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PITUITARY–TESTICULAR AXIS ABNORMALITIES IN IMMATURE MALE HYPOTHYROID RATS

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Summary—The pituitary–testicular disturbances which follow the onset of hypothyroidism were studied in immature male Wistar rats rendered hypothyroid by treatment with methimazole (MMI) given in drinking water, starting at 40 days of age. Half of the animals continued on MMI (MMI group) up to 140 days of age; the remaining rats were withdrawn MMI at 100 days and injected thereafter s.c. with 3 μg of T_3 daily, during the last 40 days (MMI + T_3 group). Ten rats were used as controls (C group). Hypothyroidism induced in immature animals significantly decreased serum T_4, T_3, LH, PRL, and testosterone levels, and also impaired the normal growth of body and sex accessory glands. T_3 replacement therapy helped to normalize serum hormonal levels, but the body and sex accessory gland weights were not fully corrected. Hypothyroidism also reduced the [^{125}I]LH/hCG binding sites of testicular homogenates. T_3 replacement was not able to improve the binding; nonetheless, the hormone–receptor affinity constant remained unaltered among the groups. Leydig cell responsiveness to hCG stimulation *in vitro* (0–82 nM) showed impaired testosterone production in the MMI group (25% of that found in the C group) and also in the MMI + T_3 group (80% of that found in the C group). These data demonstrate that induction of hypothyroidism in the immature male rat leads to alterations in serum LH, PRL and testosterone levels, and suggest that thyroid hormones have a modulating action on the testis as far as LH-mediated testosterone secretion is concerned.

INTRODUCTION

Thyroid hormone deficiency is known to be accompanied by morphological and functional alterations in the pituitary–gonadal axis [1]. During hypothyroidism, several alterations of sexual and reproductive functions in male rats have been noted. However, contradictory results regarding the effects of hypothyroidism have been observed in the levels of serum pituitary hormones and testosterone, as well as in the weights of reproductive organs. Baksi[2] and Bruni *et al.*[3] reported low serum concentration of luteinizing hormone (LH) and testosterone, whereas Kalland *et al.*[4] observed no differences between euthyroid and hypothyroid animals in this regard. In addition, conflicting data have been published on sex accessory gland weights of male hypothyroid rats [3–5]. It has been shown in thyroidectomized rats that normal prolactin (PRL) serum levels coexisted with decreased PRL synthesis by the pituitary gland [6].

Among several explanations for the above discrepancies, the following merit mention: (a) the method for inducing hypothyroidism; (b) the age at which the dysfunction is established; (c) the duration of the condition, and (d) the parameters used for the study. It is also important to observe that thyroid hormone replacement causes rapid reversal of some of those alterations [4, 5].

The precise mechanisms whereby hypothyroidism exerts its effects on testes and sex accessory glands are not fully understood. The receptor occupancy by T_3 is in some way directly correlated to metabolic responsiveness to the hormone [7], but there is little information about the mechanism of action of thyroid hormones on the endocrine function of testis. Thus, the present study was designed to evaluate the pituitary–testicular axis in immature male rats rendered hypothyroid from the age of 40 days. Particular attention was given to the responsiveness of testes to gonadotropic stimulation in terms of testosterone production.

EXPERIMENTAL

Animals

Wistar male rats maintained on Purina rat chow and water *ad libitum* were employed. Hypothyroidism was induced by adding 0.1% methimazole (MMI) to drinking water, starting at 40 days of age. While half of the animals continued on MMI ("MMI" group) until sacrificed (at 140 days of age), the remaining rats were withdrawn MMI at 100 days of age, being thereafter treated with T_3 (3 μg per rat per day, s.c.), and labeled as "MMI + T_3" group.

Euthyroid rats of the same age were similarly scheduled, injected with saline s.c. when appropriate, and used as controls ("C" group). About 16 h after the last T_3 (or saline) injection, all animals were sacrificed by decapitation, trunk blood was collected and the sera were stored at −20°C until hormone determinations. Prostates, epididymides and testes were removed, dissected free of adherent tissue and weighed to the nearest 0.1 mg.

Membrane fraction preparation for binding assays

Decapsulated testes were homogenized in 50 mM phosphate-buffered saline (PBS) pH 7.4 in a ratio 1:4 (w/v) at 4°C using a Polytron homogenizer (Brinkmann, NY, U.S.A.). The homogenates were centrifuged at 12,000 *g* for 30 min at 4°C and the supernatants discarded. The membrane-rich pellets were resuspended in the same volume of PBS, rehomogenized, then filtered through nylon cloth (Nitex 50) and used in binding studies. Protein concentration was determined by the method of Lowry *et al.*[8].

