

A mitochondrial superoxide theory for oxidative stress diseases and aging

Hiroko P. Indo,^{1,2,3} Hsiu-Chuan Yen,^{4,*} Ikuo Nakanishi,⁵ Ken-ichiro Matsumoto,⁵ Masato Tamura,⁶ Yumiko Nagano,⁶ Hirofumi Matsui,⁶ Oleg Gusev,^{7,8,9} Richard Cornette,⁹ Takashi Okuda,⁹ Yukiko Minamiyama,¹⁰ Hiroshi Ichikawa,¹¹ Shigeaki Suenaga,¹ Misato Oki,² Tsuyoshi Sato,¹ Toshihiko Ozawa,¹² Daret K. St. Clair³ and Hideyuki J. Majima^{1,2,*†}

¹Department of Oncology and ²Department of Space Environmental Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan

³Graduate Center of Toxicology and Markey Cancer Center, University of Kentucky College of Medicine, Lexington, Kentucky 40506, USA

⁴Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Kwei-Shan, Tao-Yuan 333, Taiwan

⁵Radio-Redox-Response Research Team, Advanced Particle Radiation Biology Research Program, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, Chiba 263-8555, Japan

⁶Division of Gastroenterology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

⁷Department of Invertebrates Zoology and Functional Morphology, Institute of Fundamental Medicine and Biology, Kazan Federal University, Kremevskaya str., 17 Kazan 420-008, Russia

⁸Japan Aerospace Exploration Agency, Institute of Space and Astronautical Science, ISS Science Project Office, Ibaraki 305-8505, Japan

⁹Anhydrobiosis Research Unit, National Institute of Agrobiological Sciences, Ohwashi 1-2, Tsukuba, Ibaraki 305-8634, Japan

¹⁰Food Hygiene and Environmental Health Division of Applied Life Science, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan

¹¹Department of Medical Life Systems, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto 610-0394, Japan

¹²Division of Oxidative Stress Research, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

(Received 4 March, 2014; Accepted 8 August, 2014; Published online 23 December, 2014)

Fridovich identified CuZnSOD in 1969 and manganese superoxide dismutase (MnSOD) in 1973, and proposed “the Superoxide Theory,” which postulates that superoxide ($O_2^{\cdot-}$) is the origin of most reactive oxygen species (ROS) and that it undergoes a chain reaction in a cell, playing a central role in the ROS producing system. Increased oxidative stress on an organism causes damage to cells, the smallest constituent unit of an organism, which can lead to the onset of a variety of chronic diseases, such as Alzheimer’s, Parkinson’s, amyotrophic lateral sclerosis and other neurological diseases caused by abnormalities in biological defenses or increased intracellular reactive oxygen levels. Oxidative stress also plays a role in aging. Antioxidant systems, including non-enzyme low-molecular-weight antioxidants (such as, vitamins A, C and E, polyphenols, glutathione, and coenzyme Q_{10}) and antioxidant enzymes, fight against oxidants in cells. Superoxide is considered to be a major factor in oxidant toxicity, and mitochondrial MnSOD enzymes constitute an essential defense against superoxide. Mitochondria are the major source of superoxide. The reaction of superoxide generated from mitochondria with nitric oxide is faster than SOD catalyzed reaction, and produces peroxynitrite. Thus, based on research conducted after Fridovich’s seminal studies, we now propose a modified superoxide theory; i.e., superoxide is the origin of reactive oxygen and nitrogen species (RONS) and, as such, causes various redox related diseases and aging.

Key Words: superoxide theory, MnSOD, mitochondria, ROS, oxidative stress diseases and aging

Countless harmful substances contribute to environmental problems. These substances enter the food chain, destroy living environments, and even threaten the very survival of the human race. Contact between organisms and harmful substances can lead to diseases in organisms. Some harmful substances are linked to reactive oxygen generators and ultimately cause cellular damage. However, organisms possess biological defenses against these processes. Harmful substances and biological defenses battle

inside cells, and these interactions can result in organisms maintaining their “normal” status. This review focuses on biological defenses, especially antioxidant enzyme systems.

Studies have reported that reactive oxygen causes aging and a number of diseases; for example, rheumatism, hepatitis, enteritis, and carcinogenesis.⁽¹⁾ It is now known that many neurological diseases are caused by reactive oxygen species (ROS); for example, Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS) and the like, which are caused by abnormalities in biological defenses or increased intracellular reactive oxygen levels. Increased oxidative stress on an organism causes damage to cells, the smallest constituent unit of an organism, which can lead to the onset of a variety of diseases.

The definition of a free radical and a list of reactive oxygen species are included in Table 1.⁽²⁾ Due to the emergent role of nitric oxide (NO^{\cdot}) in oxidative stress related diseases, reactive nitrogen cascades are sometimes included in reactive oxygen

Table 1. Reactive oxygen species

Free radicals: a free radical is any species capable of independent existence that contains one or more unpaired electrons. (B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 2007)

Reactive Oxygen Species	
$O_2^{\cdot-}$	superoxide
HO^{\cdot}	hydroxyl radical
1O_2	singlet oxygen
H_2O_2	hydrogen peroxide
$ONOO^{\cdot}$	peroxynitrite

*To whom correspondence should be addressed.
E-mail: hmajima@dent.kagoshima-u.ac.jp (Majima H.J.),
yen@mail.cgu.edu.tw (Yen H-C.)

[†]He received “SFRR Japan Scientific Excellence Award” in 2013 in recognition of his outstanding work.

