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Melatonin and Steroid Hormones Activate Intermembrane CU,ZN-Superoxide Dismutase by Means of Mitochondrial Cytochrome P450

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Abstract

Melatonin and steroid hormones are cytochrome P450 (CYP or P450; EC 1.14.14.1) substrates that have antioxidant properties and mitochondrial protective activities. IMS (Mitochondrial intermembrane space) SOD1 (Cu,Zn-superoxide dismutase) is activated following oxidative modification of its critical thiol moieties by superoxide anion ($O_2^{\cdot-}$). This study was aimed at investigating the potential association between the hormonal protective antioxidant actions in mitochondria and regulation of IMS SOD1 activity. Melatonin, testosterone, dihydrotestosterone, estradiol, and vitamin D induced a sustained activation over time of SOD1 in intact mitochondria showing a bell-shaped enzyme activation dose-response with a threshold at 50 nM and a maximum effect at 1 μ M concentration. Enzyme activation was not affected by furafylline, but it was inhibited by omeprazole, ketoconazole, and tiron, thereby supporting the occurrence of a mitochondrial P450 activity and $O_2^{\cdot-}$ requirements. Mitochondrial P450-dependent activation of IMS SOD1 prevented $O_2^{\cdot-}$ -induced loss of aconitase activity in intact mitochondria respiring at state 3 respiration. Optimal protection of aconitase activity was observed at 0.1 μ M P450 substrate concentration evidencing a likely oxidative effect on the mitochondrial matrix by higher substrate concentrations. Likewise, enzyme activation mediated by mitochondrial P450 activity delayed $CaCl_2$ -induced loss of trans-membrane potential, and decreased cytochrome *c* release. Omeprazole and ketoconazole abrogated both protecting mitochondrial functions promoted by melatonin and steroid hormones.

Melatonin has been widely described as an efficient antioxidant with a remarkable ability to scavenge hydroxyl radical and peroxynitrite [1, 2] and also but less potently a variety of reactive oxygen and nitrogen species including, hydrogen peroxide (H_2O_2) [3], singlet oxygen [4], nitric oxide [5], and $O_2^{\cdot-}$ [6]. Accordingly, systemic treatment with melatonin increased glutathione peroxidase activity in rats [7] as well as the gene expression and activity of antioxidant enzymes, such as SOD2 (Mn-SOD) and SOD1 (Cu,Zn-SOD) [8]. It has also been reported that melatonin improved mitochondrial function by decreasing their oxidative status, increasing the activity of the respiratory chain [9] and enhancing

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mitochondrial ATP production by stimulation of complex I and IV activities without changes in ATP synthase activity [10]. Likewise, the free radical scavenging activities of steroid hormones partly support their cellular and mitochondrial protective functions [11-13].

P450 and NADPH-cytochrome P450 reductase (NPR; EC 1.6.2.4) are components of the monooxygenase system involved in the oxidation of a variety of endogenous and xenobiotic compounds including melatonin, steroid hormones, drugs, and carcinogens in eukaryotes [14]. P450 is a super family of membrane b-type cytochrome enzymes that contain more than 200 different molecular species [15]. A widespread division of P450 monooxygenase systems into two main types, microsomal and bacterial/mitochondrial is recognized [16]. Mitochondrial P450s are located in the inner membrane and were found to participate in the biosynthesis of bile acids from cholesterol in the liver, steroid hormones in adrenal gland and gonads, and in the metabolic activation of Vitamin D3 to its active form, 1, 25-dihydroxyvitamin D3, in liver and kidney [17]. Most mitochondrial P450s show high specificity for their endogenous substrates and have negligible activity towards xenobiotic compounds [18]. During the P450 enzyme catalytic cycle, molecular oxygen reacts with ferrous P450 to yield a dioxygen adduct ($\text{Fe}^{\text{II}}-\text{O}_2$); although this complex is relatively stable, it can dissociate to Fe^{III} and $\text{O}_2^{\cdot-}$ with a rate constant of 0.01 s^{-1} at room temperature ($\text{Fe}^{\text{II}}-\text{O}_2 \rightarrow \text{Fe}^{\text{III}} + \text{O}_2^{\cdot-}$) [19]. This step is a decoupling or uncoupling reaction and is more frequent in eukaryotic P450s than in bacterial enzymes. Therefore, P450 enzymes are considered as relevant sources of $\text{O}_2^{\cdot-}$ not only as a physiological mediator in healthy conditions but also in pathophysiological situations entailing oxidative stress [20].