Preparation of collagenase-dispersed Leydig cells

Leydig cells were isolated as described by Dufau and Catt [9]. In brief, decapsulated testes were incubated in medium 199 (Difco, Detroit, U.S.A.) containing 0.3 mg/ml collagenase and 0.1% bovine serum albumin for about 15 min at 34°C using a Dubnoff metabolic incubator (Fanem, S. Paulo, Brazil). The supernatant containing the cells obtained by decantation was drained and the sediment was washed with medium 199; this procedure was repeated until the supernatant was clear. The combined supernatants were centrifuged at 250 *g* for 5 min at 4°C. The cell pellet was resuspended in the same medium (approx 1 ml per testis). Cell viability was determined by trypan blue exclusion; the yield of viable cells was in a range of 80–90%. The samples were counted in a Levy ultraplane hemacytometer, and final dilutions were made in medium 199 to give 10^6 cells/ml.

Binding assays

[^{125}I]hCG was prepared using the lactoperoxidase technique of Thorell and Johansson[10] with minor modifications and further purified, before use, as previously described [11, 12]. Specific activities of typical preparations averaged 45 μCi/μg. The membrane-rich fractions of testes homogenates (600–700 μg protein/tube) were incubated in PBS medium with increasing concentrations of [^{125}I]hCG in a final volume of 0.25 ml for 18 h at 20°C. Nonspecific binding was assessed by parallel incubates containing 5 μg of unlabelled hormone. The reaction was stopped by adding 2 ml of cold PBS and centrifuged at 5,000 *g* for 15 min at 4°C. The pellets were washed twice and the radioactivity was measured in a Packard 45–26 Gamma counter with 70% efficiency. Specific binding data were studied using the method of Scatchard[13].

Production of testosterone by Leydig cells

Isolated Leydig cells from C, MMI and MMI + T_3 groups of rats were incubated under constant shaking with increasing concentrations of hCG in medium 199 containing 0.125 mM 1-methyl-3-isobutylxanthine (Sigma, St Louis, U.S.A.) at 34°C for 4 h. Each incubation had 2×10^6 cells in a final volume of 3.2 ml. Incubations were stopped by rapid cooling and centrifugation at 2,000 *g* for 20 min at 4°C. Aliquots (1.0 ml) of the supernatants were kept frozen (−20°C) until assayed for testosterone.

Hormone assays

Testosterone was determined by RIA using a specific anti-testosterone antibody raised in rabbits and kindly donated by Dr Eduardo H. Charreau (Inst. Biologia y Medicina Experimental, Buenos Aires, Argentina). Ether extractions of sera were performed as previously described [14]. Testosterone in incubation media (see above) was determined without previous extraction. The intraassay and interassay coefficients of variation were 9.1 and 15.3% respectively.

Serum LH and PRL concentrations were determined using a double antibody RIA, with materials kindly donated by NIH; results were expressed in terms of the NIAMDD-Rat-PRL-RP-2 (for prolactin) and NIAMDD-Rat-LH-RP-1 (for LH) standards provided. In our conditions, the assay blanks for both RIAs using sera from hypophysectomized rats gave undetectable hormone levels. The intraassay and interassay coefficients of variation for PRL were 12 and 16%, and for LH were 11 and 19%, respectively.

Serum T_3 and T_4 were measured by specific RIA kits (GammaCoat, T_3 RIA, Clinical Assay, Cambridge; T_4 RIA, Diagnostic Prod. Corp., Los Angeles, U.S.A.).

Statistical analysis

Data were analysed by Dunnett's multiple range test [15].

RESULTS

Hormonal status and body parameters

The effectiveness of the treatment with MMI became apparent by the observation of lower concentrations of T_4 and T_3 in sera of MMI-treated rats than in controls (Table 1) and by marked decrease of body weight gain (Table 2).

Table 1. Effects of methimazole (MMI) and of T_3 replacement therapy of MMI-treated animals (MMI + T_3) on serum hormone levels of male rats

Hormone	Control (7)	MMI (6)	MMI + T_3 (6)
T_4 (μg/dl)	4.8 ± 0.19	1.4 ± 0.16[b]	1.9 ± 0.4[b]
T_3 (ng/dl)	54.4 ± 8.1	26.0 ± 6.5[b]	64.0 ± 9.2[c]
LH (ng/ml)	26.0 ± 2.0	21.0 ± 0.5[a]	25.6 ± 4.2
PRL (ng/ml)	30.6 ± 2.6	24.0 ± 2.7[a]	29.5 ± 3.2
Testosterone (ng/dl)	410.7 ± 57.0	237.0 ± 45.3[a]	459.0 ± 68.5[c]

Figures in parentheses indicate the number of animals. Superscript letters indicate results of statistical comparisons: [a] $P < 0.05$ and [b] $P < 0.001$ in relation to the controls; [c] $P < 0.05$ in relation to the "MMI" value.
Results are mean ± SEM.