Table 2. Antioxidant defense systems

Enzymes
• Mn Superoxide Dismutase (MnSOD) - Mitochondria
• CuZnSOD - Cytosol
• Glutathione Peroxidase - Cytosol and Mitochondria
• Peroxiredoxins - Cytosol, Extracellular Space and Mitochondria
Non-enzyme
• Vitamins A, C, E
• Glutathione
• Polyphenols
• Coenzyme Q ₁₀

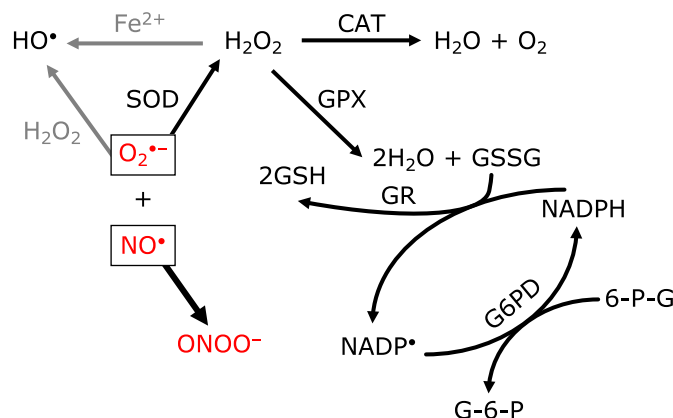


Fig. 1. Intracellular antioxidant enzymes and their chain reactions. Superoxide ($\text{O}_2^{\bullet-}$), predominantly induced from the mitochondrial electron transport chain (ETC), reacts with nitric oxide (NO^\bullet) and forms peroxynitrite (ONOO^-). Peroxynitrite, a potent oxidant, then induces apoptosis or necrosis. MnSOD, which locates in mitochondria, eliminates $\text{O}_2^{\bullet-}$ and inhibits binding with NO^\bullet .

against those oxidant reactions, biological defense systems exist in cells, including enzyme-based systems and non-enzyme-based systems, as shown in Table 2. The relationship between intracellular antioxidant systems and the functions of these systems are shown in Fig. 1.

A New Mitochondrial Superoxide Theory

In 1969, when Fridovich discovered superoxide dismutase (CuZnSOD), an enzyme that eliminates superoxide,⁽³⁾ he established that superoxide ($\text{O}_2^{\bullet-}$) is an important substance that is responsible for initiating a series of ROS. The history of this discovery is shown in Table 3. Fridovich also discovered manganese superoxide dismutase (MnSOD), in 1973.⁽⁴⁾ However, a research published approximately 10 years later showed that superoxide has low activity and poor reactivity, and concluded that the superoxide theory was invalid.⁽⁵⁾ Rate constants for the principal radicals with ascorbic acid in ion-balanced solutions, shown in Table 4,⁽⁶⁾ confirm that superoxide has low activity and poor reactivity. It was believed that this was also the case in bio-environments. In the same period, studies showing that nitric oxide, which plays an important role in the physiological activities of organisms, may participate in superoxide-mediated reactivity and received much attention.⁽⁷⁾ It was eventually established that superoxide and nitric oxide readily react to form an extremely reactive substance called peroxynitrite (ONOO^-) (Fig. 2).⁽⁸⁾ In 1997, we demonstrated that cells transfected with MnSOD genes

Table 3. History of superoxide theory of oxygen toxicity

- Discovery of Superoxide dismutase (CuZnSOD); McCord JM, Fridovich I. *J Biol Chem* 1969; 244: 6049–6055.
- Discovery of Mitochondrial Superoxide dismutase (MnSOD); Weisiger RA, Fridovich I. *J Biol Chem* 1973; 248: 4793–4796.
- Superoxide ($\text{O}_2^{\bullet-}$) is not a strong oxidant; Sawyer DT, Valentine JS. *Acc Chem Res* 1981; 14: 393–400.
- Endothelium-derived relaxing factor (EDRF) is nitric oxide (NO); Palmer RMJ, Ferrige AG, Moncada S. *Nature* 1987; 327: 524–526.
- Peroxynitrite (ONOO^-) is a potent oxidant and nitrating agent; Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA, *Proc Natl Acad U S A* 1990; 87: 1620–1624.
- MnSOD protects against cell death: Superoxide induction from mitochondria relates apoptosis; Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen H-C, Szweda LI, St. Clair D. *J Biol Chem* 1998; 273: 8217–8224.
- Nobel prize laureates: Murat F, Ignarro L, Furchgott R. 1998.

Table 4. Second-order rate constants for the reaction of the free radicals with ascorbate

Free Radicals	k_{obs} ($\text{M}^{-1}\text{s}^{-1}$)
HO^\bullet	1.1×10^{10}
RO^\bullet (tert-butoxy radical)	1.6×10^9
ROO^\bullet (alkylperoxyl radical)	$1\text{--}2 \times 10^6$
GS^\bullet (glutathionyl radical)	6×10^8
$\text{O}_2^{\bullet-}$	1×10^5

exhibited resistance to apoptotic death caused by alkaline, which is an oxidative stress condition.⁽⁹⁾ MnSOD precursor protein has a mitochondrial targeting signal (MTS) composed of 24 amino acids that transport MnSOD protein from cytosol into the matrix of mitochondria (Fig. 3). MTS is cleaved, and then mature MnSOD protein eliminates superoxide inside the matrix of mitochondria, protecting mitochondrial DNA by inhibiting superoxide from reacting with nitric oxide (Fig. 4). These results suggest that superoxide generated from mitochondria controls subsequent oxidative reactions. As a result of this finding, we propose “A Mitochondrial Superoxide Theory.”⁽¹⁰⁾

