Published in final edited form as:

J Pineal Res. 2008 November ; 45(4): 515–523. doi:10.1111/j.1600-079X.2008.00630.x.

Metabolism of melatonin by cytochrome P-450s in rat liver mitochondria and microsomes

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Abstract

In the present study we provide direct evidence for the involvement of rat microsomal cytochrome P450s in melatonin O-demethylation and hydroxylation at two different positions: 2 and 6, as well as generation of N¹-acetyl-N²-formyl-5-methoxy-kynuramine (AFMK) and two unknown products. Moreover, we found that mitochondrial cytochrome P450s also converts melatonin into AFMK, N-acetylserotonin (NAS), 2-hydroxymelatonin, 6-hydroxymelatonin and the same two unknown products. Eadie-Hofstee plots for 6-hydroxylation and O-demethylation reactions were curvilinear for all tested fractions, suggestive of involvement of at least two components, one with a high affinity and low capacity, and another with a low affinity and high capacity. Mitochondrial cytochrome P450s exhibited higher affinity (suggesting lower K_m value) and higher V_{max} for melatonin 6-hydroxylation and O-demethylation for both high-affinity and low-affinity components as compared to microsomal enzymes. The intrinsic clearance for melatonin hydroxylation by high- and low-affinity components displayed the highest values in all tested fractions, indicating that both mitochondrial and microsomal cytochrome P-450s metabolize melatonin principally by 6-hydroxylation, with O-demethylation representing a minor metabolic pathway.

Keywords

melatonin; 6-hydroxymelatonin; N-acetylserotonin; 2-hydroxymelatonin; AFMK; CYPs

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine involved in biochemical regulation of the circadian rhythms and other biological functions throughout the body [1,2]. Melatonin is also synthesized in extrapineal sites that include retina, Harderian glands, gut, ovary, testes, bone marrow, lens and skin [3–6]. In mammals, melatonin metabolic degradation occurs either directly at the site of production, or in the liver (for circulating melatonin) proceeding through complex pathways. Thus, through side chain changes melatonin can be transformed into 5-methoxyindole acetic acid or 5-methoxytryptophol [7]; alternately, through cleavage of the pyrrole ring by indoleamine 2,3-dioxygenase it can form N¹-acetyl-N²-formyl-5-methoxy-kynuramine (AFMK) [8]. Melatonin oxidation to AFMK can be also mediated by myeloperoxidase, cytochrome c (cyt c), hemoglobin, horseradish

peroxidase or by reactive oxygen species (ROS) and ultraviolet radiation [10–14]. AFMK is in turn degraded by arylamine formamidase to form N¹-acetyl-5-methoxykynuramine (AMK) [8,9]. AFMK can be also be deformylated to AMK under the action of catalase, at least *in vitro* [15]. As regards circulating melatonin, the major metabolic pathway is hepatic biotransformation through O-demethylation and 6-hydroxylation mediated by cytochrome P450 (CYPs). The latter reactions in humans are catalysed almost entirely by microsomal CYP1A2 (with minimal contributions by CYP2C19 and CYP2C9), generating 6-hydroxymelatonin and N-acetylserotonin (N-acetyl-5-hydroxytryptamine, NAS) ([16,17], Fig. 1). Under an action of sulfotransferase, 6-hydroxymelatonin is further conjugated with sulfate to form 6-sulphatoxymelatonin which is excreted in urine [18].

Previous findings have implicated cytochrome P450s in melatonin biotransformation in rat liver. Thus, rats treated with α -naphthoflavone had a 3-fold increase in melatonin conversion to 6-sulphatoxymelatonin in liver slices and microsomes and it was assumed that this was due entirely to the induction of CYP1A2 [19]. However, until now no direct evidence of CYP1A2 involvement in melatonin metabolism in rat liver microsomes has been provided. Furthermore, nothing is known about the contribution of mitochondrial CYPs in melatonin biotransformation, although the melatonin concentrations are 100 times higher in these organelles than in the blood [18], raising the question of the local CYP-mediated metabolism.

Therefore, the goals of this study are to determine the cytochrome P450 mediated metabolic pathways of melatonin in rat liver, and identify the major cytochrome P450 forms responsible for its biotransformation.

MATERIALS AND METHODS

Reagents

All reagents used in enzymatic assays and HPLC analysis were commercially available and of the highest purity. Melatonin, 6-hydroxymelatonin, NADPH, isocitrate, quinidine, sulfaphenazole, diethyldithiocarbamate, ketoconazole, furafylline, omeprazole, digitonin were purchased from Sigma Chemical Co., St. Louis, MO.

