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Sulfation of melatonin: Enzymatic characterization, differences of organs, species and genders, and bioactivity variation

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ABSTRACT

Exogenous melatonin (Mel) is widely used in clinic for multiple therapeutic purposes. In metabolism pathways of Mel, 6-hydroxymelatonin-sulfate (S-*O*-Mel) and *N*-acetylserotonin sulfate (S-NAS) are the most abundant metabolites account for over 90% of total Mel metabolites in humans, indicating that sulfation plays an important role in reflecting the functions and clearance of Mel in vivo. In the present study, we characterized Mel sulfation using various human organ cytosols (liver, lung, kidney, small intestine and brain), liver cytosols from five different animal species, and cDNA-expressed human sulfotransferase (SULT) for the first time. Our results demonstrated that liver, lung, kidney and small intestine of humans had high catalytic efficiency for Mel sulfation, however, brain contained a very low reaction rate. Interestingly, organ cytosols prepared from females exhibited higher sulfation activity than those of males. SULT isoforms 1A1, 1A2, 1A3, 1B1 and 1E1 exhibited metabolic activities toward Mel. According to kinetic parameters (K_m and V_{max}), chemical inhibition, correlation analysis, molecular docking and sulfation assays with recombinant human SULTs isoforms, SULT1A1 was determined as the major enzyme responsible for Mel sulfation. Furthermore, considerable species differences in Mel sulfation were observed, and the total intrinsic clearance rate of Mel sulfation was as follows: monkey > rat > dog > human > pig > mouse. Additionally, the anti-inflammatory effects of Mel and its sulfated metabolites were evaluated by inhibiting nitric oxide (NO) production in RAW264.7 cells, and S-*O*-Mel as a bioactive form, exhibited potent bioactivity. Our investigation provided a global view of the enzyme-dependent sulfation of Mel that can guide biomedical research on Mel.

1. Introduction

Sulfation plays a key role in the activation and detoxification of various endogenous and exogenous compounds such as steroids, thyroid hormones, catecholamines and therapeutic drugs, in humans and other mammals [1–3]. These vital metabolism reactions are catalyzed by cytosolic sulfotransferases (SULTs), which transfer a sulfonate group from active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to target substrates containing either a hydroxyl or amino group. Various SULTs are widely distributed throughout the human body and serve to inactivate and increase water solubility of xenobiotics and therapeutic drugs. Additionally, SULTs are also known to bioactivate certain prodrugs and initiate chemical carcinogenesis. In general, human cytosolic SULTs are divided into two major subfamilies, phenol SULTs (SULT1) and hydroxysteroid SULTs (SULT2), based on their specific substrate reactivity [4]. Common SULT isoforms usually exhibit widespread tissue distribution and are expressed in many tissues including the liver, lung, brain, kidney, gastrointestinal tract and even tumorous tissues [5]. Species and gender differences between human and

Abbreviations: Mel, melatonin; 6-OM, 6-hydroxymelatonin; NAS, *N*-acetylserotonin; S-*O*-Mel, 6-hydroxymelatonin-sulfate; S-NAS, *N*-acetylserotonin sulfate; SULT, sulfotransferase; MFC, Mefenamic acid; HLC, human liver cytosol; HKC, human kidney cytosol; HLuC, human lung cytosol; HIC, human small intestine cytosol; PLC, pig liver cytosol; RLC, SD rat liver cytosol; MLC, mouse liver cytosol; DLC, dog liver cytosol; CyLC, cynomolgus monkey liver cytosol; DCNP, 2,6-dichloro-p-nitrophenol; C_{max}, maximum plasma drug concentration; LPS, lipolysaccharide; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; DTT, dithiothreitol; 4-N-Ph, 4-nitrophenol; CL_{int}, intrinsic clearances.

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experimental animal species are often observed in the sulfation of chemicals, which resulting in a complexity to extrapolation of animal data to humans [6,7]. Therefore, characterization of human SULT enzymes involved in metabolism, interspecies and even gender differences are critical for elucidating the pharmacokinetic, bioactivity and toxicity profiles of target compounds in humans.

Melatonin (*N*-acetyl-5-methoxytryptamine) (Mel), a neurohormone secreted primarily by the pineal gland, performs clock and calendar function in animals, plant and microbes [8,9]. Mel serves a key role in the entrainment and regulation of circadian rhythms as a biological modulator of mood, sleep, and sexual behavior. In addition, Mel is also involved in a variety of other pathophysiological processes, including immune response modulation [10], antioxidant [11,12], neuroprotective [13] and anti-tumor activities [14,15]. The protection of Mel against inflammation has received increasing attention, due to its formation by monocytes, macrophages and lymphocytes. A number of studies have shown the ability of Mel to reduce the inflammatory response to lipopolysaccharide (LPS) [16–18]. Currently, due to its significant biological functions, exogenous Mel has been proposed for the treatment of sleep disturbances, depression, insomnia, and cardiovascular diseases in clinical settings [19–21].

