Prenatal induced chronic dietary hypothyroidism delays but does not block adult-type Leydig cell development

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Rijntjes E, Swarts HJ, Anand-Ivell R, Teerds KJ. Prenatal induced chronic dietary hypothyroidism delays but does not block adult-type Leydig cell development. Am J Physiol Endocrinol Metab 296: E305–E314, 2009. First published November 25, 2008; doi:10.1152/ajpendo.90750.2008.—Transient hypothyroidism induced by propyl-2-thiouracil blocks postpartum Leydig cell development. In the present study, the effects of chronic hypothyroidism on the formation of this adult-type Leydig cell population were investigated, using a more physiological approach. Before mating, dams were put on a diet consisting of an iodide-poor feed supplemented with a low dose of perchlorate and, with their offspring, were kept on this diet until death. In the pups at day 12 postpartum, plasma thyroid-stimulating hormone levels were increased by 20-fold, whereas thyroxine and free tri-iodothyronine levels were severely depressed, confirming a hypothyroid condition. Adult-type progenitor Leydig cell formation and proliferation were reduced by 40–60% on days 16 and 28 postpartum. This was followed by increased Leydig cell proliferation at later ages, suggesting a possible slower developmental onset of the adult-type Leydig cell population under hypothyroid conditions. Testosterone levels were increased 2–10-fold in the hypothryroid animals between days 21 and 42 postpartum compared with the age-matched controls. Combined with the decreased presence of 5α-reductase, this implicates a lower production capacity of 5α-reduced androgens. In 84-day-old rats, after correction for body weight-to-testis weight ratio, plasma insulin-like factor-3 levels were 35% lower in the hypothryroid animals, suggestive of a reduced Leydig cell population. This is confirmed by a 37% reduction in the Sertoli cell-to-Leydig cell ratio in hypothryroid rats. In conclusion, we show that dietary-induced hypothyroidism delays but, unlike propyl-2-thiouracil, does not block the development of the adult-type Leydig cell population.

Thyroxine; testosterone; insulin-like factor-3; Leydig cell proliferation

Alterations in thyroid hormone levels are well known to influence key functions in growth and development. Prenatal/neonatal neural development is particularly sensitive to the influence of insufficient thyroid hormone levels (20). The trace element iodide is essential for adequate thyroid hormone synthesis. The World Health Organization and several national Food and Nutrition organizations therefore made recommendations for adequate iodide intake. Decreased uptake of this nutrient usually results in either reduced levels of iodide in urine or elevated levels of thyroid-stimulating hormone (TSH) because of a reduced negative feedback of thyroid hormone on the pituitary release of TSH. The latter leads to deficiency disorders like goiter, decreased fertility, and retarded physical and mental development (22, 25). Although in many countries food products are fortified with iodide, still, not all humans and animals have access to or use these fortified products. For example, 15–20% of women of childbearing age in the United States have a suboptimal iodide intake (37, 49, 50, 55). The presently described experiments follow the most common cause of hypothyroidism, dietary-induced iodide deficiency, as a method to study Leydig cell development from the neonate up to adulthood.

Daily administration of high doses of the thyroid hormone tri-iodothyronine (T₃), ranging from 5 to 10 μg T₃/100 g body wt, has been shown to stimulate Sertoli cell differentiation, eventually leading to a premature cessation of Sertoli cell proliferation and an acceleration of the progression of spermatogenesis. As a consequence, the final number of Sertoli cells per testis was reduced by 50%, and concomitantly the total number of germ cells was decreased (51). The formation of Leydig cell progenitors and thus the development of the adult-type Leydig cell population were advanced under these conditions (2, 28, 46). On the other hand, continuous oral administration of thryroxine (T₄) (15 μg T₄/100 g body wt) did not result in an acceleration of testicular development. On the contrary, the progression of spermatogenesis was slightly delayed while the level of Leydig cell proliferation remained elevated for a prolonged period of time (39).