The Role of Superoxide Dismutase Enzymes

Enzymes scavenging superoxide are referred to as SODs (EC 1. 15. 1. 1). Three types of SODs have been identified in mammals: CuZnSOD (SOD1), MnSOD (SOD2) and ECSOD (SOD3, extracellular SOD).^(3,4,11,12) The oxidation-reduction active centers of these SODs are zinc and copper for CuZnSOD and ECSOD, and manganese for MnSOD. CuZnSOD is homodimeric and present in cytoplasm. The genes of CuZnSOD are present in chromosome 21 in humans and in chromosome 16 in mice. CuZnSOD is localized in cytosol, but recent studies have shown that it is also localized inside mitochondria, in intermembrane space,^(13–16) where superoxide anions are released from Complex III. SOD1 is linked to amyotrophic lateral sclerosis (ALS),^(17–20) aging⁽²¹⁾ and Alzheimer’s disease.⁽²²⁾ The enzyme MnSOD is located in mitochondria, and it is homotetrameric. Genes of this enzyme are present in chromosome 6 in humans and in chromosome 8 in mice. The enzyme has a mitochondrial localizing signal, comprised of 24 amino acids that target the protein to mitochondria. Upon reaching its destination, the signal part is cleaved and the mature protein becomes localized inside the mitochondria.^(9,10)

ECSOD is homotetrameric and a glycosylated CuZnSOD. Genes of ECSOD are present in chromosome 4 in humans and in chromosome 5 in mice. ECSOD is found predominantly in the

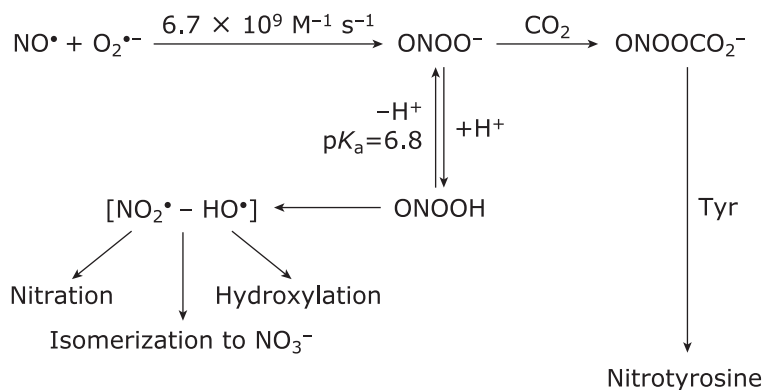


Fig. 2. Formation of peroxynitrite (ONOO⁻) and its further reactions. Nitrates and/or hydroxylates of proteins, lipids and DNAs, by formation of NO₂[•] and HO[•] (adapted from Beckman, *et al.*⁽⁶⁾).

MLSRAVCGTSRQLAPALGYLGSRQ
ATGTTGAGCCGGGCAGTGTGCGGCACCAGCAGGCAGCTG
GCTCCGGCTTTGGGGTATCTGGGCTCCAGGCAGAAGCACA
 GCCTCCCCGACCTGCCCTACGACTACGGCGCCCTGGAACC
 TCACATCAACGCGCAGATCATGCAGCTGCACCACAGCAA
 GCACCACGCGGCCTACGTGAACAACCTGAACGTCAACGA
 GGAGAAGTACCAGGAGGCGTTGGCCAAGGGAGATGTTAC
 AGCCAGATAGCTCTTCAGCCTGCACTGAAGTTCAATGGT
 GGTGGTCATATCAATCATAGCATTCTGGACAAACCTCA
 GCCCTAACGGTGGTGGAGAACCCAAAGGGGAGTTGCTGG
 AAGCCATCAAACGTGACTTTGGTTCCTTTGACAAGTTTAA
 GGAGAAGCTGACGGCTGCATCTGTTGGTGTCCAAGGCTCA
 GTTGGGGTTGGCTTGGTTTCAATAAGGAACGGGGACACT
 TACAAATTGCTGCTTGTCCAATCAGGATCCACTGCAAGG
 AACAAACAGGCCTTATTCCACTGCTGGGGATTGATGTGTGG
 GAGCACGCTTACTACCTTCAGTATAAAAATGTCAGGCCTG
 ATTATCTAAAAGCTATTTGGAATGTAATCAACTGGGAGAA
 TGTAAGTAAAAGATAACATGGCTTGCAAAAAG

Fig. 3. MnSOD has a mitochondria targeting signal (MTS). The sequence consists of an alternating pattern (amphipathic helix) composed of 24 amino acids, which includes three positively charged peptides (arginine [R]).

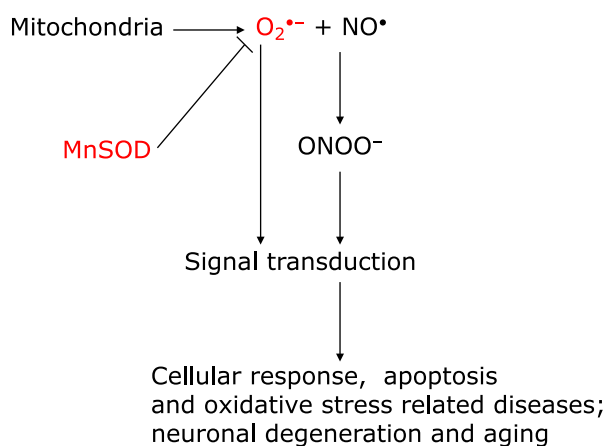


Fig. 4. A Mitochondrial Superoxide Theory. The binding reaction of superoxide (O₂^{•-}) and nitric oxide (NO[•]) forms peroxynitrite (ONOO⁻) (amendment to Motoori, *et al.*⁽⁷¹⁾).

extracellular matrix of tissues and situated to prevent cell and tissue damage initiated by extracellularly produced ROS. ECSOD may protect against pulmonary fibrosis and other extracellular superoxide mediated diseases.^(12,23,24)

