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Melatonin and its metabolites accumulate in the human epidermis *in vivo* and inhibit proliferation and tyrosinase activity in epidermal melanocytes *in vitro*

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ABSTRACT

Melatonin and its metabolites including 6-hydroxymelatonin (6(OH)M), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and 5-methoxytryptamine (5MT) are endogenously produced in human epidermis. This production depends on race, gender and age. The highest melatonin levels are in African-Americans. In each racial group they are highest in young African-Americans [30–50 years old (yo)], old Caucasians (60–90 yo) and Caucasian females. AFMK levels are the highest in African-Americans, while 6(OH)M and 5MT levels are similar in all groups. Testing of their phenotypic effects in normal human melanocytes show that melatonin and its metabolites (10^{-5} M) inhibit tyrosinase activity and cell growth, and inhibit DNA synthesis in a dose dependent manner with 10^{-9} M being the lowest effective concentration. In melanoma cells, they inhibited cell growth but had no effect on melanogenesis, except for 5MT which enhanced L-tyrosine induced melanogenesis. In conclusion, melatonin and its metabolites [6(OH)M, AFMK and 5MT] are produced endogenously in human epidermis and can affect melanocyte and melanoma behavior.

1. Introduction

Melatonin is a hormone and a bioregulator with structure of methoxyindole, which is present in almost all biological systems such as animals, plants and microbes (Fischer et al., 2008a; Hardeland et al., 2011; Lanoix et al., 2012a; Lerner et al., 1960; Reiter et al., 2007a, 2007b, 2010; Slominski et al., 2008). It is predominantly synthesized in the pineal gland through a multistep process starting from hydroxylation of tryptophan and culminating with transformation of serotonin to *N*-acetyl serotonin and further methylation to melatonin (Hardeland et al., 2006; Lerner et al., 1960; Reiter, 1991; Reiter et al., 2007a; Roseboom et al., 1998; Yu and Reiter, 1993). Melatonin is also synthesized in the brain, nerves and peripheral organs (Bubenik, 2008; Hardeland et al., 2011; Konturek et al., 2007; Lanoix et al., 2012a, 2012b; Lerner et al., 1959; Reiter et al., 2010; Tan et al., 2007; Zmijewski et al., 2009) including rodent (Kobayashi et al., 2005; Slominski et al., 1996, 2002a) and human skin (Kobayashi et al., 2005; Slominski et al., 2002b, 2005a). In the periphery and on the central levels, melatonin is metabolized through indolic and kynuric pathways (Fig. 1) with production of 6-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and 5-methoxytryptamine (5MT) as well as other metabolites (Grace et al., 1991; Hardeland et al., 2006; Hirata et al., 1974; Lerner et al., 1960; Ma et al., 2005; Rogawski et al., 1979; Semak et al., 2005, 2008; Slominski et al., 2008; Young et al., 1985).

In humans, melatonin is well known for regulating circadian rhythm. It also has many other effects including regulation of immune and endocrine functions, and it shows anti-oxidative and protective properties against the cellular toxins and internal and environmental insults (Bubenik, 2008; Fischer et al., 2008a; Hardeland et al., 2006, 2011; Lanoix et al., 2012a; Luchetti et al., 2010; Reiter, 1991; Reiter et al., 2010; Slominski et al., 2005a; Tan et al., 2007; Yu and Reiter, 1993). These effects are mediated either through binding to membrane bound melatonin receptors (MT1 and MT2), receptor independent mechanisms or through activation of putative nuclear receptors (Dubocovich and Markowska, 2005; Hardeland et al., 2011; Reiter et al., 2010; Slominski et al., 2012a; Tan et al., 2007).

Abbreviations: 6(OH)M, 6-hydroxymelatonin; AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; 5MT, 5-methoxytryptamine; yo, years old; UVB, ultraviolet B; FBS, fetal bovine serum; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; ESI, electrospray ionization; MS, mass spectrometry; LC-MS, liquid chromatography mass spectrometry; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; qTOF, quadrupole time-of-flight.

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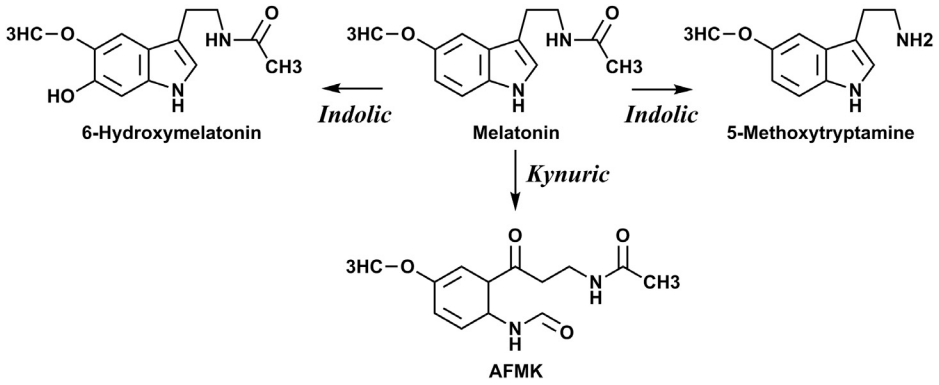

Fig. 1. Main pathways of melatonin metabolism in skin cells.

