Mitochondrial respiratory chain and thioredoxin reductase regulate intermembrane Cu,Zn-superoxide dismutase activity: implications for mitochondrial energy metabolism and apoptosis

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IMS (intermembrane space) SOD1 (Cu/Zn-superoxide dismutase) is inactive in isolated intact rat liver mitochondria and is activated following oxidative modification of its critical thiol groups. The present study aimed to identify biochemical pathways implicated in the regulation of IMS SOD1 activity and to assess the impact of its functional state on key mitochondrial events. Exogenous H_2O_2 (5 μM) activated SOD1 in intact mitochondria. However, neither H_2O_2 alone nor H_2O_2 in the presence of mitochondrial peroxiredoxin III activated SOD1, which was purified from mitochondria and subsequently reduced by dithiothreitol to an inactive state. The reduced enzyme was activated following incubation with the superoxide generating system, xanthine and xanthine oxidase. In intact mitochondria, the extent and duration of SOD1 activation was inversely correlated with mitochondrial superoxide production. The presence of TxrR-1 (thioredoxin reductase-1) was demonstrated in the mitochondrial IMS by Western blotting. Inhibitors of TxrR-1, CDNB (1-chloro-2,4-dinitrobenzene) or auranofin, prolonged the duration of H_2O_2-induced SOD1 activity in intact mitochondria. TxrR-1 inactivated SOD1 purified from mitochondria in an active oxidized state. Activation of IMS SOD1 by exogenous H_2O_2 delayed $CaCl_2$-induced loss of transmembrane potential, decreased cytochrome *c* release and markedly prevented superoxide-induced loss of aconitase activity in intact mitochondria respiring at state-3. These findings suggest that H_2O_2, superoxide and TxrR-1 regulate IMS SOD1 activity reversibly, and that the active enzyme is implicated in protecting vital mitochondrial functions.

Key words: aconitase, Cu,Zn-superoxide dismutase (SOD1), disulfide bond, mitochondrial intermembrane space, reactive oxygen species, thioredoxin reductase (TxrR-1).

INTRODUCTION

Recent studies on the subcellular distribution of SOD (superoxide dismutase) demonstrated that SOD1 (Cu,Zn-SOD) is localized not only in the cytosol [1], but also in the mitochondrial IMS (intermembrane space) of rat liver [2,3] and yeast [4]. Comparative kinetic studies, however, revealed that SOD1 is inactive in isolated intact rat liver mitochondria, unless the outer membrane is disrupted by digitonin [5–8]. The presence of alkylating agents during disruption of the outer membrane prevents activation of the enzyme, whereas exposure of intact mitochondria to low concentrations of H_2O_2 (5 μM) activated SOD1 in the IMS [8]. These findings suggest that the intra-subunit disulfide bond necessary for the active state of SOD1 has not been formed in mitochondria [8].

The biochemical pathways catalysing reversible redox activation of SOD1 are not defined in mitochondria. Activation of SOD1 following exposure of mitochondria to low levels of H_2O_2 suggests the involvement of an enzyme-mediated thiol oxidation, rather than direct oxidation of the enzyme by H_2O_2. Potential candidates to catalyse such a reaction are mitochondrial peroxiredoxins (III and/or V), a family of peroxidases that reduce H_2O_2 to water through reducing equivalents provided by thiol-containing proteins [9,10]. TxrR (thioredoxin reductase) and glutathione reductase are major mitochondrial systems catalysing reduction of protein disulfide bonds through reducing equivalents provided by Txr (thioredoxin) and GSH [11,12]; hence, they may be implicated in reduction, and as a corollary inactivation of IMS SOD1.

The role of IMS SOD1 in mitochondrial function is not known. However, its localization in the IMS suggests that the enzyme has a functional relationship with the occurrence of superoxide in this compartment. Several mitochondrial sites are primary targets of regulation by fluctuating levels of superoxide and/or H_2O_2. Enzymes containing 4Fe-4S clusters, such as aconitase and fumarase, are targets of inactivation by superoxide [13,14]. Superoxide and/or H_2O_2 also mediate the opening of PTPs (permeability transition pores), leading to the loss of Ψm (mitochondrial transmembrane potential), cytochrome *c* release and apoptosis [15,16]. Therefore IMS SOD1 activity may potentially play an important role in key mitochondrial events (e.g. energy metabolism, Ψm and cytochrome *c* release) to maintain the integrity of mitochondria. Hence, the aims of this study were to elucidate the biochemical pathways regulating IMS SOD1 activity, and to establish a functional link between its activity and key mitochondrial events.

EXPERIMENTAL

Chemicals

p-Hydroxyphenylacetate, bovine SOD1, fatty acid free BSA, XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide], digitonin, IAM (iodoacetamide), xanthine oxidase, catalase, reduced glutathione, Txr, TxrR-1, antimycin, horseradish peroxidase, $NADP^+$, isocitrate dehydrogenase,

Abbreviations used: Ψm, mitochondrial transmembrane potential; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; IAM, iodoacetamide; IMS, intermembrane space; PTP, permeability transition pore; SOD1, Cu,Zn-superoxide dismutase; Txr, thioredoxin; TxrR-1, thioredoxin reductase-1; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide.