The effects of thyroid hormone deficiency on testicular development are commonly studied using propyl-2-thiouracil (PTU) as goitrogen. Because of PTU toxicity, administration during pregnancy usually results in premature abortion. As a consequence, treatment is generally initiated around the time of delivery and stopped before the pups reach puberty. Transient neonatal-prepubertal hypothyroidism induced by postnatal PTU treatment has been shown to lead to macroorchidism in adult rats and mice. These changes were caused by a prolongation of Sertoli cell proliferation and a delay in Sertoli cell differentiation (9, 18, 42, 52). At the same time, under these hypothyroid conditions, progenitor-type Leydig cell formation was reported to be arrested up to 21 days after birth in rats (27). Following cessation of the hypothyroid condition at the age of 26 days, developing adult-type Leydig cells started to proliferate massively (Teerds KJ, unpublished data), leading to an approximate 70% increase in the Leydig cell population in adulthood (14, 46).

Whereas in rats the development of fetal-type Leydig cells starts in utero, the development of the adult-type Leydig cell

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population is initiated during prepuberty (reviewed in Ref. 13). Stem Leydig cells develop through several steps of differentiation and proliferation into the mature adult-type Leydig cell population (11, 13, 16, 23, 26, 30). In short, spindle-shaped stem Leydig cells differentiate into Leydig cell progenitors between days 10 and 13 after birth (11, 46). The presence of sufficient amounts of thyroid hormone appears to be necessary for this process to proceed (11). Subsequently, these cells undergo further proliferation and differentiate to immature adult-type Leydig cells between days 28 and 35 after birth, a developmental phase during which 5α-reduced androgens become the prime steroids produced by the developing Leydig cells (45, 56). The immature adult-type Leydig cells then gradually develop into mature, terminally differentiated adult-type Leydig cells (13, 16).

Apart from gonadotrophins, endogenously produced (growth) factors as well as thyroid hormones are of importance in the regulation of these processes (13). As indicated above, experiments by Mendis-Handagama et al. (27) using PTU as a goitrogen suggested that, under transient hypothyroid conditions, adult-type Leydig cell formation comes to a halt before the age of 21 days. In the present investigation, we expanded these in vivo studies. We selected an iodide-poor diet in combination with sodium perchlorate instead of the more toxic agent PTU to induce hypothyroidism. With this diet it was possible to expose pups already in utero to decreased thyroid hormone levels without the risk of abortions. Dams were put on the experimental diet 2 wk before mating. After parturition and weaning, the diet was continued and pups were killed at different ages up to adulthood, after which the development of the Leydig cell population was analyzed and related to the endocrine condition of the animals [plasma TSH, thyroid hormones, luteinizing hormone (LH), testosterone, prolactin, and corticosterone levels]. In contrast to studies in which PTU was used to induce a transient hypothyroid condition, the present setup of experiments mimics the most common form of hypothyroidism, dietary iodine deficiency, and allowed us to study the effects of continued hypothyroidism on fetal-type as well as adult-type Leydig cell development.

MATERIALS AND METHODS

Chemicals and antibodies. All chemicals were purchased from Sigma (Zwijndrecht, The Netherlands) unless otherwise indicated. Sodium perchlorate was purchased from VWR (Amsterdam, The Netherlands). The polyclonal antibody against 3β-hydroxysteroid dehydrogenase (3β-HSD) was a kind gift from Dr. A. H. Payne (Stanford, CA). Biotinylated horse anti-mouse antibody, alkaline phosphatase goat anti-rabbit antibody, and the Vectastain ABC kit Elite were purchased from Vector Labs (Burlingame, CA). The mouse anti-bromodeoxyuridine (BrdU) antibody was obtained from Beckton and Dickinson (Mountain View, CA). Acetylated BSA (BSA-c) was purchased from Aurion (Wageningen, The Netherlands). The radioimmunoassay (RIA) kits for the determination of total T3, free T3, total T4, free T4, testosterone, and free testosterone were obtained from DSL (Webster, TX). The RIA kit to determine corticosterone levels was purchased from MP Biomedicals (Costa Mesa, CA). SACcel, the secondary donkey anti-rabbit antibody complex used in the in-house RIA analysis, was obtained from Welcome Reagents (Beckenham, UK).

