J. Pineal Res. 2001; 30:243–247

Journal of Pineal Research
ISSN 0742-3098

Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat

Stefulj J, Hörtner M, Ghosh M, Schauenstein K, Rinner I, Wölfler A, Semmler J, Liebmann PM. Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J. Pineal Res. 2001; 30:243–247. © Munksgaard, Copenhagen

Abstract: Besides the pineal gland, melatonin is reported to be produced in a number of extrapineal sites, where it could act as an intracellular mediator or paracrine signal in addition to its endocrine effects. In view of the suggested immunoregulatory role of melatonin, we compared lymphoid organs and several other tissues of the rat for their potential to synthesize melatonin. Using the reverse transcription-polymerase chain reaction (RT-PCR) method, we determined the tissue-specific expression of mRNAs encoding two key enzymes of the melatonin biosynthesis: serotonin-N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT). The minimal number of PCR cycles required to obtain a positive signal served as a measure for the abundance of a given mRNA. NAT and HIOMT mRNAs were detected in all tested tissues at high numbers of PCR cycles (40 and 45, respectively). At 35 cycles, only gut, testis, spinal cord, raphe nuclei, stomach fundus and striatum yielded positive signals for both enzymes. In conclusion, the presence of NAT and HIOMT mRNAs in a wide range of tissues corroborates and extends the notion of extrapineal melatonin synthesis. Comparatively low levels of the HIOMT messages in lymphoid organs, however, indicate a limited significance of melatonin synthesis within the immune system.

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Key words: hydroxyindole-O-methyltransferase – melatonin synthesis – N-acetyltransferase

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Received March 9, 2000; accepted June 27, 2000.

Introduction

Melatonin was first isolated from the pineal gland. Since its discovery, there has been increasing evidence for extrapineal melatonin synthesis in a number of tissues, including retina, gastrointestinal tract, reproductive organs, skin, platelets, cells of the immune system and several brain regions [for review see Kvetnoy, 1999]. Regarding the immune system, melatonin production was demonstrated in human cultured peripheral blood mononuclear leukocytes exposed to serotonin and/or interferon-γ [Finocchiaro et al., 1988, 1991]. In view of the reported influences of melatonin on immune functions [Maestroni, 1993; Liebmann et al., 1996, 1997] a paracrine role, in addition to its endocrine effects on immune physiology, is intriguing. Locally produced (paracrine) melatonin could contribute to the numerous effects ascribed to melatonin, either by itself or by interfering with the endocrine effects of this agent. Furthermore, the local tissue concentrations of melatonin could reach much higher values than what was hitherto regarded as physiological. This issue might be of particular importance in view of described antioxidative features of melatonin, even though respective reports are not unequivocal [Abuja et al., 1997; Wölfler et al., 1999]. Therefore, studies on the physiological relevance of extrapineal melatonin formation are of importance.

Melatonin is synthesized from serotonin by the sequential action of N-acetyltransferase (NAT; EC 2.3.1.87) and hydroxyindole-O-methyltransferase (HIOMT; EC 3.1.1.4) [Wurtman and Axelrod, 1968]. In the present study, we examined the potential capacity of melatonin production in thymus, spleen, and several other tissues of the rat by determining the tissue-specific mRNA expression

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Table 1. Oligonucleotide primers used in PCR amplification of rat NAT and HIOMT cDNAs

Gene	Accession-number[a]	Primer sequences	Position (bp)[b]	PCR product length (bp)
NAT	U40803	f: 5′-CCTTCACTGAGCTGCACACTGT-3′ r: 5′-TCAGCCTGGCCTAGAGTGAG-3′	756–771 1185–1166	430
HIOMT	L78306	f: 5′-GGTAGCTCCGTGTGTGTCTT-3′ r: 5′-AGTGGCCAGGTTGCGGTAGT-3′	788–807 1150–1131	363

[a] GenBank sequence database accession number. [b] Position of the primer in the coding sequence. f = forward; r = reverse.

pattern of NAT and HIOMT with reverse transcription-polymerase chain reaction (RT-PCR).