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rotenone and rhodamine 123 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Isolation of liver mitochondria

Liver mitochondria were isolated from adult male Wistar rats by differential centrifugation as described previously [17]. Rat livers were excised, chopped into fine pieces, washed with 0.25 M sucrose and homogenized in isolation buffer (210 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, and 0.05 % BSA). The homogenate was centrifuged at 800 ***g*** for 8 min at 4 °C, the pellet was removed and then the centrifugation process was repeated. The supernatant was centrifuged at 8000 ***g*** for 10 min at 4 °C, the pellet was then washed with isolation buffer and then the centrifugation was repeated. The pellet, containing a mixture of organelles, was further fractionated by centrifugation at 8500 ***g*** for 10 min at 4 °C in a Percoll gradient consisting of three layers of 18 %, 30 % and 60 % (w/v) Percoll in sucrose/Tris/HCl buffer (0.25 M sucrose, 1 mM EDTA and 50 mM Tris/HCl, pH 7.4). Mitochondria were collected from the interface of 30 % and 60 % Percoll and washed with the sucrose/Tris/HCl buffer. The concentration of the mitochondrial proteins was determined by the Bradford assay [18].

Isolation of mitochondrial IMS contents

Mitochondrial outer membrane was selectively disrupted by treating mitochondria (40 mg/ml) with digitonin (0.11 mg/mg of protein) [19]. The IMS contents were then freed of mitoplasts by centrifugation at 10 000 ***g*** for 15 min at 4 °C.

Measurement of SOD activity

SOD activity was assayed by generating $O_2^{\bullet -}$ with the xanthine/xanthine oxidase system in the presence of sulfonated tetrazolium salt, XTT [20]. Cyanide at 5.0 mM was used to selectively inhibit SOD1 activity.

Measurement of H_2O_2

Fluorescence measurements were performed by using a PerkinElmer LS-5 spectrofluorimeter. H_2O_2 formation was measured by monitoring horseradish peroxidase-catalysed H_2O_2-dependent oxidation of *p*-hydroxyphenylacetate ($\lambda_{ex} = 320$ nm; $\lambda_{em} = 400$ nm) [21].

Temporal kinetics of IMS SOD1 activation by H_2O_2

Mitochondria (2 mg/ml) were incubated with 5 μM H_2O_2 for 5, 10, 20 and 30 min at 37 °C, washed with sucrose/Tris/HCl buffer, and then incubated with digitonin in the presence of IAM for 1 h at room temperature (20 °C). SOD1 activity was assayed in the mitochondrial IMS preparations.

Preparation of the oxidized and reduced IMS SOD1

Rat liver IMS SOD1 was purified in the oxidized state as described previously [2]. The reduced enzyme was prepared following incubation of the purified enzyme with 4 mM DTT (dithiothreitol) at 37 °C overnight. DTT was then removed by dialysis. The activity of the oxidized or reduced SOD1 was measured following incubation with various agents for the indicated times at 37 °C.

Measurement of IMS SOD1 activation by mitochondrial superoxide

Mitochondria were incubated with various concentrations of antimycin ranging from 0.01–0.1 μg/mg of mitochondria respiring at state 4 in the presence of 1 mM *p*-hydroxyphenylacetate. H_2O_2 formation was measured by monitoring horseradish peroxidase-catalysed (5 units/ml) H_2O_2-dependent oxidation of *p*-hydroxyphenylacetate. Mitochondria were also incubated with 0.01–0.1 μg/mg of antimycin for the indicated times at 37 °C, washed with sucrose/Tris/HCl buffer, and incubated with digitonin in the presence of IAM for 1 h at room temperature. SOD1 activity was assayed in the mitochondrial IMS preparations.

Detection of TxrR-1 in mitochondrial IMS

Mitochondrial IMS and cytosolic fractions were resolved on 12 % (w/v) polyacrylamide gels and immunoblotted onto nitrocellulose membranes using anti-TxrR-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Cytosolic contamination was assessed using an anti-β-actin antibody (Sigma Chemical Co.).

Measurement of aconitase activity

Mitochondria were incubated in the absence or presence of 5 μM H_2O_2 for 5 min at 37 °C. The state 3 respiration was then initiated by addition of 2 mM ADP, 4 mM $MgCl_2$, 5 mM KH_2PO_4 and 10 mM succinate. Aliquots (1 ml) were taken at indicated times to determine mitochondrial aconitase activity. Aconitase activity was assayed at room temperature by following the formation of NADPH at 340 nm for 10 min in a reaction mixture containing 50 mM Tris/HCl (pH 7.4), 30 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$ and 1 unit/ml of isocitrate dehydrogenase. One nmole of NADPH formed per min corresponded to 1 milliunit of aconitase [22].

