presence of glucose oxidase (GO), a steady generator of H₂O₂. The anti-DMPO signal was also observed using fluorescence imaging, which revealed not only that MnSOD overexpression leads to increased protein oxidation in the presence of bolus H₂O₂ but also that, contrary to neo controls, the signal is present both in the nucleus and mitochondria (Fig. 2C). No significant differences were observed by overexpression of MnSOD per se in the absence of H₂O₂ (Fig. 2). Thus, based on the requirement for H₂O₂, we conclude that MnSOD can function as a mitochondrial peroxidase in specific contexts.

MnSOD peroxidase activity promotes loss of mitochondrial membrane potential and alters mitochondrial ultrastructure

The intriguing finding that MnSOD overexpression predisposes mitochondria to oxidative stress led us to assess some of the consequences of increased peroxidative mitochondrial activity to a biological outcome. Mitochondrial membrane potential (ψ_m) is a key indicator of mitochondrial function and cellular viability as it reflects the pumping of proton ions across the inner membrane during electron transport and ATP production. Thus, we assessed the ability of neo and Mn11 cells to maintain their (ψ_m) upon challenge with exogenous H₂O₂. ψ_m measurements were carried out based on the import of the cationic probe 1H, 5H, 11H, 15H-xantheno[2,3,4-ij,5,6,7-, 'j']diquinolizin-18-ium, 9-[4-(chromomethyl)phenyl]-2,3,6,7,12,13,16,17-octahydro-chloride (CMXROS, Fig. 3A) and the fluorescent dye 5'5'6'6'-tetrachloro-1'1'3'3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), which can ratiometrically determine the amount of polarized or depolarized mitochondria in a population (Fig 3B).

Briefly, neo and MnSOD-overexpressing Mn11 cells, were exposed to H₂O₂ in serum free RPMI 1640 media for 15 minutes. After 4 h of recovery in pre-conditioned media, cells were loaded with CMXROS and the ψ_m was analyzed using a confocal microscope. CMXROS is imported to the mitochondria in a potential-dependent manner and is maintained permanently inside of the organelle due to its capacity to covalently react with mitochondrial-protein thiol and amine groups. As shown in Fig. 3A, ψ_m was markedly reduced in Mn11 cells 4h after acute H₂O₂ exposure as judged by decreased fluorescence, while only modest changes were observed in neo controls. The loss of ψ_m was dependent on the concentration of H₂O₂ in Mn11 cells (Fig. 3A right panels). Several chemotherapeutic and radiation based approaches are notorious for producing acute oxidative stress, hence, these experiments suggest that mitochondria of cells that overexpress MnSOD are more likely to lose their potential post-treatment. Similar results were obtained when probing the ability of cells to maintain the ψ_m after H₂O₂ treatment with JC-1 (Fig. 3B). Accumulation of JC-1 in mitochondria, which requires high ψ_m ,

favors the formation of oligomers that fluoresce red. The inefficient import of JC-1, associated to low ψ_m , keeps the probe in its monomeric form that fluoresces green. The use of JC-1 also indicated that overall overexpression of MnSOD *per se* modestly increase the amount of depolarized mitochondria that is accompanied by a decrease in the polarized population (Fig. 3B compare panels on first and third row, see boxed areas). Mitochondrial impairment is often accompanied by changes in organellar morphology such as swelling, loss of cristae and the appearance of megamitochondria. Thus, we next evaluated mitochondrial ultrastructure using EM. Untreated Mn11 cells showed signs of mitochondrial structural integrity loss and membrane disruption when compared to non-treated neo controls (Fig. 4, upper panels). No apparent changes in the ultrastructure of mitochondria were detected in neo cells exposed to varying concentrations of H₂O₂ (0-100 μ M) for 15 minutes. In contrast, Mn11 cells treated with H₂O₂ showed apparent mitochondrial membrane damage, cristae disorientation, and mineralization which was worsened in cells treated with higher concentrations of H₂O₂ (100-250 μ M). Cristae remodeling due to oxidative stress was extensive in cells overexpressing MnSOD while fairly modest in the controls (Fig. 4).