Male Wistar rats (180–200 g) were housed in individual cages in a room with controlled temperature and under a 12 h-light/dark cycle; food and water were made available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23). The animals were sacrificed between 10:00 and 12:00 h by cervical dislocation.

Isolation of mitochondria and mitoplasts

The mitochondria and mitoplasts were prepared as described elsewhere [21]. In brief, the rat liver was homogenized in five volumes of ice-cold sucrose-mannitol buffer (2 mM HEPES (pH 7.4), 70 mM sucrose, 220 mM D-mannitol, 2mM EDTA). The homogenate was centrifuged at 1,200 g for 10 min at 4°C. The resulting supernatant was removed and centrifuged again at 10,000 g for 20 min at 4°C. The mitochondrial pellet was resuspended in sucrose-mannitol buffer and the centrifugation step was repeated twice under the same conditions. The resulting mitochondria preparations with microsomal contamination less than 0.06% were suspended in 0.25M sucrose for immediate use as enzyme source. The purity of the mitochondrial fraction was assessed by measuring activity of glucose 6-phosphatase (marker of microsomal contamination) [22].

Mitoplasts were prepared by removal of the outer mitochondrial membrane by digitonin treatment. Briefly, the mitochondrial fraction isolated as described above was resuspended

in sucrose-mannitol buffer to a final protein concentration of 1 mg/ml and treated with 75 μ g digitonin/mg protein. The mixture was placed on ice and shaken gently for 5 min. Mitoplasts were then washed with sucrose-mannitol buffer and pelleted by centrifugation twice at 10,000 g for 10 min at 4°C. The resulting mitoplast preparations were suspended in 0.25M sucrose for immediate use as enzyme source.

Isolation of hepatic microsomal fraction

The microsomal fraction of liver was prepared as previously described [23]. Total protein concentrations in mitoplasts, mitochondrial and microsomal fractions were determined using bovine serum albumin (BSA) as the standard [24].

Metabolism of melatonin in mitochondria or mitoplasts

Stock solutions of melatonin were prepared immediately before use by dissolving the indoleamine in 45% 2-hydroxypropyl- β -cyclodextrin. Mitochondria (0.5 mg protein/ml) or mitoplasts (0.5 mg protein/ml) were preincubated (10 min at 37°C) with 50 μ M melatonin in the medium that consisted of 0.25 M sucrose, 50 mM HEPES pH 7.4, 20 mM KCl, 5 mM MgSO₄, 0.2 mM EDTA and fatty acid free BSA (1 mg/ml). Reactions were initiated by adding NADPH (final concentration 0.5 mM) and isocitrate (final concentration 5 mM), and carried out at 37°C for 60 min. Reactions were stopped by adding ice-cold methylene chloride; this was followed by two extraction steps with methylene chloride. The methylene chloride layers were combined and dried in a rotational vacuum concentrator RVC 2-18 (Christ, Germany). The residues were redissolved in methanol and subjected to HPLC analysis.

Metabolism of melatonin by microsomes

Microsomes (0.5 mg protein/ml) were preincubated (10 min at 37°C) with 50 μ M melatonin in 10 mM Tris-HCl (pH 7.4). Reactions were initiated by adding NADPH (final concentration 1 mM) and carried out at 37°C for 60 min. The reactions were stopped by adding ice-cold methylene chloride; this was followed by two extraction steps with methylene chloride. The samples for HPLC analysis were prepared as described above for mitochondria and mitoplasts.

Kinetic studies

To define optimal conditions for incubation and HPLC analysis, melatonin (50 μ M) was incubated with mitochondria or microsomes for 0 to 180 min across a range of microsomal (mitochondrial) protein concentrations (0.1–2.5 mg protein/ml). For determination of the apparent *K_m* and *V_{max}* values for 6-hydroxylation and O-demethylation reactions in microsomes and mitochondria, melatonin concentrations were varied from 0.1 to 1.5 mM. At least 9 (10) concentrations were routinely used, and the experiments were repeated three times. A HPLC analysis with fluorescence detection (an excitation wavelength of 285 nm and an emission wavelength of 365 nm) was used for quantification of the 6-hydroxymelatonin and NAS (see below).

