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Melatonin in the rat testis: Evidence for local synthesis

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The vertebrate pineal gland rhythmically produces melatonin, a hormone involved in regulation of several physiological and behavioral processes. Melatonin is synthesized from serotonin essentially by two enzymatic steps involving N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase activities. We have previously demonstrated the presence of melatonin binding sites in the rat testes, and an inhibitory effect of melatonin on testicular gonadotrophin-stimulated androgen production. It is unknown whether these effects are mediated by melatonin synthesized locally or by melatonin from pineal origin. To assess the potential capacity of melatonin production by the testis, we used radiolabeled precursors to measure the activities of N-acetyltransferase and hydroxyindole-O-methyltransferase. The production of N-acetylserotonin was time-dependent during over 10 min of incubation. Melatonin had a linear increase throughout the 30 min incubation period with S-adenosyl-L-[methyl-^{14}C]methionine. Identities of melatonin and N-acetylserotonin were confirmed by thin-layer chromatography. The ability of the testis to synthesize melatonin during sexual maturation was also analyzed. When activity of NAT was expressed per mg of protein, the maximal activity was observed on day 40. In contrast, when activity of NAT is expressed by the testis, the amount of NAT increased to peak on day 40 and remained elevated through day 70. We determined that both activities were predominantly localized in interstitial cells. NAT activity in seminiferous tubules was substantially decreased, representing 6.4% of NAT activity in interstitial cells. We concluded that rat testes are capable of synthesizing melatonin due to the presence of the enzymes necessary for the transformation of serotonin to melatonin. (Steroids **61**:65–68, 1996)

Keywords: *N*-acetyltransferase; hydroxyindole-*O*-methyltransferase; melatonin; testis; testosterone

Introduction

Melatonin, an indole derivative chemically identified as *N*-acetyl-5-methoxytriptamine, is a neurohormone secreted by the mammalian pineal gland.[1] The pathway for melatonin synthesis involves a hydroxylation and decarboxylation process.[2] The enzymes involved in the conversion of serotonin to melatonin, *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) have been studied in retina, harderian, and pineal gland from rodent and avian species.[3–5] It is generally believed that melatonin exerts an antigonadotrophic effect mainly at the level of the hypothalamus and pituitary,[6,7] but a peripheral action has not been excluded. In previous studies, we found that melatonin has a direct inhibitory effect on testicular gonadotropin-stimulated testosterone production in immature rats (Moraga et al., data submitted for publication). Moreover, we have recently reported the presence of melatonin binding sites in 40-day-old rat testis[8]; however, on the basis of melatonin concentration necessary for the maximal inhibitory effect (100 ng/L)[9] and the concentration of melatonin in rat plasma,[10] the effects of this hormone may be mediated by its local synthesis and not by melatonin of pineal origin. Bearing this in mind we investigated the activities of both enzymes, NAT and HIOMT, in homogenate rat testis.

Experimental

Immature Wistar rats (35–40 days old) were obtained from our colony. Rats were kept under a light:dark cycle of 14:10 h, and food and water were provided ad libitum. Animals were killed by decapitation between 10:00 and 12:00 h and their testes were removed quickly. After removal of the tunica albuginea, the testes were separated into seminiferous tubules and interstitial fraction by the wet dissection method of Christensen et al.,[11] in the presence of 0.1 mM PMSF. For the determination of NAT activity, the fractions were homogenized in cold 0.05 M phosphate buffer pH 6.8. Twenty microliters of the homogenate was assayed according to methods developed by Champney et al.[12] Each sample was

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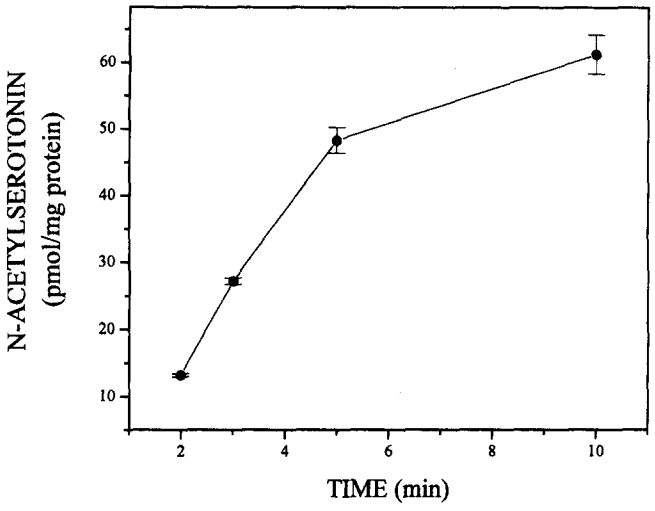