IMS (intermembrane space) SOD1 is inactive in isolated intact rat liver mitochondria [21, 22] but it is activated following oxidative modification of its critical thiol groups by $\text{O}_2^{\cdot-}$ [23, 24, 25, 26]. The exact mechanism is not known and it could be completed by a specific mediator as mitochondrial CCS [24, 25, 26]. Melatonin and steroid hormones are also substrates of P450-catalyzed-mitochondrial covalent modifications. This study was aimed at investigating whether mitochondrial oxidation of P450 substrates could induce the activation of IMS SOD1, as a contribution of their protective actions in mitochondria, and at examining its consequence on vital mitochondrial functions, such as intermediate metabolism, trans-membrane potential, and apoptosis.

Materials and Methods

Chemicals

Melatonin, estradiol, ketoconazole, omeprazole, fatty acid free bovine serum albumin (BSA), 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT), digitonin, iodoacetamide (IAM), xanthine oxidase, NADP⁺, isocitrate dehydrogenase, rotenone, and rhodamine 123 were from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone, dihydrotestosterone, estradiol, and vitamina D were from Fluka (Buchs, Switzerland),

Isolation of liver mitochondria

Liver mitochondria were isolated from adult male Wistar rats by differential centrifugation as described previously [27]. Rat livers were excised, chopped into fine pieces, washed with 0.25 M sucrose and homogenized using an isolation buffer consisted of 210 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, and 0.05% BSA. The homogenate was centrifuged at 800g for 8 min, the pellet was removed, and the centrifugation process was repeated. The supernatant was centrifuged at 8,000g for 10 min, the pellet was washed with the isolation buffer, and the centrifugation was repeated. The pellet containing mixture of organelles were

further fractionated by centrifugation at 8,500 g for 10 min in Percoll gradient consisted of three layers of 18%, 30%, and 60% (w/v) Percoll in sucrose-Tris buffer (0.25 M sucrose, 1 mM EDTA, and 50 mM Tris), pH 7.4. Mitochondria were collected from the interface of 30% and 60% Percoll and washed with the sucrose-Tris buffer. Mitochondrial proteins were determined by Bradford assay [28].

Isolation of IMS contents

Mitochondrial outer membrane was selectively disrupted by treating mitochondria (40 mg/ml) with 0.11 mg of digitonin per mg of protein [29]. The IMS contents were then freed of mitoplasts by centrifugation at 10,000 g for 15 min.

Assays of marker enzymes

The purity of isolated mitochondria was characterized enzymatically by measuring, in both granule and Percoll-purified mitochondrial fractions, the activities of acid phosphatase (a lysosomal marker), urate oxidase (a peroxisomal marker), and fumarase and sulphite oxidase (mitochondrial matrix and mitochondrial IMS markers, respectively), as described previously [22]. The activities of the marker enzymes in the mitochondrial fraction were expressed as percentages of total activities observed in the granule fraction.

Measurement of SOD activity

SOD activity was assayed by generating $O_2^{\cdot-}$ with the xanthine/xanthine oxidase system in the presence of sulphonated tetrazolium salt, XTT [30]. Cyanide at 5.0 mM was used to selectively inhibit SOD1 activity. SOD activity was also assayed in native gels following electrophoresis by NBT (Nitro Blue Tetrazolium) staining (data not shown) [31].

IMS SOD1 activation induced by hormones and its inhibition

Mitochondria (2 mg/ml) were incubated with 1 μ M of melatonin, steroid hormones, and vitamin D for 5, 10, 20, 30 minutes at 37°C. Likewise mitochondria were incubated 0.05, 0.1, 0.5, 1, 5, 10, and 20 μ M of melatonin and steroid hormones and vitamin D for 5 minutes at 37°C. Inhibition assays were developed with 1 μ M of melatonin, steroid hormones, and vitamin D for 5 minutes at 37°C in the presence of 20 μ M of furafylline, 100 μ M of ketoconazole and omeprazole, 1 mM of tiron, 5 μ M of amiodarone, 50 μ M of amitriptyline, and 250 μ M of Mg^{+2} . Then mitochondria were washed with sucrose-Tris buffer, and then incubated with digitonin in the presence of iodoacetamide (IAM) for 1 hour at room temperature. SOD1 activity was assayed in the mitochondrial IMS preparations.

