Abstract. Aim: The aim of this study was to investigate the expression of melatonin receptor MTNR3 and nuclear receptors in murine lymphocytes and their dependence on lighting conditions and circadian time. Materials and Methods: The mRNA levels of melatonin receptors were investigated in cells isolated from thymus, spleen, lymph nodes and bone marrow during the day or during the night. Results: The expression of MTNR3 in B-cells and bone marrow cells was much higher than in thymocytes and T-cells. Retinoic acid receptor-related orphan receptor A (Rora) was found mostly in thymocytes and cluster of differentiation 4 positive (Cd4+) T cells. Rorc was detected in thymocytes; its expression in peripheral T-cells was very low. Rorb was not detected in lymphocytes. MTNR3 transcripts in B-cells and Rorc transcripts in thymocytes increased during the day and decreased during the night. Conclusion: Circadian time and lighting could be involved in the regulation of the expression of melatonin receptors MTNR3 and Rorc.

Melatonin is a hormone that regulates the circadian day-night rhythm and seasonal biorhythms. It also modulates immune defence responses, and influences the functions of lymphocytes (1). Melatonin can bind to three types of receptors: membrane receptors MTNR1 and MTNR2, cytosolic receptor MTNR3 and nuclear receptors (2). MTNR3 is expressed in the various tissues of hamster, rabbit, monkey, mouse, and dog (3). MTNR3 is the human cytosolic enzyme quinone reductase 2 (NQO2) (4) that can activate quinines, leading to production of reactive oxygen species (5). Melatonin can inhibit catalytic activity of NQO2 and therefore protect cells from highly reactive oxygen species (6). Acting via MTNR3, melatonin can inhibit the adhesion of rat leukocytes to the vascular endothelium (7). However, the expression of MTNR3 in the cells of the immune system has not been thoroughly investigated.

The nuclear receptors of melatonin belong to the subfamily of retinoic acid receptor-related orphan receptors (ROR). This subfamily includes the products of three genes: RORA, RORB, and RORC (8). Four human RORα (RORα1-4) and only two murine (Rorα1 and Rorβ4) isoforms have been identified (9). RORα is expressed in different organs and cells (2), including the cells of the immune system (10, 11) and controls the immune responses. RORα regulates thymopoiesis, lymphocyte development (9, 12), T helper 17 (Th17) T-cell differentiation (13), cytokine production (12-14) and antibody production (12).

The murine Rorb gene encodes two isoforms, β1 and β2, while only the RORβ1 isoform was found in humans (9). RORβ is specifically expressed in brain, pineal gland, retina, and bone marrow. RORβ has been implicated in bone formation, neurogenesis, and stress response (15). RORβ has not been detected in the immune system.

The mouse and human RORC gene encodes two isoforms, γ1 and γ2 (9). RORγ1 is expressed in skeletal muscles, liver, kidney, thymus, brain, heart, and lung (16). RORγ2 is detected in thymus, lymph nodes, and Peyer's patches. It has been reported that RORγ2 plays a critical role in thymopoiesis and in the development of secondary lymphoid tissues (9). It is also required for development of lymph nodes and Peyer’s patches, but not splenic follicles (17). RORγ2 inhibits interleukin-2 production (16) and similar to RORα regulates the differentiation of Th17 T-cells (18).

However, despite extensive investigations on different types of melatonin receptors, the expression of MTNR3 has not yet been examined in the immune system of any organism. The amount of melatonin in the blood and the immunological response varies during the day and night (19, 20), but there is no knowledge on the influence of circadian time and lighting conditions on the expression of melatonin receptors in mice. Therefore, we studied the influence of circadian time and lighting conditions on the expression of MTNR3 and nuclear melatonin receptors in murine lymphocytes.
Materials and Methods

Experimental animals. BALB/c mice were bred and housed in our animal facility. The experiments with animals were performed according to international ethical standards. The research protocol was submitted to and approved by a Lithuanian State Food and Veterinary Service (permission number 0225). The rodents were given ad libitum access to food and water and maintained under a 12/12-h light/dark cycle. One group of mice (LD) was kept under normal light/dark conditions; another group (LL) was kept under constant artificial lighting (around 50 lux) for about one week before experiments. The animals were sacrificed by cervical dislocation 4 h after beginning light (LD day) or dark (LD night) time. The mice kept at constant lighting (LL day) were sacrificed at the same time as LD day mice.

Cell preparation. Thymocytes, splenocytes, lymph node cells and bone marrow cells were isolated from 8- to 9-week-old BALB/c mice. Splenocytes were stained with phycoerythrin (PE)-labelled antibodies to mouse B220 (BD Biosciences, San Jose, CA, USA). Lymph node cells were stained with PE-labelled antibodies to mouse Cd4 (BD Biosciences). The stained cells were separated using positive selection with anti-R-(PE) Magnetic Particles-DM (BD Biosciences, San Jose, CA, USA). The cell isolation was performed according to the manufacturer’s recommendations. Cell purity was estimated by flow cytometry.

RNA isolation, and cDNA synthesis. RNA was extracted using GeneJET™ RNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according the manufacturer’s instructions. cDNA was synthesized using Maxima Reverse Transcriptase and random hexamer primers (Thermo Fisher Scientific Baltics, Vilnius, Lithuania).