Figure 1 Time course of *N*-acetylation of serotonin accumulation in 40-day-old rat testes. Twenty microliters of homogenized samples was incubated for the indicated times, as described in Experimental, in the presence of 25 µmol of acetyl [l-^{14}C]-coenzyme A. Values shown are the means ± SD of three determinations.

incubated in triplicate at 34°C in the presence of 5 µL of 5.6 mM tryptamine-HCl or serotonin (Sigma, Chemical Co., St. Louis, MO, USA), 10 µL of 25 µmol/L of acetyl[l-^{14}C]coenzyme A (55.6 mCi/mmol, NEN DuPont Research Products, Boston, MA, USA) and 10 µL of phosphate buffer. The reaction was stopped with the addition of 1 mL of chloroform, agitated for 3 min, and centrifuged in a microfuge. The aqueous phase was removed and the organic phase was washed with 100 µL of 0.2 M borate buffer. A 0.6 mL aliquot of organic phase was evaporated to dryness under an air stream in a glass vial. Ten milliliters of toluene:triton X-100 (3:1, v/v) (Sigma, Chemical Co., St. Louis, MO, USA) was added prior to liquid scintillation spectrometry. Hydroxyindole-*O*-methyltransferase activity was measured by a modification of the method of Axelrod.[13] The separated fractions were homogenized in 0.05 M phosphate buffer pH 7.9. Homogenized samples (50 µL) were incubated for 30 min at 34°C in polypropylene tubes containing 30 µL of 3 mM *N*-acetylserotonin and 10 µL of 14 nmol/L of *S*-adenosyl-L-[methyl-^{14}C]methionine (50–60 mCi/mmol, Amersham, International, Bucks, UK) in a final volume of 100 µL. The reaction was stopped by addition of 0.3 mL of 0.2 M sodium borate buffer pH 10.0, followed by 2 mL of chloroform. Tubes were agitated for 15 seconds and the aqueous phase was removed by aspiration. The organic phase was washed again with borate buffer and then agitated. The aqueous phase was removed and 1 mL of chloroform was transferred into a glass vial and evaporated to dryness. Radioactivity was measured in a Beckmann LS 5000 TD counter. Protein concentrations of the tissue homogenates were determined by the Lowry et al. method.[14] In some experiments, the identities of *N*-acetyl-[^{14}C]serotonin and [^{14}C]melatonin, were identified by thin-layer chromatography (TLC, Gelman Science, Ann Arbor, MI, USA) using ethyl acetate as solvent.[15]

