МЕЛАТОНИН В ДИФФЕРЕНЦИРОВКЕ Th17/Treg: ВКЛАД СОБСТВЕННОЙ ПРОДУКЦИИ ГОРМОНА Т-ЛИМФОЦИТАМИ

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Резюме. Гормон мелатонин обладает широким спектром биологических эффектов и регулирует работу практически всех органов и систем организма. В иммунной системе важнейшей мишенью мелатонина являются основные эффекторы адаптивного иммунитета – Т-лимфоциты: они экспрессируют специфические мелатониновые рецепторы – мембранные, MT1 и MT2, и ядерный, RORα (все с разной аффинностью к гормону), а также ряд внутриклеточных молекул, неспецифически связывающих мелатонин в высоких концентрациях. Более того, в исследованиях *in vitro* многими авторами показана собственная продукция мелатонина Т-лимфоцитами в ответ на поликлональную активацию, а также участие такого эндогенного мелатонина в качестве аутокринного или паракринного фактора в индукции синтеза Т-клетками IL-2 и IL-2-рецептора (IL-2R), причем в реализацию данных эффектов были вовлечены как мембранные, так и ядерный рецепторы для мелатонина. Поскольку IL-2/IL-2R-зависимый сигнал является ключевым событием в индукции пролиферативного ответа Т-лимфоцитов, собственный мелатонин, по-видимому, напрямую задействован как минимум в клональной экспансии этих клеток. Мы в настоящей работе исследовали вклад Т-клеточного мелатонина в регуляцию следующего этапа активации Т-лимфоцитов, а именно, в дифференцировку Т-хелперных популяций Th17 и Treg. Показано, что блокада и мембранных, и ядерного мелатониновых рецепторов не вызывает статистически значимых изменений в дифференцировке Th17, хотя тенденция к снижению фиксировалась. В то же время, уровень $CD4^+FoxP3^+$Т-клеток снижался на фоне неселективной блокады мембранных рецепторов для гормона, а концентрация соответствующего Treg-ассоциированного цитокина TGF-β в супернатантах активированных культур снижалась как в случае неселективной блокады MT1/MT2, так и при селективной блокаде MT2. Полученные данные свидетельствуют о том, что мелатонин, продуцируемый Т-лимфоцитами в культуре, может вносить вклад в контроль дифференцировки наивных $CD4^+$Т-клеток в Treg *in vitro*, причем действие гормона опосредуется мембранными мелатониновыми рецепторами. Наличие у Т-лимфоцитов большого количества разноаффинных мишеней для мелатонина определяет ключевую роль концентрации гормона в его эффектах в отношении этих клеток. Поэтому важно учитывать собственную продукцию гормона лимфоцитами, поскольку Т-клеточный мелатонин может маскировать эффекты экзогенного гормона или препятствовать его действию за счет конкурентного связывания с гормональными рецепторами.

Ключевые слова: мелатонин, мелатониновые рецепторы, Т-лимфоциты, Th17, Treg, дифференцировка

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MELATONIN IN Th17/Treg DIFFERENTIATION: THE CONTRIBUTION OF THE HORMONE'S OWN PRODUCTION BY T LYMPHOCYTES

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Abstract. The hormone melatonin is involved in regulation of functioning of almost all organs and systems of the organism. In the immune system, T lymphocytes are an important target of melatonin: they express specific melatonin receptors with different affinities – membrane MT1 and MT2 and nuclear RORα, as well as intracellular molecules that nonspecifically bind melatonin at high concentrations. Moreover, many *in vitro* studies reveal their own production of melatonin by T lymphocytes in response to polyclonal activation and its involvement as autocrine or paracrine factor in the induction of IL-2 and IL-2 receptor (IL-2R) synthesis by T cells, with melatonin receptors involvement in implementation of these effects. Since IL-2/IL-2R-dependent signal is a key event in T lymphocytes proliferative response induction, intrinsic melatonin seems to be directly involved at least in the clonal expansion of these cells. In this work, we investigated the contribution of T cells' melatonin to regulation of the next stage of T lymphocyte activation, namely, the differentiation of T helper populations Th17 and Treg. It was shown that blockade of both membrane and nuclear melatonin receptors did not cause statistically significant changes in Th17 differentiation, although the trend was fixed for a decrease. Simultaneously, $CD4^+FoxP3^+$T cells level decreased under the nonselective blockade of membrane hormone receptors, and Treg-associated cytokine TGF-β concentration in activated cultures supernatants decreased both in case of MT1/MT2 nonselective blockade and MT2 selective blockade. The data indicate that melatonin produced by T lymphocytes in culture can contribute to the control of naive $CD4^+$T cell differentiation into Treg *in vitro*, and the hormone effects are mediated by membrane melatonin receptors. The presence of a large number targets with different affinities for melatonin in T lymphocytes determines the key role of the hormone concentration in its effects on these cells. And when interpreting data on melatonin-dependent regulation of Treg, it is important to take into account the hormone's own production by lymphocytes, since T cells' melatonin can mask the exogenous hormone effects or interfere with its action due to competitive binding to hormone receptors.