Animals and treatment. The animal experiment was approved by the Animal Welfare Committee of Wageningen University. Wistar WU (HsdCpbWU) rats were obtained from Harlan (Horst, The Netherlands) at the age of 7 wk (females) or 10 wk (males) and kept at a room temperature of 20.5–21.5°C, humidity of 55–65%, and light regime of 60–80 lux; lights were on from 0300 to 1700 (24-h clock). The female rats were individually housed after arrival. Cage enrichment was provided in the form of 10 cm of sisal rope. Two weeks after arrival, the female rats were put on an iodide-poor diet based on AIN 1993 requirements (Research Diet Services, Wijk bij Duurstede, The Netherlands) (38, 41). This control diet was supplemented with 7 μg of iodide per 100 g dry wt of the diet to fulfill the normal iodide requirements of rats. At the age of 10 wk, the rats were put on the experimental diets: the control diet (as indicated above) or the iodide-poor diet supplemented with 0.75% sodium perchlorate in the drinking water. At the age of 12 wk, the rats were mated. Pups were weaned on postnatal day 28. The male offspring was group housed (3–4 animals together) up to the age of 73 days after birth, after which the remaining animals were housed in pairs until death.

Body temperature was monitored using an IPTT-100 Pocket Scanner (BioMedicData Systems). At weaning, 8 males per group received a subcutaneous transponder under isoflurane anesthesia (3% isoflurane vaporized in 1:1 O2:N2O). Body temperature was measured once a week until the animals were killed.

Groups of 6–20 pups were killed at the age of 12, 15, 21, 28, 35, 42, 49, 63, 77, 84, 100, or 120 days postpartum. The experimental diets were continued throughout pregnancy until death of the offspring. Two hours before the rats were killed, each animal received a subcutaneous injection of BrdU (150 mg/kg body wt) in saline. BrdU is a compound that is specifically incorporated in the DNA of cells in the S-phase of the cell cycle and gives an indication of the proliferative activity of a tissue. Rats were anesthetized using carbon dioxide and oxygen (flow, 2:1). Blood was collected by heart puncture (5 IU heparin/ml blood). Rats were killed by decapitation. After collection of the testes, wet weights were determined. Plasma was stored at −20°C until further analysis.

Histology and immunohistochemistry. The right testes were fixed in Metacarnoy’s fluid for 2 h and alcohol (100%) for 30 min and stored in alcohol (70%) until embedding in paraffin. To determine the proliferation of the developing adult-type Leydig cell population, testis sections were incubated with a monoclonal antibody against BrdU and then with the polyclonal antibody against 3β-HSD, a marker for Leydig cells, as described previously (39). In short, 5-μm sections 100 μm apart were deparaffinized, transferred to a 0.01 M PBS (pH 7.4) buffer, and rinsed with 0.06 mg/ml PBS plus 0.03% H2O2. Next, sections were rinsed in PBS, incubated with 10% normal goat serum in PBS (30 min), and incubated overnight with the polyclonal rabbit anti-3β-HSD antibody (diluted 1:300) at 4°C, followed by incubation with an alkaline phosphatase-labeled secondary goat anti-rabbit antibody (diluted 1:200) for 60 min. Alkaline phosphatase activity was demonstrated using naphthol [1 mg of naphthol AX MS phosphate with 50 μl of N,N-dimethylanilinomethane in 8 ml of Tris-HCl, 0.1 M (pH 8.5)] and Fast Blue BB Base (2 mg of Fast Blue BB Base with 50 μl of 0.2 M HCl and 50 μl of 4% NaNO2) and levamisole (5 mg) at 37°C. Slides were rinsed, counterstained with Mayer’s hematoxylin, and mounted.