Measurement of Ψm and cytochrome *c* release from mitochondria

Mitochondria (1 mg/ml) were incubated in a buffer containing 250 μM sucrose, 10 mM Hepes/KOH (pH 7.4), 0.1 % fatty-acid free BSA, 6 mM succinate, 1 μg/ml rotenone and 1 μM Rhodamine 123 for 5 min at 25 °C in order to equilibrate mitochondria with the cationic dye. Mitochondria were incubated with 5 μM H_2O_2 for 3 min at 25 °C. $CaCl_2$ (12 or 25 μM) was then added to induce dissipation of Ψm. Ψm was assessed fluorimetrically by measuring the Ψm-dependent uptake of Rhodamine 123 (1 μM) with excitation at 505 nm and recording at 534 nm [23]. Pellets and supernatants were treated with SDS sample buffer [2.5 % (w/v) SDS, 20 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 0.1 mg/ml Bromophenol Blue and 20 mM Tris/HCl, pH 6.8] and boiled, and aliquots were resolved by SDS/PAGE and immunoblotted on to nitrocellulose membranes. Cytochrome *c* levels were measured using anti-(cytochrome *c*) antibodies (BD Bioscience, San Jose, CA, U.S.A.).

Data presentation

Results are shown as means $\pm$ S.D. for at least three independent experiments performed in triplicate. Analysis of variance and Duncan's multiple range test was used to analyse the difference among group means. $P < 0.05$ was considered statistically significant.

RESULTS

Superoxide and H_2O_2 promote activation of IMS SOD1

In order to determine the biochemical processes involved in the activation of IMS SOD1, a series of experiments were conducted with intact mitochondria and the purified inactive enzyme. Low micromolar concentrations (5 μM) of exogenous H_2O_2 activated

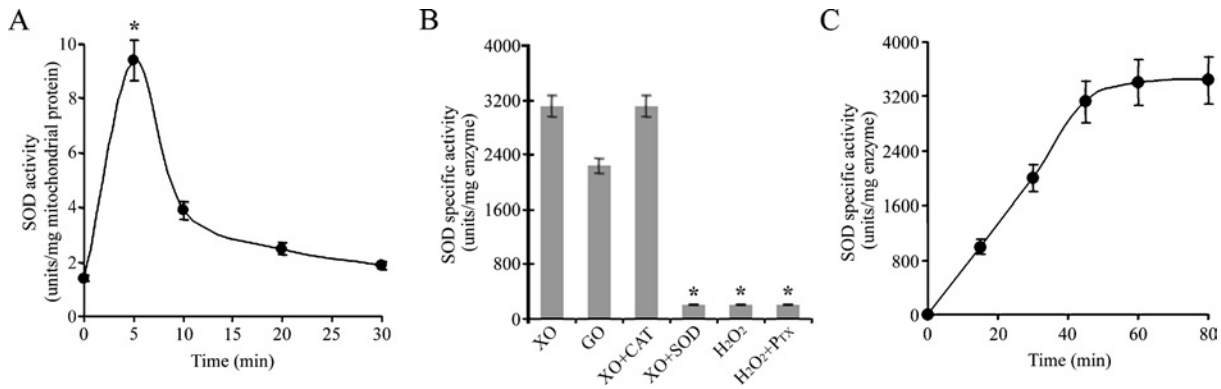


Figure 1 H_2O_2 and superoxide promote activation of IMS SOD1

(**A**) Temporal kinetics of SOD1 activation by exogenous H_2O_2 in intact mitochondria. Mitochondria were incubated with H_2O_2 (5 μM) for the indicated times at 37 °C, washed with sucrose/Tris/HCl buffer, and incubated with digitonin in the presence of IAM for 1 h at room temperature. *$P < 0.05$, compared with the activity at 0 min. (**B**) Superoxide, but not H_2O_2, directly activates purified IMS SOD1. SOD1 was purified from mitochondria and subsequently reduced by DTT to the inactive state. DTT was then removed by dialysis. The reduced enzyme (3 μM) was incubated with xanthine (50 μM) and xanthine oxidase (XO; 5 milliunits/ml), glucose (2 mM) and glucose oxidase (GO; 2 milliunits/ml), peroxiredoxin III (Prx; 20 μM) and H_2O_2 (5 μM), or H_2O_2 alone in the presence or absence of catalase (CAT; 1000 units/ml) or bovine erythrocyte SOD (300 units/ml) for 1 h at 37 °C. *$P < 0.05$ compared with the activity after incubation with xanthine oxidase. (**C**) Temporal kinetics of the reduced SOD1 activation by superoxide. The reduced IMS SOD1 (3 μM) was incubated with xanthine (50 μM) and xanthine oxidase (5 milliunits/ml) for the indicated times at 37 °C. (**A**–**C**) SOD1 activity was measured by following the reduction of XTT at 470 nm.