Measurement of H₂O₂ production by melatonin-treated mitochondria

Mitochondria (0.5 mg/ml) were incubated with various concentrations of melatonin alone ranging from 0.1–10 μ M and with 1 mM tiron or 30 μ M omeprazole in the presence of 1 mM *p*-hydroxyphenylacetate. H₂O₂ formation was measured by monitoring horseradish peroxidase catalysed (25 units/ml) H₂O₂-dependent oxidation of *p*-hydroxyphenylacetate.

Measurement of aconitase activity

Mitochondria were incubated in the presence or absence of 5 μ M H₂O₂ for 5 minutes at 37°C. The state 3 respiration was then initiated by addition of 2 mM ADP, 4 mM MgCl₂, 5 mM KH₂PO₄, and 10 mM succinate. Aliquots were taken at indicated times to determine mitochondrial aconitase activity. Aconitase activity was assayed at room temperature by following formation of NADPH at 340 nm for 10 minutes in a reaction mixture containing 50 mM Tris HCl, pH 7.4, 30 mM sodium citrate, 0.6 mM MnCl₂, 0.2 mM NADP⁺ and 1 U/ml of isocitrate dehydrogenase. One nmole NADPH formed per min was corresponded to 1 mU of aconitase [32].

Measurement of Ψ_m and cytochrome c release from mitochondria

Mitochondria (1 mg/ml) were incubated in a buffer containing 0.250 mM sucrose, 10 mM Hepes/KOH, pH 7.4, 0.1% fatty-acid free bovine serum albumin, 6 mM succinate, 1 μ g/ml rotenone, and 1 μ M Rhodamine 123 for 5 min at 25°C in order to equilibrate mitochondria with the cationic dye. Mitochondria were incubated with 15 μ M melatonin, steroid hormones, vitamin D, ketoconazole, and omeprazole for 5 minutes at 25°C. CaCl_2 25 μ M was then added to induce dissipation of Ψ_m . Ψ_m was assessed fluorimetrically by measuring the Ψ_m dependent uptake of Rhodamine 123 (1 μ M) with excitation at 505 nm and recording at 534 nm [33]. Pellets and supernatants were treated with SDS sample buffer and boiled, and aliquots were immunoblotted using anti-cytochrome c antibodies.

Statistical analysis

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 were used to analyse the difference among group means. $P < 0.05$ was considered statistically significant.

Results

Isolation and purity of mitochondrial preparations

The activities of marker enzymes for lysosomes, peroxisomes, and mitochondria were measured both in the granule- and Percoll purified mitochondrial preparations. The activities of selected marker enzymes in mitochondrial preparations were expressed as percentages of total activity observed in the granule fraction before centrifugation in Percoll gradients (Table 1). Purified mitochondria preparation was well separated from microsomes showing that glucose-6-phosphatase activity, a typical microsomal marker in mammalian cells, was not present (data not shown). The identity of isolated mitochondrial fractions was confirmed by the presence of high levels of fumarase and sulphite oxidase activities, located in mitochondrial matrix and intermembrane space respectively.

Melatonin, steroid hormones, and vitamin D activate IMS SOD1 via mitochondrial P450 activity and inner membrane anion channel (IMAC)

Incubation of mitochondria with low micromolar concentrations of melatonin, testosterone, dihydrotestosterone, estradiol, and vitamin D, induced the activation of IMS SOD1 (Fig. 1, panels A, B, C, D, and E, respectively). Maximal activation was observed at 5 min of exposure to hormones and vitamin D; this maximal activation was sustained for about 30 min. Activation of IMS SOD1 under these experimental conditions showed a bell-shaped dose response with a threshold at about 50 nM and maximal effect at about 1 μ M (Fig. 2). Enzyme activation was not affected by furafylline, a selective inhibitor of P450 1A2 isoform, but it was inhibited by omeprazole and ketoconazole, which have been reported as selective inhibitors of rat liver P450 3A subfamily [34] (Fig. 3). Moreover, induced enzyme activation was markedly inhibited by tiron, a O_2^- specific scavenger (Fig. 3). Likewise, amiodarone, amitriptyline, and Mg^{+2} , reported specific IMAC inhibitors [35], were also able for abrogating IMS enzyme activation. These observations suggest that in rat liver intact mitochondria P450 substrates were able to activate IMS SOD1 by means of O_2^- produced and released into mitochondrial matrix by $\text{Fe}^{\text{II}}-\text{O}_2$ complex dissociation ($\text{Fe}^{\text{II}}-\text{O}_2 \rightarrow \text{Fe}^{\text{III}} + \text{O}_2^-$) during P450 catalytic cycle, .