Fetal-type Leydig cells, Leydig cell progenitors, and immature and mature adult-type Leydig cells were counted in at least three randomly chosen sections per testis. Only those 3β-HSD-positive Leydig cells (blue cytoplasm) in which the nucleus was present were counted. Proliferating Leydig cells were identified by the presence of BrdU (brown) staining of the nucleus. Per testis, 1,500 Leydig cells (3β-HSD labeled and 3β-HSD plus BrdU labeled) were counted, and the percentage of Leydig cells in S-phase of the cell cycle was calculated.
In the case of young animals when less than 1,500 3β-HSD-positive cells were present, all Leydig cells in the cross sections were counted. The size of the Leydig cell population was estimated by determining the Leydig cell-to-Sertoli cell ratio in the testes of 84-day-old control and hypothyroid rats. Leydig cells were scored in random testicular areas until a minimum of 1,000 Sertoli cell nuclei were counted. Leydig cell numbers were expressed per 1,000 Sertoli cell nuclei according to the method of Heller et al. (17). RIA s. RIA s for total T4 (DSL-3200), free T4 (DSL-40100), total T3 (DSL-3100), free T3 (DSL-41100), testosterone (DSL-4100), free testosterone (DSL-4900), and corticosterone (ImmucChem Double Antibody Corticosterone RIA kit) were assayed according to the manufacturer’s protocol. LH, TSH, and prolactin levels were determined by validated in-house double-antibody RIA s for rat serum analysis (24, 31, 32) using materials supplied by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Bethesda, MD). For LH, rLH-I-9 was used as label and anti-rLH-S-11 as antiserum; for TSH, rTSH-I-9 as label and anti-TSH-RIA-6 as antiserum; and for prolactin, rPRL-I-5 as label and anti-rPRL-415 as antiserum. SACcel (donkey anti-rabbit) was used as a secondary antibody. The levels of hormones were expressed in terms of NIDDK antiserum. The detection limit of the assays were as follows: 5 ng/ml antibody. The levels of hormones were expressed in terms of NIDDK standards. The detection limit of the assays were as follows: 5 ng/ml for total T4; 2 pg/ml for free T4; 0.25 ng/ml for total T3; 0.6 pg/ml for free T3; 25 ng/ml for corticosterone; 0.03 ng/ml for LH; 0.1 ng/ml for testosterone, TSH, and prolactin; and 0.25 pg/ml for free testosterone. The intra- and interassay variation was determined using several pools of rat serum and was < 11% for all purchased RIAs and < 9.5% for all in-house RIAs.

Rat insulin-like factor-3 (Insl3) in serum was measured using a time-resolved fluorescent immunoassay (TRFIA) essentially as described by Anand-Ivell et al. (1), except that the Eu+ + labeled tracer made use of pure chemically synthesized rat Insl3, and the primary antibody had been raised in rabbits against the same molecule. The lower and upper limits of detection were 20 pg/ml and 10 ng/ml, respectively, with intra- and interassay coefficients of variation in the midrange of 9.5% for all purchased RIAs and 11% for all purchased RIAs.

Statistical analysis. Data are expressed as means ± SE. Previous experiments suggested that testicular development was dependent on the founders (Rijntjes E, unpublished data); hence per age group only 1 pup was used per dam. Statistical analysis was carried out using SAS 9.1.3 for Windows. Data were tested using repeated measurements two-way ANOVA (time × thyroid status) followed by Tukey’s post hoc analysis. A Mann-Whitney U-test was used when, even after log transformation of the data, normality could not be assumed. Values of P < 0.05 were considered to be significantly different.

RESULTS

Two weeks after the start of the experimental diets, at the time of mating, the body weights of the dams were comparable for both the hypothyroid and control groups. The day before parturition, however, the body weight of the hypothyroid dams was on average 20 g lower than for the control dams (245 ± 12 g, n = 24, vs. 268 ± 11 g, n = 21, in the control dams), although this difference did not reach the level of significance. Litter size and gender ratio of the offspring were not affected by the hypothyroid condition. In the hypothyroid pups, the eyes opened 1 day later compared with the age-matched controls. Body temperature was significantly decreased in the hypothyroid offspring by 1.3°C from the start of measurements at day 28 postpartum. The observed reduction in body temperature of the hypothyroid rats was in line with earlier studies (53, 57).