Results

The production of *N*-acetylserotonin by homogenates of immature rat testes is shown in Figure 1. There was a time-dependent increase in the formation of *N*-acetylserotonin during the 10 min incubation period. Moreover, the *N*-acetylserotonin formed was proportional to the amount of proteins per incubate: 9.2 ± 1.1, 16.1 ± 1.8, 28.4 ± 2.9, and 34.5 ± 3.7 pmol with 0.25, 0.5, 0.75, and 1 mg of proteins, respectively. TLC of the radioactive material in the organic phase represented 90% of *N*-acetylserotonin. In order to determine the hydroxyindole-*O*-methyltransferase (HIOMT) activity, the time-course formation of melatonin was examined (Figure 2). The production of [^{14}C]melatonin increased linearly during the 30 min incubation period of testis homogenates with *S*-adenosyl-L-[methyl-^{14}C]-methionine. The radioactive product from this reaction had the same R_f value as purified melatonin by TLC (data not shown). The ability of the testis to synthesize melatonin during sexual development was also analyzed. Age-dependent changes in the levels of NAT were investigated by comparing the activity of NAT expressed as pmol of *N*-acetylserotonin per mg of protein or pmol of *N*-acetylserotonin per testis (Figure 3). Mean NAT on day 16 was 10.7 ± 1.04 pmol/mg protein/10 min. Activity increased to 30.2 ± 6.9 pmol on day 40 and decreased to 9.0 ± 0.5 pmol and 5.8 ± 0.3 pmol on days 50 and 70, respectively. When activity is expressed by testis, the amount of NAT on day 16 was 30.5 ± 3.0 pmol. Activity increased to peak at 326.2 ± 46.0 pmol on day 40 and remained elevated through day 70. In order to localize the cellular site of melatonin synthesis in the testis, the activity of NAT and HIOMT was determined in seminiferous tubules and interstitial cells (Figure 4). Substantial NAT activity was observed in interstitial cells (143.2 ± 18.9 pmol/mg protein) during 10 min incubation. In contrast, the NAT activity in the tubule fraction was less than 6.4%. HIOMT activity was 43.4 ± 4.2 pmol/mg protein in interstitial cells, compared to 1.91 ± 0.3 pmol/mg protein in seminiferous tubules.

Discussion

Since the isolation and identification of melatonin by Lerner et al.,[16] melatonin research in higher vertebrates has been almost exclusively associated with the pineal gland. The present study provides the first evidence that the rat testis

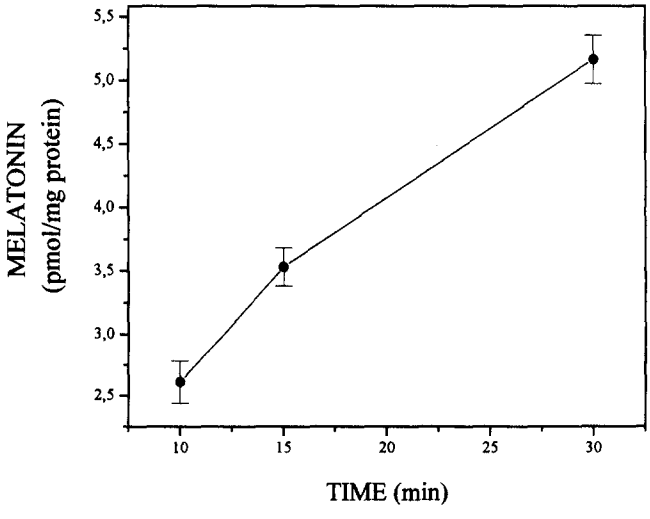

Figure 2 Time course of melatonin accumulation in 40-day-old rat testes. Fifty microliters of homogenate samples was incubated, in the presence of 3 mM *N*-acetylserotonin and 10 µL of *S*-adenosyl-L-[methyl^{14}C]methionine. Values shown are the means ± SD of three determinations.

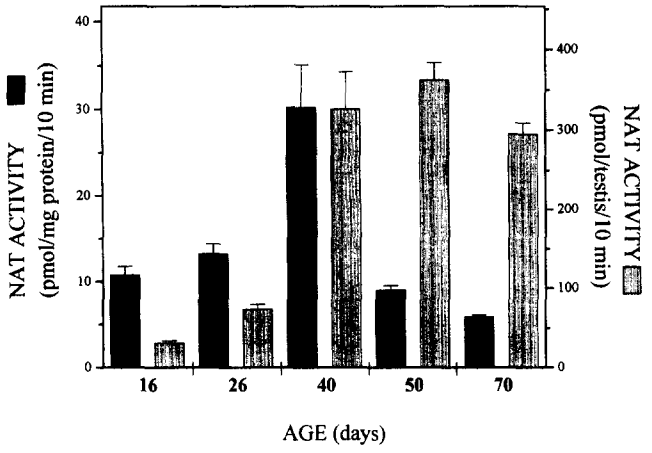

Figure 3 *N*-acetyltransferase (NAT) activity during sexual development. NAT activity is expressed as pmol/mg protein or pmol/testis. Values shown are the means ± SD of five determinations.