Mitochondrial dysfunction has been linked to aging and a wide range of degenerative and metabolic diseases, including cancer.⁽²⁵⁾ In cells, superoxide is produced from oxygen molecules by xanthine oxidase, NADPH oxidase and mitochondrial electron transfer systems. Superoxide produced in mitochondria is generated by electrons leaking from the electron transfer system, which is located in the inner membrane of mitochondria. These electrons are then captured by molecular oxygen and become superoxide.⁽²⁶⁻²⁹⁾ It is estimated that an adult at rest utilizes 3.5 ml O₂/kg/minute or 352.8 L/day (assuming 70 kg body mass) or 14.7 mol/day.⁽³⁰⁾ If 1% makes superoxide, a human produces 0.147 mol/day or 53.66 moles/year or about 1.72 kg/year of superoxide. During physical exertion, this would increase up to 10-fold, assuming that the 1% still applied. Therefore, superoxide generated from mitochondria in normal cells is present in large quantities in cells. SODs eliminate these superoxides. The importance of these SODs can be seen from the results of experiments using genetic knockout mice.^(31,32) Cutler reported on the correlation between SOD activity and survival time using 2 rodents and

12 primate species of maximum lifespan potentials (MLSPs) ranging from 3.5 to 95 years.⁽³³⁾ Recently, Page *et al.*⁽³⁴⁾ reported no correlation between antioxidant enzyme activities and longevity among 14 mammalian and avian species with MLSPs ranging from 3 years to over 100 years. However, they also found that MnSOD and catalase positively correlated with MLSP only for brain tissue. Although the Mitochondrial Free Radical Theory of Aging (MFRTA) remains unproven due to various complex data,⁽³⁵⁾ it seems that MnSOD could play an important role in aging. In other words, a control of ROS generated from mitochondria could be an important factor for the aging process. These reports, then, suggest that superoxide has an important effect in cells, which in turn implies that “the superoxide theory” is still valid. The regulation of reactive oxygen has implications for many types of degenerative conditions, including aging.

Mitochondria-localized Manganese Superoxide Dismutase (MnSOD)

While CuZnSOD is present in the cytoplasm and forms a dimer, MnSOD forms a tetramer (homotetrameric), as described previously. The gene encoding MnSOD is located on chromosome 6 in humans and chromosome 8 in mice.⁽³⁶⁾ The cDNA of the MnSOD gene consists of 666 bps, the first 72 bps of which correspond to 24 amino acids MTS for translocation of the precursor protein into the mitochondrion (Fig. 3).⁽³⁷⁾ MTS is followed by the start signal codon (AUG; methionine). This mitochondrial translocation signal contains many basic amino acids and thus is a cation as a whole. Because the mitochondrial outer membrane is negatively charged, the speculation was that it electrically attracts MTS. Subsequent studies identified TOM and TIM in the mitochondrial outer and inner membranes, which were found to attract proteins into the mitochondrion.⁽³⁸⁻⁴⁰⁾ To attract proteins into the mitochondrion, these molecules have a stable primary structure via the binding of a chaperone protein to the amino acid chain.⁽³⁸⁻⁴⁰⁾ An MnSOD molecule attracted into the mitochondrial matrix is cleaved at the MTS portion and assumes a three-dimensional structure, which then forms a tetramer with manganese at the active center to form the mature and active form of the molecule.⁽³⁸⁻⁴⁰⁾

Several studies conducted in the 1970s demonstrated a leakage of electrons from some proteins present in the mitochondrial inner membrane, particularly complexes I and III, and the resulting production of superoxide.^(28,29,41) However, the exact percentage of all intracellularly generated active oxygen species accounted for by mitochondria-derived superoxide remains unclear. The intracellular systems that produce superoxide include peroxidases (non-specific),⁽⁴²⁾ xanthine oxidase,^(43,44) nitric oxide synthase (NOS),⁽⁴⁵⁻⁵⁰⁾ aldehyde oxidase,^(51,52) NADPH oxidase,⁽⁵³⁾ fumarate reductase,⁽⁵⁴⁾ heme proteins⁽⁵⁵⁾ and the mitochondrial electron transport system.⁽⁵⁶⁾ We have demonstrated that the mitochondrion is the most abundant source of superoxide of all these systems.⁽⁵⁶⁾ Generation of active oxygen ($O_2^{\cdot-}$) from the mitochondrial electron transport system causes oxidative stress to the cell and subsequently induces oxidative-related diseases.^(26,57,58) Although other antioxidant enzymes play important roles in mitochondria, e.g., peroxiredoxin (Prx)^(59,60) and glutathione peroxidase (GPx),^(61,62) as mentioned above, MnSOD, an enzyme localized in the mitochondrion, and scavenging superoxide, has several important roles.⁽⁴⁾ The following findings of MnSOD suggest the critical role of MnSOD in the survival of aerobic life: (1) *Escherichia coli* and yeasts lacking the MnSOD gene are highly sensitive to oxidative stress.⁽⁶³⁻⁶⁵⁾ (2) MnSOD gene knockout mice can only survive 10–18 days after birth, with pathological findings of cardiomyopathy, fatty liver, skeletal muscle acidosis and degeneration of neurons in the central nervous system due to mitochondrial disorder, suggesting a critical role of the enzyme.^(31,32) Superoxide can directly oxidize [4Fe-4S] of aconitase, etc., to form hydrogen

peroxide (H_2O_2) with subsequent release of Fe^{2+} . The cluster is also in complex I, and so aconitase and complex I are inactivated in SOD2 KO mice, which is important for superoxide toxicity.^(66,67) (3) Cells transfected with MnSOD cDNAs are resistant to paraquat,⁽⁶⁸⁾ tumor necrosis factor,^(69,70) doxorubicin,⁽⁶⁹⁾ mitomycin C,⁽⁶⁹⁾ irradiation,^(69,71-76) alkaline treatment,⁽⁹⁾ hypoxic condition,⁽⁷⁷⁾ ischemic reperfusion,⁽⁷⁸⁾ smoking toxicity⁽⁷⁹⁾ and radiation carcinogenesis.⁽⁸⁰⁾ (4) Human MnSOD gene transgenic mice show reduced severity of hyperbaric oxygen-induced pulmonary damage⁽⁸¹⁾ and adriamycin-induced myocardial damage.⁽⁸²⁾ Free radicals generated from mitochondria could play roles in any kind of cell death; i.e., apoptosis, necrosis and autophagy.^(83,84)