Body and testes weights. The body weights of the hypothyroid animals were significantly decreased at all ages by at least 30% on day 12 after birth up to 70% on day 63 after birth and 50% on day 120 postpartum compared with respective age-matched control animals (Fig. 1A). Wet testis weights on the contrary were significantly decreased from day 16 to day 77 after birth in the hypothyroid animals, but showed a marked increase between the age of 49 and 84 days, when control levels were reached. At that age, the testis weight of the age-matched control rats had reached a plateau (Fig. 1B). By calculation of the testis weight-to-body weight ratio, the testis weight is corrected for the difference in body weight. From day 49 postpartum onward, we noted a significant relative macroorchidism for the hypothyroid rats (Fig. 1C).

Thyroid hormone and TSH levels. To assess the thyroid status of the hypothyroid animals, plasma TSH and total free T4 and T3 concentrations were determined. After 2 wk on the experimental diet, the hypothyroid dams had significantly
increased TSH levels (9.21 ± 0.58 vs. 0.55 ± 0.02 ng/ml in the control dams). TSH levels reached a plateau at the time of parturition (25.11 ± 3.27 ng/ml in the hypothyroid dams vs. 0.81 ± 0.10 ng/ml in the control dams). Total T₄ and T₃ levels in the dams were significantly reduced at the time of mating to 13 and 71% of the control levels, respectively.

TSH levels in the hypothyroid offspring were significantly increased at all measured ages (from day 12 to day 120 after birth) compared with the age-matched controls (Fig. 2A). Concomitantly, both plasma total T₄ and free T₄ levels were decreased to below the detection limit of the assay in the hypothyroid animals at all ages vs. levels that ranged from 14 to 45 ng/ml for total T₄ per milliliter and from 16 to 26 pg/ml for free T₄ in the respective age-matched control rats. Plasma T₃ levels were significantly reduced by 20–40% from 16 days after birth onward in the hypothyroid rats compared with the age-matched euthyroid control rats (Fig. 2B). At day 12 postpartum, no difference in total T₃ levels was observed between the hypothyroid animals and the controls because of nonshivering thermogenesis (enhanced thyroid function to control body temperature). Free T₃ levels were reduced by 50–85%, depending on the age of the animals (Fig. 2C).

Leydig cell proliferation. To evaluate Leydig cell proliferation and differentiation, BrdU incorporation in 3β-HSD-positive Leydig cells was determined. In the strain of rats used in the present study, the first Leydig cell progenitors were formed around days 13 to 14 after birth. The BrdU-3β-HSD-positive cells observed in the 12-day-old testes were thus of fetal origin. These fetal-type Leydig cells were often localized in characteristic clusters (reviewed in Ref. 13). There were no differences in proliferative activity between the treatments at this age. At the age of 16 days, proliferating adult-type Leydig cell progenitors were detected in the euthyroid control rats. Histologically, the proliferating progenitor cells were identified by their spindle-shaped to oval nucleus and their location in close vicinity to either the seminiferous tubules or blood vessels (Fig. 3) (reviewed in Ref. 13). In the 16-day-old hypothyroid testis, progenitor-type Leydig cells could be identified, although their number seemed to be lower compared with the euthyroid controls. The percentage of proliferating Leydig cells was initially, at the age of 16 days, significantly lower in the hypothyroid testis compared with the age-matched controls, was not different from control levels on day 21, and significantly decreased again on day 28 (Fig. 4). In euthyroid rats, most progenitor-type Leydig cells differentiated into immature-type Leydig cells between the ages of 28 and 35 days (16). Because of the absence of specific markers for either progenitor cells or immature Leydig cells, it was not possible to discriminate between these two Leydig cell types. By the age of 49 days in the control group, proliferation of immature Leydig cells had ceased, and these cells had started to differentiate into mature adult-type Leydig cells. In the hypothyroid animals, however, the proliferative activity of the adult-type Leydig cells remained elevated up to the age of 63 days (Figs. 3 and 4).