has the capacity to synthesize melatonin and that this capacity is mainly localized in interstitial cells. The results of this study clearly demonstrate the presence of both the metabolites and the enzymes involved in melatonin synthesis. Melatonin is a well known pineal secretory hormone.[17] In the pineal gland it is formed directly from serotonin by the action of NAT and HIOMT. The patterns of NAT activity in the pineal gland have been reported to differ from those observed in the testes. In the pineal rat, NAT exhibits a nyctohemeral profile with a progressive increase in its activity after the onset of the dark period.[18] This nocturnal activity varies from 20- to 100-fold depending on the species.[19] By contrast, no significant difference was found for NAT activity in testes during the light:dark period (Tijmes and Valladares, unpublished results). When NAT activity is expressed on a milligram of protein basis for 10 min, the pineal (dark on) has 27 nmol,[20] while the testis has 0.031 ± 0.005 nmol; however, when NAT activity is expressed on a total organ basis, the testis has 0.33 ± 0.05 nmol and the

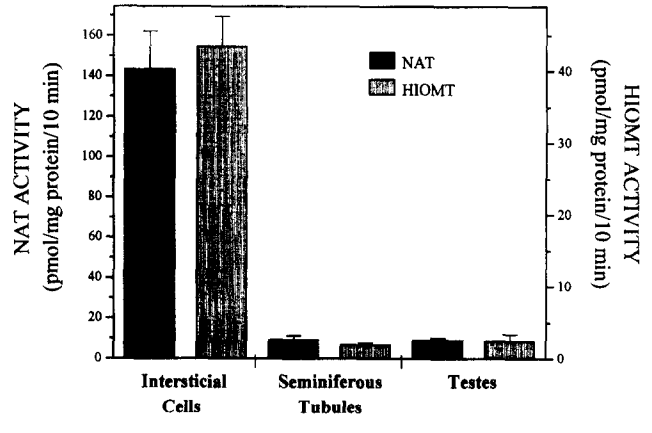

Figure 4 *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) activities, expressed as pmol/mg protein/10 min in different compartments of 40-day-old rat testis. Interstitial cells and seminiferous tubules were obtained as described in Experimental. Values shown are the means ± SD of five determinations.

pineal has 0.53 nmol.[12] The transformation of *N*-acetylserotonin to melatonin is catalyzed by HIOMT.[21] Rat pineal HIOMT activity has been shown to be constant throughout the light:dark period.[22] In the present study we did not investigate the activity of the methylating enzyme during darkness. Our results with testis HIOMT differ from those observed in the pineal gland.[23] The radioenzymatic analysis revealed that HIOMT activity is 36.0 ± 14.1 pmol melatonin/testis/10 min in 40-day-old rats. This value is approximately 63% higher than that reported for HIOMT activity in the pineal rat. The physiological role of melatonin in regulating testicular function remains to be elucidated. Previously, we have found that 100 nmol/L of melatonin produces a direct inhibitory effect of HCG-induced testosterone production only in immature rats (Moraga et al., data submitted for publication). Furthermore, in initial studies using 2-[^{125}I]-Iodomelatonin we also demonstrated high affinity melatonin binding sites (K_d = 100 pmol/L) in interstitial testicular cells from immature rats.[8] On the basis of peripheral concentration of melatonin in the rat (10–40 pmol/L),[10] the direct effect observed in testis is probably not related to circulating levels, and in this respect, the NAT and HIOMT activities could be important. Although melatonin was originally considered as a pineal hormone which modifies functions of the central nervous system,[22] it has recently been reported that other tissues have receptors for this hormone.[24–26] Therefore, the possibility exists that melatonin may be synthesized within the testis. Other authors have also reported extrapineal sites of melatonin synthesis.[27] We could speculate a model in which testicular cells provide the substrate (serotonin) for melatonin synthesis and then this hormone interacts with its receptor in Leydig cells for regulation of testosterone production. It is interesting to consider that interstitial cells produce high levels of serotonin,[28] and the results of the present study are consistent with these observations. The relationship between NAT and HIOMT activities and the intratesticular production of melatonin, as well as the regulation of both enzymes, remains to be elucidated.

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