How SOD Works to Remove Oxidative Stress in Mitochondria

Several hypotheses describe the important role MnSOD plays as an antioxidant.⁽⁷⁶⁾ Hydroxyl radicals are produced from the Fenton reaction (1) or Haber-Weiss reaction. Thus, they are formed H_2O_2 and superoxide ($O_2^{\cdot-}$).

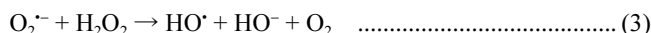
The Fenton reaction is defined as:



The Haber-Weiss reaction is initiated by



The overall reaction is:



Cells with more MnSOD should generate more H_2O_2 due to more substrate being available for reactions, and more HO^{\cdot} could be produced by the Fenton and Haber-Weiss reactions. However, the absence of superoxide prevents the first step of the Haber-Weiss reaction and thus HO^{\cdot} formation is reduced. This is consistent with the general observation that the level of ROS (HO^{\cdot}), subsequent lipid peroxidation and apoptosis are decreased by MnSOD overexpression. Thus, the Fenton reaction alone does not correspond with these results and does not explain the observed reduced amounts of ROS (mostly HO^{\cdot}) by MnSOD transfection. The excess production of H_2O_2 by MnSOD could be quickly detoxified by GPx by reducing it to water.⁽⁸⁵⁾ This reaction could be accompanied by glutathione, of which the level for most cells is ~5 mM, an excess amount for the reaction.⁽⁸⁵⁾

Superoxide radicals can react with NO^{\cdot} to form $ONOO^{-}$ with a diffusion-controlled rate, because NO^{\cdot} has an unpaired electron.⁽⁸⁶⁾ $ONOO^{-}$ is a potent biological oxidant that has recently been implicated in diverse forms of free radical-induced tissue injury.^(8,87) The reaction of $ONOO^{-}$ with membrane lipids induces a phospholipid membrane peroxidation product even without iron being present.⁽⁸⁸⁾ Various aldehydes are generated as final products when lipid hydroperoxides break down. Among them, HNE is a highly toxic nine-carbon α,β -unsaturated aldehyde that can be generated by the peroxidation of ω -6 unsaturated fatty acids, such as arachidonic acid and linoleic acid.^(89,90) Peroxynitrous acid (protonated forms of $ONOO^{-}$: $ONOOH$) subsequently produces HO^{\cdot} and nitrogen dioxide radicals (NO_2^{\cdot}), resulting in oxidation and nitration, respectively.⁽⁸⁾ Thus, MnSOD inhibits the formation of $ONOO^{-}$ by decreasing superoxide levels that prevent the peroxidant effects produced by $ONOO^{-}$ (Fig. 2 and 4).

Free radicals generated from mitochondria could play a role in many kinds of cell death; i.e., apoptosis, necrosis, autophagy.^(83,84) In our published paper that reported the relationship between ROS generated from mitochondria and apoptosis,⁽⁹⁾ we showed an electron microscopic picture of mitophagy impacted by an alkaline condition, whereas MnSOD transfected cells seemed normal, as

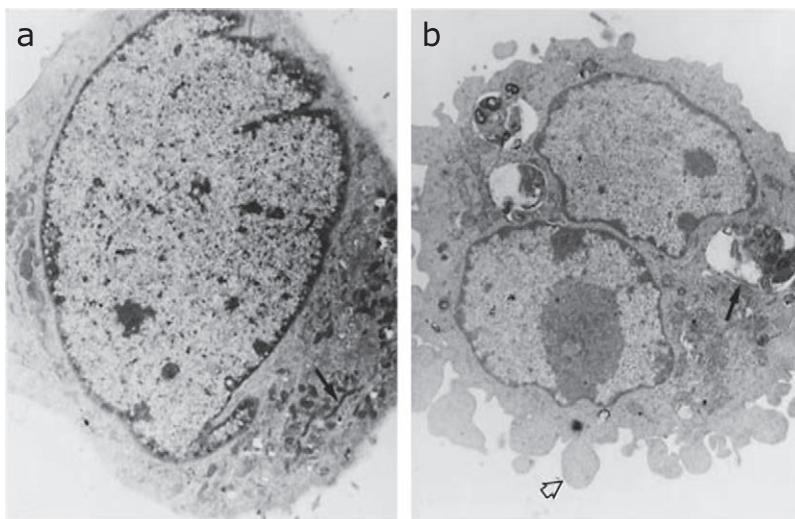


Fig. 5. Ultrastructural analysis of cells cultured at pH 8.3 for 6 h. Electron microscopy of (a) SOD transfected cells that appear normal with normal mitochondria (arrow) and (b) NEO cells show accumulation of lysosomes with membrane debris observed internally (arrow). The cell surface demonstrates prominent membrane blebs (arrowhead). Mitochondria show focal swelling and loss of cristae. Focal condensation of chromosomal material is present. This research was originally published in *J Biol Chem.*, Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen H-C, Szveda LI, St Clair DK: Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J Biol Chem* 1998; 273: 8217–8224. Reprint from ref. 9 with permission.

shown in Fig. 5. These findings indicate that MnSOD is essential for maintenance of life and cellular resistance to oxidative stress in the presence of oxygen, and suggest that superoxide generated from mitochondria plays an important role in oxidative stress and its related diseases, including aging.

