Effects and mechanisms of melatonin on immune responses in mice of different months

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KEY WORDS melatonin; immunity; GTP-binding proteins; cyclic AMP; enkephalins

ABSTRACT

AIM: To study the effects and mechanisms of melatonin (MT) on immune responses in mice of different months. **METHODS:** Thymocyte proliferation and IL-2 activity were assayed by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and activated mouse splenocyte proliferation methods, respectively; cAMP and methionine-enkephalin (met-Enk) level was determined by competitive protein binding assay and radioimmunoassay, respectively. **RESULTS:** The function of lymphocytes, obtained from BALB/c mice aged 6 and 11 months were decreased, which was restored by melatonin at the dose of 5 mg/kg or 30 mg/kg. *In vitro*, proliferation of lymphocytes in 11-month-old mice was decreased and cAMP level was increased. Melatonin (0.1 nmol/L or 1 μmol/L) had negative regulation to this. Forskolin (10 μmol/L) enhanced the cAMP level of lymphocytes in 2- and 11-month-old mice (P<0.01), which was antagonized partially by melatonin and this effect of melatonin was also abolished by pertussis toxin (1 mg/L) completely. Melatonin (1 μmol/L and 0.1 nmol/L) increased the content of met-Enk of lymphocytes in 2- and 11-month-old mice, respectively (P<0.01), which was blocked by nifedipine (1 μmol/L). **CONCLUSION:** Melatonin exerted an effect on immune responses in mice of different months, which might be mediated by G protein-AC-cAMP signal transduction pathway and regulation of met-Enk level.

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine), secreted by the pineal gland of vertebrates including the humans[1], participates in several important physiological functions, including the circadian rhythms regulation[2], and normal patterns of sleep[3,4], and also plays a fundamental role in neuroimmunomodulation in rodents and humans[5-9]. At the same time, it has analgesic and sedative actions, and slows the processes of aging. The original interest in melatonin derives from the suggestion that the pineal gland may act as a key regulator in aging and senescence[10-15]. Some studies showed that cAMP signal transduction played an important role in regulating lymphocyte function, mediated mainly inhibiting and anti-proliferation signal[16]. To further elucidate the question, we have reported the effects of melatonin on pain and the relation with enkephalin[17,18]. The present study was designed to investigate the relationship between the effects of melatonin on immune responses and both G protein-adenylate cyclase-cAMP signal transduction and met-Enk release from lymphocytes.

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MATERIALS AND METHODS

Animals Kunming mice (20 g±2 g, ♂ or ♀, 2-month age) and BALB/c mice (20 g±2 g, ♀, 2- month age, Grade II, Certificate No 96001), were obtained from the Experimental Animal Center of Anhui Medical University.

Materials Melatonin, concanavalin A (ConA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), forskolin, nifedipine, and Hepes were obtained from Sigma (St, MO); RPMI-1640 medium and pertussis toxin were purchased from Gibco Laboratories (Life Technologies Inc); met-Enk and ^{125}I-met-Enk kit were obtained from Incstar Corporation, USA; [^{3}H]cAMP kit was purchased from the Basic Medical Institute of Chinese Academy of Medical Sciences; other chemicals used in these experiments were analytical grade from commercial sources.

Thymocyte proliferation assay[19] The proliferation of thymocytes was determined by MTT assay. Rats were killed by cervical dislocation. Thymus was removed in sterile condition. Thymocytes were isolated by routine method. Then the cells were suspended in RPMI-1640 medium at a concentration of 1×10^{10} cell/L. The cell suspension (100 μL) and ConA (100 μL, with final concentration of 5 mg/L) were seeded to 96-well flat-bottomed culture plate respectively. The cultures were incubated at 37 °C, 5 % CO_2 for 48 h. MTT (5 g/L, 10 μL) was added to each well and oscillated for 1 min on oscillator, at 37 °C, 5 % CO_2 for 2 h continuously. After incubation the cultures were centrifuged (760×*g*, 10 min). The supernatants were aspired, then 120 μL of isopropanol was added to each well and oscillated for 30 s again. The absorbance (*A*) was measured on EJ301 ELISA Microwell Reader at 570 nm. The results were described as the average of *A*.

Production and analysis of IL-2[20] IL-2 activity was estimated by the activated mouse splenocyte proliferation. Thymus of rat was removed in sterile condition. Thymocyte was collected by routine method. Then the cells were suspended in RPMI-1640 medium at a concentration of 5×10^{10} cell/L. The 100 μL of suspension, 100 μL of ConA with final concentration of 5 mg/L, and 800 μL RPMI-1640 medium were added to each well of 24-well culture plate, respectively, final volume 1 mL. The cultures were centrifuged (500×*g*, 10 min) after incubation at 37 °C, 5 % CO_2 for 48 h. The supernatants were collected, and reserved at -20 °C.