PCR. PCR was performed in a real-time PCR cycler Rotor-Gene RG 6000-time PCR instrument. The sequences of Rora primers (GGAAAGAGCTCAGCAGAATAACG and GCTGACATCAGTACGAATGCAG) were as described by Carrillo-Vico et al. (1). The other primers were designed using Lasergene software (DNASTAR, Madison, WI, USA): MTNR3 primers: CAATGGGTCCCTGAAGAACGTGG and CCCCCGTTAAGGAAAGGAGAC; Rorb primers: AATCAAGGCGTATCAAAGCAAGTC and TGGGGATATCAGAACACCTGC. All primers are expressed in the brain. Therefore, brain cDNA served as a calibrator. A five-fold dilution of brain cDNA was prepared and the PCR was performed with each dilutions of calibrator cDNA and tested samples. We accepted that brain cDNA has certain amount of relative units of target gene. The relative standard curve was constructed adding relative units to the Y axis and threshold values (Ct) to the X axis. Target quantity was determined from the standard curve using Rotor-Gene RG 6000 Ver. 1.7 software. Ubiquitously expressed Hprt mRNA was used to monitor the quality of RNA and the efficiency of the RT and the PCR processes. The relative quantities of each target gene were normalized to Hprt as a reference gene and were carried out according to the ABI PRISM 7700 Sequence Detection System bulletin #2 (21).

Results

Expression of MTNR3 receptor in lymphocytes. We found MTNR3 mRNA in all analyzed cells: thymocytes, splenic B-cells, Cd4+ T-cells and bone marrow cells (Figure 1). The expression of MTNR3 was much stronger (up to 100 times) in B-cells and bone marrow cells as compared to thymocytes and Cd4+ T-cells and it was dependent on circadian time and lighting conditions. B-Cells prepared from the LD day mice had significantly more (p<0.005) MTNR3 transcripts compared to B-cells isolated from LL day and LD night mice. Thymocytes prepared from LD day mice showed a trend towards higher levels of MTNR3 transcripts. The circadian time and lighting conditions were not important for MTNR3 expression in T-cells and bone marrow cells.

Expression of Rora, Rorb and Rorc in lymphocytes. The highest expression of Rora mRNA was found in thymus and Cd4+ T-cells (Figure 1). Transcripts of Rora in bone marrow cells were about 10 times less as compared to that in thymocytes and Cd4+ T-cells. Rora was barely detectable in B-cells. The circadian time and lighting conditions did not influence the expression of Rora.

Rorb was not expressed in lymphocytes (data not shown). Rorc was expressed in thymocytes and Cd4+ T-cells (Figure 1). The levels of the transcripts in thymocytes were 10-100 times higher than in Cd4+ T-cells. The expression of Rorc in thymocytes depends on circadian time. Transcripts were significantly (p<0.005) higher in thymocytes from LD day mice as compared to LL day and LD night mice. We found more transcripts in Cd4+ T cells prepared from LD night mice to compare with the T cells obtained from LL day and LD day mice; however these differences were not statistically significant. Rorc was barely detectable in B-cells and bone marrow cells.

Discussion

In the present study, we investigated the expression of melatonin receptors in the lymphocytes of BALB/c mice and their dependence on circadian time and lighting
conditions. The cells were isolated during the day (LD day mice) and at night (LD night mice), which corresponds to low and high melatonin concentrations in the blood, respectively (20). In order to determine the influence of disrupted circadian clock on the expression of melatonin receptors, we also isolated cells from mice kept under constant lighting conditions (LL mice). We show that the levels of MTNR3, and Rorc mRNA depend on the circadian time or lighting conditions. Although some authors argue that the expression of Rora in the lung of Perdicula asiatica (22) and Jurkat cells (23) depends on the melatonin concentration, we did not find a significant variation of Rora expression under different circadian time and lighting conditions. Our data demonstrate that Rora is expressed mostly in T-cells (thymocytes and peripheral Ca4+ T-cells) and at a lower level in bone marrow cells; it was barely detectable in splenic B-cells. The primers we used can amplify both isoforms of murine Rora. Rora mRNA was found in murine thymus, spleen, peripheral Cd4+ T-cells (1, 12). Rora expression in murine B cells was detected at a very low level (12). The expression of RORA was detected in human Th17 T-helper cells, CD8+ T-cells, monocytes, granulocytes, natural killer cells and in contrast with the mouse, in B-cells (1, 13).

Rorb nuclear receptor has not been detected in the immune system so far. We also did not find Rorb mRNA in lymphocytes.

The expression of MTNR3 has not yet been investigated in the cells of immune system. We have found that MTNR3 was expressed in thymocytes, splenic B-cells, Cd4+ T-cells and bone marrow cells. The expression of MTNR3 differed depending on the type of the cells. The T-cells (thymocytes and CD4+ T-cells) have less MTNR3 than B-cells and bone marrow cells. Splenic B-cells isolated during the light time had higher expression of MTNR3 than the cells isolated at the dark time.

Taken together, the amount of MT3R mRNA in B cells and the amount of RORγ mRNA in thymocytes depends on circadian time. The expression of RORα in murine lymphocytes does not depend on circadian time and lighting conditions.
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References


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