At the age of 84 days postpartum, at the time the testis did not grow any further, Ins13 levels, a Leydig cell-specific marker, were determined in plasma to obtain an impression of the number of Leydig cells in the testis as described by Anand-Ivell et al. (1) with minor modifications. Ins13 levels did not differ significantly between the two groups of animals (2.2 ng/ml in the euthyroid control animals, and 3.0 ng/ml in the hypothyroid rats). However, if one takes into account that Ins13 is considered to be specifically secreted by mature Leydig cells in the adult testis and constitutively expressed without any feedback regulation (Ref. 40; R. Ivell, personal communication), the reduced body weight of the hypothyroid rats might overestimate the plasma Ins13 value. Assuming that the blood volume relates to a fixed portion of the body weight, one can hypothesize thus that the Ins13 concentration as a proportion of blood volume is artificially elevated in the case of the hypothyroid animals. If one corrects for the body weight-to-testis weight ratio, plasma Ins13 levels appeared, actually, to be 35% lower in the hypothyroid rats compared with the age-matched controls, suggesting that in the hypothyroid animals, the Leydig

Fig. 2. A: plasma thyroid-stimulating hormone (TSH) levels were 4- to 20-fold increased in the hypothyroid pups. B: plasma total tri-iodothyronine (T3) levels were ~30% reduced. C: plasma free T3 concentrations were reduced by 50–85%. Values represent means ± SE. *P < 0.05; n = 10–15 from 12 to 63 days postpartum, n = 5–8 from 77 to 120 days postpartum. ND, not determined.
cell population was reduced in size. Another way to obtain an impression of the size of the Leydig cell population in the testis is by determining the Sertoli cell-to-Leydig cell ratio (17). The Sertoli cell number in the testis is indicative of the number of germ cells present in the testis, both of which together determine testis weight (5). Since, by the age of 84 days, there appears to be no significant difference in testis weight between the hypothyroid and control animals, nor in seminiferous tubule diameter, it does not seem likely that the number of Sertoli cells in the hypothyroid testis would differ significantly from that in the control testis. In the control rats at the age of 84 days, 931 ± 26 Leydig cells were counted per 1,000 Sertoli cells, whereas in the hypothyroid testis, significantly fewer (594 ± 65 Leydig cells per 1,000 Sertoli cells) were counted, a reduction of 37% in the size of the Leydig cell population. This confirms the reduction in plasma Insl3 levels in the hypothyroid rats following correction for differences in the body weight-to-testis weight ratio between hypothyroid and control animals.

Hormone levels. LH levels, involved in stimulating adult-type Leydig cell proliferation and differentiation, peak between days 21 and 35 after birth in the euthyroid control animals. In the hypothyroid animals, such a clear peak in plasma LH levels was not observed (Fig. 5A).

In early life, when progenitor-type Leydig cells develop and the first immature-type Leydig cells are formed, the 5α-reduced androgens 5α-dihydrotestosterone and 3α-androstanediol are the main steroids produced by the testis. Dihydrotestosterone is metabolized to 3α-androstanediol, which can then be glucuronidated into 3α-androstanediol glucuronide, which is excreted via the urine (36, 45). When immature-type Leydig cells undergo further differentiation, testosterone becomes the most abundant androgen. Plasma total testosterone and free testosterone levels were significantly increased between the ages of 21 and 42 days in the hypothyroid animals (Fig. 5, B and C). Plasma free testosterone levels were highly linearly correlated ($R^2 = 0.95$) to total testosterone levels.
In general, prolactin levels were significantly decreased by about threefold in the hypothyroid rats compared with the age-matched controls in all age groups from day 21 onward (Fig. 6A). Corticosterone levels did not differ between the groups at any of the ages investigated (Fig. 6B).

