DELETERIOUS ROLE OF SUPEROXIDE DISMUTASE IN THE MITOCHONDRIAL INTERMEMBRANE SPACE

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This paper demonstrates how increased activity of Cu,Zn superoxide dismutase (SOD1) paradoxically boosts production of toxic reactive oxygen species (ROS) in the intermembrane space (IMS) of mitochondria. Even though SOD1 is a cytosolic enzyme, a fraction of it is found in the IMS, where it is thought to provide protection against oxidative damage. We found that SOD1 controls cytochrome c catalyzed peroxidation in vitro when superoxide is available. The presence of SOD1 significantly increased the rate of ROS production in mitoplasts that are devoid of outer membrane and IMS. In response to inhibition of respiration with Antimycin A, isolated mouse wild-type mitochondria increased ROS production, but the mitochondria from SOD1 mice lacking SOD1 (SOD1⁻/⁻) did not. Also, lymphocytes isolated from SOD1⁻/⁻ mice produced significantly less ROS than wild-type cells and were more resistant to the apoptosis induced by inhibition of respiration. Moreover, increased amount of toxic mutant G93A SOD1 in the IMS increased ROS production. The mitochondrial dysfunction and cellular damage paradoxically induced by the SOD1-mediated ROS production may be implicated in chronic degenerative diseases.

Mitochondria are the major intracellular source of superoxide, the primary reactive oxygen species (ROS). Superoxide anion radical is generated by one electron reduction of oxygen. The two major pathways of superoxide production in mitochondria are autooxidation or complex III catalyzed oxidation of ubisemiquinone (1) and complex I catalyzed reduction through reversed electron flow upon the respiratory chain (2). Despite of its name, superoxide is not a superoxidant but rather a strong nucleophile and week reductant, with the ability to actively react with a number of cellular targets. Instead of reverse oxidation of superoxide to oxygen, the detoxifying mechanism for superoxide includes dismutation to hydroperoxide and oxygen. There are three dedicated enzymes catalyzing this dismutation reaction: Cu,Zn-superoxide dismutase (SOD1), which is mainly localized in the cytosol, Mn-superoxide dismutase (SOD2), which is found in the mitochondrial matrix, and extracellular superoxide dismutase (SOD3) (3).

Unlike hydroperoxide which freely diffuses through the membranes, superoxide cannot cross the mitochondrial inner membrane. In the matrix SOD2 converts superoxide to hydroperoxide, which in turn is reduced to water by the matrix glutathione peroxidase (4). In the mitochondrial intermembrane space (IMS) superoxide is produced presumably by complex III (1). The fate of superoxide in this compartment is expected to be determined by SOD1 and cytochrome c, which is present there in millimolar concentrations (5,6). Recently, cytosolic SOD1 was demonstrated in the yeast (7) and rat (8) IMS, where it has been suggested to be an important part of the mitochondrial superoxide scavenging system by detoxification of ROS. Cytochrome c is a heme containing protein, which functions as an electron carrier between complex III and cytochrome c.
oxidase in the respiratory chain. Cytocrome c can also efficiently oxidize superoxide to oxygen. In this respect cytochrome c functions as a true antioxidant, scavenging superoxide without production of secondary ROS (9).

However cytochrome c also has a potential to catalyze oxidation by hydroperoxide. Importantly, hydroperoxide oxidizes the prosthetic heme in the cytochrome c molecule to oxoferryl heme, forming so called peroxidase compound I-type intermediate, a highly reactive oxidant that is able to react with a number of intracellular targets including proteins, nucleic acids and lipids, causing cell damage (10). Cytochrome c peroxidase activity is controlled by the coordination state of heme iron, particularly by the sulfur ligand of methionine-80 (Met-80), which can be easily displaced by hydroperoxide (11,12). The peroxidase activity of cytochrome c is increased by unfolding and post-translational modifications, such as proteolytic cleavage, nitration and oxidation (13) (14,15).

We hypothesized that upon mitochondrial stress SOD1 might compete with cytochrome c for superoxide in the IMS and generate hydroperoxide, which then could react with cytochrome c and form peroxidase compound I-type intermediate, eventually leading to a paradoxical increase in ROS production and cellular injury (Fig. 1). Our results demonstrate how the presence of SOD1 activity in the IMS causes paradoxically augmented ROS production upon the mitochondrially stress. Understanding the deleterious role of SOD1 in mitochondrial IMS sheds light on cell death mechanisms of the diseases, in which mitochondrial pathology is implicated, including atherosclerosis, diabetes and neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Animals - The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland. Eight weeks old, male C57BL mice were decapitated under carbon dioxide anesthesia and mitochondria from liver tissues were isolated. The SOD1 knock-out (SOD1−/−) mice with the 129/CD1 background were bred with the CD1 strain of mice for at least eight generations. G93A-SOD1 transgenic mice [B6.Cg-Tg(SOD1-G93A)1Gur/J, The Jackson Laboratory, Bar Harbor, ME] carrying a high copy number of the mutant human G93A-SOD1 were sacrificed at the age of eight weeks.

