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Research Article

MELATONIN RECEPTOR (MT1 AND MT2) PROTEINS EXPRESSION IN THYROID GLAND AND LEVEL OF THYROXIN (T_4), THYROTROPIN (TSH) HORMONE DURING PROGRESSION OF AGE IN MALE SWISS ALBINO MICE

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ABSTRACT

Advancement of age is related with a progressive declining in most of the physiological activities. The activity of thyroid gland is also disturbed with increment of age in the organism. Melatonin regulates most of the physiological activities through its membrane bound MT1 and MT2 receptors in mammals. Melatonin receptors were localized on thyroid gland but their abundance on thyroid gland along with progression of age was unknown. In present study we investigated the age related changes in serum T_4, TSH level and the abundance of melatonin receptor (MT1 and MT2) proteins in thyroid gland following the immunohistochemical study and western blot analysis. Decreased serum T_4 level was noted in aged mice whereas serum TSH level was remained unchanged. Both MT1 and MT2 melatonin receptor proteins abundance was decreased with advancement of age in mice. Our present study may suggest that aging modulates the responsiveness of thyroid gland to melatonin through modulation of melatonin receptor (MT1 and MT2) proteins on thyroid gland. Further, this modulation may be responsible for contribution in the declining of physiological status of the thyroid in the organism.

Keywords: aging, melatonin receptor (MT1 and MT2), thyroid, T_4, TSH

INTRODUCTION

Aging is a slow and complex physiological process that reflects the sum total of all changes occurs in living organisms with the passage of time with an increased risk of disease. Aging is related with a progressive deterioration of many mechanisms in the body that leads to functional impairment and increased pathology. Aging is also associated with decreased ability to stress responsiveness[1], a progressive increase in free radical generation and also a diminished capability of immune system[2]. In common with other physiological system, the endocrine system is also affected by aging. Progression of age modifies the pituitary set point or comparably reduced response to thyroxin, resulting in lesser serum TSH elevation in older individuals[3]. Aging is linked with alterations of pituitary-thyroid axis activity as well as an increased occurrence of autoimmune and nodular thyroid disease[4]. Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine secreted from the pineal gland and is derived from the amino acid tryptophan. Melatonin is involved in the regulation of circadian rhythm event in mammals and other vertebrates. In mammals, melatonin performs its cellular activity via activation of two high-affinity G-protein coupled receptors, the MT1 and MT2[5-7]. Specific membrane and nuclear binding sites for melatonin have been detected in different tissues in different species[8]. Although, the pineal gland is considered the main site of melatonin synthesis, many extra-pineal tissues have been identified as melatonin synthesizers, such as retina[9-11], Harderian gland[12], gut[13,14], ovary[15,16], immune system[17-20], skin[21,22] and testes[23]. Moreover, melatonin has also been localized in the thyroid gland[24]. Inter-relationship between the pineal gland hormone melatonin and the thyroid have for long time been a subject of intensive research. Melatonin may regulate the thyroid gland activity through stimulatory effect on hypothalamus-pituitary axis, playing a modulatory action on HPT axis and performing as a servo-mechanism that diminishes or increases responses when stimuli are respectively too strong or too low[25]. Besides, melatonin has a direct inhibitory effect on T_4 secretion and also, suppresses the response of the thyroid to TSH[26,27]. Furthermore, melatonin has also play a protective role against oxidative stress in the rat thyroid gland[28-30]. It was established in numerous studies that melatonin reduced oxidative stress by its free radical eliminating and direct antioxidant effects[31]. Melatonin also helps to inhibited lipid peroxidation stimulated by fenton in the thyroid gland[28]. An age-related decline in melatonin receptor binding sites in several tissues of different species including the rat, Syrian hamster and mice supra chiasmatic nucleus (SCN), rat brain[32-34] suggested declined melatonin activity in those tissues during aging. In thyroid gland melatonin receptors binding sites were also reported but till date age related patterns of melatonin receptor proteins expression was unknown. Therefore to understand the melatonin receptor (MT1 and MT2) proteins responsiveness in thyroid gland and status of T_4, TSH hormone level along with progression of age in mice the present study was conducted. We made the basic approaches to achieve the aimed objectives.

MATERIAL AND METHODS

Animal Procurement and Maintenance

Healthy laboratory Swiss albino mice were housed at ambient laboratories conditions. Mice were kept in groups of seven in polycarbonate cages (43 cm x 27 cm x 14 cm) to avoid the crowding effect and fed with mice feed and water *ad libitum*. Mice were procured and acclimatized for 1 month at ambient laboratory conditions before experimentation in normal day-light (12L : 12D) condition. All the experiments on the animals were conducted in accordance with institutional practice and within the framework of the revised Animal (Specific Procedure) Act of 2007 of Govt. of India on animal welfare. The

study protocol was approved by institutional animal ethics committee with ethical clearance no. TU/IAEC/2013/V/5-3.