IMS SOD1 at a rate of 2 units/min per mg of mitochondrial protein (Figure 1A). The enzyme activity reached its maximum level of 10 units/mg of mitochondrial protein after 5 min of mitochondrial exposure to H_2O_2. The levels then decreased sharply at 10 min and ultimately returned to the basal levels at 30 min (Figure 1A). The possibility of direct interaction between IMS SOD1 and H_2O_2 was assessed by exploiting SOD1, which was purified from mitochondria and subsequently reduced by DTT to its inactive state. The specific activity of the purified IMS SOD1 was determined as 3200 ± 270 units/min per mg of enzyme following the rupture of the outer membrane with digitonin alone, but prior to reduction with DTT. Neither H_2O_2 alone nor H_2O_2 in the presence of mitochondrial peroxiredoxin III activated the reduced SOD1 (Figure 1B). However, the enzyme was markedly activated following incubation with the superoxide generating system, xanthine and xanthine oxidase, and to a lesser extent with glucose and glucose oxidase, a system producing superoxide, in addition to H_2O_2, with a rate dependent on the pH and O_2 concentration [24]. The lower efficiency of the glucose/glucose oxidase system is possibly due to a lower rate of superoxide production compared with the xanthine/xanthine oxidase system. The presence of bovine erythrocyte SOD (300 units/ml) in the assay mixture abolished this activation, whereas catalase was ineffective (Figure 1B). Examination of the kinetics of the enzyme activation revealed that steady state levels of superoxide (0.7–0.8 nmol/ml per min) generated by xanthine/xanthine oxidase activate the reduced IMS SOD1 at a rate of 66 units/min per mg of enzyme (Figure 1C). The enzyme specific activity ultimately reached a plateau of 3200 units/mg of enzyme at 45 min. These findings suggest that both H_2O_2 and superoxide may act as activators of IMS SOD1. Although superoxide can act directly upon the enzyme, H_2O_2 requires additional mitochondrial component(s) to trigger activation of SOD1.

Superoxide generated by mitochondrial respiration activates SOD1 in intact mitochondria

In order to verify whether superoxide generated during mitochondrial respiration can activate SOD1 in the IMS, superoxide production was promoted by antimycin in mitochondria respiring at state 4 (Figure 2A). Mitochondrial superoxide production was

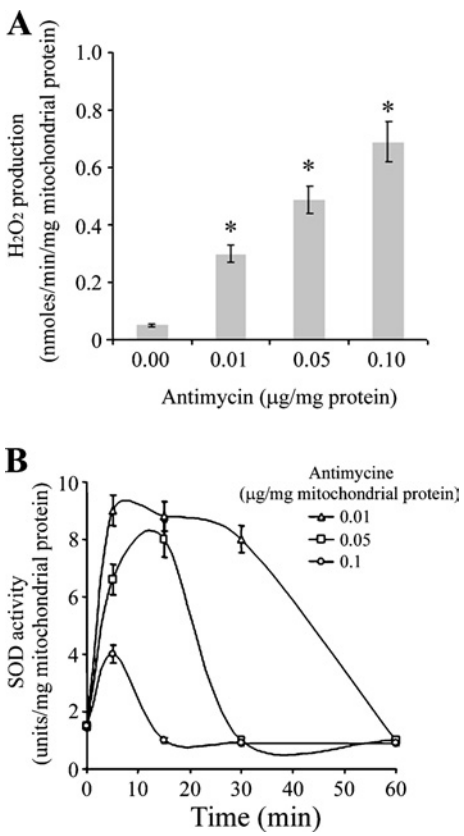


Figure 2 Mitochondrial respiration regulates SOD1 activity in the IMS

(**A**) Kinetics of superoxide production by antimycin-treated mitochondria. Mitochondria (2 mg/ml) respiring at state 4 were incubated with the indicated concentrations of antimycin in the presence of 1 mM *p*-hydroxyphenylacetate. Superoxide formation was measured indirectly by monitoring horseradish peroxidase-catalysed (5 units/ml) H_2O_2-dependent oxidation of *p*-hydroxyphenylacetate. *$P < 0.05$ compared with H_2O_2 production in the absence of antimycin. (**B**) Temporal kinetics of SOD1 activation by superoxide generated during mitochondrial respiration. Mitochondria (2 mg/ml) respiring at state 4 were incubated with the indicated concentrations of antimycin for the specified times at 37 °C. Mitochondria were subsequently washed with sucrose/Tris/HCl buffer and incubated with digitonin in the presence of IAM for 1 h at room temperature. SOD1 activity was measured by following the reduction of XTT at 470 nm.