Isolation of mitochondria - The liver tissue was homogenized with all-glass dounce homogenizer (Kontes) in ice-cold isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris-HCl pH 7.4). The homogenate was centrifuged for 3 min 2000× g and the supernatant was transferred to a new tube and centrifuged for 10 min 10000 g. The resulting supernatant was discarded and the pellet containing mitochondrial fraction was washed once with wash buffer (0.2M sucrose, 20mM HEPES pH 7.2, 0.1mM EGTA, 4mM KH2PO4) and resuspended to the same buffer to a protein concentration of 10 mg/ml. The protein concentrations were determined by Protein assay dye (Bio-Rad). For the ROS production studies, mitochondria were resuspended to a protein concentration of 10 mg/ml in standard medium, containing 0.3M mannitol, 10mM KCl, 10mM KH2PO4, 5mM MgCl2, 1mg/ml BSA, pH 7.4. The purity of mitochondrial fraction obtained was assessed by western blotting (Supplemental Fig. 1). The intactness and functional integrity of the isolated mitochondria were confirmed by measuring mitochondrial membrane potential with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl-benzimidazolylcarbocyanine iodide) dye and mitochondrial respiration with an oxygraph method (Supplemental Fig. 2).

Isolation of mitoplasts - In order to obtain mitoplasts, mitochondria were incubated with 5 × volume of cold hypotonic buffer (10mM Tris, pH 7.4, 1mM EDTA and 1mM DTT ) for 10 min on ice. After 10 min 150mM NaCl was added and mitoplast were incubated 10 min on ice and centrifugated 18000 × g for 20 min, 4°C. Mitoplasts were washed and resuspended back to original volume in standard medium containing 0.3M mannitol, 10mM KCl, 10mM KH2PO4, 5mM MgCl2, 1mg/ml BSA.

Measurements of mitochondrial ROS production - Measurements were carried out in 96-well plates by mixing 20 μl mitochondria suspension,
containing 150 μg mitochondrial protein, with 140 μl standard medium and adding substrate mixture to final concentrations of 1.3 mM succinate and 10μM 2,7-dichlorodihydrofluorescein diacetate (DCF) (Fluka). Antimycin A (Sigma, with 3 μM final concentration) was added in experiments where respiratory chain Complex III was inhibited. Oxidized DCF fluorescence was recorded with a Victor multilabel reader (Wallac). DCF oxidation rate in the absence of mitochondria was also measured, an even though the reagents, especially SOD1 and cytochrome c increased DCF oxidation rate in the absence of mitochondria, the rate was only up to 10% of the rate measured in the presence of mitochondria (Supplemental Fig. 3). All samples were run in triplicates.

**Measurements of cytochrome c catalysed peroxidation** - To study the role of SOD1 in cytochrome c-catalysed DCF oxidation in presence of superoxide source, 15 μl of xanthine oxidase suspension (Sigma), diluted 1:100 in 50 mM phosphate buffer (pH 7.8), was mixed with xanthine solution to 0.4 mM final concentration and complemented with 10 μM DCF. The total volume of the reaction mixture was 200 μl. DCF oxidation was recorded on a Victor multilabel reader (Wallac). The fluorescence kinetics was recorded in the presence of 10 μM Cytochrome c (Sigma) and together with purified human erythrocyte SOD1 (15, 30, 60, 250 nM). The human SOD1 was isolated from erythrocytes by fractionated precipitation and DEAE ion exchange chromatography (16). The purity and identity of isolated SOD1 was confirmed by SDS-PAGE and immunoblotting. All samples were run in triplicates.

**IMS isolation and measurement of SOD1 activity** - SOD1 activity was measured in the mitochondrial IMS preparation, obtained in the presence of iodoacetamide, by NBT reduction assay, and was expressed as percentage of activated enzyme in the absence of iodoacetamide. In order to isolate the contents of the IMS, mitochondria (10mg/ml) were treated with 0.1 mg of digitonin per mg of mitochondria for 1 h at room temperature. 100mM iodoacetamide was added to samples in order to prevent SOD1 activation upon the disruption of outer membrane. After the centrifugation at 10 000 × g for 10 min the supernatant was assayed for SOD activity as quenching of NBT reduction by xanthine oxidase/xanthine reaction-generated superoxide anion radical (17,18). SOD1 activity was measured in the mitochondrial IMS preparation, obtained in the presence of iodoacetamide, and was expressed as percentage of activated enzyme in the absence of iodoacetamide (8).