Keywords: melatonin, melatonin receptors, T lymphocytes, Th17, Treg, differentiation

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Introduction

The hormone melatonin, synthesized at the system level mainly by the pituitary gland, has multifunctional biological and pharmacological effects, including antioxidant, antitumor, anti-inflammatory, antiviral, antibacterial, neuroprotective ones [5]. It determines the functioning of the nervous and endocrine systems of the organism, and is also able to effectively regulate immune responses due to receptor-dependent and receptor-independent mechanisms [5]. The obvious target of the hormone in the immune system is the main effectors of the adaptive immunity, namely, T lymphocytes: they express at least two high-affinity membrane receptors for melatonin, MT1 and MT2 [15], and for the T helper population Th17, as well as for some regulatory T lymphocytes (Treg), the presence of a nuclear receptor RORα is shown [11].

Furthermore, at micromolar concentrations, melatonin is able to bind other intracellular targets, such as quinone reductase 2, identified as another cytoplasmic receptor for melatonin, MT3 [1], or calmodulin [13] – both factors are also present in T lymphocytes and are involved in the activation signaling. Consequently, melatonin effectively regulates T lymphocytes; its action has been convincingly demonstrated both for the early stages of activation of these cells, including IL-2 production, proliferative response [2, 6, 8] and apoptosis [3], as well as for the functional differentiation of T helper populations, such as Th1, Th2 [6, 14], Th17 and Treg [4, 7, 9].

Moreover, *in vitro* studies, many authors have shown the production of melatonin by T lymphocytes themselves in response to polyclonal activation, in amounts exceeding its upper physiological level, as well as the involvement of such endogenous melatonin as an autocrine or paracrine factor in the induction of IL-2 synthesis and IL-2 receptor (IL-2R) expression by T cells: blockade of hormone synthesis in the cell with a tryptophan hydroxylase inhibitor caused a decrease in the level of both factors, which was reversed by the addition of exogenous melatonin [2, 10, 12]. The involvement of both membrane (MT1/MT2)

and nuclear (RORα) receptors for melatonin in the implementation of these effects has been shown [2]. Since the IL-2/IL-2R-dependent signal is a key event in the induction of T lymphocyte proliferative response, intrinsic melatonin seems to be directly involved at least in the early stages of these cells' activation. And given the ability of the hormone to effectively regulate the functional differentiation of T lymphocytes, in particular, the development of T helper populations, it was important to evaluate the possible involvement of own T cell's melatonin in this process.

The aim of this work is to determine the contribution of intrinsic melatonin produced by T lymphocytes to the differentiation of Th17 and Treg cells.

Methods and materials

We used leukocytes from healthy donors (n = 10, mean age 38.30±1.95 years). All donors signed the informed consent form for participation in the study. The study was approved by the Ethics Committee of the IEGM Ural Branch of the Russian Academy of Sciences (protocol No. 15 dated May 20, 2022) and conducted in accordance with the provisions of the Declaration of Helsinki on research involving humans. Leukocytes were isolated from heparinized venous blood by density gradient centrifugation in ficoll-verografin (ρ = 1.077 g/cm^3). We used naive $CD4^+$T lymphocytes fractionated with commercial isolation systems (BioLegend, USA). $CD4^+$T cells (1×10^6 cells/mL) were cultured for 48 hours in RPMI 1640 medium (Gibco, UK) with 1 mM HEPES (Sigma-Aldrich, USA), 2 mM L-glutamine (Serva, Germany), and 40 U/ml gentamicin (Pharmacia, Sweden) at 37 °C and 5% CO_2 without activator (spontaneous variant) and under the polyclonal activation (activation system based on monoclonal antibodies to CD3/CD28, Invitrogen, USA).