Inhibition study

To define the CYPs responsible for the production of NAS and 6-hydroxymelatonin microsomes and mitochondria (mitoplasts) were incubated in the presence of CYP inhibitors: 5 μ M of quinidine (CYP2C6, CYP3A family), 10 μ M of sulfaphenazole (CYP2C6), 50 μ M of diethyldithiocarbamate (CYP2E1), 5 μ M of ketoconazole (CYP3A1/2, CYP1A2 and CYP2C6 inhibitor) and 20 μ M of furafylline (CYP1A2), 100 μ M omeprazole (CYP3A subfamily) [25,26]. Inhibitors were screened for their effects on melatonin O-

demethylation and 6-hydroxylation in mitochondria (mitoplasts) or microsomes at a melatonin concentration of 50 μ M.

The inhibitors were dissolved in methanol, and then the alcohol was removed by evaporating to dryness under reduced pressure with a rotational vacuum concentrator RVC 2-18 (Christ, Germany). After evaporation the incubation mixture with subcellular fraction and melatonin was added to test tubes, and the samples were preincubated at 37°C for 10 min. Reactions were initiated by adding NADPH (final concentration 1 mM) to microsomes or NADPH/isocitrate (final concentration 0.5 mM and 5 mM, respectively) to mitochondria (or mitoplasts), and carried out at 37°C for 60 min. Furafylline and diethyldithiocarbamate, the mechanism-based inhibitors, were preincubated at 37°C for 10 min with microsomes or mitochondria in the presence of NADPH or NADPH/isocitrate, respectively, before adding melatonin. After a 60 min incubation at 37°C, reactions were stopped by adding ice-cold methylene chloride. Sample preparation for HPLC analysis was carried out as described above.

HPLC analysis

Aliquots (20 μ l) of sample were separated on an LC-MS QP8000a (Shimadzu, Japan) equipped with diode array, fluorimetric and single quadrupole mass-spectrometric detectors. A Restec Allure C18 reverse-phase column (150 $\times$ 4.6 mm; 5 μ m particle size; and 60 Å pore size) was used with a mobile phase that consisted of 15% acetonitrile and 0.1% acetic acid. Elution was carried out isocratically at flow rate of 0.75 ml/min and temperature of 40°C. The column eluent was monitored by absorbance at 265 nm and by fluorescence with an excitation wavelength of 285 nm and an emission wavelength of 365 nm. The concentrations of 6-hydroxymelatonin and NAS were calculated from standard curves prepared in the range 10–500 pmoles of each metabolite.

The eluent was also routed to the mass-spectrometric electrospray interface (ESI) set in positive mode using nitrogen as the nebulizing gas. Mass-spectrometry parameters were as follows: nebulizer gas flow rate, 4.5 l/min; electrospray voltage, 4.5 kV; and curved desolvation line (CDL) heater temperature, 250°C. Analyses were carried out in the scan mode from m/z =160 to m/z =300 or in the SIM (selected ion monitoring) mode for specific detection of ions at m/z =219 (NAS); m/z =249 (hydroxymelatonin); m/z =265 (AFMK) and m/z =233 (melatonin). System control and data acquisition were performed with the LC-MS workstation Class-8000 software (Shimadzu, Japan).

Data analysis

The data were analyzed with Student's *t*-test and are presented as means $\pm$ SEM. The kinetics of melatonin O-demethylation and 6-hydroxylation by liver microsomes and mitochondria were fitted to equations for different enzyme models, using a non-linear regression program Prism 4.01 (GraphPad Software, San Diego, CA). The choice of the best-fitted enzyme model was based on the examination of Michaelis-Menten plots, Eadie-Hofstee plots, and the residual sum of squares. The best-fitted enzyme model and the respective equation was:

$$v = [(V_{max1}[S]) / (K_{m1} + [S]) + (V_{max2}[S]) / (K_{m2} + [S])] \quad (1)$$

where apparent V_{max1} and K_{m1} are related to the high-affinity, low-capacity site, and apparent V_{max2} and K_{m2} are the corresponding parameters for the second low-affinity, high-capacity site. Intrinsic clearance (CL_{int}) was calculated as a ratio of the V_{max} to the K_m . The total intrinsic clearance (CL_{total}) was then determined by the summation of the individual CL_{int} values:

$$CL_{total}=CL_{int}(1)+CL_{int}(2) \quad (2)$$

RESULTS

Incubation of microsomes with melatonin and NADPH resulted in the formation of six melatonin metabolites detected at 265nm. The metabolites had retention time (RT) of 6.5 min (metabolite 1), 9.14 min (metabolite 2), 9.91 min (metabolite 3), 11.52 min (metabolite 4), 16.32 min (metabolite 5), 28.61 min (metabolite 6), respectively, and were not detected when melatonin and NADPH were incubated with boiled microsomes (Fig. 2). Incubation of mitochondria with melatonin and NADPH/isocitrate resulted in the formation of the same six metabolites (data not shown). Preparations of mitoplasts fully retained the capability to metabolize melatonin (data not shown).