**DISCUSSION**

In the present study, hypothyroidism was induced by feeding rats an iodide-poor diet combined with sodium perchlorate in the drinking water to limit iodide uptake by the thyroid. Hypothyroidism was induced 2 wk before pregnancy and continued after birth. Increased plasma TSH levels confirmed the hypothyroid condition of the dams and their offspring, while both total and free T₃ and T₄ levels were considerably reduced. Leydig cell proliferation was decreased in the hypothyroid rats at the ages of 16 and 28 days and clearly elevated from day 42 to day 63 postpartum, suggesting a possible slower onset of adult-type Leydig cell formation and proliferation compared with the euthyroid control animals. The development of the adult-type Leydig cell population was not completely inhibited as has been suggested by Mendis-Handagama et al. (27) in the presence of the goitrogen PTU. Quite surprisingly, analysis of the size of the Leydig cell population (number of Leydig cells per 1,000 Sertoli cells) showed that this cell population was reduced in the adult hypothyroid testis compared with the euthyroid controls.

Development of the fetal-type Leydig cell pool starts in utero; cell numbers increase throughout gestation, reaching the highest level close to the day of birth (14, 19, 29). In the youngest age group included in the present experiment, day 12 after birth, no significant differences in testis weight were seen between the hypothyroid animals and age-matched control group, nor were there any differences seen in Leydig cell proliferation of these fetal-type Leydig cells. Our data suggest that, from fetal life up to at least 12 days of age, Leydig cell development was not altered under hypothyroid conditions. These observations expand the elaborate studies of Mendis-Handagama et al. (27), who have shown that hypothyroidism induced by PTU from the day of birth onward does not affect the postnatal development of the fetal-type Leydig cell population.

The development of the adult-type Leydig cell population is initiated during prepuberty (reviewed in Ref. 13). In the strain of rats used in many studies, spindle-shaped stem Leydig cells differentiate into 3β-HSD/LH receptor-positive Leydig cell progenitors around day 10 after birth (3, 11, 26). In the strain of rats used in the present study, the first progenitor cells were detected around day 13 postpartum (47). In the rats killed at day 16 postpartum, progenitor Leydig cells were identified based on their spindle-formed shape and peritubular position in both the euthyroid as well as the hypothyroid rats, although the number of progenitor-type Leydig cells seemed to be lower in the hypothyroid testis, suggesting that differentiation of Leydig cell progenitors from spindle-shaped stem Leydig cells may have been delayed under hypothyroid conditions. This needs to be further investigated using specific stem Leydig cell markers (11). These findings are in contrast to studies using PTU to induce hypothyroidism, where adult-type Leydig cell development was suggested to be completely arrested during goitrogen treatment (3, 27).
Subsequently, under euthyroid conditions, Leydig cell progenitors undergo further differentiation into immature adult-type Leydig cells between days 28 and 35 after birth. After this age, the proliferative activity of the cells is decreasing rapidly, and the immature Leydig cells gradually develop into mature terminally differentiated adult-type Leydig cells. By the end of puberty, the development of the adult population is completed (4, 13). Concomitantly with the delay in progenitor formation, however, chronic hypothyroidism also resulted in a delay in the peak in adult-type Leydig cell proliferation, which has been described to occur around day 28 postpartum in untreated rats (14). The present investigation showed that, in the hypothyroid animals, the proliferative activity of the developing adult-type Leydig cells appeared to follow a different pattern compared with the respective age-matched control groups. Leydig cell proliferation did not decline after day 35 postpartum but increased even further to reach a maximum level between days 42 and 49 postpartum, 2 full wk after Leydig cell proliferation began to decrease in the age-matched euthyroid controls. This suggests that the onset of progenitor-type Leydig cell proliferation and differentiation could be postponed under hypothyroid conditions, and subsequently immature-type Leydig cell development may be delayed by 1–2 wk. Because of the absence of specific markers for progenitor and immature Leydig cells, however, it cannot be excluded that, besides progenitor Leydig cells, some newly formed immature Leydig cells also contributed to this broad peak in cell proliferation in the developing hypothyroid testis. Nevertheless, this prolonged period of Leydig cell proliferation does not seem to be enough for the formation of a normal-sized mature adult-type Leydig cell population, since in the 84-day-old hypothyroid testis, the size of the Leydig cell population is reduced by 37%, as became apparent by the lower number of Leydig cells (per 1,000 Sertoli cells) compared with the age-matched controls. At present the cause of this reduced cell number is not clear and needs to be further investigated.

