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Original Contributions

The peroxidase activity of mitochondrial superoxide dismutase

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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 H_2O_2 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 activity along with increases in H_2O_2 further increase cancer risk in the face of MnSOD overexpression. These facts led us to hypothesize that, like its Cu,ZnSOD counterpart, MnSOD may work as a peroxidase, utilizing H_2O_2 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 $O_2^{\bullet -}$, producing H_2O_2 and O_2 at a 1:1 ratio. Because $O_2^{\bullet -}$ in fairly specific contexts can act as an oxidant ($E^{\circ}O_2^{\bullet -}/H_2O_2 = +0.9$ V vs $E^{\circ}O_2^{\bullet -}/O_2 = -0.16$ V) [4,5], the capacity of MnSOD to act as a superoxide dismutase has been regarded as protective of mitochondria against oxidative damage. Further studies on the consequences of MnSOD downregulation for mitochondrial function confirmed that at normal, levels MnSOD is a first-line mitochondrial antioxidant defense against electron transport chain-derived collateral oxidative stress [6–8]. In support of this idea, studies by several authors showed that mild (two- to threefold) MnSOD overexpression effectively reduces mitochondrial $O_2^{\bullet -}$, generally correlating with improved mitochondrial function [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 dehydrogenase 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 1990s when studies by Oberley et al. [13–17], St Clair et al. [18,19], Melendez et al. [20–26], and others [27–29] showed that MnSOD expression imposes 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 influences cell proliferation [13,30] and bidirectionally regulates p53 [31–36], many groups have contributed to showing 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 the alanine-containing isoform enzyme becomes an important prostate cancer risk factor when cellular antioxidant systems that detoxify H_2O_2 are deactivated or overwhelmed [38,39].

Abbreviations: CAT1H, 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride; Cu, ZnSOD, copper, zinc-dependent superoxide dismutase; DMPO, 5,5-dimethyl pyrroline-*N*-oxide; EM, electron microscopy; EPR, electron paramagnetic resonance; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; MnSOD, manganese superoxide dismutase.

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Together with findings that MnSOD overexpression sensitizes cells and in particular mitochondria to H_2O_2 [18] these epidemiological observations led us to surmise that in addition to its well-documented superoxide dismutase activity, MnSOD might possess an undocumented peroxidase activity that would enable the enzyme to interact directly with its product H_2O_2. We also hypothesized that such activity would be especially evident when MnSOD is upregulated, an intriguing possibility that would be in accordance with the observation that MnSOD overexpression can either protect or worsen [18,40,41] mitochondrial functions in a context-dependent manner. This hypothesis is based on the premise that when overexpressed, MnSOD is enabled to outcompete H_2O_2-detoxifying systems in mitochondria. 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 to be critical in determining cellular outcomes. Our novel findings should contribute to the understanding of the multiple roles of MnSOD in cells and, importantly, to the elucidation of 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, USA); the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). All antibodies against mitochondrial electron transport chain complex components were purchased from Invitrogen (Grand Island, NY, USA). Anti-MnSOD was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Sodium phosphate was purchased from Mallinckrodt Baker (Paris, KY, USA). Chelex 100 resin was purchased from Bio-Rad Laboratories (Hercules, CA, USA). 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, USA) and were analytical grade or better.

Reconstitution of MnSOD with $MnCl_2$

MnSOD was reconstituted by mixing recombinant human MnSOD (10 mg/ml) with phosphate-buffered saline (PBS) and 10 mM $MnCl_2$. After 30 min, the protein was desalted using polyacrylamide spin desalting columns from Pierce Thermo Scientific (Rockford, IL, USA) according to the manufacturer's instructions. The desalted MnSOD was diluted 10 times with Chelex-treated 100 mM phosphate buffer, pH 7.4, and transferred to a protein concentrator (Pierce Thermo Scientific). 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 to effect total buffer exchange and to wash the remaining $MnCl_2$ from the protein.

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 medium supplemented with 10% fetal bovine serum, 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 medium for 15 min before replenishment with preconditioned medium.

Confocal microscopy

Cells were plated onto MatTek glass-bottomed culture dishes (1.5 mm thickness) and allowed to adhere overnight. After treatments were performed, the cells were washed with PBS and fixed 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, USA) 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 weights on 4–12% Bis–Tris gels under reducing and denaturing conditions (NuPAGE system; Invitrogen (Grand Island, NY, USA).) followed by electroblotting on nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-T (TBS, pH 7.4, with 0.05% Tween). After being blocked, the membranes were washed once with TBS-T and incubated with primary antibody, rabbit anti-DMPO serum, 1:5000, or rabbit anti-MnSOD, 1:1000 (Abcam, Cambridge, MA, USA). After three washes with TBS-T, secondary antibody, anti-rabbit IgG–alkaline phosphatase, 1:5000 (Pierce Chemical Co., Rockford, IL, USA) in washing buffer, was added and incubated for 60 min. After three more washes with TBS-T, the antigen–antibody complexes were analyzed with a chemiluminescence system (CDP-Star; Roche Molecular Biochemicals, Indianapolis, IN, USA). Gels were stained with the Coomassie-based stain Simply-Blue (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Electron microscopy

Samples from neo and Mn11 cells treated or not with H_2O_2 were evaluated using electron microscopy (ER) to detect mitochondrial structural changes. Sections (approximately 1-mm cubes) were rapidly fixed in diluted Karnovsky's fixative and processed for EM. Embedded sections (0.5 μm) 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, The Netherlands). Structurally damaged mitochondria were operationally defined as having loss or dissolution of $\geq$25% of cristae; alterations in size and number of mitochondria per cell and vacuolization were also considered.