IL-2 was estimated using the activated mouse splenocyte proliferation. Spleen cells of mice were suspended at concentration of 2×10^9 cell/L. ConA 5 mg/L was added, incubated at 37 °C, 5 % CO_2 for 4 d, and washed 3 times with 5 % bovine-Hank's. The suspension of activated mouse splenocyte was adjusted to 1×10^9 cell/L with RPMI-1640 medium. Suspension (100 μL) and IL-2 supernatants (100 μL) were added to each well of 96-well culture plate, respectively, then were incubated at 37 °C, 5 % CO_2 for 24 h (similar to thymocyte proliferation assay).

Met-Enk radioimmunoassay (RIA) Rats were killed by cervical dislocation. Thymus was removed in sterile condition. Thymocytes were isolated by routine method. Then the cells were suspended in RPMI-1640 medium at a density of 2×10^9 cell/L and incubated at 37 °C, 5 % CO_2 for 24 h. Suspension of thymoctes was adjust to 1×10^{10} cell/L with RPMI-1640 medium (1000 kU aprotinin was added), incubated for 30 min, and centrifuged (760×*g*, 15 min). Supernatants were collected and reserved at -20 °C, then HCl (1 mol/L) 100 μL was added to 1 mL sample, pH 3-4. ODS silica gel column (Sep-Pak) was washed with 5 mL methanol and 20 mL sterile water , then the sample was injected to the column slowly for less than 1 min. The column was washed with 4 % acetic acid so as to eliminate foreign matter. Methanol 4 mL was injected to the column slowly and the filter liquid was collected with 16 mm×100 mm tube for no less than 3 min. The sample was placed on 37 °C water bath and blowed with nitrogen. Then the met-Enk was assayed according to the procedures described by instruction manual of methionine-enkephalin ^{125}I-RIA kit.

Assay for lymphocytic cAMP The experiment was done by competitive protein binding assay (CPBA) according to the procedures described[21].

Rats were killed by cervical dislocation. Thymus was removed in sterile condition. Thymocytes were isolated by routine method. Then the cells were suspended in RPMI-1640 medium at a density of 1×10^9 cell/L and centrifuged (1500×*g*, 10 min). The supernatant was quickly removed and cell precipitates were boiled at 100 °C for 5 min and reserved at -20 °C. Then the cAMP was assayed using [^{3}H]cAMP kit following manufacturer's instructions.

Statistical analysis Data were shown as mean±SD. The analysis of variance (ANOVA) and Student's *t*-test were used to determine significant differences between groups. A level of $P<0.05$ was ac-

cepted as statistically significant.

RESULTS

Effects of melatonin (ig) on thymocyte proliferation and IL-2 production in mice of different months The lymphocyte proliferation and IL-2 production decreased in 6-month-old BALB/c mice, and melatonin (30 mg/kg, ig×7 d) restored it. Especially, at the age of 11 months, lymphocyte proliferation and IL-2 production of mice decreased significantly (P<0.01). Melatonin (5 mg/kg, ig×7 d) enhanced the function of thymocytes (Tab 1).

Tab 1. Effects of melatonin (ig) on lymphocyte proliferation and IL-2 production in mice. n=5. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* 2-month-old mice. ^fP<0.01 *vs* 6- or 11-month-old mice.

Mice/ months	Dose/mg· kg^{-1}	Absorbance of Proliferation	Absorbance of IL-2 production
2	-	0.69±0.08	0.62±0.15
2	30	1.04±0.12^c	0.81±0.09^c
5	-	0.77±0.06	0.61±0.09
6	-	0.47±0.06^c	0.44±0.06^b
6	30	0.70±0.12^f	0.63±0.05^f
11	-	0.31±0.06^c	0.35±0.05^c
11	5	0.72±0.09^f	0.68±0.06^f

Effects of melatonin on lymphocyte proliferation and cAMP production in mice of different months *in vitro* The thymocyte proliferation in 11-month-old mice decreased significantly, while cAMP production increased (P<0.01). Proliferation function of thymocytes in 11-month-old mice was improved by melatonin (0.1 nmol/L, 1 μmol/L), accompanied with the decrease of cAMP level, but the effect of melatonin on that in 2-month-old mice appeared only at large concentration (1 μmol/L) (Tab 2).

The G protein coupled mechanisms of melatonin on cAMP production of thymocytes in mice of different months Forskolin (F, 10 μmol/L), a selective adenylate cyclase (AC) activator, enhanced markedly the cAMP level of thymocytes of mice aging 2 and 11 months, which were antagonized partially by melatonin. And the decreased level of cAMP of lymphocytes by melatonin could be abolished by pertussis toxin (PT, 1 mg/L) completely (Tab 3).

Tab 2. Effects of melatonin *in vitro* on lymphocyte proliferation and cAMP production in mice. n=3-4. Mean±SD. ^cP<0.01 *vs* 2-month-old mice. ^fP<0.01 *vs* 11-month-old mice.