Sample Collection

Seven (N = 7) healthy male Swiss-albino mice of each age group (2 months and 8 months) were selected. Mice were decapitated without anaesthesia. Thyroid glands were dissected out and fixed in Bouin's solution. For western blot analysis thyroid glands were dissected out and immediately kept at -40°C. The trunk blood was collected and separated serum was stored at -40°C.

Hormonal Analysis

Serum T_4 and TSH hormone analysis was done by commercial ELISA Kits (Diagnostic Automatation Inc, CA, USA). For T_4, detection range 0-30 µg/dl, specificity 96.30 % and sensitivity was 0.05 µg/ml. For TSH, detection range 0-40 µIU/ml, specificity 100 % and sensitivity was 0.20 µIU/ml.

Immunohistochemical Staining

Immunohistochemical study of thyroid gland was done following the procedure of Savaskan *et al*[35]. 5 µm thick paraffin sections were mounted on 3 % gelatine coated slide. Sections were deparaffinised and rehydrated with alcohol grades. The sections were placed in PBS for 30 minutes and endogenous peroxide activity was blocked by 0.3 % H_2O_2 in methanol for 30 minutes at room temperature. Sections were washed thrice with PBS and placed in blocking solution (horse blocking serum, diluted 1:100 in PBS, PK-6200, Vector Laboratories, Burlingame, CA). Then sections were incubated with primary antibodies (Mel 1AR; sc-13186 and Mel 1BR; sc-13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:100) overnight at 4°C. Sections were washed thrice with PBS and were incubated with biotinylated secondary antibody (Vectastain ABC Universal Kit, PK-6200, Vector Laboratories, Burlingame, CA, dilution 1:200). Sections were washed thrice with PBS and incubated with preformed AB complex reagent for 30 minutes. The antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB; Sigma-Aldrich Chemicals, St. Louis, USA) and counter stained with Ehrlich's haematoxylin. Sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 40X objective of Olympus microscope. To test the specificity of the used antibodies, the primary antibodies were not added in control sections. The control sections were incubated with same dilution of normal serum for overnight at 4^0C and following morning the immunohistochemical protocol was followed under the same condition.

Western Blot Analysis

Thyroid samples were homogenized and lysed in RIPA buffer (1 % (v/v) NP-40, 0.1 % w/v sodium dodecyl sulphate (SDS) in PBS containing aprotonin, sodium orthovanadate and phenyl methyl sulphonyl fluoride (PMSF) and quantified by Lowry method (1951). Aliquots containing 100 µg proteins were resolved by 10 % (w/v) SDS polyacrylamide gel electrophoresis followed by electro transfer to nitrocellulose membrane (Santa Cruz Biotech, USA). Immune detection was carried out by using anti-Mel 1AR, anti-Mel 1BR (Mel 1AR; sc-13186 and Mel 1BR; sc-13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:200) and β-actin antibody (sc-130656, rabbit polyclonal, Santacruz Biotech, USA, diluted 1:500) diluted in PBS contained 5 % skimmed milk and 0.01 % Tween-20 followed by incubation with horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG for β-actin antisera; diluted 1:1000 and rabbit anti-goat IgG for Mel 1AR and Mel 1BR antisera; diluted 1:1000). The immune interactions were detected by using Super Signal West Pico Chemiluminescent Substrate (# 34080, Thermo Scientific, Rockford, USA). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as ratio of the density of the specific signal to β-actin signal and expressed as the % control value[36]. Each sample corresponds to tissue from a single animal and at least four gels corresponding to each subunit and experimental conditions were analyzed.

Statistical Analysis

Statistical analysis of the data was performed by one way ANOVA followed by Student's Newman-Keul's multiple range tests. The differences were considered significant when $p < 0.05$.

RESULTS

Estimation of T_4 and TSH

Hormone analysis (Table 1) showed changes in circulatory T_4 level with advancement of age. Significant decrease of serum T_4 hormone level was noted in 8 months old mice in compare to 2 months old mice. In 8 months old mice, serum TSH level was remaining unchanged in compare to 2 months old mice.