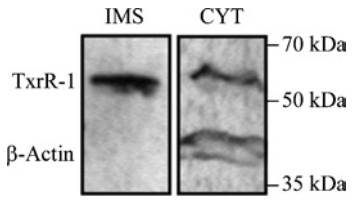


Figure 3 TxrR-1 is localized in the IMS of rat liver mitochondria

TxrR-1 was detected by Western blotting in both IMS and cytosolic fractions. Cytosolic contamination of IMS fractions was assessed by detection of β-actin.

increased in a dose-dependent manner with antimycin concentrations between 0.01 and 0.1 μg/mg of mitochondrial protein, generating H_2O_2 ranging from 0.30 ± 0.03–0.65 ± 0.04 nmoles/min per mg of protein (equivalent to superoxide ranging from 0.60 ± 0.06–1.35 ± 0.08 nmoles/min per mg of protein). An inverse relationship was, however, found between the levels of mitochondrial superoxide production and the extent and duration of SOD1 activation in the IMS. SOD1 was more potently activated for a significantly longer duration when mitochondria generated lower levels of superoxide (0.60 ± 0.06 nmol/min per mg of mitochondrial protein) compared with a lesser and shorter duration of the enzyme activity when mitochondria generated high levels of superoxide (1.35 ± 0.08 nmoles/mg of mitochondrial protein per min; Figure 2B). These findings suggest that mitochondrial respiration regulates SOD1 activity in the IMS.

TxrR-1 inactivates IMS SOD1

The presence of TxrR-1 in the IMS of rat liver mitochondria was detected by Western blotting (Figure 3). In order to determine potential biochemical pathway(s) implicated in the inactivation of IMS SOD1, the enzyme was purified from mitochondria in an active, oxidized state by rupturing the outer membrane with digitonin alone. Incubation with TxrR-1 from rat liver significantly decreased activity of the oxidized SOD1 (Figure 4A). The initial rate of inactivation of the oxidized enzyme was estimated at 60 units/min per mg of enzyme (Figure 4B). The presence of Txr in the assay mixture did not potentiate inactivation of the oxidized enzyme by TxrR-1 (Figure 4A). Furthermore, neither Txr nor GSH alone affected the superoxide scavenging activity of the oxidized form of SOD1. These findings suggest that mitochondrial TxrR-1 is implicated in the inactivation of IMS SOD1.

CDNB (1-chloro-2,4-dinitrobenzene) is an alkylating agent that selectively inhibits TxrR-1 at low concentrations (250 μM). Pre-incubation of purified IMS SOD1, which was reduced with DTT and subjected to dialysis to remove excess DTT, with CDNB at 37 °C for 10 min did not affect activation of the reduced enzyme using the xanthine/xanthine oxidase system (results not shown). This finding suggests that CDNB, at the concentration used here, is unable to alkylate and inhibit IMS SOD1. Addition of CDNB to intact mitochondria after 4 min of H_2O_2 treatment substantially prolonged the duration of H_2O_2-induced IMS SOD1 activity (Figure 4C). In a similar set of experiments, auranofin, a potent inhibitor of selenocystein-containing enzymes [25], comparably prolonged the enzyme activity induced by H_2O_2 (results not shown). These findings suggest that TxrR-1 plays a role in inactivation of SOD1 in intact mitochondria. However, pre-incubation of intact mitochondria with CDNB completely abolished activation of IMS SOD1 by H_2O_2 (Figure 4C). It is likely that mitochondrial factor(s) involved in H_2O_2-induced activation of IMS SOD1 are sensitive to alkylation by CDNB under this experimental condition.

IMS SOD1 protects against oxidative loss of aconitase activity in the mitochondrial matrix

Rat liver mitochondria are known to produce superoxide when respiring with complex II-linked substrates in state 3 [26,27]. Incubation of rat liver mitochondria respiring at state 3 at 37 °C resulted in loss of aconitase activity by 33 ± 5 % and 42 ± 4 % after 5 and 15 min respectively (Figure 5). However, activation of IMS SOD1 following incubation of intact mitochondria with H_2O_2 (5 μM) significantly prevented loss of aconitase activity in mitochondrial matrix (Figure 5).

IMS SOD1 diminishes the apoptotic capacity of mitochondria

$CaCl_2$ progressively promoted dissipation of Ψm in mitochondria respiring at state 4 (Figure 6A). Loss of Ψm is known to