**Kinetics of DCF oxidation by isolated mitoplasts** - The ROS production by mitoplasts was measured in the presence of 1.3mM succinate, 10μM DCF, 3 μM antimycin A and 10 μM cytochrome c, as described for the ROS production by mitochondria. 100 nM purified human erythrocyte SOD1 was added in order to study the effect of SOD1 on ROS production.

**Flow cytometry** - Peroxide production in mouse blood lymphocytes in the presence of 3 μM Antimycin A, loaded with 10 μM DCF and analysed by flow cytometry (FL1 – DCF fluorescence). Mouse (wt and SOD1−/−) blood lymphocytes were isolated with differential centrifugation using Ficoll Paque PLUS (GE Healthcare) according manufacturer's instructions. Lymphocytes were washed and suspended in HBSS containing 10 μM DCF. Ten minutes later 3 μM antimycin A was added and the samples were analyzed with FACSCalibur flow cytometer (Becton-Dickinson) immediately, at 30 min and at 90 min time points. 10 000 cells were counted altogether.

Kinetics of Antimycin A-induced apoptosis in lymphocytes was measured by AnnexinV-FITC binding. Mouse (wt and SOD1−/−) blood lymphocytes were isolated with differential centrifugation using Ficoll Paque PLUS (GE Healthcare) according manufacturer's instructions. Lymphocytes were washed and suspended in HBSS containing 20 μM antimycin A. Non-treated cells and antimycin A-treated lymphocytes after 30, 60 and 90 min were sedimented by centrifugation and resuspended in the binding buffer containing 10mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4. Annexin V-FITC (Sigma) was added to the final concentration of 0.45μg/ml, and after incubation for 10 min, propidium iodide (PI) was added to the final concentration of 2 μg/ml. After another 10 min incubation the
samples were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson). 10 000 cells were counted altogether.

**Zymography** - SOD activity in the IMS preparations was assessed as described (18). Briefly, 10 μl of each sample, containing 0.2 μg/μl of total protein, was loaded onto a 10% native PAA gel. After electrophoresis the gels were washed in 50 mM phosphate buffer (pH 7.8) for 10 min, and then incubated in 1 mg/ml NBT solution in the same buffer for 15 min. After the incubation the gels were briefly washed in phosphate buffer and incubated in 0.25% TEMED solution containing 30 μM riboflavin for 15 min. The gels were rinsed in phosphate buffer and illuminated for 15 min with a fluorescent light source. SOD activity appeared as clear bands on blue background. The gels were scanned with a GelDoc (BioRad) scanner and the bands were quantified with ImageQuant (GE Healthcare) software. In case of mitoplast analysis, equal loading was assured by equal SOD2 activity bands. Samples were run in triplicates.

**Statistical analyses** - Results are shown as mean +/- standard deviation. Differences between the groups were determined by Student’s t-test. * p<0.05 was considered statistically significant, ** p<0.01.

**RESULTS**

*SOD1 controls mitochondrial hydroperoxide production and its substrate oxidation is dependent on cytochrome c* - Isolated mouse liver mitochondria contained a considerable amount of SOD1. When analyzing the distribution of SOD1 in different subcellular fractions, SOD1 concentration in the mitochondria was about 30% (per unit of total protein) of the cytosolic SOD1 concentration. Within mitochondria, SOD1 concentration was about six times higher in the IMS fraction than in the mitoplast fraction (Supplemental Fig. 4). To investigate whether SOD activity in the IMS contributes to hydroperoxide production, mitochondria respiring in the presence of succinate were challenged with antimycin A, an inhibitor of complex III. Antimycin A has previously been shown to cause prompt superoxide production as determined by electron paramagnetic resonance (19). SOD activity in an IMS preparation was rapidly increased as a function of time in response to antimycin A (Fig. 2A). Inhibition of mitochondrial respiration also resulted in increased hydroperoxide production as determined in parallel by measurement of the fluorescence of 2,7-dichlorodihydrofluorescein (DCF), a widely used hydroperoxide-sensitive probe (20). DCF is strongly oxidized to a fluorescent derivative in the presence of heme-containing catalysts, such as cytochrome c (10). In parallel, the hydroperoxide produced by mitochondria was measured with luminol/HRP assay and found to linearly correlate with the amount of mitochondrial proteins (Supplemental Fig. 5). The possibility that DCF could also be oxidized by peroxynitrite after reaction of superoxide with nitric oxide, was ruled out, because inhibition of nitric oxide synthase by L-NNA (Nω-nitro-L-arginine) did not alter DCF fluorescence rate (Supplemental Fig. 6). The monitored fluorescence is thus a measure of the relative oxidative damage to cellular constituents. An increase in DCF fluorescence was observed after a lag period in respiring isolated mitochondria. The lag period was remarkably shortened by antimycin A (Fig. 2C) and the response coincided with the maximal increase in SOD activity. In agreement with previous studies (8), these kinetics of hydroperoxide production and SOD1 activity suggest a redox control of the dismutase activity in the IMS. SOD1 was essential for the increased hydroperoxide production, because adding SOD1 inhibitors ammonium tetrathiomolybdate (TTM) or ammonium diethyldithiocarbamate, significantly and dose-dependently reduced DCF fluorescence in isolated mitochondria (Fig. 2B and Supplemental Fig. 7).