Subsequent mass-spectrometric and spectrophotometric analysis using commercially available standards identified the first two metabolites as NAS (metabolite 1) and as 6-hydroxymelatonin (metabolite 2).

Metabolite 3 had a molecular mass of 230 (molecular ion $[M+H]^+$ at $m/z=231$), indicative of a dehydrogenated derivative of melatonin.

Metabolite 4 had a molecular mass of 248 (molecular ion $[M+H]^+$ at $m/z=249$), indicative of a monohydroxylated derivative of melatonin. The UV spectrum of metabolite 4 ($\lambda_{max}=296$ and 257 nm) was similar to that of the keto tautomer of 2-hydroxymelatonin ($\lambda_{max}=298$ and 258 nm) [27].

Metabolite 5 had a molecular ion $[M+H]^+$ at $m/z=265$, indicating incorporation of two oxygen atoms into the melatonin molecule. Metabolite 5 was identical to commercially available AFMK standard by both LC-MS and spectrophotometry criteria.

The available mass-spectrometric and spectrophotometric assays were not selective and sensitive enough to identify Metabolite 6.

To study the effect of protein concentration in the reaction mixture on the accumulation of NAS and 6-hydroxymelatonin, incubations were performed at varying enzyme concentrations (data not shown). The rate of product formation increased linearly with enzyme concentration up to 0.75 mg protein/ml. Metabolite accumulation remained linear along the tested time points between 10–90 min (data not shown).

To study the effect of substrate concentration on the rate of 6-hydroxylation and O-demethylation, incubations were performed at varying concentrations of melatonin. Since standard of 2-hydroxymelatonin was not commercially available, the kinetic parameters were calculated only for 6-hydroxymelatonin and NAS. Formation of both products was found to follow to Michaelis-Menten kinetics within 0.1–1.5 mM melatonin (Figs. 3, 4).

The Eadie-Hofstee plots for melatonin hydroxylation and demethylation in all tested fractions were biphasic, indicating the involvement of more than one CYP450 isoform in both metabolic pathways (Figs. 3, 4). Therefore kinetic parameters for the formation of NAS and 6-hydroxymelatonin were obtained by nonlinear regression analysis, using a dual-enzyme Michaelis-Menten model (Eq. (1)). Intrinsic clearance (CL_{int}) was calculated as a ratio of the V_{max} to the K_m . The total intrinsic clearance (CL_{total}) was then determined by the summation of the individual CL_{int} values (Eq. (2)).

The estimated apparent K_m and V_{max} values for NAS and 6-hydroxymelatonin formation together with the intrinsic clearance are presented in Table. The K_{m1} values were significantly smaller than K_{m2} values in all cases, evidencing the apparent differences in affinity between low- and high-affinity components in mitochondria and microsomes. The intrinsic metabolic clearance of the high affinity component (V_{max1}/K_{m1}) was significantly higher than that of the low-affinity component (V_{max2}/K_{m2}) in all cases. The contribution of the $CL_{int}(2)$ for the low-affinity enzyme to the CL_{total} for the formation of 6-hydroxymelatonin and NAS in microsomes was 24.5% and 4.2%, respectively; whereas, the contribution of the same parameter in mitochondria was 6.1% and 9.2% for 6-hydroxylation and O-demethylation, respectively.

Mitochondrial CYPs exhibited higher affinity (suggesting lower K_m value) and higher V_{max} for melatonin 6-hydroxylation and O-demethylation for both high-affinity and low-affinity components as compared to microsomal enzymes.

To evaluate the role of different CYP isoforms in melatonin metabolism, we tested the effects of cytochrome P450 inhibitors (furaflavine, ketoconazole, quinidine, omeprazole, sulfaphenazole and diethyldihydrocarbamate) on 6-hydroxylation and O-demethylation of melatonin. Their actions on formation of NAS and 6-hydroxymelatonin are shown in Figure 5. The inhibition studies were performed at low melatonin concentration (50 μM), where high affinity activity predominated.

Furaflavine, a relatively selective inhibitor for rat CYP1A2, strongly inhibited mitochondrial 6-hydroxylation and O-demethylation, by 64% and 63%, respectively. Although NAS and 6-hydroxymelatonin formation in rat liver microsomes was also inhibited by furaflavine, the action was weaker than in mitochondria (27% and 38%, respectively).