The metabolism and elimination pathways of Mel and its major metabolites directly reflect its fate and functions in humans. Previous studies have indicated that Mel is mainly metabolized to 6-hydroxymelatonin (6-*O*-Mel) in the liver via hydroxylation mediated by cytochrome P450s (CYP450) CYP1A1, 1A2, 1B1, and 2C9, and the plasma concentration of 6-*O*-Mel was 37-fold greater than that of Mel [22,23]. Additionally, melatonin *O*-demethylation derivative (NAS), a minor phase I metabolite, is catalyzed by CYP2C19 and CYP1A2 in humans [23]. After this phase I metabolism, 6-*O*-Mel and NAS are further metabolized to form sulfate or glucuronide conjugates as the final excreted products in human urine. Importantly, 6-hydroxymelatonin-sulfate (S-*O*-Mel) and *N*-acetylserotonin sulfate (S-NAS) were found at higher levels than those of glucuronide-conjugates in human subjects treated with Mel, and almost 80% of the detected conjugates in human urine were S-*O*-Mel [23–25] (Fig. 1). In addition, Mel can be metabolized by free radicals and is converted to cyclic 3- and 6-hydroxymelatonin such as N^1-Acetyl-N^2-formyl-5-methoxykinuramine (AFMK) and N^1-acetyl-5-methoxy-kinuramine (AMK) [22,26]. However, AFMK and AMK were found to be trace or undetectable metabolites in humans. Therefore, all of these previous reports strongly suggested that sulfation is vital for Mel metabolism in humans. Additionally, Mel could also be sulfated by breast cancer cells [27]. Urinary levels of S-*O*-Mel and S-NAS have been closely associated with breast cancer risk in premenopausal woman [28,29]. SULT conjugation pathways and the detailed kinetic profiles of 6-*O*-Mel in humans and experimental animals have not been fully elucidated yet, although they play a critical role in the clearance, clinical risk prediction and rational selection of suitable animal models for the further development of Mel and its related derivatives.

The objectives of present study were **1**) to identify the human SULT enzymes responsible for Mel sulfation; **2**) to determine the sulfation kinetic parameters, gender differences and pH dependences for 6-*O*-Mel and NAS using recombinant SULT isoforms, as well as the subcellular fractions from male and female human tissues including the liver, kidney, lung, brain and intestine; **3**) to determine the interaction between 6-*O*-Mel and NAS sulfation in human liver cytosol (HLC); **4**) to elucidate species differences of Mel sulfation among human, monkey, pig, dog, rat and mouse; and **5**) to compare the anti-inflammatory activities of Mel and its major sulfation metabolites in RAW 264.7 macrophage cells. All of these findings will provide important information for elucidating the enzyme-dependent metabolism pathways of both endogenous and exogenous Mel, and present beneficial guidance for its rational use in the clinic.

2. Materials and methods

2.1. Materials

3′-Phosphoadenosine-5′-phosphosulfate (PAPS), 4-nitrophenol, 4-nitophenylsulfate, nimesulide, ibuprofen, mefenamic acid (MFC), 2,6-dichloro-p-nitrophenol (DCNP), quercetin, estrone, dithiothreitol (DTT), 6-hydroxymelatonin (6-*O*-Mel), N-acetylserotonin (NAS), and lipolysaccharide (LPS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-iNOS and mouse monoclonal anti-β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). NF-κB and p65 were obtained from Cell Signaling Technology, Inc. (Danvers, USA). Pooled human liver cytosol (HLC), 16 individual HLCs, liver cytosols from different species, and male or female human kidney, lung, intestine, and brain cytosols were all purchased from Rild Research Institute for Liver Diseases (Shanghai, China), Recombinant human SULTs were obtained from BD Gentest (Woburn, MA, USA).

2.2. Incubation system and analysis method

The standard incubation system for SULT reactions included HLC (0.5 mg/mL) or 0.1 mg/mL protein (recombinant human SULTs), DTT (8 mM), PAPS (4 mM), potassium phosphate buffer (50 mM PH 7.4), $MgCl_2$ (5 mM), 25 μg/mL alamethicin, and substrates in a final volume of 200 μL [30]. The volume of organic solvent did not exceed 1% of the total volume. After 30 min of incubation at 37 °C, reactions were terminated by the addition of 100 μL of methanol, followed by centrifugation at 20,000 × *g* for 20 min to obtain the supernatant for liquid chromatography–mass

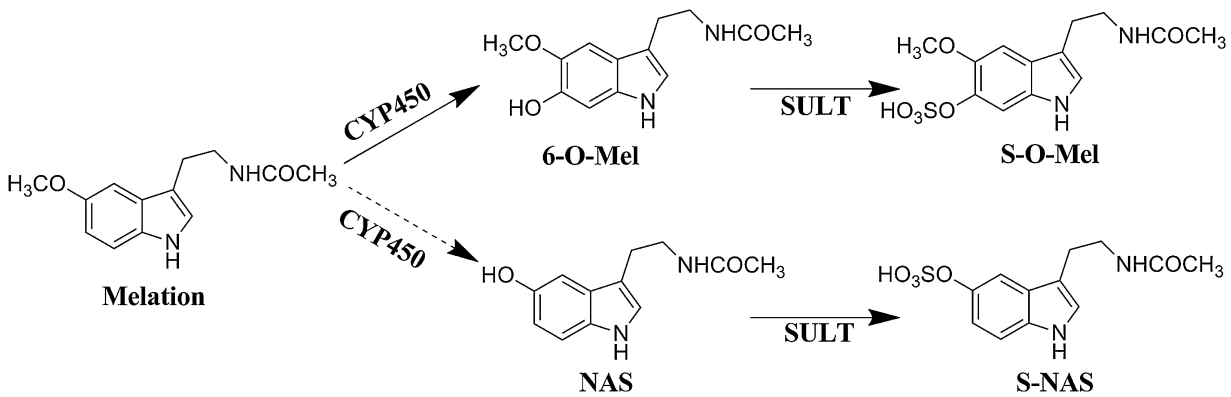

Fig. 1. Chemical structures of 6-*O*-Mel, NAS and their sulfated metabolites, S-O-Mel and S-NAS.