These results led us to the paradoxical hypothesis that mitochondria lacking SOD1 should produce less hydroperoxide when stressed by inhibiting respiration, resulting in lower cytochrome c catalysed DCF oxidation. To test this hypothesis we isolated intact mitochondria from wild-type (wt) and *SOD1*−/− mice. Mitochondrial peroxide production was measured again by DCF oxidation in the presence and absence of antimycin A. Indeed, the mitochondria isolated from SOD1-deficient (SOD1−/−) mice produced substantially less DCF fluorescence and did not show any
response to inhibition of complex III by antimycin A (Fig. 2C).
These results indicate that upon inhibition of mitochondrial respiration elevated SOD1 activity is responsible for increased hydroperoxide production in the IMS, resulting in cytochrome c catalyzed DCF oxidation. To test this hypothesis further, we used mitoplasts, e.g. mitochondria devoid of outer membrane and the IMS, to reconstitute conditions for hydroperoxide production. No significant DCF oxidation could be detected in respiring mitoplasts even after inhibiting complex III by antimycin A (Fig. 2D). Addition of cytochrome c caused an increase in DCF fluorescence, possibly due to the reconstituted respiration and hydroperoxide escaping from the mitochondrial matrix. Importantly, addition of SOD1 at a 100 nM concentration more than doubled the rate of DCF oxidation in the presence of cytochrome c (Fig. 2D).

To model the interaction of superoxide, cytochrome c, and SOD1 in the IMS, we reconstituted a reaction where superoxide was generated through xanthine oxidase/xanthine (XO/X). A slow oxidation of DCF occurred in the presence of this enzyme substrate pair. The rate of DCF oxidation was slightly elevated by 5 μM cytochrome c (Fig. 2E). However, adding increasing concentrations of SOD1 in the reaction mixture strongly and dose-dependently increased the rate of DCF oxidation, indicating that SOD1 significantly enhances cytochrome c-catalyzed peroxidation. The effect of SOD1 was hydroperoxide dependent, since addition of 5U/ml catalase completely abolished it (Supplemental Fig. 8).

**SOD1 increases mitochondrial peroxide production in lymphocytes and contributes to apoptosis** - To investigate whether SOD1 also controls hydroperoxide production in the IMS of intact cells, we isolated lymphocytes from wt and SOD1−/− mouse blood by differential centrifugation, and loaded them with DCF before adding antimycin A. As seen in Fig. 3A,B, flow cytometric analysis showed that antimycin A-induced DCF oxidation was attenuated in SOD1−/− lymphocytes at 90 min of incubation compared with hydroperoxide production in wt lymphocytes. The difference in hydroperoxide production was confirmed to be statistically significant by K-S test.

In addition to increased superoxide production in mitochondria, inhibition of complex III by antimycin A has also been shown to induce apoptosis (21). To test whether SOD1 activity is important for apoptosis, SOD1−/− and wt lymphocytes were challenged with antimycin A for 90 min and apoptosis was determined by Annexin V binding using flow cytometry. SOD1−/− lymphocytes showed significantly less apoptosis than wt lymphocytes (Fig. 3C,D, Supplemental Fig. 9).

**Increased SOD1 activity associated with G93A SOD1 in spinal cord mitoplasts leads to elevated ROS production** - Mutations in SOD1 are known to cause familial form of amyotrophic lateral sclerosis (ALS) (22), a disease characterized by loss of motor neurones in the spinal cord leading to muscle atrophy, paralysis of voluntary muscles and death in 3-5 years (23). Although the nature of toxic gain of function in mutant SOD1 has not been identified, it is believed that altered generation of free radical and reactive oxygen species (ROS) is a leading contributor to the destruction of motor neurons. Most recent studies of familial ALS animal models indicate that mutant SOD1 causes selective mitochondrial dysfunction resulting in increased ROS production. We hypothesized that the increased amount of SOD1 may be associated with SOD1 activity in the mitochondrial IMS and thereby boost ROS production.