Conclusion

Mitochondrial ETC generates superoxide under physiological conditions, and oxidative stress increases ROS production. Mitochondria are the major source of intracellular superoxide production, and damage of mtDNA appears to damage mitochondrial DNA encoded proteins in ETC, causing more superoxide to be produced. Reducing excess amounts of mitochondria-generated superoxide seems important to protecting against oxidative stress related diseases. The reactivity of superoxide is relatively low, as shown in Table 4. However, when elevated levels of NO[•] are present, nitric oxide binds to mitochondrial ETC-generated superoxide. Subsequently, ONOO⁻ is formed with the rate constant close to that of the reaction between hydroxyl radical (HO[•]) and ascorbic acid (Table 4, Fig. 2). Then, ONOO⁻ produces hydroxyl radicals and nitrogen dioxide, and oxidizes and nitrates DNAs,

lipids and proteins, etc., and induces apoptosis, autophagy, mitophagy and necrosis (Fig. 2 and 5). MnSOD exists in mitochondria (Fig. 3) to block the binding of mitochondrial ETC-generated superoxide with nitric oxide (Fig. 4). This theory, called “A Mitochondrial Superoxide Theory” (Fig. 4), could explain the initiation of numerous chronic diseases, such as aging and carcinogenesis.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 22592093 to H.J.M.) and the Strategic Promotion Program for Basic Nuclear Research (to H.J.M.) of the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) (to H.J.M.) by the Ministry of Agriculture, Forestry and Fisheries of Japan.

Conflict of Interest

No potential conflicts of interest were disclosed.

References

- Halliwell B, Gutteridge JMC. Reactive species and disease: fact, fiction or filibuster? In: Halliwell B, Gutteridge JMC, eds. *Free Radicals in Biology and Medicine (4th ed.)*, Oxford: Oxford University Press, 2007; 488–613.
- Halliwell B, Gutteridge JMC. Oxygen is a toxic gas—an introduction to oxygen toxicity and reactive species. In: Halliwell B, Gutteridge JMC, eds. *Free Radicals in Biology and Medicine (3rd ed.)*, Oxford: Oxford University Press, 2007; 19–21.
- McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocyte hemocuprein. *J Biol Chem* 1969; **244**: 6049–6055.
- Weisiger RA, Fridovich I. Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. *J Biol Chem* 1973; **248**: 4793–4796.
- Sawyer DT, Valentine JS. How super is superoxide? *Acc Chem Res* 1981; **14**: 393–400.
- Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 1996; **145**: 532–541.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; **327**: 524–526.
- Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990; **87**: 1620–1624.
- Majima HJ, Oberley TD, Furukawa K, et al. Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J Biol Chem* 1998; **273**: 8217–8224.
- Majima HJ, Indo HP, Suenaga S, et al. Mitochondria as Source of Free Radicals. In: Naito Y, Suematsu M, Yoshikawa T, eds. *Free Radical Biology*