Coincubation of melatonin with omeprazole reduced the microsomal demethylation and hydroxylation rates by 78% and 54%, respectively, suggesting the involvement of the CYP3A subfamily. Omeprazole exhibited the similar degree of inhibition of O-demethylation and 6-hydroxylation reactions in mitochondria (81% and 68%, respectively), as compared with microsomes.

Diethyldihydrocarbamate effectively blocked mitochondrial production of NAS and 6-hydroxymelatonin by 74% and 73%, respectively, demonstrating an essential role of CYP2E1 in melatonin metabolism in mitochondria, and had lesser effect on both reactions in microsomes (38% and 54%, respectively).

Ketoconazole (CYP3A1/2, CYP1A2- and CYP2C6 inhibitor) reduced the formation of 6-hydroxymelatonin and NAS in microsomes by 36% and 52%, respectively. In mitochondria, ketoconazole caused an inhibition of 6-hydroxylation and O-demethylation reactions of 44% and 49%, respectively.

Quinidine (CYP2C6, CYP3A subfamily) decreased the production of 6-hydroxymelatonin in microsomes by 64%, while a lesser degree of inhibition (38%) was observed for the O-demethylation reaction. In mitochondria, quinidine was much less potent at inhibiting melatonin hydroxylation (34%) than in microsomes, although it had similar inhibitory effect on O-demethylation (33%).

Since CYP2C6 is one of the cytochromes inhibited by quinidine, we also tested sulfaphenazole, a relatively selective CYP2C6 inhibitor. In microsomes, sulfaphenazole effectively blocked (by 49%) the 6-hydroxylation of melatonin and did not affect the O-demethylation reaction; in mitochondria, sulfaphenazole inhibited moderately melatonin hydroxylation (17%), with stronger effect on demethylation (27%).

DISCUSSION

In the present study we provide direct evidence for the involvement of rat microsomal cytochrome P450s in melatonin O-demethylation and hydroxylation at two different positions: 2 and 6, as well as generation of AFMK and 2 unknown products. Moreover, we found that mitochondrial cytochrome P450s are also able to convert melatonin into AFMK, NAS, 2-hydroxymelatonin, 6-hydroxymelatonin and the same 2 unknown products.

Our kinetic analysis indicates that more than one enzyme appeared to participate in the formation of melatonin metabolites in both microsomes and mitochondria. Eadie-Hofstee plots for 6-hydroxylation and O-demethylation reactions were curvilinear for all tested fractions, suggestive of involvement of at least two components, one with a high affinity and low capacity, and another with a low affinity and high capacity (Figs. 3, 4). Moreover, the calculated kinetic parameters indicate that both high- and low-affinity components responsible for melatonin metabolism saturate much faster in mitochondria than in microsomes (Table). Lastly, the total intrinsic clearance (CL_{int}), an index of enzymatic efficiency at substrate concentration below K_m levels, was significantly higher for both melatonin 6-hydroxylation and O-demethylation in mitochondria as compared with the microsomes. Because physiological concentrations of melatonin are much lower than the corresponding K_m values, CL_{int} is a physiologically important parameter and it indicates that melatonin metabolism *in vivo* is carried out more effectively in mitochondria than in microsomes.

Thus, our data suggest that mitochondria are organelles with high-capacity for melatonin metabolism. The intrinsic clearance for melatonin hydroxylation by high- and low-affinity components displayed the highest values in all tested fractions, indicating that both mitochondrial and microsomal cytochrome P-450s metabolize melatonin principally by 6-hydroxylation, with O-demethylation representing a minor metabolic pathway.

Currently CYP1A2 is thought to be primarily involved in the biotransformation of melatonin by human liver microsomes [16]. It has been also reported that furafylline inhibited markedly the conversion of melatonin to 6-sulphatoxymelatonin by rat liver postmitochondrial supernatant [19]. This is consistent with our results showing that melatonin 6-hydroxylation and O-demethylation in rat microsomes are inhibited by furafylline, suggesting CYP1A2 mediation.

Cytochrome P450s that cross-react with antibodies against the microsomal forms 1A1/2, 2B1/2, 3A1/2 and 2E1 have been detected in mitochondria [28]. Distinctive features of the mitochondrial forms are their location on the matrix side of the inner membrane and the requirements for specific elements to express catalytic activity; those include ferredoxin, a small soluble iron-sulfur protein localized to the mitochondrial matrix, and NADPH-ferredoxin reductase, a flavoprotein bound to the inner mitochondrial membrane. In spite of these differences with the microsomal enzyme, mitochondrial CYP1A2 metabolizes melatonin to NAS and 6-hydroxymelatonin, similar to its microsomal congener. Moreover, furafylline inhibition effect on melatonin metabolism was significantly higher (2-fold) in mitochondria than in microsomes, suggesting a stronger CYP1A2 contribution to melatonin clearance in mitochondria than in microsomes.