Materials and methods

Oligonucleotide primers

Oligonucleotides used in PCR amplification were synthesized at Life Technologies (Vienna, Austria). Primers specific for the detection of rat HIOMT and NAT genes were designed using Primer Design 2.0 software (National Center for Biotechnical Information, Bethesda, MD), according to published cDNA sequences (Table 1). A pair of primers specific for the ubiquitously expressed β-actin mRNA was used to monitor the quality of RNAs and the efficiency of reverse transcription.

Tissues and RNA preparation

Male Sprague-Dawley rats weighing 200–300 g were used. Animals were kept at a 12 hr light/12 hr dark cycle, with the lights on at 07.00 hr. Tissue samples to be analyzed (i.e. spleen, thymus, platelets, lung, heart, kidney, muscle, liver, stomach fundus, gut, testis, spinal cord, brain cortex, striatum, raphe nuclei and pineal gland) were taken at 09.00 hr (light phase) from four to six animals (Table 2). In the case of pineal gland, whole organs from two animals were pooled, while the other samples were processed individually. Cell suspensions were prepared from spleen and thymus by passing tissue fragments through a nylon sieve. Platelets were obtained by centrifugation of the blood as described [Jernej et al., 1988]. Other tissue samples were frozen in liquid nitrogen immediately after excision. Total cellular RNA was isolated from either 10^7 cells or 20–30 mg of tissue according to the guanidinium method [Chomczynski and Sacchi, 1987]. All RNA preparations were additionally treated with DNAse I to eliminate residual genomic DNA contamination and were analyzed for the amount and integrity of RNA by agarose gel electrophoresis.

cDNA synthesis and polymerase chain reaction

One microgram of each RNA was reverse transcribed in a final volume of 20 μL with 15 U of AMV reverse transcriptase (Promega) and 0.5 μg oligo $(dT)_{15}$ primer (Promega), for 30 min at 42°C, followed by 5 min at 99°C. A control reaction lacking reverse transcriptase was also prepared from each RNA sample (tissue negative control). PCR amplification was performed with 1.5-μL aliquots of cDNA in a total volume of 25 μL with 0.6 U of AmpliTaq Gold DNA-polymerase (Perkin-Elmer) per sample and 0.2 mM dNTP (Promega). The final concentrations of primers were 0.8 μM for NAT and HIOMT, and 0.5 μM for β-actin. The maximal sensitivity/specificity of the reaction was obtained for NAT and HIOMT at 2.0 mM $MgCl_2$, and for β-actin at 1.5 mM $MgCl_2$. One PCR cycle consisted of 45 s at 94°C, 1 min at 60°C and 50 s at 72°C. Beta-actin PCR was run at 29 cycles, whereas the number of amplification cycles for NAT and HIOMT ranged from 30 to 45 in steps of five.

Table 2. PCR amplification of NAT and HIOMT cDNAs derived from various extrapineal tissues of the rat. Results are marked (−) when positive signals were obtained after 45 PCR cycles only, (+) when positive signals were obtained after 40, and (++) after 35 PCR cycles.

	N	NAT	HIOMT
Thymus	6	++	−
Spleen	6	++	−
Platelets	5	+	−
Lung	4	++	−
Heart	4	++	−
Kidney	4	+	++
Muscle	4	+	++
Liver[a]	4	+	+
Stomach fundus	4	++	++
Gut	5	++	++
Testis	4	++	++
Spinal cord	4	++	++
Brain cortex	6	++	++
Raphe nuclei	5	++	++
Striatum	5	++	++

[a] Positive signals at 35 PCR cycles were obtained only in two of four rats.