- in *Digestive Diseases, Front Gastrointest Res (Vol. 29)*, Basel: Karger, 2011; 12–22.
- 11 von Ahsen O, Renken C, Perkins G, Kluck RM, Bossy-Wetzel E, Newmeyer DD. Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. *J Cell Biol* 2000; **150**: 1027–1036.
 - 12 Fukai T, Ushio-Fukai M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal* 2011; **15**: 1583–1606.
 - 13 Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* 2001; **276**: 38388–38393.
 - 14 Kawamata H, Manfredi G. Import, maturation, and function of SOD1 and its copper chaperone CCS in the mitochondrial intermembrane space. *Antioxid Redox Signal* 2010; **13**: 1375–1384.
 - 15 Suzuki Y, Ali M, Fischer M, Riemer J. Human copper chaperone for superoxide dismutase 1 mediates its own oxidation-dependent import into mitochondria. *Nat Commun* 2013; **4**: 2430.
 - 16 Dröse S, Brandt U. Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Adv Exp Med Biol* 2012; **748**: 145–169.
 - 17 Beal MF. Mitochondrial dysfunction in neurodegenerative diseases. *Biochim Biophys Acta* 1998; **1366**: 211–223.
 - 18 Okado-Matsumoto A, Fridovich I. Amyotrophic lateral sclerosis: a proposed mechanism. *Proc Natl Acad Sci U S A* 2002; **99**: 9010–9014.
 - 19 Tan W, Nanche N, Bogush A, Pedrini S, Trotti D, Pasinelli P. Small peptides against the mutant SOD1/Bcl-2 toxic mitochondrial complex restore mitochondrial function and cell viability in mutant SOD1-mediated ALS. *J Neurosci* 2013; **33**: 11588–11598.
 - 20 Solsona C, Kahn TB, Badilla CL, et al. Altered thiol chemistry in human Amyotrophic Lateral Sclerosis-linked mutants of superoxide dismutase 1. *J Biol Chem* 2014; **289**: 26722–26732.
 - 21 Kostrominova TY. Advanced age-related denervation and fiber-type grouping in skeletal muscle of SOD1 knockout mice. *Free Radic Biol Med* 2010; **49**: 1582–1593.
 - 22 Oladzad Abbasabadi A, Javanian A, Nikkhab M, Meratan AA, Ghiasi P, Nemat-Gorgani M. Disruption of mitochondrial membrane integrity induced by amyloid aggregates arising from variants of SOD1. *Int J Biol Macromol* 2013; **61**: 212–217.
 - 23 Gao F, Kinnula VL, Myllärmiemi M, Oury TD. Extracellular superoxide dismutase in pulmonary fibrosis. *Antioxid Redox Signal* 2008; **10**: 343–354.
 - 24 Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. *Free Radic Biol Med* 2009; **47**: 344–356.
 - 25 Wallace DC, Fan W, Procaccio V. Mitochondrial energetics and therapeutics. *Annu Rev Pathol* 2010; **5**: 297–348.
 - 26 Wallace DC. Mitochondrial DNA in aging and disease. *Sci Am* 1997; **277**: 40–47.
 - 27 Beyer RE. An analysis of the role of coenzyme Q in free radical generation and as an antioxidant. *Biochem Cell Biol* 1992; **70**: 390–403.
 - 28 Takeshige K, Minakami S. NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochem J* 1970; **180**: 129–135.
 - 29 Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 1973; **134**: 707–716.
 - 30 Halliwell B. Free radicals and antioxidants: a personal view. *Nutr Rev* 1994; **52**: 253–265.
 - 31 Li Y, Huang TT, Carlson EJ, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995; **11**: 376–381.
 - 32 Lebovitz RM, Zhang H, Vogel H, et al. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci U S A* 1996; **93**: 9782–9787.
 - 33 Tolmasoff JM, Ono T, Cutler RG. Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. *Proc Natl Acad Sci U S A* 1980; **77**: 2777–2781.
 - 34 Page MM, Richardson J, Wiens BE, et al. Antioxidant enzyme activities are not broadly correlated with longevity in 14 vertebrate endotherm species. *Age (Dordr)* 2010; **32**: 255–270.
 - 35 Sanz A, Stefanatos RK. The mitochondrial free radical theory of aging: a critical view. *Curr Aging Sci* 2008; **1**: 10–21.
 - 36 Church SL, Grant JW, Meese EU, Trent JM. Sublocalization of the gene encoding manganese superoxide dismutase (MnSOD/SOD2) to 6q25 by fluorescence *in situ* hybridization and somatic cell hybrid mapping. *Genomics* 1992; **14**: 823–825.
 - 37 Ho YS, Crapo JD. Isolation and characterization of complementary DNAs encoding human manganese-containing superoxide dismutase. *FEBS Lett* 1988; **229**: 256–260.
 - 38 Mihara T, Onuma T. Cytoplasmic chaperons in precursor targeting to mitochondria: the role of MSF and hsp 70. *Trends Cell Biol* 1996; **6**: 104–108.
 - 39 Bohnert M, Pfanner N, van der Laan M. A dynamic machinery for import of mitochondrial precursor proteins. *FEBS Lett* 2007; **581**: 2802–2810.
 - 40 Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N. Importing mitochondrial proteins: machineries and mechanisms. *Cell* 2009; **138**: 628–644.
 - 41 Boveris A, Cadenas E. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett* 1975; **1**: 311–314.
 - 42 Smith AM, Morrison WL, Milham PJ. Oxidation of indole-3-acetic acid by peroxidase: involvement of reduced peroxidase and compound III with superoxide as a product. *Biochemistry* 1982; **21**: 4414–4419.
 - 43 Dockrell HM, Playfair JH. Killing of plasmodium yoelii by enzyme-induced products of the oxidative burst. *Infect Immun* 1984; **43**: 451–456.
 - 44 Thomas MJ, Mehl KS, Pryor WA. The role of superoxide in xanthine oxidase-induced autooxidation of linoleic acid. *J Biol Chem* 1982; **257**: 8343–8347.
 - 45 Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A* 1997; **94**: 6954–6958.
 - 46 Kaesemeyer WH, Ogonowski AA, Jin L, Caldwell RB, Caldwell RW. Endothelial nitric oxide synthase is a site of superoxide synthesis in endothelial cells treated with glyceryl trinitrate. *Br J Pharmacol* 2000; **131**: 1019–1023.
 - 47 Pou S, Keaton L, Surichamorn W, Rosen GM. Mechanism of superoxide generation by neuronal nitric-oxide synthase. *J Biol Chem* 1999; **274**: 9573–9580.
 - 48 Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 1992; **267**: 24173–24176.
 - 49 Xia Y, Roman LJ, Masters BS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J Biol Chem* 1998; **273**: 22635–22639.
 - 50 Vásquez-Vivar J, Kalyanaram B, Martásek P, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 1998; **95**: 9220–9225.
 - 51 Zarepour M, Simon K, Wilch M, et al. Identification of superoxide production by Arabidopsis thaliana aldehyde oxidases AAO1 and AAO3. *Plant Mol Biol* 2012; **80**: 659–671.
 - 52 Kundu TK, Velayutham M, Zweier JL. Aldehyde oxidase functions as a superoxide generating NADH oxidase: an important redox regulated pathway of cellular oxygen radical formation. *Biochemistry* 2012; **51**: 2930–2939.
 - 53 Nakamura Y, Ohtaki S, Makino R, Tanaka T, Ishimura Y. Superoxide anion is the initial product in the hydrogen peroxide formation catalyzed by NADPH oxidase in porcine thyroid plasma membrane. *J Biol Chem* 1989; **264**: 4759–4761.
 - 54 Imalay JA. A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. *J Biol Chem* 1995; **270**: 19767–19777.
 - 55 Dershwitz M, Novak RF. Generation of superoxide via the interaction of nitrofurantoin with oxyhemoglobin. *J Biol Chem* 1982; **257**: 75–79.
 - 56 Indo HP, Davidson M, Yen H-C, et al. Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage. *Mitochondrion* 2000; **7**: 106–118.
 - 57 Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* 2000; **1**: 120–129.
 - 58 Mattson MP, Duan W, Pedersen WA, Culmsee C. Neurodegenerative disorders and ischemic brain diseases. *Apoptosis* 2001; **6**: 69–81.
 - 59 Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 2005; **38**: 1543–1552.
 - 60 Cao Z, Lindsay JG, Isaacs NW. Mitochondrial peroxiredoxins. *Subcell Biochem* 2007; **44**: 295–315.
 - 61 Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. *Biochem J* 2000; **351**: 183–193.
 - 62 Margis R, Dunand C, Teixeira FK, Margis-Pinheiro M. Glutathione peroxidase family - an evolutionary overview. *FEBS J* 2008; **275**: 3959–3970.
 - 63 Carlizoz A, Touati D. Isolation of superoxide dismutase mutants in *Escherichia*