Table 2. Comparison of body and sex accessory gland weights of rats, control and treated with methimazole (MMI) or with methimazole and further with T_3 (MMI + T_3)

Weight	Control		MMI		MMI + T_3	
	Total	Relative	Total	Relative	Total	Relative
Body (g)	282 ± 19 (7)	—	183 ± 9.4[a] (8)	—	222 ± 7.1[a,b] (7)	—
Testis (mg)	1637 ± 30 (10)	0.58 ± 0.02	1258 ± 20[a] (12)	0.69 ± 0.02[a]	1231 ± 10[a] (10)	0.55 ± 0.01[b]
Epididymis (mg)	586 ± 25.2 (10)	0.21 ± 0.01	308 ± 53.8[a] (12)	0.17 ± 0.02	457 ± 48.8[a] (10)	0.21 ± 0.01
Prostate (mg)	554 ± 67.4 (7)	0.20 ± 0.02	198 ± 33.1[a] (8)	0.11 ± 0.01[a]	279 ± 59.6[a] (7)	0.13 ± 0.02[a]

Data are mean ± SEM.
Figures in parentheses indicate the number of animals or organs used. Organ weights are wet weights. Relative weights are organ wet weights per 100 g body weight. Superscript letters indicate results of statistical comparisons: [a]$P < 0.05$ in relation to the controls, and [b]$P < 0.05$ in relation to the corresponding "MMI" value.

Table 3. *In vitro* testosterone production* by isolated Leydig cells from control, MMI- and MMI + T_3-treated rats, upon stimulation with increasing concentrations of hCG

Group	hCG concentration				
	0	10.3	20.6	41.2	82.4 nM
	0	250	500	10^3	10^4 ng/inc.
Control	2.39 ± 0.24	3.49 ± 0.37	8.16 ± 0.88	8.55 ± 0.94	15.74 ± 1.76
MMI	1.67 ± 0.15	2.05 ± 0.23[a]	2.52 ± 0.37[a]	2.72 ± 0.29[a]	4.04 ± 0.44[a]
MMI + T_3	2.20 ± 0.24	6.69 ± 0.99[a]	7.40 ± 0.67	12.10 ± 1.79	12.57 ± 1.89

*Results (mean ± SEM) are given as ng testosterone produced per 2×10^6 cells; testosterone was determined by RIA in triplicate for every incubation medium (see Experimental). [a]$P < 0.05$ in relation to controls.

Table 1 also shows that serum concentrations of LH, PRL and testosterone fell significantly in the MMI group of rats. Hypothyroidism caused a significant decrease in the weight of testes and sex accessory glands, as shown in Table 2. The hormone replacement therapy (MMI + T_3 group) was effective in restoring T_3, LH, PRL and testosterone levels to the normal range, being less effective with regard to body weight and the weight of testes and sex accessory glands.

Testosterone production

Leydig cell responsiveness to hCG stimulation is shown in Table 3. Testosterone production by cells isolated from the MMI group was markedly decreased, to 25% of that found for the C group at the highest concentration of hCG used. T_3 replacement was able to fully restore the response to hCG.

LH/hCG receptors

Figure 1 shows the Scatchard plots of binding studies of [^{125}I]hCG to the membrane fraction of testes from C, MMI and MMI + T_3 groups of rats. No alterations in the affinity constant were detected among the groups tested, but MMI rats showed a marked reduction of the number of binding sites (31%). T_3 replacement therapy was ineffective in restoring binding site levels to the control value.

DISCUSSION

The effectiveness of MMI treatment in inducing hypothyroidism could be seen by the substantial decrease in serum T_3 and T_4 levels. Though a food intake control of the rats was not carried out, the duration of our experiment was long enough to ensure a well-established hypothyroid state [see 16, 17].

