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Original Contribution

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. The mitochondrial intermembrane space (IMS) Cu,Zn-superoxide dismutase (SOD1) is activated after oxidative modification of its critical thiol moieties by superoxide anion ($O_2^{\bullet-}$). This study was aimed at investigating the potential association between the hormonal protective antioxidant actions in mitochondria and the 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 μM 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 $O_2^{\bullet-}$ requirements. Mitochondrial P450-dependent activation of IMS SOD1 prevented $O_2^{\bullet-}$-induced loss of aconitase activity in intact mitochondria respiring in State 3. Optimal protection of aconitase activity was observed at 0.1 μM 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 transmembrane potential and decreased cytochrome *c* release. Omeprazole and ketoconazole abrogated both protecting mitochondrial functions promoted by melatonin and steroid hormones.

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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 $O_2^{\bullet-}$ [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 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 (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 superfamily of membrane *b*-type cytochrome enzymes that contains more than 200 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 and that of 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 substrate and have negligible activity toward xenobiotic compounds [18]. During the P450 enzyme catalytic cycle, molecular oxygen reacts with ferrous P450 to yield a dioxygen adduct (Fe^{II}–O_2); although this complex is relatively stable, it can dissociate to Fe^{III} and $O_2^{\bullet-}$ with a rate constant of 0.01 s^{-1} at room temperature (Fe^{II}–$O_2 \rightarrow Fe^{III} + O_2^{\bullet-}$) [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 relevant sources of $O_2^{\bullet-}$ 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 after oxidative modification of its critical thiol groups by $O_2^{\bullet-}$ [23–26]. The exact mechanism is not

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known and it could be completed by a specific mediator such as the copper chaperone mitochondrial CCS [24–26]. 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, transmembrane 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 vitamin 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 consisting of 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, the pellet was removed, and the centrifugation process was repeated. The supernatant was centrifuged at 8000 *g* for 10 min, the pellet was washed with the isolation buffer, and the centrifugation was repeated. The pellet containing a mixture of organelles was further fractionated by centrifugation at 8500 *g* for 10 min in Percoll gradient consisting 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

The mitochondrial outer membrane was selectively disrupted by treating mitochondria (40 mg/ml) with 0.11 mg of digitonin per milligram 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 sulfite 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 $O_2^{\bullet -}$ with the xanthine/xanthine oxidase system in the presence of the sulfonated tetrazolium salt XTT [30]. Cyanide at 5.0 mM was used to selectively inhibit SOD1 activity. SOD activity was also assayed in native gels after electrophoresis by nitroblue tetrazolium staining (data not shown) [31].

IMS SOD1 activation induced by hormones and its inhibition

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

Measurement of H_2O_2 production by melatonin-treated mitochondria

Mitochondria (0.5 mg/ml) were incubated with various concentrations of melatonin alone ranging from 0.1 to 10 μM and with 1 mM tiron or 30 μM omeprazole in the presence of 1 mM *p*-hydroxyphenylacetate. H_2O_2 formation was measured by monitoring horseradish peroxidase-catalyzed (25 units/ml) H_2O_2-dependent oxidation of *p*-hydroxyphenylacetate.

Measurement of aconitase activity

Mitochondria were incubated in the presence or absence of 0.1 μM P450 substrate 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 were taken at the 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 isocitrate dehydrogenase. One nanomole of NADPH formed per minute corresponded to 1 mU of aconitase [32].

Measurement of membrane potential (Ψm) 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 μg/ml rotenone, and 1 μM rhodamine 123 for 5 min at 25 °C to equilibrate mitochondria with the cationic dye. Mitochondria were incubated with 15 μM melatonin, steroid hormones, vitamin D, ketoconazole, and omeprazole for 5 min at 25 °C. $CaCl_2$ (25 μM) was then added to induce dissipation of Ψm. The 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 [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 ± SD for at least three independent experiments performed in triplicate. Analysis of variance and Duncan's multiple-range test were used to analyze the differences 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 in the Percoll-purified mitochondrial preparations. The activities of selected marker

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