- coli*: is superoxide dismutase necessary for aerobic life? *EMBO J* 1986; **5**: 623–630.
- 64 Farr SB, D'Ari R, Touati D. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proc Natl Acad Sci U S A* 1986; **83**: 8268–8272.
- 65 van Loon AP, Pesold-Hurt B, Schatz G. A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc Natl Acad Sci U S A* 1986; **83**: 3820–3824.
- 66 Flint DH, Tuminello JF, Emptage MH. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem* 1993; **268**: 22369–22376.
- 67 Liang LP, Patel M. Iron-sulfur enzyme mediated mitochondrial superoxide toxicity in experimental Parkinson's disease. *J Neurochem* 2004; **90**: 1076–1084.
- 68 St Clair DK, Oberley TD, Ho YS. Overproduction of human Mn-Superoxide dismutase modulates paraquat-mediated toxicity in mammalian cells. *FEBS Lett* 1991; **293**: 199–203.
- 69 Hirose K, Longo DL, Oppenheim JJ, Matsushima K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB J* 1993; **7**: 361–368.
- 70 Wong GH, Elwell JH, Oberley LW, Goeddel DV. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. *Cell* 1989; **58**: 923–931.
- 71 Motoori S, Majima HJ, Ebara M, et al. Overexpression of mitochondrial manganese superoxide dismutase protects against radiation-induced cell death in the human hepatocellular carcinoma cell line HLE. *Cancer Res* 2001; **61**: 5382–5388.
- 72 Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkeisan RB. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. *Cancer Res* 2001; **61**: 3894–3901.
- 73 Sun J, Chen Y, Li M, Ge Z. Role of antioxidant enzymes on ionizing radiation resistance. *Free Radic Biol Med* 1998; **24**: 586–593.
- 74 Epperly MW, Gretton JE, Sikora CA, et al. Mitochondrial localization of superoxide dismutase is required for decreasing radiation-induced cellular damage. *Radiat Res* 2003; **160**: 568–578.
- 75 Majima HJ, Indo HP, Tomita K, et al. Intracellular oxidative stress caused by ionizing radiation. In: Singh K, ed. *Oxidative Stress, Disease and Cancer*. London: Imperial College Press, 2006; 61–83.
- 76 Indo HP, Inanami O, Koumura T, et al. Roles of mitochondria-generated reactive oxygen species on X-ray-induced apoptosis in a human hepatocellular carcinoma cell line, HLE. *Free Radic Res* 2012; **46**: 1029–1043.
- 77 Kinningham KK, Oberley TD, Lin S, Mattingly CA, St Clair DK. Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. *FASEB J* 1999; **13**: 1601–1610.
- 78 Hirai F, Motoori S, Kakinuma S, et al. Mitochondrial signal lacking manganese superoxide dismutase failed to prevent cell death by reoxygenation following hypoxia in a human pancreatic cancer cell line, KP4. *Antioxid Redox Signal* 2004; **6**: 523–535.
- 79 St Clair DK, Jordan JA, Wan S, Gairola CG. Protective role of manganese superoxide dismutase against cigarette smoke-induced cytotoxicity. *J Toxicol Environ Health* 1994; **43**: 239–249.
- 80 St Clair DK, Wan XS, Oberley TD, Muse KE, St Clair WH. Suppression of radiation-induced neoplastic transformation by overexpression of mitochondrial superoxide dismutase. *Mol Carcinog* 1992; **6**: 238–242.
- 81 Wispé JR, Warner BB, Clark JC, et al. Human Mn-superoxide dismutase in pulmonary epithelial cells of transgenic mice confers protection from oxygen injury. *J Biol Chem* 1992; **267**: 23937–23941.
- 82 Yen HC, Oberley TD, Vichitbandha S, Ho YS, St Clair DK. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J Clin Invest* 1996; **98**: 1253–1260.
- 83 Majima HJ, Indo HP, Tomita K, et al. Bio-assessment of risk in long-term manned space exploration—cell death factors in space radiation and/or microgravity: a review. *Biol Sci Space* 2009; **23**: 43–53.
- 84 Majima HJ, Indo HP, Suenaga S, Matsui H, Yen HC, Ozawa T. Mitochondria as possible pharmaceutical targets for the effects of vitamin E and its homologues in oxidative stress-related diseases. *Curr Pharm Des* 2011; **17**: 2190–2195.
- 85 Davies KJ. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life* 2000; **50**: 279–289.
- 86 Blough NV, Zafriou OC. Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. *Inorg Chem* 1985; **24**: 3502–3504.
- 87 Keller JN, Kindy MS, Holtsberg FW, et al. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci* 1998; **18**: 687–697.
- 88 Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991; **288**: 481–487.
- 89 Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde, and related aldehydes. *Free Radic Biol Med* 1991; **11**: 81–128.
- 90 Pryor WA, Porter NA. Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radic Biol Med* 1990; **8**: 541–543.