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2′,3′-Cyclic nucleotide 3′-phosphodiesterase as a messenger of protection of the mitochondrial function during melatonin treatment in aging

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ABSTRACT

The process of aging is considered to be tightly related to mitochondrial dysfunction. One of the causes of aging is an increased sensitivity to the induction of mitochondrial permeability transition pore (mPTP) opening in the inner membrane of mitochondria. Melatonin, a natural antioxidant, is a hormone produced by the pineal gland. The role of melatonin whose level decreases with aging is well understood. In the present study, we demonstrated that long-term treatment of aged rats with melatonin improved the functional state of mitochondria; thus, the Ca^{2+} capacity was enhanced and mitochondrial swelling was deaccelerated in mitochondria. Melatonin prevented mPTP and impaired the release of cytochrome *c* and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) from mitochondria of both young and aged rats. Our data suggest that melatonin retains CNPase inside mitochondria, thereby providing the protection of the protein against deleterious effects of 2′,3′-cAMP in aging.

1. Introduction

Mitochondria are dynamic, plastic organelles linked to the cellular biochemical powerhouse, since they produce the greatest part of ATP through oxidative phosphorylation and carry out several other crucial functions in cells [1]. The damage to the mitochondrial function is considered as the main factor in aging, ischemia/reperfusion, septic shock, and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington disease [2]. Mitochondria are the major intracellular source of ROS, which cause oxidative stress [3,4]. The factors responsible for mitochondrial function damage enhance the generation of reactive oxygen species (ROS) and activation of inducible NO-synthesis as well as the down-regulation of respiratory chain enzyme activities and induction of the mitochondrial permeability transition pore (mPTP). In response to oxidative stress, or when the mitochondrial matrix is overloaded by calcium, an increase of inner membrane permeability (permeability transition) occurs, and the nonspecific pore (mPTP) forms. The composition of mPTP and the regulation of its functioning are not yet completely understood [5]. The mPTP is a multicomponent protein complex formed in the inner membrane of mitochondria, which allows the passage of solutes with a molecular weight of up to 1.5 kDa [6]. The Ca^{2+}-induced pore opening leads to the depolarization of the inner membrane, the uncoupling of respiration, the swelling of mitochondria, and the release of proapoptotic factors. In mitochondria isolated from the liver, heart, and brain of aged animals, an increased sensitivity to mPTP opening was found [7–10]. The functioning of mPTP is considered to be an important factor related to aging, though the exact mechanism underlying the age-dependent mitochondrial changes has not yet been established [4]. Therefore, a lot of experiments are performed to study the protective properties of antioxidants with the aim of suppressing oxidative stress and diminish the age-related oxidative cellular damage and mitochondrial dysfunction [11]. In this connection, melatonin (MEL) is considered as an effective antioxidant, a natural compound, and a neuroendocrine hormone produced by the pineal gland (epiphysis) [12], where the enzyme tryptophan hydroxylase converts tryptophan to 5-hydroxytryptophan and further to MEL. Recently it has been established that MEL is produced not only by the pineal gland but also in many other tissues [13–16]. Being a highly lipophilic molecule, MEL penetrates cell membranes, easily reaching the subcellular structures [17]. MEL is found in vertebrates, invertebrates, plants, unicellular eukaryotes, algae, and even in bacteria [14,18,19]. The content of MEL decreases sharply in aging [20,21].

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MEL accomplishes its action via a number of pathways. Many cells are provided with membrane receptors, which allow them to respond to the circadian MEL message [22,23]. In addition to being a broad spectrum antioxidant [24], MEL is a ligand of several G protein-coupled receptors [25]. There are two mammalian isoforms of the MEL receptor in the brain and peripheral tissues, MT1 and MT2 [25–27].

Chronic treatment with MEL in a pharmacological dose affects the mitochondrial function and prevents mitochondrial dysfunction under experimental diabetes and intoxication, demonstrating the mitochondrion-specific activity of MEL [28–31]. It was shown that, in addition to scavenging mitochondrial ROS, MEL targets mitochondrial Ca^{2+}-induced mPTP in astrocytes for the protection during Ca^{2+}-mediated apoptosis. It maintained the mitochondrial membrane potential ($\Delta\Psi_m$) and not only prevented mPTP induction but also retained $\Delta\Psi_m$-dependent ATP formation [32].

Recently, a number of neuroscientists have demonstrated that MEL influences the morphological features of the nerve tissue. In particular, it was shown using various experimental injury models that MEL has positive effects on the number of axons and myelin sheath [33] and promotes myelination in the white matter [34].

Recently a neuroprotective protein has been identified as 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) in rat brain mitochondria (RBM), and it was shown that CNPase protects mitochondria from mPTP opening [35]. The enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP, EC 3.1.4.37) catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to form the corresponding 2′-monophosphates [36]. The functions of CNPase in mitochondria are unclear nowadays. CNPase substrates (2′, 3′-cAMP and 2′,3′-cNADP) were found to be able to enhance Ca^{2+}-activated and CsA-sensitive pore opening in RBM as well as in liver mitochondria [35] which indicates potential interaction of CNPase with modulators of mPTP in mitochondria. CNPase interacts with RNA, calmodulin, and the cytoskeleton. CNPase was also shown to inhibit translation, to modulate mitochondrial membrane permeability, and to have putative ATP/GTPase activity. The addition of anti-CNP antibody to a mitochondrial suspension was found to protect mitochondria from mPTP opening [37,38]. Interestingly, MEL interacts with calmodulin, G-proteins, protein kinase-alpha, and adenylyl cyclase, which are involved in calcium signaling and the cAMP-signaling cascade [39]. Moreover, recently we have shown that, under mPTP stimulation, CNPase itself can be released from mitochondria in parallel with cytochrome *c*, AIF, and Endo G [40]. These data allowed us to raise a question about the synergistic effect of CNPase and MEL on the parameters of mPTP opening in rat liver mitochondria (RLM). Therefore, the goal of the present study was to examine the effect of 2′,3′-cAMP (CNPase substrate) on mPTP opening in RLM isolated from young and aged rats treated and untreated with MEL.