H_2O_2 generated by mitochondrial P450 activity

In order to assess quantitatively H_2O_2 generation with an original source from mitochondrial P450 catalytic activity, mitochondria were incubated in the presence of increasing concentration of melatonin (Fig. 4). Mitochondrial H_2O_2 production was increased in a

dose-dependent manner with melatonin concentrations between 0.1 μ M and 10 μ M, generating H_2O_2 ranging from 0.28 ± 0.03 – 1.05 ± 0.10 nmoles/ min per mg of protein (equivalent to O_2^- ranging from 0.56 ± 0.06 – 2.10 ± 0.20 nmoles/min per mg of protein). H_2O_2 production from melatonin treated mitochondria was abrogated by tiron, a O_2^- scavenger, and by omeprazole, a reported selective inhibitor of rat liver P450 3A subfamily [34]

Effect of P450 substrates on protection against oxidative loss of aconitase activity in mitochondrial matrix

Incubation of rat liver mitochondria at 37°C while respiring on state 3 (in the presence of ADP and succinate) resulted in loss of aconitase activity by $33 \pm 5\%$ and $42 \pm 4\%$ after 5 and 15 min, respectively (Fig. 5). However, activation of IMS SOD1 following incubation of rat liver intact mitochondria with P450 substrates significantly prevented loss of aconitase activity in the mitochondrial matrix (Fig. 5). The bell-shaped dose response observed on aconitase protection showed an optimal effect at 0.1 μ M, a significant lower P450 substrate concentration than that required for maximal induced enzyme activation (Fig. 6). The antioxidant scavenger properties of P450 substrates such as melatonin have been established [1, 2]; however, a possible interfering and perturbing respiratory effect was observed at higher doses of substrates that do not induce enzyme activation but are also apparently protective of aconitase activity (Fig. 6).

P450 substrates reduce apoptotic capacity of mitochondria

Loss of membrane potential in intact mitochondria respiring on state 4 by $CaCl_2$ is known to precede the release of mitochondrial apoptotic factors, such as cytochrome *c* (Fig. 7A). Analysis of the mitochondria incubated with $CaCl_2$ for 30 min showed significant loss of intermembrane space cytochrome *c* (Fig. 7B, upper panel). Prior exposure of intact mitochondria to P450 substrates (at a concentration entailing the activation of IMS SOD1) delayed loss of membrane potential and prevented $CaCl_2$ -induced cytochrome *c* release. Omeprazole –a selective inhibitor of P450 – abolished the delay of membrane potential loss and did not prevent cytochrome *c* release induced by $CaCl_2$. These findings suggest that the expression and activity of the mitochondrial P450 system along with substrate availability may contribute to mitochondrial function regulation via activation of IMS SOD1.

Discussion

Data in this study show that the mitochondrial P450-dependent metabolism of melatonin, steroid hormones, and vitamin D is associated with the activation of IMS SOD1. This notion is strengthened by the effect of selective inhibitors of the P450 system, which failed to activate the SOD1. The effect of tiron suggests the involvement of O_2^- in activation of the dismutase. However, the exact mechanism is still certainly not known and it can not be rule out that O_2^- induced IMS SOD1 activation could be eventually made by another enzyme or molecule, most likely through mediation of mitochondrial CCS or glutathione [26]. Bell-shaped dose response observed in hormone induced activation of IMS SOD1 under these experimental conditions could be sustained because of uncontrolled oxidation of SOD1 thiol groups that could be detrimental to its activity [26]. From a physiological perspective, it has been suggested that melatonin may accumulate in some cellular structures such as cell membrane, cytosol and nuclei; intramitochondrial melatonin levels are 100-fold higher than 1 nM nocturnal plasma concentration [36]. It has long been known that of P450 subfamily is expressed in rat liver microsomes and brain mitochondria [37, 38]. Interestingly, it has been recently reported that melatonin metabolism by P450 *in vivo* is carried out more efficiently in mitochondria than in rat liver microsomes [39]. Likewise, mitochondrial P450 has been also described to catalyze the steroid hormones hydroxylation and the metabolic