The present study shows that when hypothyroidism is induced at an immature age, both impairment of body growth and atrophy of sex accessory glands become evident. As seen in Table 2, hypothyroid animals showed reduced total testicular weight. This finding is not commonly seen when hypothyroidism is induced at adulthood [4], but confirms data reported by Leathem[17] that the reproductive system of immature rats is much more influenced by thyroid dysfunction than that of adult animals. On the other hand, when these results are calculated relatively to the body weight, an increase of testicular weight is detected (Table 2). This phenomenon is well known to occur in the hypothyroid state, and can be viewed as a consequence of impaired general body growth, being testicular growth somewhat less sensitive.

Under our experimental conditions, MMI-treated rats showed a decrease of serum LH, PRL and testosterone levels, and T_3 replacement re-established the cell metabolic processes, but conceivably not those processes related to trophic phenomena. This appears to confirm the observation by Larsen and Frumess[16] that T_4 and T_3 may have clearly separate actions.

It is now firmly established that not only testosterone but also PRL are important regulators in the maintenance and function of the testes and male sex accessory glands [19, 20]. The low serum levels noted

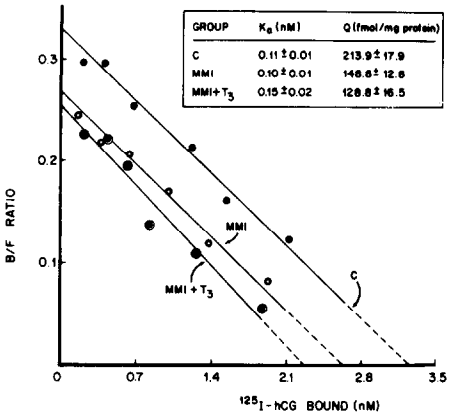

Fig. 1. Scatchard plot analysis of data obtained in saturation binding studies of [^{125}I]hCG to the membrane fraction of Leydig cells from rats treated with MMI (hypothyroid group), with MMI + T_3 (T_3-recovered group), or control (C). K_a = apparent affinity constant; Q = maximal binding capacity.

of these hormones likely have a direct relationship to the reduction of prostate and epididymal weights. The decrease of testosterone concentration correlates not only with the low response of Leydig cells to the gonadotropic stimulation, but also with the low number of testicular LH/hCG receptors.

So far, the serum PRL levels during hypothyroidism have been only partially explained [1]. High levels of PRL observed in human hypothyroidism may be explained by a reduction metabolic clearance rate of PRL [21]. On the other hand, not only does TRH seem not to be the physiological regulator of PRL secretion [22], but also pituitaries from hypothyroid animals did not prove to synthesize increased amounts of PRL *in vitro* [23]. Overall, it can be postulated that the age at which thyroid dysfunction is induced is crucial to the patterns of PRL secretion [24].

It was somewhat surprising to find low prostatic weight in MMI + T_3 animals, since normally this organ responds to the synergistic action of PRL and testosterone [24, 25]. As the concentrations of these hormones were normalized in this group, one of the possible explanations is depressed peripheral response to PRL at the receptor level [20, 26].

The diminished number of LH/hCG receptors in testes of MMI + T_3 animals (Fig. 1) may appear to be inconsistent with their normal testosterone response to graded doses of hCG (Table 3). However, it is open to question whether reduced LH receptor content would necessarily imply reduced testosterone production. It has been shown that a decrease in testicular LH receptor content does not always result in substantial loss of the steroidogenic capacity of Leydig cells [27], a finding that could be explained, at least in part, by the concept of "spare receptors". In fact, it is now well-recognized that only a minute fraction of the total number of LH receptors in the testis need be occupied to elicit the steroidogenic response [27]. Recent studies in our laboratory indicate that hypothyroid rats have reduced testicular content of testosterone, even after treatment with thyroid hormone and PRL (unpublished data). On the other hand, it can not be discarded that the fall in testicular LH receptors would result from a significant decrease in the bioactivity of serum LH, coupled to a concomitant fall in serum PRL.

Since T_3 receptors in testes are less than 1% of those found in liver [7], the correction of hypothyroidism by T_3 replacement in general suggests that T_3 has a modulating action in testes [6, 26], as far as LH-mediated testosterone secretion is concerned.

Our results indicate that hypothyroidism induced at an immature age leads to alterations of the pituitary–testicular axis, and also that such alterations can be better defined in this condition than in mature animals. In addition, we found that some of these dysfunctions did not prove to be relievable by T_3. It is likely that severe, "irreversible" changes could explain why these animals, at adulthood, behave so differently from others reported in the literature [2–6]. In fact, although they shared identical hormonal dysfunctions, they were completely different regarding to the response to hormonal replacement therapy.

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