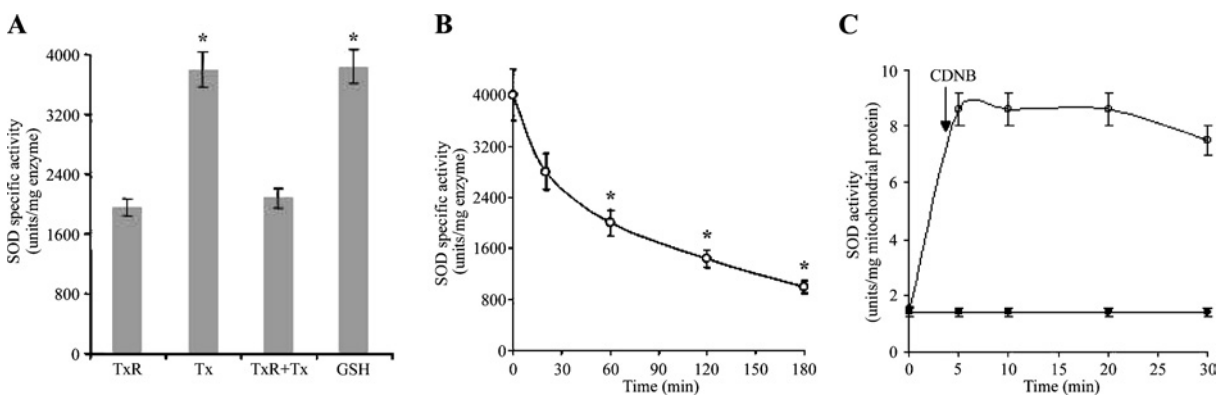


Figure 4 TxrR-1 inactivates the oxidized IMS SOD1

SOD1 was purified from mitochondria in an active, oxidized state by rupturing the outer membrane with digitonin. (**A**) Inactivation of IMS SOD1 by TxrR-1. The oxidized enzyme (3 μM) was incubated with TxrR-1 (20 units/ml), Txr (15 units/ml) alone or together, or with glutathione (5 mM) for 60 min at 37 °C. NADPH (0.25 mM) was added to the reaction mixture when TxrR-1 was present. SOD1 activity was assayed by following the reduction of XTT at 470 nm. *$P < 0.05$ compared with the activity in the presence of TxrR-1 and Txr. (**B**) Temporal kinetics of SOD1 inactivation by TxrR-1. The oxidized enzyme (3 μM) was incubated with TxrR-1 (20 units/ml) and NADPH (0.25 mM) for the indicated times at 37 °C. *$P < 0.05$ compared with the activity at 0 min. (**C**) CDNB prolongs the duration of IMS SOD1 activity in intact mitochondria. Mitochondria were incubated with H_2O_2 (5 μM) for the indicated times at 37 °C, washed with sucrose/Tris/HCl buffer and incubated with digitonin in the presence of IAM for 1 h at room temperature. CDNB (250 μM) was pre-incubated with mitochondria for 10 min (●) or it was added after 4 min of H_2O_2 incubation (○). (**A**–**C**) SOD1 activity was measured by following the reduction of XTT at 470 nm.

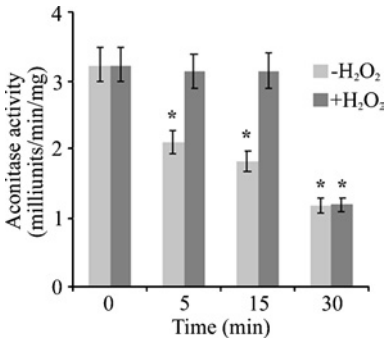


Figure 5 IMS SOD1 prevents oxidative loss of aconitase activity in the mitochondrial matrix

Mitochondria (2 mg/ml) were incubated in the absence or presence or H_2O_2 (5 μM) at 37 °C for 5 min. State 3 respiration was then initiated following addition of ADP (2 mM), $MgCl_2$ (4 mM), KH_2PO_4 (5 mM) and succinate (10 mM) for the indicated times. Aconitase activity was subsequently measured as described in the Experimental section. *$P < 0.05$ compared with the activity at 0 min.

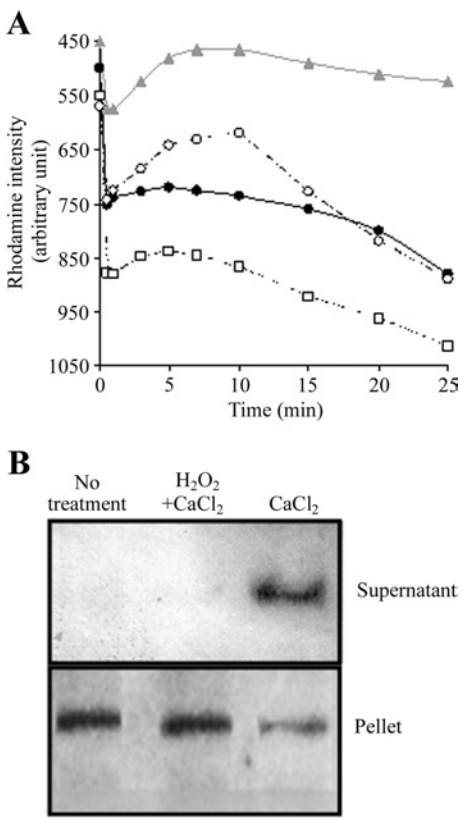


Figure 6 IMS SOD1 decreases the apoptotic capacity of mitochondria

(**A**) Delay in the loss of Ψm induced by $CaCl_2$. Mitochondria (1 mg/ml) were incubated at 37 °C for 3 min in the presence (●) or absence (○, ▲ and □) of H_2O_2 (5 μM) followed by addition of vehicle (▲) or 12 μM (○) or 25 μM (● and □) $CaCl_2$ to dissipate Ψm. Rhodamine 123 uptake was monitored to measure Ψm. (**B**) Prevention of mitochondrial cytochrome *c* release induced by $CaCl_2$. Mitochondria (1 mg/ml) were incubated at 37 °C for 3 min in the presence or absence of H_2O_2 (5 μM) followed by the addition of 25 μM $CaCl_2$ for 30 min. The supernatants were then concentrated 10-fold and cytochrome *c* was detected by Western blotting in both the mitochondrial pellets (lower panel) and the supernatants (upper panel).