activation of vitamin D3 to its active form, 1, 25-dihydroxyvitamin D3. Moreover, according with our study, melatonin has been widely described as a very powerful and efficient cellular protective molecule by preserving mitochondrial homeostasis and function [1-10]. Steroid hormones and vitamin D have been also described as compounds that support mitochondrial function; hence, the specific metabolism of these compounds by mitochondrial P450 may be considered as a component of mitochondrial physiology.

We have recently demonstrated that active IMS SOD1 protects matrix aconitase activity against $O_2^{\cdot-}$ -mediated inactivation [23]. This observation is in agreement with current findings that suggest that $O_2^{\cdot-}$ released into the intermembrane space may cross the mitochondrial inner membrane and damage 4Fe-4S cluster-containing enzymes even in the presence of SOD2 [40]. $O_2^{\cdot-}$ (pK 4.8) is likely to be either protonated ($HO_2^{\cdot}$) at the external surface of inner membrane due to the H^+ extrusion and diffuse into the matrix [41] or react with nitric oxide ($\cdot NO$) to yield peroxynitrite ($ONOO^-$) (pK 6.8), which can be diffuse into the matrix [42]. Hence, direct aerobic inactivation of enzymes, such as aconitase, homoaconitase, and succinate dehydrogenase, containing $O_2^{\cdot-}$ -sensitive 4Fe-4S clusters was observed in yeast mutant lacking or deficient in SOD1. In this study, mitochondrial P450-dependent activation of IMS SOD1 results in significant protection of aconitase activity. Maximal protection of aconitase was observed at concentrations 0.1 μM , a considerable more physiological substrate concentration in these organelles. The free radical-scavenging properties of some P450 substrates, such as melatonin, have been extensively investigated [1, 2]; however, a perturbing effect on mitochondrial respiration could be exerted at higher doses that do not promote or induce enzyme activation [43].

Local increase in mitochondrial production of reactive oxygen species by Ca^{2+} overload promotes loss of membrane potential (Ψ_m) and the opening of PTP [44, 45, 46] leading to cytochrome *c* release into cytosol and activation of the intrinsic apoptotic pathway [47]. We have recently reported that transient activation of IMS SOD1 significantly decreased Ψ_m dissipation and cytochrome *c* release induced by mitochondrial Ca^{2+} overload, thus suggesting that intermembrane space Cu,Zn-SOD may function to prevent apoptosis during episodes of increased mitochondrial $O_2^{\cdot-}$ levels [23]. Likewise, activation of IMS SOD1 in intact mitochondria by P450 activity significantly delayed loss of Ψ_m and blocked release of cytochrome *c* induced by mitochondrial Ca^{2+} overload.

These findings suggest that mitochondrial P450-dependent metabolism of melatonin, steroid hormones, and vitamin D is an effective source of $O_2^{\cdot-}$, which could mediate potentially by means of mitochondrial CCS or other mediators [25, 26], the activation of IMS SOD1 upon oxidation of critical thiols; this may be part of a protective mechanism inherent in the aforementioned compounds and of significance for vital mitochondrial functions (Fig. 8).

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Fig. 1. Temporal kinetics of SOD1 activation by melatonin, steroid hormones and vitamin D in intact mitochondria

Mitochondria were incubated with 1 μ M of melatonin, testosterone, dihydrotestosterone, estradiol, and vitamin D (panels A, B, C, D, and E, respectively) separately for indicated times at 37°C, washed with sucrose-Tris buffer, and incubated with digitonin in the presence of iodoacetamide for 1 hour at room temperature. After incubations SOD1 activity was measured by following reduction of XTT at 470 nm. * P <0.05 compared with the activity at 0 min.

Fig. 2. Dose response of SOD1 activation by melatonin, steroid hormones and vitamin D in intact mitochondria

Mitochondria were incubated with 0,05, 0,1, 0,5 ,1, 5, 10, 20 μ M of melatonin, steroid hormones and vitamin D separately for 5 min at 37°C, washed with sucrose-Tris buffer, and incubated with digitonin in the presence of iodoacetamide for 1 hour at room temperature. After incubations SOD1 activity was measured by following reduction of XTT at 470 nm.