MnSOD overexpression dampens mitochondria respiratory rate and maximum respiratory capacity

Further experiments using extracellular flux analyzer (Seahore Biosciences) showed that mitochondrial respiration and mitochondrial maximum respiratory capacity were reduced by MnSOD overexpression. Mitochondrial respiration and the maximum respiratory capacity were further reduced by H₂O₂ treatment especially in MnSOD overexpressing cells. Consistent with data presented in Figs. 3 and 4, ATP-linked respiration was also reduced in Mn11 cells as compared to neo, confirming that MnSOD overexpression *per se* negatively impacts mitochondrial function. H₂O₂ did not further reduce this parameter in either cell type, suggesting that the cells can maintain their ATP production upon oxidative stress. However, the decreased respiratory capacity together with the low ATP levels in Mn11 already at basal states indicate the mitochondria of these cells are likely uncoupled due to the significant level of oxidative damage promoted by MnSOD overexpression especially in the presence of H₂O₂.

MnSOD-overexpression increases mitochondrial DNA (mtDNA) damage

Lastly, to further support the findings that MnSOD overexpression is detrimental to mitochondria, we used gene-specific quantitative PCR (QPCR) to evaluate mtDNA integrity in the cells at basal states and upon oxidative stress. Given the proximity of the mitochondrial genome to the main site of reactive oxygen species (ROS) generation, it is generally accepted that the mtDNA is a critical target for oxidative damage. Once damaged, mtDNA amplifies oxidative stress due to decreased expression of critical protein components of the electron transport chain. Such effects lead to a vicious cycle of increasing ROS generation and organelle deregulation that can eventually trigger apoptosis [49],[50]. Thus, mtDNA integrity is an adequate indicator of endogenous oxidative stress and proper mitochondrial function. Results presented in Fig. 6 show that basal levels of mtDNA damage were about 8 fold higher by overexpression of MnSOD *per se* as compared to neo controls (Fig. 6 bars on the left). H₂O₂ exposure increased mtDNA damage in both cell

types, which was significantly exacerbated in Mn11. These results show that overexpression of MnSOD sensitized mtDNA to oxidative damage. Taken together our results indicate that when overexpressed MnSOD gain of function as a peroxidase contributes to mitochondrial dysfunction.

DISCUSSION

MnSOD has long been recognized to be important against mitochondria-generated oxidants because of its well-known superoxide dismutase activity. Indeed, several studies have established that many of the cellular effects of MnSOD can be attributed to the superoxide scavenging ability of the enzyme that restricts superoxide-induced iron release from iron-sulfur components of mitochondrial enzymes thereby limiting loss of catalytic function and deleterious Fenton-reactions [5,51]. However, recent observations have implied that MnSOD may function as a mediator of numerous cellular processes beyond its superoxide dismutase activity [13-26]. The effects of MnSOD are complex and interdependent of multiple regulatory mechanisms in opposition to the general assumption that increased oxidative stress resulting from inefficient superoxide scavenging accounts for all abnormalities resulting from MnSOD deficiency [[52], this study]. In the current study, we show that in addition to its superoxide dismutase, MnSOD possesses an intrinsic peroxidase activity that is clearly observed when the enzyme is overexpressed. Although relatively low when compared to that of other peroxidases (i.e. horseradish peroxidase) was demonstrated in this study to impact mitochondria especially in conjunction with H_2O_2 . Hence, the prevailing net result of MnSOD activity in defending or sensitizing mitochondria to oxidative stress (by acting as a dismutase or peroxidase) was demonstrated to depend on its expression levels which will drastically change the capacities of the enzyme to impact numerous redox cellular processes. Because MnSOD governs mitochondrial redox status and the outflow of H_2O_2 from mitochondria, understanding its interactions with its signaling active product is of importance. Based on the capacity of MnSOD to differently regulate H_2O_2 production and disposition depending on specific scenarios, we hypothesized that the H_2O_2 dependent effects of MnSOD relied on an undocumented peroxidase activity. Consistent with this hypothesis we demonstrated in vitro that MnSOD is capable of utilizing H_2O_2 to execute typical peroxidase reactions (Fig. 1). It is also noteworthy that the treatment with $MnCl_2$ led to an increase in the specific dismutase activity of the recombinant purified MnSOD in the same magnitude of the increase in its peroxidase activity, which corroborates that MnSOD has a direct peroxidase activity (Fig. 1C). We also showed that the peroxidase activity is operative in mitochondria in cells (Fig. 2), and that it significantly impairs mitochondrial function especially in the presence of H_2O_2 . For the presented experiments we utilized exogenous H_2O_2 addition or exposure to glucose/glucose oxidase which generates steady flows of H_2O_2 certainly above of what is typically found *in vivo*. However, the fact that MnSOD overexpression *per se* in most cases sufficed to dampen mitochondrial energetic functions argues for the importance of MnSOD overexpression as a contributing factor to mitochondrial failure. These findings may have implications in pathologic cases where MnSOD overexpression is observed. For instance, there is a broad body of literature reporting marked overexpression of MnSOD in conjunction with oxidative stress in a variety of advanced cancers [53-56]. Interestingly, in advanced cancers mitochondrial failure