Extensive studies have been focused on melatonin's role in general regulation of body homeostasis (Hardeland et al., 2011; Lanoix et al., 2012a; Luchetti et al., 2010; Reiter, 1991; Reiter et al., 2010; Tan et al., 2007; Yu and Reiter, 1993). Skin with subcutaneous adipose tissue is the largest organ in the human body playing an important role in the regulation of local and body homeostasis (Slominski and Wortsman, 2000; Slominski et al., 2012b). Locally produced melatonin plays an important role in the regulation of skin functions (Fischer et al., 2008a, 2008b; Kleszczynski et al., 2011; Kobayashi et al., 2005; Slominski et al., 2005a, 2005b, 2008). Although the role of melatonin and of AFMK in the functions of the epidermis has been extensively investigated (Fischer et al., 2006a, 2008c, 2013; Kim et al., 2013; Kleszczynski et al., 2012, 2013; Slominski et al., 1994, 2003), there is a lack of similar information on functions of other melatonin metabolites. The literature on the role played by melatonin and its metabolites in regulation of behavior of human melanocytes is limited.

Previously we have shown that exogenously applied melatonin in cultured immortalized epidermal (HaCaT) keratinocytes and melanoma cells is metabolized through indolic and kynuric pathways with production of 2-hydroxymelatonin, 4-hydroxymelatonin, 6-hydroxymelatonin, AFMK, and 5MT (Fischer et al., 2006b; Kim et al., 2013; Slominski et al., 2002c). Production of AFMK in HaCaT keratinocytes can be stimulated by ultraviolet radiation (UVB) (Fischer et al., 2006b), and AFMK can also be generated from melatonin through pseudoenzymatic or non-enzymatic processes mediated by free radicals or through photocatalysis induced by UVB (Fischer et al., 2006b; Hardeland et al., 2006; Semak et al., 2005). We have also characterized metabolism of melatonin in normal human primary epidermal keratinocytes, melanocytes, dermal fibroblasts and melanoma cells and show that 6-hydroxymelatonin is the main product of metabolism with lower production of AFMK and 5MT (Kim et al., 2013).

Originally melatonin was defined as lightening agent based on its action on amphibian skin (Lerner, 1960; Lerner et al., 1960). In mammalian system, melatonin's role in fur pigmentation has been well established (Logan and Weatherhead, 1979, 1980) and reviewed (Fischer et al., 2008b; Slominski et al., 2004, 2005c). Also tumorostatic activity of melatonin has been well documented in rodent and human melanomas [reviewed in (Fischer et al., 2006c; Slominski et al., 2005b; Yu and Reiter, 1993)]. However, the role of melatonin in human skin pigmentation is unclear (Slominski et al., 2004) as indicated by lack of effect of orally delivered melatonin on skin melanin pigmentation (McElhinney et al., 1994).

To better understand the role of melatoninergic system in human epidermis we investigated accumulation of melatonin and its metabolites in the human epidermis from healthy donors of different race, age and sex, and evaluated their effects on proliferation and melanogenesis in human normal epidermal melanocytes in comparison with human melanoma cells.

2. Materials and methods

2.1. Chemicals

Charcoal stripped fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Lawrenceville, GA, USA. Melatonin, 6-hydroxymelatonin and 5-methoxytryptamine (5MT) were purchased from Sigma-Aldrich, St Louis, MO, USA and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) was purchased from Cayman chemical, Ann Arbor, MI, USA. Acetonitrile, water and acetic acid (Fisher scientific, Pittsburgh, PA) were used for HPLC. For LC-MS system, acetonitrile, water and formic acid (Sigma-Aldrich, St Louis, MO, USA) were used. Trichloroacetic acid (TCA) was purchased from Sigma-Aldrich, St Louis, MO, USA and [^{3}H]-thymidine was purchased from Moravek Biochemicals Inc., Brea, CA, USA.

2.2. Human skins and epidermis preparation

The use of human tissues was approved by UTHSC Institutional Review Boards as an exempt protocol #4. Human skin samples were obtained from the Methodist University Hospital in Memphis, TN. The skin specimens ($n = 13$) were obtained from both males and females (30–90 years old) of African-American and Caucasian races. The specimens were incubated at 60 °C for 1 h and the epidermis was peeled out and stored at –80 °C for further experiments.

2.3. Extraction of melatonin and its metabolites from human epidermis

The epidermis collected as above was mixed with 3.2 volume (v/w) of PBS and homogenized using Poly Tron PT 2100 (Kinematica, Switzerland). Additional homogenization was performed in 75% acetonitrile. After centrifugation at 4000 rpm, the supernatant was filtered using syringe filter (PES, 0.45 μm, 30 mm; Celltreat, Shirley, MA, USA) and then dried by speedvac drier (Savant Instruments, Inc., Holbrook, NY, USA).

2.4. Detection of melatonin and its metabolites

In order to detect melatonin and its metabolites, the epidermal samples was re-dissolved in methanol. The UPLC (ultra-performance liquid chromatography) separation was performed on a Waters ACQUITY I-Class UPLC system (Waters, Milford, MA, USA) consisting of a binary pump, an autosampler, a column manager, a degasser and a diode-array detector (DAD). An Agilent Zorbax Eclipse Plus