Table 1

Age group	Thyroxin (T_4), ng/ml	Thyrotropin (TSH), µIU/ml
2 month	18 ± 1.6	0.9 ± 0.03
8 month	12 ± 1.4**	0.85 ± 0.045

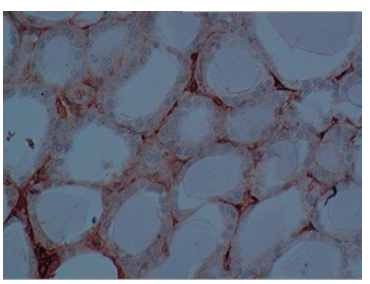

Figure 1 (A): Immunostaining of MT1 melatonin receptors in thyroid gland of 2 months old mice. Microphotographs were taken by Olympus Microscope under 40X objective

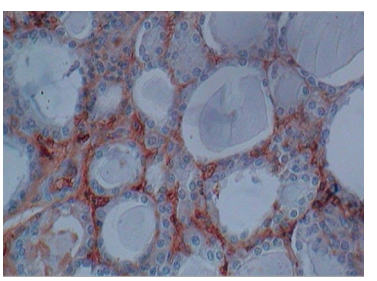

Figure 1 (B): Immunostaining of MT1 melatonin receptors in thyroid gland of 8 months old mice. Microphotographs were taken by Olympus Microscope under 40X objective

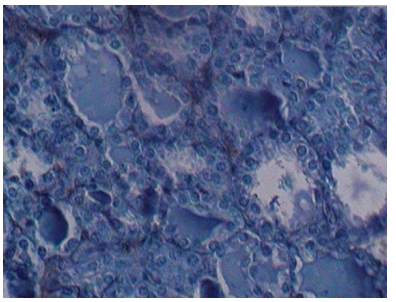

Figure 1 (C): Negative control section, DAB reaction was not detected. Microphotographs were taken by Olympus Microscope under 40X objective

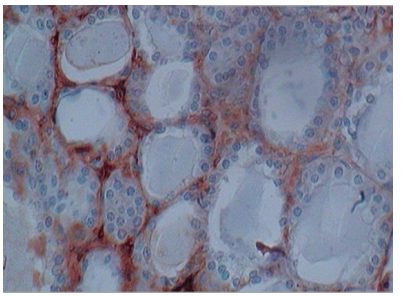

Figure 2 (A): Immunostaining of MT2 melatonin receptors in thyroid gland of 2 months old mice. Microphotographs were taken by Olympus Microscope under 40X objective

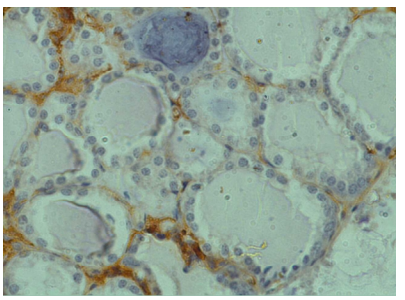

Figure 2 (B): Immunostaining of MT2 melatonin receptors in thyroid gland of 8 months old mice. Microphotographs were taken by Olympus Microscope under 40X objective

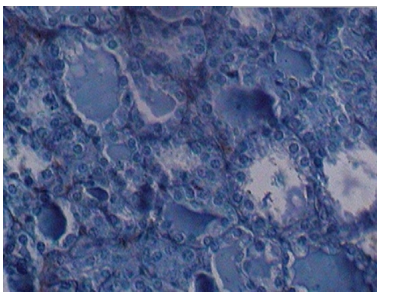

Figure 2 (C): Negative control section, DAB reaction was not detected. Microphotographs were taken by Olympus Microscope under 40X objective

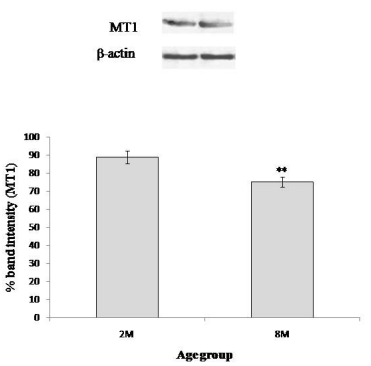

Figure 3: Western blot analysis of MT1 receptor in thyroid gland of 2 month and 8 month old mice. β-actin was used as loading control. Lower panel shows percent band intensity of MT1 receptor following Scion Image analysis. Histogram represents Mean ± SEM. 8 months differences from 2 month were considered when p < 0.05
** = P < 0.01

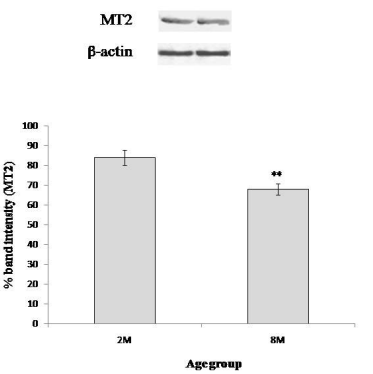

Figure 4: Western blot analysis of MT2 receptor in thyroid gland of 2 month and 8 month old mice. β-actin was used as loading control. Lower panel shows percent band intensity of MT2 receptor following Scion Image analysis. Histogram represents Mean ± SEM. 8 months differences from 2 month were considered when p < 0.05
** = P < 0.01

Immunohistochemical Observation

Immunohistochemical staining showed both MT1 and MT2 melatonin receptors immune reactivity in the thyroid gland of studied age group (2 months and 8 months). Both MT1 and MT2 immuno reactivity was noted on follicular cells as well as on C-cells or parafollicular cells of the thyroid gland.