accompanies the shift in cellular metabolism towards aerobic glycolysis, which maintains cells viable in the face of mitochondrial collapse. The mechanisms underlying such phenotypes are still ill defined but it is possible that the novel peroxidase activity of MnSOD uncovered in this study contributes to such state. Our studies offer an additional mechanism that likely supports the known progressive mitochondrial dysfunction known to be a hallmark of cancer cell transitioning to advanced stages (Fig. 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge Dr. Ann Motten and Mrs. Mary J. Mason for their valuable assistance in the preparation of this manuscript, Dr. Larry Oberley (in memoriam) for the generous gift of neo and Mn11 cells. We are also indebted to Mrs. Deloris Sutton (NIEHS/NIH) for the acquisition of the electron microscopy images. This work was supported in part by the intramural research program of the National Institutes of Health (NIEHS), by funds of the College of Medicine, University of Illinois at Chicago and American Heart Association Grant No. 09SDG2250933 to M.G.B. K. A. is supported by a NIH T32 training grant HL072742-08

ABBREVIATIONS

CAT1H	1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride
Cu, Zn-SOD	Copper, zinc-dependent superoxide dismutase
DMPO	5,5-dimethyl-pyrroline- <i>N</i> -oxide
EM	electron microscopy
EPR	electron paramagnetic resonance
MnSOD	manganese superoxide dismutase

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Figure 1. Peroxidase activity of MnSOD characterized *in vitro*

Reactions were performed in Chelex-treated phosphate buffer (100 mM, pH 7.4) at room temperature. In the panels A, B the generation of the resulting Amplex Red oxidation-product resorufin was followed spectrophotometrically at 571 nm. **Panel A** shows the H₂O₂ concentration dependence (0.2 to 2.0 mM) on the oxidation of Amplex Red (200 μ M) catalyzed by MnSOD (1 mg/mL). **Panel B** shows the enzyme concentration dependence (0.25 to 1 mg/mL) on the oxidation of Amplex Red (200 μ M) in the presence of H₂O₂ (1 mM). In **panel C**, samples of recombinant human MnSOD (120 μ g/mL) and preincubated MnSOD with MnCl₂ (120 μ g/mL) were submitted to the peroxidase activity assay with Amplex Red (200 μ M) and H₂O₂ (1 mM). From the initial rates of the resorufin generation ($\epsilon_{571\text{nm}} = 54,000 \text{ M}^{-1}\text{cm}^{-1}$) the specific peroxidase activities were calculated as 0.94 and 1.69 mU/mg of protein. In the **panel D** samples of MnSOD (0.5 mg/mL) were incubated with 50 mM DMPO and 1 mM H₂O₂ for 1h. The samples were diluted and submitted to electrophoresis and western blotting anti-DMPO (5 μ g of protein/lane) as describe in Experimental Procedures. **Panel E**, shows electron paramagnetic resonance experiments

using the hydroxylamine probe CAT1H incubated with either MnSOD (0.25 mg/ml), H₂O₂ or both at the indicated concentrations of H₂O₂.