2. Material and methods

2.1. Animals and treatment

In experiments, twenty-four young and aged male were used, six animals in a group. For each separate experiment, one rat was used; thus, six replicates were done for each experimental group. Animals were maintained in a temperature controlled room (22 °C) (two individuals in a cage) and kept on a standard diet with full access to water and food. A group of young and aged animals was given orally melatonin for 2 months starting at 1 month for young and 16 months for aged animals after which rats were sacrificed. Animals were sacrificed at the same time, at 10.00 a.m. All experiments were performed according to the European Community Guide for animal care. Melatonin was dissolved in sterile water diluted to 100 μg/mL, which was made available to animals *ad libitum* as drinking water. The volume of daily water intake was 33 ± 3 mL per rat, which made up approximately 7 mg of melatonin per kg body weight per day [41]. After two months, RLM were isolated from the liver of rats in each group.

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from rats by a standard method [42] using a homogenization medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.05% bovine serum albumin fraction V, and 10 mM Tris (pH 7.3). The homogenate was centrifuged at 800*g* for 10 min to pellet nuclei and damaged cells. The supernatant containing mitochondria was centrifuged for 10 min at 9000*g*. Sedimented mitochondria were washed twice in medium containing EGTA and BSA for 10 min at 9000*g* and resuspended in the same medium. The protein concentration was determined using the Bradford assay.

2.3. Evaluation of mitochondrial functions

The Ca^{2+} capacity of RLM was determined with a Ca^{2+}-sensitive electrode (Nico, Russia), and the oxygen consumption rate was measured with a Clark-type O_2 electrode in a 1-mL measuring chamber [43].

Mitochondria (1 mg protein/mL) were incubated in a medium containing 125 mM KCl, 10 mM Tris, 2 mM K_2HPO_4, pH 7.4, at 25 °C. In experiments, succinate (5 mM) was used as a substrate, and rotenone (5 μM) was added to the measuring medium in order to block Complex I dehydrogenases. The respiratory control index (RCI) was measured in a closed chamber after the addition of 200 μM ADP. The RCI of mitochondria from young rats (1st group) was used as a control. The mPTP opening in RLM was induced by a threshold Ca^{2+} load. A threshold Ca^{2+} load is that load of calcium added to a mitochondrial suspension at which calcium ions (when accumulating in mitochondria) induce the mPTP opening. For Ca^{2+}-loading of RLM, each addition was 50 nmol of Ca^{2+} per mg of protein with 1.0 mg protein/mL in the chamber. The Ca^{2+} capacity was calculated as the amount of calcium loaded before mPTP opened and Ca^{2+} was released from RLM.

The swelling of RLM was measured as a change in light scattering in a mitochondrial suspension at 540 nm (A540) using a Tecan I-Control infinite 200 spectrophotometer at 25 °C. The standard incubation medium for the swelling assay contained 125 mM KCl, 10 mM Tris, 2 mM KH_2PO_4, 5 mM succinate, and 5 μM rotenone. The concentration of the mitochondrial protein in a well was 0.5 mg protein/mL. Swelling was initiated by the addition of 300 nmol of Ca^{2+} per mg of protein. The swelling process was characterized by the time needed to reach the half-maximal light scattering signal ($T_{1/2}$).

2.4. Sample preparation

Aliquots (100 μL) were taken from the chamber, placed in an Eppendorf tube, and centrifuged for 5 min at 15,000 × *g*. Then, 60 μL of the supernatant was placed in another Eppendorf tube, and 20 μL of 4× Laemmli buffer was added. Samples were heated to 95 °C for 5 min and applied to the gel. 40 μL of a sample with the supernatant was applied and subjected to electrophoresis followed by Western blot analysis.

2.5. Electrophoresis and immunoblotting of mitochondrial proteins

Supernatant samples were separated under denaturing conditions in 12.5% SDS-PAGE gels and transferred to a nitrocellulose membrane. Precision Plus Pre-stained Standards from Bio-Rad Laboratories (Hercules, CA, USA) were used as markers. After overnight blocking, the membrane was incubated with the appropriate primary antibody. Anti-melatonin related receptor antibody (rabbit polyclonal) was used at the 1:1000 dilution (abcam, USA). The monoclonal anti-CNP antibody (anti-CNP Ab) was obtained as described [44] and used at the 1:10,000 dilution, the monoclonal anti-cytochrome *c* antibody (# DLN 06724 from Dianova; Hamburg, Germany) was used at the 1:2000 dilution, and the Tom20 antibody (Cell Signaling, USA) was used to rule out the contamination of the supernatant with mitochondria and as a loading control (1:1000 dilution). The immunoreactivity was detected using the appropriate secondary antibody conjugated to horseradish