precede the release of mitochondrial apoptotic factors, such as cytochrome *c*. Analysis of the mitochondria incubated with $CaCl_2$ for 30 min demonstrated significant loss of IMS cytochrome *c* accompanied by its release into the extra-mitochondrial space (Figure 6B, upper panel). Prior activation of IMS SOD1, by exposing mitochondria to H_2O_2 (5 μM), significantly delayed loss of Ψm and prevented cytochrome *c* release induced by $CaCl_2$ (Figure 6). These findings suggest that the active state of IMS SOD1 may regulate events leading to the loss of mitochondrial integrity and cell death.

DISCUSSION

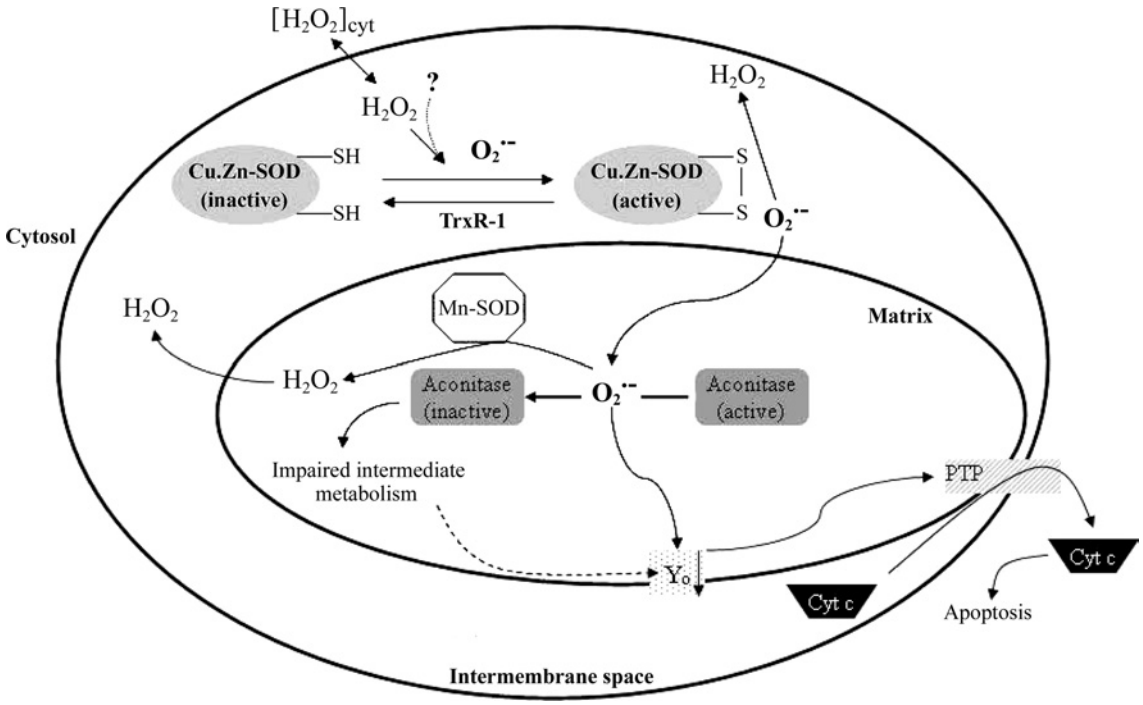
The findings of the present study suggest that the mitochondrial respiratory chain and TxrR-1 regulate IMS SOD1 activity. Scheme 1 illustrates a network of biochemical pathways, which may regulate or be affected by IMS SOD1 activity. It appears that IMS SOD1 is implicated in protecting intermediate metabolism against oxidative damage and regulating apoptosis.

Regulation of SOD1 activity in the IMS

In order to elucidate the biochemical pathways involved in reversible activation of IMS SOD1, a series of experiments were conducted with intact mitochondria and with purified SOD1 reduced by DTT to an inactive state. Although H_2O_2 transiently activated SOD1 in intact mitochondria, it did not affect the activity of the reduced enzyme once it was purified from the IMS, suggesting that additional mitochondrial component(s) are necessary to catalyse the intra-subunit disulfide-bond formation. In spite of localization of the peroxiredoxin III in the mitochondrial matrix and in the IMS, our findings suggest that peroxiredoxin III is not implicated in the activation of SOD1. However, given that H_2O_2 is readily diffusible across membranes, these findings suggest that IMS SOD1 is responsive to fluctuations of physiologically relevant concentrations of H_2O_2 in a cell.