Since furafylline did not produce complete inhibition in all tested fractions, melatonin metabolism cannot be attributed solely to CYP1A2, and others CYPs expressed in rat liver may also be involved.

To further clarify the CYPs responsible for melatonin metabolism, various inhibitors, such as ketoconazole, quinidine, omeprazole, sulfaphenazole and diethyldithiocarbamate, were

screened for their effects on CYP-mediated reactions in rat microsomes, mitochondria and mitoplasts (Fig. 5).

In mitochondria (mitoplasts) the largest inhibition of melatonin metabolism was seen with diethyldithiocarbamate and omeprazole, indicating the involvement of CYP2E1 and CYP3A family, respectively. In microsomes omeprazole strongly inhibited NAS production, and, to a lesser extent the hydroxylation reaction. This indicates that the CYP3A family is primarily responsible for melatonin O-demethylation in microsomes, and, to a lesser degree, for melatonin 6-hydroxylation. In contrast, diethyldithiocarbamate exerted smaller inhibitory effect on NAS production in microsomes as compared with mitochondria, suggesting that microsomal CYP2E1 probably plays a smaller role in melatonin O-demethylation in rat liver. The rat CYP2E1 associated with the mitochondrial membrane compartment is almost identical to microsomal CYP2E1, except that the former is phosphorylated at a higher level and its activity is supported exclusively by ferredoxin and NADPH-ferredoxin reductase [29]. The different phosphorylation state of the mitochondrial CYP2E1 (P450 MT5) could be the likely cause of its preference for melatonin, as compared with the microsomal enzyme.

Since the inhibition profiles and contributions of low-affinity component clearance to the total intrinsic clearance were similar for O-demethylation and 6-hydroxylation reactions in mitochondria, it is likely that the same CYPs are involved in both metabolic pathways in these organelles.

This is in contrast with microsomes, where, the inhibitory effects of sulfaphenazole and quinidine indicated that CYP2C6 was a major mediator of 6-hydroxymelatonin formation, whereas the greatest inhibition of NAS production achieved with omeprazole clearly indicated that melatonin O-demethylation was primarily catalyzed *via* CYP3A. Although quinidine showed moderate inhibition of melatonin O-demethylation in microsomes, the absence of inhibition of NAS formation by sulphaphenazole ruled out the participation of CYP2C6 in this reaction. The involvement of different CYP isoforms in metabolic pathways in microsomes was further confirmed by the finding of significant differences in contribution of low-affinity component clearance to the formation of 6-hydroxymelatonin and NAS (Table 1).

The results of our study of melatonin metabolism in rat microsomes and mitochondria agree with earlier reports on the importance of CYP1A2 in melatonin 6-hydroxylation and O-demethylation, in both humans and rodents [16,19]. Furthermore, we also demonstrate that at least in rats, CYP3A and CYP2E1 provide additional contribution to melatonin metabolism in the mitochondria, while CYP3A and CYP2C6 are the main mediators of melatonin biotransformation in the microsomes.

It should be noted that some of inhibitors used in this work could affect mitochondrial CYP reactions indirectly. Thus, ketoconazole have been shown to produce a dose-dependent inhibition of NADH oxidase in isolated rat liver mitochondria [30]. Diethyldithiocarbamate, a potent copper chelating agent, could induce oxidative stress and subsequent mitochondrial dysfunction via inhibition of Cu/Zn superoxide dismutase localized in the intermembrane space [31]. Therefore, further investigation using specific anti-CYP antibodies is needed to confirm the roles of individual P450s in melatonin metabolism by mitochondria.

Interestingly, that quinidine and ketoconazole were only weak inhibitors of melatonin metabolism in human liver microsomes [16]. However, these data do not interfere with our interpretations, because CYP inhibitors do not exhibit the same selectivity in human and rat liver microsomes. Thus, ketoconazole, which is a potent inhibitor of human CYP3A4, inhibited CYP3A1/2, CYP1A2- and CYP2C6- mediated activities in rats [25]. Omeprazole,

a selective inhibitor of human CYP2C19, has also been reported as a potent inhibitor of the CYP3A family in rat [26]. Quinidine, which is one of the most potent inhibitors of human CYP2D6, appears to be a relatively selective inhibitor for rat CYP2C6 and to have a weak inhibitory effect on members of CYP3A subfamily [25].