Thus, we studied mitochondrial SOD1 activity in transgenic (tg) mice expressing mutant G93A-SOD1, a model of familial amyotrophic lateral sclerosis (ALS) (24). The mutant SOD1 accumulates in mitochondria of the spinal cord, where it is associated with the inner membrane (25-27). The spinal cord is also the most affected tissue in ALS. SOD1 activity of IMS preparations from the spinal cord was 6-fold greater in the G93A-SOD1 mice than in the wt mice (Fig. 4 A,D). In addition, IMS SOD1 activity in the spinal cord was clearly higher in comparison with non-affected brain tissues of the same animals (Fig. 4B,E). SOD1 activity of the mitoplast preparations isolated from the tg spinal cord was twice as high as from the tg brain (Fig. 4C,F). To investigate whether the elevated activity and accumulation of
mutant G93A-SOD1 in mitoplasts results in increased ROS production, we measured DCF oxidation in the mitoplast preparations in the presence of cytochrome c. We found that the mitoplasts isolated from the tg spinal cord produced significantly more DCF fluorescence compared with the mitoplasts that derived from the tg cortex, indicating that the increased amount of SOD1 in the IMS is also associated with an increase in hydroperoxide production (Fig. 4G).

**DISCUSSION**

Our data indicate that inhibition of electron transfer at the level of complex III leads to an increase in SOD1 activity in the IMS, paradoxically resulting in increased hydroperoxide production and, consequently, cytochrome c-catalyzed peroxidation.

This could trigger a vicious circle where oxidative damage to mitochondrial respiratory components leads to further ROS production and peroxidation. Indeed, we demonstrate that inhibition of mitochondrial respiration at the level of complex III causes SOD1-dependent ROS production and apoptotic death of isolated blood lymphocytes. Moreover, accumulation of mutant human G93A-SOD1 in the IMS that is observed in the tg animal models of ALS, leads to elevated SOD1 activity and increased cytochrome c-catalyzed oxidation in the IMS.

SOD is generally thought to protect cells from oxidative damage. Accordingly, as a cytosolic antioxidant SOD1 provides protection in models of transient myocardial (28) and brain (29) ischemia and Parkinson's disease (30). Some other studies, however, suggest that the increased SOD1 activity promotes injury. For instance, immature mouse brains overexpressing SOD1 show an increased propensity for injury and accumulate more hydroperoxide after hypoxia-ischemia than wt mouse brains (31). Also, elevation of SOD1 increases acoustic trauma from noise exposure (32), and mice deficient in SOD1 are resistant to acetaminophen toxicity (33). Even though SOD1 as a cytosolic antioxidant protects against mitochondrial dysfunction in a mouse model of transient focal cerebral ischemia (34), SOD1 deficiency, rather than overexpression, is associated with enhanced recovery and attenuated activation of NF-kappaB after brain trauma in mice (35). Moreover, a superoxide generator, menadione, produces significantly increased DCF fluorescence and greater death in SOD1 tg neurons than in wt neurons, suggesting increased hydroperoxide formation in the SOD1 tg cells (36). This apparent discrepancy concerning the role of SOD1 in cellular injury can be explained by the results presented here showing that increased SOD1 activity in the IMS paradoxically produces peroxides which are converted to highly toxic ROS.

Previous studies, including electron paramagnetic resonance (EPR) studies (12,37-39) have demonstrated that the reaction of cytochrome c with hydroperoxide results in formation of oxoferryl cytochrome c (peroxidase compound I-type intermediate) and corresponding protein-derived tyrosyl radical, which is highly reactive and has a potential to oxidize proteins, DNA, and lipids, as well as endogenous antioxidants such as glutathione, NADH, and ascorbate (10). In particular, oxidation of cardiolipin, a phospholipid which is in complex with cytochrome c on the surface of the inner mitochondrial membrane, causes the release of proapoptotic factors from mitochondria (37,40). This leads to a scenario where the hydroperoxide produced by increased SOD1 activity in the IMS, would thus serve as a substrate for cardiolipin-bound cytochrome c and consequently switch on very early proapoptotic processes, inducing consecutive programmed cell death (38).

