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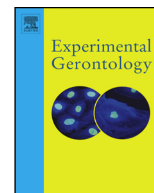

The influence of ageing on the extrapineal melatonin synthetic pathway

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ABSTRACT

Ageing affects various physiological and metabolic processes in a body and a progressive accumulation of oxidative damage stands out as often used explanation. One of the most powerful scavenger of reactive oxygen species (ROS) in all organs is melatonin. A majority of melatonin supplied to the body via blood originates from the pineal gland. However, we have been interested in a locally produced melatonin.

We have used 2.5- and 36-months-old Wistar rats. Tissues were collected and gene expression of AA-NAT and ASMT, the two key enzymes in a synthesis of melatonin, was determined in brain, liver, kidney, heart, skin, and intestine. Since melatonin can influence antioxidant enzymes, the activity of superoxide dismutase (SOD) and catalase (CAT), and the level of GSH were measured in liver. In addition, Copper (Cu), Zinc (Zn), and Manganese (Mn) were also determined in liver since these microelements might affect the activity of antioxidant enzymes.

The expression of AA-NAT and ASMT was increased in liver and skin of old animals. A positive correlation in AA-NAT and ASMT expression was observed in liver, intestine and kidney. Moreover, the activity of CAT enzyme in liver was increased while SOD activity was decreased. SOD and CAT were probably affected by the observed decreased amount of Cu, Zn, and Mn in liver of old animals.

In our model, extrapineal melatonin pathway in ageing consisted of complex interplay of locally produced melatonin, activities of SOD and CAT, and adequate presence of Cu, Zn and Mn microelements in order to defend organs against oxidative damage.

1. Introduction

Ageing is unstoppable, complex biological process that affects various physiological and metabolic processes in a body, resulting in a decrease of their function (Barton et al., 2016). However, ageing is not a uniform process, since different organs, tissues and cell types may age at different rates (Hamezah et al., 2017; Bonomini et al., 2010). There are a few suggested mechanisms of ageing, and progressive accumulation of oxidative damage stands out as probably the most used explanation (Flores et al., 2012). Not all organs are equally exposed to the damaging factors, such as to the reactive oxygen species (ROS), since different organs have different functions. In that respect, liver and skin are, in general, more exposed to possible oxidative damaging factors than some other organs.

A defense mechanism against ROS consists of antioxidant enzymes and various scavenging molecules. One of the most powerful scavenger of ROS in all organs, with more effective properties than classical antioxidant, is melatonin (Baydas et al., 2002). A majority of melatonin supplied to the body via blood originates from the pineal gland. However, we have here focused on a locally produced melatonin in various organs. A predominant melatonin synthesis in both animals and humans occurs via serotonin-*N*-acetylserotonin-melatonin pathway (Tan et al., 2016) and is regulated by the two key enzymes: arylalkyamine-*N*-acetyltransferease (AA-NAT) and acetylserotonin *O*-methyltransferase

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(ASMT) / hydroxyindole-*O*-methyltransferase (HIOMT). In addition to a dominant role of AA-NAT in acetylation of serotonin, alternative enzymes, arylamine-*N*-acetyltransfereases (NAT-1, NAT-2), were detected in rodent (C57BL/6 mouse) and human skin (Debiec-Rychter et al., 1996; Slominski et al., 2003; Kawakubo et al., 2000). An inactive AA-NAT enzyme, due to a specific gene insertion or deletion, is functionally replaced by NAT-1 enzyme during acetylation of serotonin (Slominski et al., 2005). Higher activity of these enzymes could suggest higher production of melatonin.

It has been reported that melatonin levels were much higher in peripheral organs, especially in skin and liver, compared to the serum melatonin (Slominski et al., 2008; Bubenik, 2002). Apart from its important role in protection against ultraviolet and X-ray radiation-induced oxidative stress, melatonin and its metabolites in the skin may have other numerous functions, such as: induction of DNA repair and anticancer activity, blocking apoptosis, maintaining mitochondrial membrane potential and ATP synthesis, anti-inflammatory and immunostimulatory actions, regulation of melanin pigmentations, thermoregulations, and wound healing (Slominski et al., 2018; Slominski et al., 2017a). The melatonin activity in the skin is determined by a topography, health status of the skin, involved cell type, and the animal strain (Slominski et al., 2017a). Moreover, as it was first noticed in rat liver and later in other organs, melatonin can be metabolized by cytochrome 450 (CYP) into powerful antioxidant factors (AFMK and AMK), which have a role in attenuating of ROS and RNS (reactive nitrogen species) elements in mitochondria (Semak et al., 2008; Slominski et al., 2017b).

It has been shown that activity of both enzymes, AA-NAT and ASMT, is in correlation with the expression of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Rodriguez et al., 2004). Also, certain essential microelements may affect the activity of antioxidant enzymes. Namely, manganese (Mn), zinc (Zn), and copper (Cu) inhibit activity of AA-NAT (Zhan-Poe and Craft, 1999) and they are also cofactors of CuZnSOD/MnSOD complexes. Moreover, Cu and Zn block CAT activity (Xianyong et al., 2017) and Cu and Mn may possess pro-oxidative properties (Valko et al., 2005).