There are similarities and differences between biotransformation of melatonin in the rat and human microsomes. The same two major metabolites are produced by each species (6-hydroxymelatonin and NAS), but apparently with notable differences in kinetic parameters [16].

Interestingly, that rat cytochromes P450 are able to metabolize melatonin to 2-hydroxymelatonin and AFMK, a feature not described for human enzymes. The production of 2-hydroxymelatonin and AFMK *in vivo* and their excretion in the urine of mice has been documented by Ma et al. [32]. These oxidation products of melatonin are thought to arise mainly from its nonenzymic reactions with ROS, RNS and HOCl or from its pseudoperoxidase oxidation by hemoglobin and cytochrome c [12,33–36]. Cytochrome P450-dependent production of 2-hydroxymelatonin and AFMK had not been reported earlier and it is unknown whether or not this enzymatic pathway exists *in vivo*. However, according to our *in vitro* studies melatonin is not only metabolized to 6-hydroxymelatonin and NAS by rat CYP450, but also undergoes additional cytochrome P450 – mediated reactions to yield 2-hydroxymelatonin and AFMK (Fig. 2). Thus, melatonin oxidation by cytochrome P450s could be one of the mechanisms of 2-hydroxymelatonin and AFMK generation *in vivo*.

Overall, it appears that the differences in the kinetic parameters for melatonin metabolism and hydroxylation specificity between human and rat microsomal CYPs are most likely due to the involvement of completely different CYP isoforms in the two species and/or to differences in the catalytic properties of the same isoforms between the two species.

CYP1A subfamily shows a strong conservation among species and the rat and human CYP1A2 have similar substrate specificity and share common amino acid sequences at the active site [37]. Nevertheless, furafylline inhibits rat CYP1A2 at concentration higher than it is required to inhibit the human isoenzyme, suggesting a major difference in active site geometry between the human and rat CYP1A2 orthologues [38]. There are also marked species differences in hepatic level of CYP1A2. In human liver CYP1A2 accounts for 13% of the total CYP content being expressed at significantly lower levels (2%) in rat liver [39].

The hepatic metabolism of melatonin in humans involves in addition to microsomal CYP1A2, CYP2C19 and CYP2C9 isoforms that are not expressed in rat liver. Our results indicate a major involvement for CYP2C6, and perhaps other members of the CYP2C subfamily, in melatonin hydroxylation by rat microsomes. Further, the data of inhibitory analysis provide strong support for the suggestion that in contrast to humans melatonin metabolism in rat hepatic microsomes is mediated by the CYP3A subfamily and CYP2E1.

In summary, a number of novel findings are reported here. Previous studies have reported that only 6-hydroxymelatonin and NAS are products of cytochrome P450 mediated metabolism of melatonin in the liver. The present investigation demonstrates for the first time that 2-hydroxymelatonin and AFMK are also formed in reactions catalysed by liver cytochrome P450 (Fig. 1). Further, it is generally accepted that melatonin is predominantly metabolised in microsomes. In contrast to this general view, our studies showed that mitochondrial cytochrome P450s are also participating in melatonin metabolism in rat liver. At present, mitochondria have been identified as a target for melatonin actions. Melatonin increases the activity of the respiratory chain complexes I and IV; inhibits mitochondrial pathway of apoptosis; participates in the circadian oscillations of oxidative phosphorylation [40,41]. These melatonin activities in mitochondria would require precise regulation of its

local level and such control function could be served by the mitochondrial cytochrome P450s.

Acknowledgments

The paper is dedicated to the memory of Aaron Lerner. Partial support from NIH (Grant # AR052190), intradepartmental funds (SUB) and Ministry of Education of Belarus are acknowledged. We thank Dr. Jacobo Wortsman, MD, for his editorial work, valuable comments and suggestions and careful review of the manuscript.

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Fig. 1.
Melatonin metabolism mediated by cytochrome P450.

Fig. 2.