* $P < 0.05$ compared with the activity after incubation in the absence of P450 substrate.

Fig. 3. P450 and IMAC inhibitors abrogate SOD1 activation by melatonin, steroid hormones and vitamin D in intact mitochondria

Mitochondria were incubated with 1 μ M of melatonin, steroid hormones and vitamin D separately for 5 min at 37°, in the presence of furafylline (20 μ M), omeprazole (100 μ M), ketoconazole (100 μ M), tiron (1mM), amiodarone (5 μ M), amitriptyline (50 μ M) and Mg⁺² (250 μ M) washed with sucrose-Tris buffer, and incubated with digitonin in the presence of iodoacetamide for 1 hour at room temperature. After incubations SOD1 activity was measured by following reduction of XTT at 470 nm. **P*<0.05 compared with the activity of control.

Fig. 4. H₂O₂ production by melatonin-treated mitochondria

Mitochondria (0.5 mg/ml) were incubated with the indicated concentrations of melatonin alone and with omeprazole (30μM) in the presence of 1mM p-hydroxyphenylacetate. O₂– formation was measured indirectly by monitoring horseradish peroxidase-catalyzed (25 units/ml) H₂O₂-dependent oxidation of p-hydroxyphenylacetate. **P*<0.05 compared with H₂O₂ production in the absence of melatonin.

Fig. 5. P450 substrates prevent oxidative loss of aconitase activity in matrix

Mitochondria (2 mg/ml) were incubated in the presence or absence of P450 substrates (0.1 μ M) 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 Material & Methods.

* $P < 0.05$ compared with the activity at 0 min.

Fig. 6. Dose response of aconitase activity protection by P450 substrates in intact mitochondria Mitochondria (2 mg/ml) were incubated in the presence or absence of several P450 substrate concentrations (0.0, 0.1, 2.0 and 20 μM), at 37°C for 5 min. State 3 respiration was then initiated following addition of ADP (2 mM), MgCl₂ (4 mM), KH₂PO₄ (5 mM) and succinate (10 mM) for the indicated times. Aconitase activity was subsequently measured as described in the Material & Methods. **P*<0.05 compared with the activity of control.

Fig. 7. P450 substrates can decrease apoptotic capacity of mitochondria

(A) Delay in the loss of transmembrane potential induced by CaCl_2 . Mitochondria (1 mg/ml) were incubated at 37°C for 5 min in the presence (♦), in the presence plus $100\mu\text{M}$ omeprazole (●), or absence (▲, and ■) of P450 substrates ($10\mu\text{M}$) followed by addition of vehicle (▲) or $25\mu\text{M}$ (♦, ● and ■) CaCl_2 to dissipate transmembrane potential. Rhodamine 123 uptake was monitored to measure transmembrane potential. (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 P450 substrates ($10\mu\text{M}$) followed by addition of $25\mu\text{M}$ CaCl_2 for 30 min. The supernatants were then concentrated by 10-fold and cytochrome c was detected by western blotting in both the mitochondrial pellets (lower panel) and the supernatants (upper panel).

Fig. 8. General mechanism for the SOD1 activation mediated by P450 enzymes

Mitochondrial IMS SOD1 is activated following oxidative modification of its critical thiol moieties by $\text{O}_2^{\cdot-}$ that comes from catalytic cycle of P450 enzymes. Molecular oxygen reacts with ferrous cytochrome P450 to produce a stable dioxygen adduct: one electron from the iron (II) center and one from the triplet oxygen pair create an iron (III)-oxygen bond; this oxygen-iron complex is relatively stable but can dissociate to an iron (III) and $\text{O}_2^{\cdot-}$.

Table 1
Marker proteins and their activity in isolated rat liver mitochondria

Assay conditions are described in the Experimental section. IMS, intermembrane space preparation; n.d., not detected.

Enzyme	Location	Activity (%)	
		Granule Fraction	Mitochondrial Fraction
Acid Phosphatase	Lysosomes	100	0.5±0.1
Urate Oxidase	Peroxisomes	100	n.d.
Fumarase	Mitochondrial Matrix	100	95±4
Sulfite Oxidase	Mitochondrial IMS	100	80±