During ageing, many processes are affected, leading to diminished expression of antioxidative factors (Reiter et al., 2002; Karasek, 2004) and changed regulation of the essential microelements like Cu, Zn, and Mn (Rotter et al., 2015). Therefore, it could be assumed that these events are related to the disorders of many systems. Since not all members of the same community have the same life span and all organs in an individual do not age at the same rate (Acuña-Castroviejo et al., 2014), we decided to use rats as old as possible to compare the expression of the AA-NAT and ASMT, the activity of SOD, CAT, and GSH-Px, and the level of Mn, Zn, and Cu between young adult and very old animals in various tissues. The role of serum melatonin in advance age is well known (Hardeland et al., 1995), but the role of locally produced melatonin is not firmly established. The goal of this work was to better understand antioxidant processes related to the melatonin pathway in various organs of very old individuals.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from the Military Medical Academy Animal Research Facility (Belgrade, Serbia). Experimental animals were divided into two groups, younger animals (7 rats) that were 2.5 months (10 weeks) old, and older ones (7 rats) that were 36 months (3 years) old. In each group, each animal was housed in its own cage and kept under controlled room condition (temperature of 20 °C, humidity of 70%, light-dark cycle of 12–12 h). Food (Veterinary Institute, Subotica, Serbia) and water were available ad libitum. Experiments were conducted in accordance with the procedures described in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Washington, DC, USA).

At appropriate time, animals were sacrificed between 12 a.m. to 3 p.m. The parts of organs were quickly removed, transferred into liquid CO_2 and then stored at −80 °C. Organ samples were used for a) RNA extraction, followed by real-time PCR analysis, b) estimation of enzymatic activity, and c) determination of Cu, Zn, and Mn concentration.

2.2. RNA extraction and cDNA synthesis

Cellular RNA for each sample, was isolated from 50 mg of extrapineal tissues (liver, skin, kidney, brain cortex, heart, and intestine), using Trisol Isolation Reagent (Invitrogen, Carlsbad, CA, USA) and chloroform (Serva, Heidelberg, Germany) as a denaturing solution. Following denaturation, extraction of RNA was conducted by a precipitation with isopropanol (Serva, Heidelberg, Germany). The obtained RNA pellet was dissolved in 10 μl of RNAase-free water, and the samples were proceeded to quantification by spectrophotometer (Eppendorf, Hamburg, Germany) at 260 nm. RNA samples were stored at −80 °C. To reduce a risk of DNA contamination, isolated RNA was purified before cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reaction volume (10 μl) for a purification consisted of: 1 μg of RNA, 1 μl of 10× Reaction buffer with $MgCl_2$, 1 μl of DNase I-RNase-free, and a nuclease-free H_2O. Following incubation at 37 °C for 10 min, 1 μl of 50 mM EDTA was added into mix that was incubated at 65 °C for 10 min in order to inactivate enzymes. The prepared RNA was then used for reverse transcription.

Single-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit. The components added to total volume of 20 μl were as follows: 1 μg of total RNA, 1 μl of random hexamer primer, 4 μl of 5× Reaction Buffer, 1 μl of RiboLock RNase inhibitor, 2 μl of 10 mM dNTP, 1 μl of RevertAid M-MuLV RT, and 12 μl a nuclease-free H_2O. Reaction was conducted at 25 °C for 5 min, followed by incubation at 42 °C for 60 min, and terminated at 70 °C for 5 min. Reaction products were stored at −20 °C until they were used in PCR reaction. To verify the results of the first strand cDNA synthesis, positive (GAPDH amplification) and negative control (RT- and NTC) reactions were performed.

2.3. Quantitative real time-qRT-PCR of AA-NAT and ASMT in extrapineal tissues

To estimate the expression level of AA-NAT and ASMT genes, real time quantitative PCR was performed using a Line Gene PCR machine (Hangzhou Bioer Technology, Shanghai, China). The cDNA amplification was conducted by adding the following components: 12.5 μl of 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 μM of forward and 0.2 μM of reverse primers (AA-NAT, ASMT), or 0.1 μM primers of housekeeping gene (HPRT), 2 μl of the first strand cDNA synthesis reaction, and a nuclease-free H_2O to 25 μl. Primer sequences were employed from the previously published paper by Sanchez-Hidalgo et al., 2009. The temperature profile was as follows: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 10 s; 57 °C for 20 s (AA-NAT) or 58 °C for 45 s (ASMT); 72 °C for 20 s, and finally extension at 72 °C for 5 min. In each reaction, tested samples were amplified in two replicates, and the NTC control was included for the assessment of a possible contamination. The specificity of amplification for each target was confirmed by melting curve analysis. A level of AA-NAT and ASMT expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data of 36-months-old group were normalized to data of 2.5-months-old animals and represented by fold change values.

2.4. Determination of SOD and CAT enzymatic activity in liver

The total superoxide dismutase (SOD) activity was determined by

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