The contribution of specific melatonin receptors to the implementation of the effects of the hormone was determined by inhibitory analysis, using the corresponding antagonists for membrane receptors – non-selective (luzindole – for MT1/MT2) and selective (4-P-PDOT – for MT2; both Tocris Bioscience, USA), and for the nuclear melatonin receptor RORα, small interfering RNAs (siRNA, OriGene, USA: three types of RORα-specific siRNA and scrambled siRNA as a negative control). siRNA transfection was carried out using lipofectamine (Invitrogen, USA), and the effectiveness of their action was confirmed by assessing the expression of RORα in cells, both at the mRNA level, by polymerase chain reaction (RT-qPCR), using the SingleShot™ SYBR® Green One- Step Kit (Bio-Rad, USA) and at the protein level by flow cytometry using anti-RORα*PE monoclonal antibodies (R&D Systems, USA).

Cell expression of transcription factors RORγt (Th17 differentiation marker) and FoxP3 (Treg marker) was determined at the end of 48-hour cultivation (by flow cytometry using monoclonal antibodies: anti-CD4*FITC, anti-RORγt*PerCP, anti-FoxP3*PE (Novus Biologicals, R&D Systems, BioLegend, USA)). The synthesis of the key cytokines of the studied subpopulations, IL-17A and TGF-β, was assessed by their level in culture supernatants (enzymatic immunoassay, R&D Systems, BioLegend, USA). Statistical analysis was carried out using STATISTICA 10.0 software. Significance of differences between groups was assessed using Student's t-test. The results are presented as the mean and its standard error (M±m). The reported percentages of Th17 and Treg cells, as well as the concentration of IL-17A and TGF-β in culture supernatants, are normalized with respect to the corresponding control (unstimulated or stimulated).

Results and discussion

Blockade of specific membrane receptors for melatonin did not reveal statistically significant changes in the differentiation of Th17 lymphocytes when assessing both the content of $CD4^+ROR\gamma t^+$T cells in culture and the level of IL-17 in culture supernatants, although a downward trend was recorded. At the same time, the level of $CD4^+FoxP3^+$T cells decreased in the presence of non-selective hormone membrane receptor antagonist (percentage of $CD4^+FoxP3^+$T cells normalized to control: luzindole – 0.72±0.07 *vs* control – 1.0; $p < 0.05$), but only in a spontaneous variant. The concentration of the corresponding Treg-associated cytokine TGF-β in the supernatants of cultures of activated $CD4^+$T cells decreased as in the case of non-selective blockade of MT1/MT2 (the level of TGF-β in the supernatants of $CD4^+$T cell cultures, normalized with respect to control: luzindole – 0.90±0.02 *vs* control – 1.0; $p < 0.05$), and with selective blockade of MT2 (the level of TGF-β concentration in supernatants of $CD4^+$T cell cultures, normalized with respect to control: 4-P-PDOT – 0.89±0.03 *vs* control – 1.0; $p < 0.05$). Under the blockade of intracellular melatonin receptor RORα using siRNA specific to RORα-mRNA, no statistically significant differences in the percentage of $CD4^+ROR\gamma t^+$ and $CD4^+FoxP3^+$ T cells in culture were found.

Conclusion

We have shown that melatonin produced by T lymphocytes in culture can contribute to the control of differentiation of naive $CD4^+$T cells into Treg *in vitro*, and the effects of the hormone are mediated by membrane melatonin receptors. The presence in lymphocytes of a large number of targets for melatonin with different affinities for the hormone indicates that the key factor determining the presence and direction of hormone effects in these cells is its concentration. And when interpreting the results, it is important to take into account self production of melatonin by lymphocytes, which increases upon activation and exceeds its physiological levels in experiments *in vitro* [2, 10, 12], since melatonin synthesized by lymphocytes can mask the effects of both an endogenous hormone secreted by the pineal gland and an exogenous one.

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