On the other hand, oxidized cytochrome c (Fe$^{3+}$) can efficiently scavenge superoxide (Supplemental Fig.10). Upon high physiological concentration of cytochrome c in the IMS the superoxide released into this compartment might become oxidized to oxygen without production of secondary ROS (hydroperoxide). Also, export of superoxide via voltage-dependent anion channels (41) would serve as a safe pathway for the superoxide produced in the IMS. In contrast, competitive superoxide dismutation catalyzed by SOD1 or occurring non-enzymatically (42), leads to hydroperoxide production in the IMS, which most likely is harmful to the mitochondria and the cell. Altogether, hydroperoxide the in presence of peroxidase activity may play a key role in oxidation of biological targets in the IMS. Thus,
SOD1 activity (Supplemental Fig.11) and other factors that may lead to increased hydroperoxide production in this compartment can be regarded as deleterious to the mitochondria and the cell. Mitochondrial dysfunction, including altered function of respiratory complexes, has been described in arteriosclerosis, diabetes mellitus, and a number of acute and degenerative brain diseases such as stroke, Parkinson's disease, and ALS. Increased ROS production is also a characteristic of these diseases (43). Increased SOD1 activity accompanied by high hydroperoxide production in the IMS may be one mechanism of neurodegeneration. Importantly, the toxicity of ALS-linked SOD1 mutants originates from their selective recruitment to spinal cord mitochondria (25). Even though we clearly showed that the mutant G93A SOD1 is able to associate with mitochondrial inner membrane specifically in the spinal cord, the molecular reason for this effect remains unknown, but may be dependent on oxidation of Cys residues in mutant SOD1 (44).

In conclusion, we suggest that SOD1 activity in the IMS may be a relevant therapeutic target for many degenerative diseases involving mitochondrial pathogenesis.
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**FOOTNOTES**

The abbreviations used are: SOD1, Cu,Zn superoxide dismutase 1, ROS, reactive oxygen species; IMS,
intermembrane space.
FIGURE LEGENDS

FIGURE 1. Deleterious role of superoxide dismutase in the mitochondrial intermembrane space (IMS). Superoxide (SO, \(O_2^-\)) is released in IMS by one electron reduction of oxygen at a site in inner membrane (I). Cytocrome c (CytC) oxidizes SO to oxygen (II). Cu/Zn Superoxide dismutase (SOD1) in IMS is activated by oxidation of sulfhydryls, leading to formation of intramolecular S=S bounds (III). SOD1 produces hydroperoxide (\(H_2O_2\)) by dismutating SO. Hydroperoxide oxydizes CytC to oxoferryl-CytC (CytC(Fe^{4+}=O)), an exceptionally strong oxidant, able to oxidize a number of vital biological targets (V).

FIGURE 2. SOD1 in IMS controls cytochrome c catalyzed peroxidation. (A) The burst of hydroperoxide production in wt mitochondria (trace ■) coincides with the peak of SOD1 activity in the IMS (trace ▲). SOD1 activity was measured in the mitochondrial IMS preparation, obtained in the presence of iodoacetamide, by NBT reduction assay and is expressed as percentage of fully activated enzyme in the absence of iodoacetamide. (B) Inhibition of SOD1 activity reduces mitochondrial hydroperoxide production. Mitochondrial hydroperoxide production by mitochondria isolated from the liver of C57BL male mice was measured in the presence of 0, 10 and 50 \(\mu\)M TTM. (C) Mitochondrial hydroperoxide production, measured as DCF oxidation, in wt and SOD1-/- mitochondria. In wt mitochondria hydroperoxide production (trace ◆) is accelerated by antimycin A (trace ■). In contrast, SOD1-/- mitochondria (trace ▼) do not respond to antimycin A (trace ▲) and produce significantly less hydroperoxide. (D) Rate of DCF oxidation by isolated mouse liver mitoplasts in the presence of 10 \(\mu\)M cytochrome c, and when 100 nM SOD1 was added. The addition of SOD1 causes a significant increase in the hydroperoxide production rate (p=0.007). In the absence of cytochrome c, SOD1 only slightly increases DCF oxidation rate. (E) SOD1 controls cytochrome c (CytC) -catalyzed peroxidation in vitro in a concentration dependent manner. Slow DCF oxidation by superoxide, generated by XO/X (trace ◄), was slightly enhanced by 5 \(\mu\)M CytC (trace ◆). SOD1 in concentrations of 15 nM (trace ▼), 30 nM (trace ▲), 60 nM (trace ●), and 250 nM (trace ■) significantly increases the rate of DCF oxidation.

FIGURE 3. SOD1-/- lymphocytes produce less ROS and are more resistant to antimycin A induced apoptosis. (A) Peroxide production in mice blood lymphocytes in the presence of 3 \(\mu\)M antimycin A, loaded with 10 \(\mu\)M DCF and analyzed by flow cytometry (FL1–DCF fluorescence). (B) SOD1-/- lymphocytes produce significantly less hydroperoxide after 90 min of incubation than wt lymphocytes (confirmed by K-S test). 10 000 cells were counted altogether. (C) Kinetics of antimycin A induced apoptosis in lymphocytes, measured by AnnexinV-FITC binding. (D) After 90 min of incubation in the presence of 20 \(\mu\)M antimycin A, SOD1-/- lymphocytes show only 30% apoptotic cells as measured by Annexin V-FITC binding in contrast to 55% for wt lymphocytes.