Within one PCR assay, various samples were simultaneously examined for the presence of a particular cDNA. Each PCR assay included a water control to rule out template contamination of PCR reagents, as well as a tissue-negative control for each sample to exclude genomic DNA contamination. All RT-PCR results were confirmed in at least two independent reactions.

Detection and analysis of the PCR products

Ten-microliter aliquots of the RT-PCR products were electrophoretically separated on 1.6% agarose gels. Bands were detected by staining with ethidium bromide and their lengths were determined using the standard DNA molecular ladder (Boehringer-Mannheim) and the analysis software package ONE-Dscan 1.0. The PCR products were sequenced by DNA-Sequencing System 373 (Perkin-Elmer) and a commercially available kit (Perkin-Elmer).

Results

The synthesized primers were pre-tested by PCR with cDNA from a tissue known to express HIOMT and NAT genes, i.e., pineal gland. After the optimal concentrations of primers and $MgCl_2$ (see Materials and methods) were determined, a single band of the expected size (Table 1) was obtained with each primer pair. The identity of the PCR products was confirmed by direct sequencing.

The quality of RNAs and the efficiency of reverse transcription were monitored by the housekeeping gene (β-actin) RT-PCR (Fig. 1A). RT-PCR products corresponding to the NAT and HIOMT genes were obtained from all tested tissues after 40 and 45 PCR cycles, respectively (Fig. 1C,E). After 35 cycles of amplification, no NAT message was detected in platelets, kidney and muscle (Fig. 1B), while spleen, thymus, platelets, lung and heart were negative for HIOMT (Fig. 1D). RNAs from all rats gave approximately identical results, except with liver, where NAT and HIOMT mRNAs were positive at 35 PCR cycles in only two of four rats (Table 2).

Extrapineal tissues, which yielded the strongest HIOMT and NAT bands after 35 cycles, i.e., gut, testis, spinal cord and raphe nuclei, were also tested at 30 PCR cycles, in comparison with pineal gland cDNA (Fig. 2). Strong RT-PCR signals were obtained from the pineal gland for both enzymes. The NAT signal was also robust in testis, distinctly weaker in spinal cord and raphe nuclei, and faint in gut. HIOMT signal was moderate in testis and faint in spinal cord, while gut and raphe nuclei gave no clearly positive results.

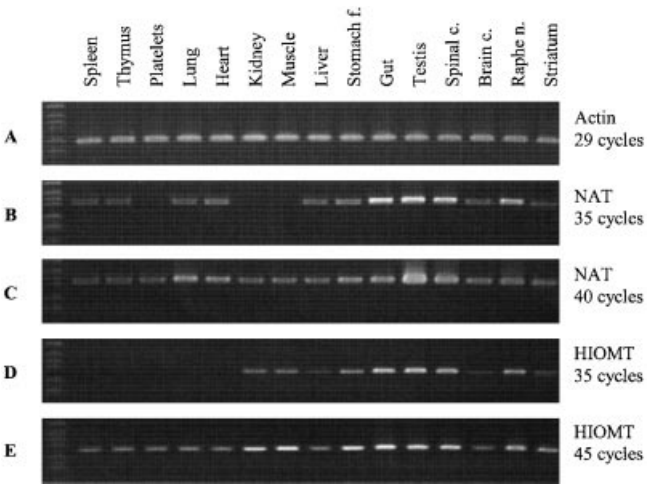

Fig. 1. Agarose gel electrophoresis of RT-PCR products obtained from various tissues of the rat. As control for the absence of genomic DNA contamination all RT-PCRs were performed with and without (negative results, not shown) reverse transcriptase added to the cDNA synthesis reaction. (**A**) To test the quality of RNA and efficiency of reverse transcription, all samples were subjected to the RT-PCR of housekeeping gene (β actin). (**B**); (**C**) NAT RT-PCR products obtained at 35 and 40 PCR cycles respectively. (**D**), (**E**) HIOMT RT-PCR products obtained at 35 and 45 PCR cycles respectively. Stomach f. = stomach fundus. Spinal c. = spinal cord. Brain c. = brain cortex. Raphe n. = raphe nuclei.