Mice/ months	Concentration of melatonin	Absorbance of proliferation	cAMP/pmol per 1×10^6 cells
2	-	0.51±0.08	2.5±0.4
2	0.1 nmol· L^{-1}	0.48±0.09	2.5±0.5
	1 μmol· L^{-1}	0.9±0.09^c	1.17±0.24^c
11	-	0.24±0.04^c	4.8±0.4^c
11	0.1 nmol· L^{-1}	0.43±0.05^f	3.6±0.3^f
	1 μmol· L^{-1}	0.64±0.04^f	3.0±0.4^f

Tab 3. Effects of melatonin and PT on F-induced lymphocytes cAMP production. n=3-4. Mean±SD. ^cP<0.01 *vs* control mice. ^fP<0.01 *vs* F-treated mice. ^iP<0.01 *vs* (MT+F)-treated mice.

Mice/ months	Treatment	Concentration	cAMP/pmol per 1×10^6 cells
2	-	-	2.5±0.4
2	F	10 μmol· L^{-1}	7.7±0.5^c
2	MT+F	1 μmol· L^{-1}+10 μmol· L^{-1}	5.8±0.7^f
2	PT+MT+F	1 mg· L^{-1}+1 μmol· L^{-1}+10 μmol· L^{-1}	7.4±0.5^i
11	-	-	4.8±0.4
11	F	10 μmol· L^{-1}	8.6±0.6^c
11	MT+F	0.1 nmol· L^1+10 μmol· L^{-1}	6.5±0.6^f
11	PT+MT+F	1 mg· L^{-1}+0.1 nmol· L^{-1}+10 μmol· L^{-1}	8.2±0.9^i

F: Forskolin; PT: pertussis toxin; MT: melatonin.

Effects of melatonin on content of met-Enk of lymphocytes in mice of different months The content of thymocyte met-Enk of mice aging 6 and 11 months old was lower than that of 2 months old. Melatonin improved significantly the content of met-Enk of thymocytes in 11-month-old mice at physical concentration (0.1 nmol/L), which was blocked by nifedipine (Nif, 1 μmol/L), a Ca^{2+} channel antagonist. Meanwhile melatonin enhanced the content of lymphocyte met-Enk in 2-month-old mice at high concentration (1 μmol/L). Forskolin (F, 10 μmol/L), a selective adenylate cyclase (AC) activator, had no effect on the content of lymphocyte met-Enk in mice of different months (Tab 4).

DISCUSSION

In this study, it was found that melatonin restored

Tab 4. Effects of melatonin on lymphocyte met-Enk production in mice. *n*=3-4. Mean±SD. [c]*P*<0.01 *vs* 2-month-old mice. [f]*P*<0.01 *vs* control 11-month-old mice. [i]*P*<0.01 *vs* MT (11-month-old mice).

Mice /month	Treatment	Concentration	met-Enk/pmol per 1×10^6 cells
2	-	-	9.3±0.3
2	MT	1 μmol·L^{-1}	16.9±0.8[c]
2	F	10 μmol·L^{-1}	9.0±2.2
6	-	-	7.3±1.2[c]
11	-	-	6.79±0.10[c]
11	MT	0.1 nmol·L^{-1}	9.8±0.4[f]
11	Nif+MT	1 μmol·L^{-1}+0.1 nmol·L^{-1}	5.26±0.24[i]

F: Forskolin; Nif: nifedipine.

the decrease of lymphocyte proliferation and IL-2 production in BALB/c mice aging 6 and 11 months, which suggested that melatonin played an important role in keeping the normal immune functions. The present study provided clear evidence that proliferation function of 11-month-old mice decreased, accompanied with the increase of cAMP level *in vitro*, which was inhibited by melatonin.

The further observation found that the G protein-AC-cAMP signal transduction might be abnormal in immune cells of aged mice, melatonin antagonized partially the cAMP level of lymphocytes stimulated by forskolin which was abolished by pertussis toxin completely. It is suggested that a pertussis toxin (PT)-sensitive GTP-binding protein (G-protein) is involved in the signal transduction mechanism and the G_i protein coupled AC-cAMP signal pathway may be one of important mechanisms for the inflammatory-immunoregulation of melatonin. Rafii-El-Idrissi *et al*[22] had studied the effect of melatonin on cAMP production of splenocytes in rat, and the result showed that melatonin alone could not improve or inhibit cAMP release, but at high concentration (10 μmol/L) could inhibit partially cAMP level of lymphocytes stimulated by forskolin (10 μmol/L). The same result had also been found in hamster at the concentration (0.01 nmol/L-0.01 μmol/L), which was mediated by G protein-coupled-receptor sensitive to pertussis toxin[23].

The immunoregulation of melatonin was mainly mediated by melatonin -induced immuno-opioid (MIIO), and MIIO had the cross-response with β-End and met-Enk[24,25]. Melatonin enhanced significantly the content of lymphocyte met-Enk in 2- and 11-month-old mice, the effect was blocked by nifedipine, a Ca^{2+} channel antagonist. It was suggested that melatonin promoted the production of met-Enk of mouse lymphocytes, which might contribute to regulation of immune responses via L-type Ca^{2+} channel.

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