Mitochondria are the major source of superoxide. The mitochondrial inner membrane vectorally releases superoxide into the IMS [28] upon autoxidation of the ubisemiquinone at the outer coenzyme Q pool. Direct activation of IMS SOD1 by superoxide constitutes a typical example of enzyme regulation in response to changes in the level of its substrate. The rate of SOD1 activation was, however, different following exposure to H_2O_2 and superoxide. Superoxide, generated by the xanthine/xanthine oxidase system or by mitochondrial respiration, activated SOD1 at a lower rate (0.6 μM/min) than H_2O_2 (5 μM/min). The higher rate of enzyme activation by H_2O_2 may reflect the enzyme-catalysed nature of this reaction. However, temporal kinetics of the enzyme activation by H_2O_2 and superoxide were similar in the intact mitochondria. Both H_2O_2 and superoxide elicited a transient activation of SOD1, although the extent and duration of enzyme activation was longer with superoxide compared with H_2O_2. It is noteworthy that the activity of SOD1 was inversely correlated with the level of mitochondrial superoxide production; a similar phenomenon was observed following exposure of intact mitochondria to exogenous H_2O_2. These findings suggest that IMS SOD1 is active in a narrow window of physiologically relevant concentrations of superoxide or H_2O_2. The underlying reason for the lower level of the enzyme activity in the presence of higher concentrations of superoxide or H_2O_2 remains to be elucidated.

The transient nature of the enzyme activation could also be attributed to the biochemical pathways inactivating IMS SOD1. One such enzyme detected in the IMS was TxrR-1. The rate of SOD1 inactivation (0.1 μM/min) was, however, slower than its activation with either H_2O_2 or superoxide. Whether additional enzymatic pathways are implicated in inactivation of IMS SOD1 remain to be determined. Alternatively, faster inactivation of SOD1 in intact mitochondria could be potentially due to the



Scheme 1 Putative biochemical pathways regulating IMS SOD1 activity and the impact of its active state on mitochondrial functions

SOD1 is inactive in the IMS of rat liver mitochondria. An increase in the tonus of superoxide and/or H_2O_2 in mitochondrial matrix and/or cytosol activates SOD1 in the IMS. Upon activation, IMS SOD1 prevents oxidative loss of matrix aconitase activity, delays dissipation of mitochondrial inner membrane potential, and prevents cytochrome *c* release into the extra-mitochondrial space, possibly by scavenging the excess superoxide, and hence decreasing intra-mitochondrial superoxide levels. TxrR-1 catalyses reduction of the critical disulfide group and inactivates IMS SOD1.

substantially higher IMS TxrR-1 concentration compared with that used in the assay described for Figure 4 (20 units/ml). Moreover, CDNB and auranofin, which are selective TxrR-1 inhibitors, prolonged the duration of H_2O_2-induced IMS SOD1 activation. These findings demonstrate that TxrR-1 is implicated in the inactivation of IMS SOD1. In agreement with these findings, eukaryotic SOD1 expressed in prokaryotic organisms was found to be active only when the cytosolic expression of TxrR-1 was prevented [29].

IMS SOD1 protects vital mitochondrial functions

Yeast mutants lacking, or deficient in, SOD1 present direct aerobic inactivation of enzymes, such as aconitase, homoaconitase and succinate dehydrogenase, which contain superoxide sensitive matrix 4Fe-4S clusters. These findings suggest that superoxide released into the IMS may pass through the mitochondrial inner membrane and damage matrix 4Fe-4S-cluster-containing enzymes, even in the presence of Mn-SOD [30]. Superoxide (pK 4.8) is likely to be protonated at the external surface of the inner membrane, due to proton extrusion, and diffuse into the matrix [31], or it may react with mitochondrial nitric oxide to produce peroxynitrite (pK 6.8), which can then diffuse into the matrix [32]. Our findings indicate that active IMS SOD1 protects the matrix aconitase activity against superoxide-mediated inactivation.

It has long been known that Ca^{2+} overload leads to the state of mitochondrial membrane permeability transition [15,16] and the opening of PTPs following increased mitochondrial production of reactive oxygen species [33]. The opening of PTPs leads to cytochrome *c* release into the cytosol, which then triggers the apoptotic process [34]. Transient activation of IMS SOD1 significantly decelerated dissipation of Ψm and decreased release of cytochrome *c*, suggesting that IMS SOD1 may function to prevent apoptosis during episodes of increased mitochondrial superoxide levels.

Taken together, these findings suggest that H_2O_2, superoxide and TxrR-1 reversibly regulate IMS SOD1 activity, and that the enzyme is implicated in protecting vital mitochondrial functions.

This work was supported by grants from IberCaja–Universidad de Zaragoza and from the Gobierno de Aragón.

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Received 5 December 2006/22 March 2007; accepted 30 March 2007
Published as BJ Immediate Publication 30 March 2007, doi:10.1042/BJ20061809