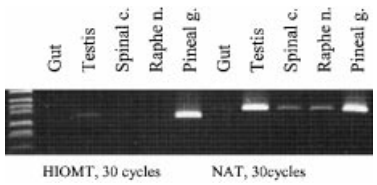

Fig. 2. Agarose gel electrophoresis of HIOMT and NAT RT-PCR products obtained at 30 PCR cycles from gut, testis, spinal cord (Spinal c.), raphe nuclei (Raphe n.) and pineal gland (Pineal g.) of the rat.

Discussion

With high amplification cycle numbers (i.e., 40 and 45, respectively) it was possible to generate positive mRNA signals for both NAT and HIOMT in all tested tissues. Because at such large numbers of cycles it is possible to detect randomly expressed mRNAs that are without functional significance, the cycle number was reduced to 35, whereby certain tissues, i.e. stomach fundus, gut, testis, spinal cord, brain cortex, raphe nuclei and striatum, still gave positive signals for both enzymes.

Our results on the presence of NAT and HIOMT mRNAs in gut, stomach fundus and

testis support results showing NAT gene expression in rat testis [Borjigin et al., 1995] and melatonin synthesis in the gastrointestinal [Huether et al., 1992] and reproductive tract of the rat [Tijmes et al., 1996; Itoh et al., 1997] and human ovary [Itoh et al., 1999]. Regarding the expression of NAT and HIOMT mRNAs in neural tissue, we demonstrate here for the first time the presence of both messages in rat spinal cord, brain cortex, raphe nuclei and striatum. The translation of the detected messages into functionally active proteins remains to be demonstrated.

At 35 PCR cycles, NAT mRNA was not detected in rat platelets, kidney, muscle and liver (Fig. 1B), while HIOMT mRNA was undetectable in the spleen, thymus, platelets, lung, heart and liver (Fig. 1D). In previous studies, the absence of NAT mRNA was demonstrated in human heart, spleen and liver [Coon et al., 1996], as well as in rat [Borjigin et al., 1995] and ovine [Coon et al., 1995] heart, liver, and muscle. According to our present data, rat platelets, kidney, muscle, spleen, thymus, lung and heart cells do not contain reliable amounts of either NAT or HIOMT mRNAs, and are therefore not equipped with the necessary tool for melatonin synthesis. These results may question the relevance of findings of others showing HIOMT mRNA in human platelets [Champier et al., 1997] and in human cell lines of immune origin [Conti et al., 2000], both using extremely sensitive analysis techniques.

Nuclei and purified membranes from spleen and thymus express high-affinity binding sites for melatonin [Lopez-Gonzalez et al., 1993; Rafii-El-Idrissi et al., 1998]. Inconsistencies with regard to the immunomodulatory effects of melatonin, as reported in the literature [Liebmann et al., 1997], may be, at least partly, due to different melatonin concentrations employed in the respective studies. In order to get a better understanding of the mechanisms of melatonin action, a re-definition of physiological/pharmacological melatonin concentrations is needed. As mentioned above, our results show that resting lymphoid cells of the rat do not express key enzymes for the melatonin synthesis. Our preliminary results (not shown) demonstrate the same with Con A-activated rat lymphocytes. Therefore, a significant paracrine role of melatonin in immunoregulation is questionable. Further studies, however, are needed to delineate extrapineal melatonin synthesis under physiological and pathological conditions, which may finally lead to a more differentiated view of the role of this hormone in the regulation of immune functions, and its physiological roles in general.

Acknowledgments

This study was supported by the Austrian Science Foundation (FWF P12679-MED) and the "Jubiläumsfonds der Oesterreichischen Nationalbank" (P #5692). J.S. and M.G. were recipients of fellowships granted by the ÖAD (Österreichischer Akademischer Austauschdienst)

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