FIGURE 4. Increased SOD1 activity in the mitochondria of G93A mice spinal cord leads to elevated ROS production. SOD1 activity was measured by in-gel zymography. (A,D) SOD1 activity in the IMS of mitochondria isolated from the spinal cord of G93A mice increased nearly by 600% in comparison to the wt animals. *, p=0.02. (B,E) SOD1 activity in the mitochondrial IMS from the spinal cord (sc) of G93A mice is increased in comparison to the SOD1 activity in the IMS from the brain (br) tissue of the same animals. **, p=0.0068. (C,F) SOD1 activity associated with isolated mitoplasts from the spinal cord of G93A mice is significantly increased when compared to the mitoplasts from the brain of the same animals. *, p=0.017. (G) Isolated mitoplasts from the spinal cord of G93A mice produce significantly more ROS in the presence of cytochrome c when compared to the mitoplasts from the brain of the same animals. *, p=0.037.
**Figure 1**

oxidation of biological targets in IMS

CytC(Fe$^{4+}$=O)

H$_2$O

CytC(Fe$^{2+}$)

O$_2$

O$_2^{-}$

SOD1$_{S=S, active}$

SOD1$_{S-H, inactive}$

$\text{O}_2$

e$^-$

$\text{O}_2$
Figure 2

A

B

C

D

E
Figure 4

A

B

C

D

E

F

G

Sc IMS

IMS

Mitoplast

Tg Wt

Sc Br

Sc Br

Sc Br

SOD1 activity, RU

*  **

160  120  80  40  0

50

120

40

0

10

40

R/min

40

30

10

**
**Supplemental Figure 1.** WB analysis of mitochondrial (mito) and cytosolic (cyto) fractions. Isolated mouse liver mitochondria (mito) were characterized by the presence of COX4 and absence of cytosolic actin. SOD1 was also evident in mito fraction.

Electrophoresis was carried out on Mini-Protean 3 (Bio-Rad) device at 200 v constant voltage according to manufacturer’s recommendations. For the Western blotting proteins were transferred onto Hybond P membrane (GE Healthcare) in a Mini TransBlot (Bio-Rad) chamber. Membranes were blocked in 5% skimmed milk solution in PBST and incubated with primary antibodies against SOD1 (rabbit polyclonal, 1:5000 dilution, Stressgen Bioreagents), β-actin (mouse monoclonal, 1:4000 dilution, Sigma, St. Louis, MO) and COX IV (mouse monoclonal, 1:1000 dilution, Molecular Probes). The blots were developed by incubating with anti-mouse Cy5-labelled antibodies (Jackson ImmunoResearch, 1:800) and with anti-rabbit HRP-labelled secondary antibodies (GE-Healthcare, 1:4000) and ECL+ detection kit (GE-Healthcare).
Supplemental Figure 2. Functional integrity of the isolated mitochondria. A. Mitochondrial membrane potential ($\Delta\Psi$) was measured using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) dye. The dye undergoes a reversible change in fluorescence emission from green to red as mitochondrial membrane potential increases. JC-1 accumulates as aggregates in the mitochondria, resulting in red fluorescence. The brightness of red fluorescence is proportional to $\Delta\Psi$. Succinate addition caused the expected rise in $\Delta\Psi$, whereas uncoupling by CCCP (carbonyl cyanide 3-chlorophenylhydrazone) led to the depolarisation, indicating normal function of mitochondrial membrane.