MT1 Melatonin Receptor Immuno-reactivity

Strong immune-reactivity of MT1 melatonin receptors was noted in 2 months old mice thyroid gland. In 8 months old mice weak immune-reactivity of MT1 melatonin receptors was noted in thyroid gland (Figure 1).

MT2 Melatonin Receptor Immunoreactivity

Strong immune-reactivity of MT2 melatonin receptors was noted in thyroid gland of 2 months age group of male mice. Weak immune-reactivity of MT2 receptors was noted in thyroid gland of 8 months old mice (Figure 2).

Western Blot Analysis
MT1 Melatonin Receptor Proteins Expression

Significant decrease of MT1 receptor proteins expression in thyroid gland was noted in 8 months old mice in comparison with 2 months old mice (Figure 3).

MT2 Melatonin Receptor Proteins expression

In 8 months age group of thyroid gland, significant decrease of MT2 receptor proteins expression was observed in comparison with 2 months age group (Figure 4).

DISCUSSION

Aging is irretrievable process that linked with progressive deteriorating of many physiological activities. Along with many physiological systems, endocrine system also exhibit changes with development of aging. Aging alters the secretion of somatotropin, IGF-I, dehydroepiandrosterone and testosterone[37] and also influenced on the function of pituitary and thyroid gland[38]. In this study we observed decreased in serum T_4 concentration in aged mice whereas, serum TSH concentration remained unchanged. Evidences suggested the lowering of circulatory T_4 concentration in male rats during aging but serum TSH level remain unaltered, suggesting a disturbance in the pituitary-thyroid feedback mechanism during older age[39]. Normal circulating levels of TSH in spite of low serum thyroid hormone levels in aged rats have also been reported by many investigators[40-42]. Development of aging is associated with alteration of number of morphological and functional changes of thyroid gland[43-45]. Moreover, aging also causes the declining of the melatonin hormone which is well known for maintenance of immune function, contributing to the onset of the immunosenescence and age-related diseases[46]. Melatonin also plays an important role in intra-thyroid hormone production[27,47]. Melatonin exerts many physiological actions through its two high affinity membrane bound receptors, MT1 and MT2 in mammals. But it is not known how melatonin receptors were responding during advancement of aging in thyroid gland, therefore the present study was designed. The functional relationship between melatonin and thyroid gland was well established by many investigators. Further, presence of melatonin receptors on thyroid gland strengthens this relation. In present study immune-histochemical staining showed presence of MT1 and MT2 melatonin receptors in follicular and parafollicular cells of thyroid gland of mice in all studied age groups. Melatonin receptor immune-positivity in follicular and parafollicular cells in thyroid gland of rat was already reported[48]. Further report was also suggested melatonin synthesis by parafollicular cells which secreted locally and might be protecting follicular cells from oxidative damage[48]. However, many researchers have suggested the role of melatonin in the protection of thyroid gland against oxidative damage during both physiological and pathological processes[28,30,49]. Physiological aging is associated with the onset of immunological problems as well as metabolic disturbances. Our western blot analysis showed the significant decrease in MT1 and MT2 melatonin receptors during aging in thyroid gland. Melatonin receptors were shown to be declined with progression of age in many different tissues. MT1 receptor protein expression was significantly decreased in extra-pineal tissues such as spleen, kidney, liver and heart tissues during physiological aging[50]. A significant decreased in MT1 receptor protein and mRNA level in SCN of mice during aging was also reported[32,51]. MT2 melatonin receptor expression in extra-pineal tissues was also decreased during physiological aging[50]. The reduced expression of MT2 melatonin receptor in hippocampal region of Alzheimer's patients was also mentioned[52]. Further age-related lowering in the density of melatonin binding sites was reported in discrete hypothalamic and hippocampal region of rat brain[33]. Declining expression of melatonin receptors with age in caudal artery of hypertensive rats was also reported[53]. The organisms seem to suffer a defective response to melatonin during physiological aging. Our study showed that MT1 and MT2 melatonin receptor proteins expression decreases in thyroid gland during aging along with decreasing in serum T_4 concentration. Therefore, the findings of the present study may suggest that aging modulates the responsiveness of thyroid gland to melatonin through mediation of melatonin receptors (MT1 and MT2) on thyroid gland. Age related decrease of melatonin receptors expression in thyroid gland may be a consequence of a generalized deterioration of the thyroid gland function during aging. However, relationship between melatonin receptor protein abundances on thyroid gland and thyroid hormone production needs more investigation.

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