Figure 2. MnSOD peroxidase activity in mitochondria

(A) MnSOD was immunopurified from whole cell lysates by immunoprecipitation. Immunoblot with anti-DMPO antibody shows the formation of MnSOD-DMPO adducts only in Mn11 cells exposed to the H_2O_2 generating system glucose/glucose oxidase. (B) MnSOD staining (red) in neo and Mn11 cells. (C) Control and MnSOD overexpressing cells were stained and subjected to confocal microscopy. Green staining shows mitochondrial complex III core and red stain shows DMPO-nitron adducts. Neo and Mn11 cells were exposed for 4 h to DMPO (40 mM) in the presence and in the absence of glucose (5mM)/glucose oxidase (0.01 U/ml). Staining of DMPO-protein nitron adducts reveals oxidation of mitochondrial proteins in MnSOD overexpressing Mn11 cells compared to control neo cells. Inset, shows the formation of MnSOD-DMPO nitron adducts in mitochondria of Mn11 cells.

Figure 3. H₂O₂ causes mitochondrial dysfunction in cells overexpressing MnSOD

Panel A- Cells were exposed to CMXRos after incubation with H₂O₂ to measure remaining mitochondrial membrane potentials. CMXRos 10μM was added to the media 4h after H₂O₂ treatments. After 10 minutes cells were washed 2 × with PBS prior to fixation. Mn11 cells overexpressing MnSOD showed a dose dependent decrease in membrane potential with H₂O₂ treatment, while the neo cells were minimally affected by the treatments. **Panel B –** JC-1 (25 μM) was added to culture media 4h post H₂O₂ (50 μM) challenge. Cells were exposed to H₂O₂ for 15 minutes in serum free media and then returned to pre-conditioned media for 3:45min. JC-1 was allowed to be in contact with cells for 20 minutes prior to live cell imaging.

Figure 4. Electron microscopy of neo and Mn11 cells exposed to H₂O₂

Cells were exposed to H₂O₂ for 15 minutes in serum free media prior to recovery in preconditioned media for 3:45minutes. After H₂O₂ challenge cells were subjected to electron microscopy. Red arrows indicate that mitochondria in MnSOD overexpressing cells suffered extensive mineralization (dark spots enveloped by the double mitochondrial membranes). Images show that MnSOD overexpression *per se* induces significantly damage to mitochondria as shown by the numerous discontinuations and ruptures in mitochondrial double membrane even in the untreated group. Significant cristae disorientation and shrinkage is noticeable especially in Mn11 cells exposed to higher concentrations of peroxide. In the case of neo milder to more severe swelling is observed in H₂O₂ treated groups with some cristae disorientation evident at the highest concentration of H₂O₂ 250 μM.

Figure 5. MnSOD overexpressing cells have altered cellular bioenergetics

Neo and Mn11 cells were compared with or without H₂O₂ treatment using an extracellular flux analyzer. Mn11 cells showed loss of (A) basal respiration, (B) ATP turnover and (C) maximal reserve capacity

Figure 6. MnSOD overexpression sensitized the mtDNA to H₂O₂

QPCR was performed to evaluate mtDNA integrity in neo and Mn11 cells exposed or not to glucose oxidase. Briefly, total genomic DNA was isolated and mtDNA integrity analyzed using primers that amplify a 8.9 Kb fragment of the mitochondrial genome. Data were normalized to mtDNA content based on amplification of a small 221 bp fragment as described previously (Details in Materials and Methods). Results represent 3 independent experiments, standard errors reflect $\pm$ SEM.

Figure 7.

Schematic representation of our hypothesis that optimal levels of MnSOD warrant health whereas diminishment or overexpression of MnSOD in mitochondria result in oxidative stress and damage.