Chromatographic analysis of products of melatonin metabolism by rat liver microsomes. **A** - Boiled microsomes (0.5 mg protein/ml) incubated at 37°C for 60 min with 1 mM NADPH and 50 μ M melatonin. **B** - Microsomes (0.5 mg protein/ml) incubated at 37°C for 60 min with 1 mM NADPH and 50 μ M melatonin. See Methods section for detail description of incubation conditions and sample preparation for HPLC analysis. A Restec Allure C18 column (150 $\times$ 4.6 mm) was used for separation of melatonin metabolites. Elution was carried out isocratically with 15% acetonitrile and 0.1% acetic acid at flow rate of 0.75 ml/min and temperature of 40°C. The column eluent was monitored by absorbance at 265 nm. The insets show eluent fluorescence with an excitation wavelength of 285 nm and an emission wavelength of 365 nm. Peaks designated as 1, 2, 3, 4, 5, 6 and 7 correspond to Metabolite 1 (NAS; $[M+H]^+$ at $m/z=219$; $\lambda_{\max}=278$ nm), Metabolite 2 (6-hydroxymelatonin; $[M+H]^+$ at $m/z=249$; $\lambda_{\max}=300$ nm), Metabolite 3 (oxidation product of melatonin with $m/z=231$; $\lambda_{\max}=273$ and 293 nm), Metabolite 4 (2-hydroxymelatonin; $[M+H]^+$ at $m/z=249$, $\lambda_{\max}=257$ and 296 nm), Metabolite 5 (AFMK; $[M+H]^+$ at $m/z=265$, $\lambda_{\max}=234$, 262 and 342 nm), Metabolite 6 (unknown product) and melatonin ($[M+H]^+$ at $m/z=233$, $\lambda_{\max}=278$ nm), respectively. See Methods section for detail description of LC-MS analysis.

Fig. 3.

Effect of melatonin concentration on rate of 6-hydroxymelatonin (A) and NAS (B) formation in microsomes. The melatonin concentrations ranged from 0.1 to 1.5 mM. The concentration of microsomal protein in the reaction was 0.5 mg/ml. Reactions were conducted at 37°C for 60 min. A HPLC analysis with fluorescence detection (excitation: 285 nm, emission: 365nm) was used for quantification of the 6-hydroxymelatonin and NAS. Means $\pm$ SEM of three replicates are shown. Points are experimentally determined values, and the solid line is the computer-generated curve of best fit for a two-enzyme model. The insets show Eadie-Hofstee plots for O-demethylation and 6-hydroxylation of melatonin.

Fig. 4. Effect of melatonin concentration on rate of 6-hydroxymelatonin (A) and NAS (B) formation in mitochondria. The melatonin concentrations ranged from 0.1 to 1.5 mM. The concentration of mitochondrial protein in the reaction was 0.5 mg/ml. Reactions were conducted at 37°C for 60 min. A HPLC analysis with fluorescence detection (excitation: 285 nm, emission: 365nm) was used for quantification of the 6-hydroxymelatonin and NAS. Means $\pm$ SEM of three replicates are shown. Points are experimentally determined values, and the solid line is the computer-generated curve of best fit for a two-enzyme model. The insets show Eadie-Hofstee plots for O-demethylation and 6-hydroxylation of melatonin.

Fig. 5. Effect of cytochrome P450 inhibitors on the transformation of melatonin to 6-hydroxymelatonin and N-acetylserotonin by microsomes (A), mitochondria (B) and mitoplasts (C). The concentrations of protein and melatonin in the reaction were 0.5 mg/ml and 50 μ M, respectively. Incubations were performed at 37°C for 60 min with 5 μ M of quinidine, 10 μ M of sulfaphenazole, 50 μ M of diethyldithiocarbamate, 5 μ M of ketoconazole, 20 μ M of furafylline and 100 μ M omeprazole. See Methods section for detail description of incubation conditions. A HPLC analysis with fluorescence detection (excitation: 285 nm, emission: 365nm) was used for quantification of the 6-hydroxymelatonin and NAS. Means $\pm$ SEM of three separate experiments are shown. For all of the inhibitors, a comparison was made to the activity of control incubations without inhibitor

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Significantly different from the corresponding values of control incubations).

Table 1
Kinetic parameters for 6-hydroxymelatonin and NAS formation in microsomes and mitochondria

Subcellular fractions	Product of reaction	KINETIC PARAMETERS					
		High affinity component			Low affinity component		
		K_m^1 ^a	V_{max}^1 ^b	$CL_{int(1)}$ ^c	K_m^2 ^a	V_{max}^2 ^b	$CL_{int(2)}$ ^c
Microsomes	6-HMel	48.33 ± 9.17	5.783 ± 0.99	0.119	496.8 ± 53.78	18.56 ± 0.74	0.0374
	NAS	25.86 ± 1.39	0.668 ± 0.019	0.025	953.3 ± 85.95	1.059 ± 0.021	0.0011
Mitochondria	6-HMel	5.37 ± 1.06	8.92 ± 0.483	1.661	406.6 ± 19.84	43.76 ± 0.4172	0.108
	NAS	13.96 ± 2.37	1.53 ± 0.15	0.109	253.5 ± 27.59	2.761 ± 0.12	0.011

Expressed as μM^a , pmoles/min^b, $\mu l/min^c$