B. An oxygraph trace of mitochondria respiring on succinate. Addition of 2.5 $\mu$M ADP causes increased oxygen consumption rate, coupled to oxidative phosphorylation and yielding mean respiratory control index (RCI) of 5.38, indicating normal tightness (RCI between 3 and 10) of the coupling between oxidative phosphorylation and respiration.
Supplemental Figure 3. DCF oxidation rate in the absence of mitochondria. Assessment of contribution of DCF autooxidation on DCF fluorescence in the presence of different experimental reagents used. DCF autooxidation rate is low, about 100 RU/min, in comparison to the peroxidase compound catalyzed DCF oxidation in the presence of mitochondria (> 10000 RU/min, see Fig. 2D). Addition of SOD1 increases the rate of DCF oxidation, most likely because of the peroxide produced from superoxide, which is generated when DCF is autooxidized. This interpretation is in line with the effect of cytochrome c addition, which inhibits DCF oxidation by oxidizing superoxide. Antimycin A and succinate have little effect on DCF oxidation. The highest oxidation rate is observed when both, SOD1 and cytochrome c are present, complying with the conclusions made in the current study. The rate of DCF oxidation is between 100 and 300 RU/min in the absence of mitochondria, and between 1000 - 4000 RU/min in the presence of mitochondria.
Supplemental Figure 4. Distribution of SOD1 in subcellular fractions of the mouse liver. Mouse liver tissue was fractionated according to the methods described in experimental procedures. Fractions were boiled with sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.06M Tris pH 6.8) and 5 μg of total protein was loaded onto a 12 % SDS-PAGE and blotted for SOD1 immunostaining. After electrophoresis, proteins were transferred to PVDF membrane, immunostained with anti-SOD1 antibody (rabbit polyclonal, 1:4,000 dilution, Stressgen Bioreagents, Ann Arbor, MI, USA) and detected with HRP -labeled anti-rabbit IgG (dilution 1:3,000, GE-Healthcare), followed by ECL+ detection (Amersham). Quantifications were carried out on STORM fluoroimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Bars represent relative amounts of SOD1 per unit of total protein (SOD1 concentration) originating from the respective fraction.
Supplemental Figure 5. Linear production of hydroperoxide by mouse liver mitochondria. The released hydroperoxide was measured in the supernatant after removal of mitochondria suspension by luminol chemiluminescence in the presence of horseradish peroxidase according to the method described in Nakamura M, Nakamura S. One- and two-electron oxidations of luminol by peroxidase systems. Free Radic Biol Med. 1998 Mar 1;24(4):537-44. In brief, 75 µl of supernatant were added to the 125µl of mixture containing 45 nM HRP, 100 µM luminol and 0.25 mM sodium bibarbonate in 50 mM phosphate buffer pH 7.8. Luminescence was measured on Victor II multilabel counter.
Supplemental Figure 6. The effect of L-NNA (Nω-Nitro-L-arginine, Sigma Cat.No. N5501) on mitochondrial hydroperoxide production. Mitochondrial hydroperoxide production by the mitochondria isolated from the liver of C57BL male mice (for details see Experimental procedures) was measured in the presence of 0, 10, 50 and 100 μM L-NNA, an inhibitor of nitric oxide synthase. L-NNA has no effect on mitochondrial hydroperoxide production.
Supplemental Figure 7. Inhibition of SOD1 activity reduces mitochondrial hydroperoxide production. Mitochondrial hydroperoxide production by the mitochondria isolated from the liver of C57BL male mice was measured in the presence of 0, 10 and 50 μM diethyldithiocarbamate.
Supplemental Figure 8. Addition of SOD1 significantly increases cytochrome c catalyzed peroxidation in vitro. DCF oxidation in the presence of superoxide, generated by X/XO, is enhanced by 5 μM CytC. SOD1 in 15 nM concentration significantly increases the rate of DCF oxidation. Addition of catalase (5U/ml) completely abolishes these effects, indicating the role of hydroperoxide.
Supplemental Figure 9. After 90 min of incubation in the presence of 20 µM antimycin A, SOD1−/− lymphocytes show only 30% apoptotic cells as measured by Annexin V-FITC binding (left panel, lower right quadrant) in contrast to 55% for wt lymphocytes (right panel).
Supplemental Figure 10. Oxidized cytochrome c (Fe$^{3+}$) efficiently scavenges superoxide. A. The commercially available cytochrome c used in this study was from equine heart (Fluka #30396) and purchased from Sigma-Aldrich Co. As stated in the product certificate, it is produced by a procedure which completely avoids TCA and yields highly native form of cytochrome c. The stated content of reduced form of cytochrome c is less than 5%. The absorption spectra of 50 μM cytochrome c solution in PBS confirms virtual identity of the cytochrome c used and the cytochrome c completely oxidized with potassium ferricyanide (K$_3$Fe(CN)$_6$) form (the lack of peak at 550 nm). Green trace represents absorption spectrum of the cytochrome c that is completely reduced with sodium hydrosulfite (Na$_2$S$_2$O$_4$).

B. Cytochrome c in 10 μM (trace ●) and 20 μM (trace ▲) concentrations inhibits NBT reduction by oxidizing superoxide. In the absence of cytochrome c regeneration (oxidation), NBT reduction starts when the oxidized form of cytochrome c is consumed. Superoxide was generated by 15 μl xanthine oxidase suspension (Sigma) diluted 1:100 in 50 mM phosphate buffer pH 7.8 were mixed with xanthine solution to final concentration 0.4 mM and complemented with 80 μM NBT and cytochrome c. Total volume of reaction mixture was 200 μl. NBT reduction was recorded on Victor multilabel reader (Wallac) at 560 nm.
Supplemental Figure 11. The DCF oxidation rate by isolated mitoplasts, measured in the presence of 1.3 mM succinate, 10µM DCF, 3 µM antimycin A and 10 µM cytocrome c. Addition of 10 nM horseradish peroxidase significantly increases DCF oxidation rate, indication hydroperoxide release. Addition of 100 nM purified human erythrocyte SOD1 to this sample significantly increases this rate even more, demonstrating the contribution of superoxide dismutation.