In line with earlier studies, in euthyroid rats a slow decrease in testosterone levels up to the fifth week after birth was noted, after which the testosterone levels started to increase again (45). In the hypothyroid animals, testosterone levels went up from day 21 onward, reaching peak levels on day 28, levels not seen in the control rats up to 120 days of age. The high testosterone levels at this age are in contrast to the studies using the more toxic goitrogen PTU (3, 15, 27, 46). One explanation for the differences between the present experiment and the PTU studies is that PTU also acts directly on developing rat Leydig cells, diminishing testosterone production. These effects are most likely to be mediated by inhibition of the cytochrome P-450 side-chain cleavage enzyme and the steroidogenic acute regulatory protein (StAR), a protein that mediates the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane protein (8, 43).

Ge and Hardy (12) showed that basal production of testosterone in immature and mature adult-type Leydig cells was, respectively, 4 and 30 times higher compared with that of the progenitor Leydig cells present from day 16 postpartum onward. Although in most studies testosterone is measured, the major androgens produced by progenitor and immature Leydig cells between the ages of 16 and 42 days are the 5α-reduced androgens dihydrotestosterone (DHT), androstenedione, and 3α-androstenediol (12, 45). We performed an experiment using immunohistochemistry to investigate the presence of 5α-reductase in the testis, to obtain more insight in the possible role of 5α-reductase in the observed increased levels of testosterone. Viger and Robaire (54) investigated the immunolocalization of the 5α-reductase protein in the rat testis during development and showed that 5α-reductase levels peak at postnatal day 28, around the time when immature-type Leydig cells begin to develop. These authors further showed that 5α-reductase activity was highest in immature-type Leydig cells, as progenitor-type Leydig cells have 50% of the 5α-reductase activity compared with immature-type Leydig cells. In our immunohistochemical experiment, the number of progenitor cells staining positively for 5α-reductase type 1 was reduced by ~50% in the hypothyroid animals compared with the age-matched controls at days 28 and 35 postpartum. The number of progenitor cells positive for 5α-reductase type 2 was ~30% lower at days 21 and 28 postpartum in the hypothyroid animals but did not differ at any of the other ages. This suggests that 5α-reductase activity might be reduced in the hypothyroid testis, at least partially inhibiting testosterone to be converted into DHT and its metabolites.

At the age of 84 days, plasma testosterone levels of the hypothyroid animals and their controls did not differ significantly. The fact, however, that the size of the Leydig cell population is reduced by 37% in the hypothyroid animals suggests that testosterone production per Leydig cell may be increased under these conditions. Whether this influences Insl3 transcription is at present not clear. Recently, the relationship between Insl3 expression and androgens has been investigated in fetal-type Leydig cells (6) as well as in a mouse cell line and in primary immature Leydig cells isolated from 35-day-old rat...
testes (21), cell types not completely comparable to the mature adult-type Leydig cells in the 84-day-old hypothyroid testis. Which factor is responsible for the possible increased testosterone production per Leydig cell in the 84-day-old testis is at present not clear. Plasma LH levels did not differ significantly between the hypothyroid and control groups from the age of 49 days onward; thus LH does not seem to be involved. Other possible explanations could be an increased expression of StAR, the rate-limiting step in androgen synthesis, under hypothyroid conditions; an increased number of LH receptors on the surface of the Leydig cells, enhancing the sensitivity of the Leydig cells to LH, thus influencing testosterone production; or, because of the hypothyroid condition, other factors released in the testis that together with LH enhance androgen production per Leydig cell. Further research is necessary to obtain more insight into Leydig cell testosterone production under hypothyroid conditions and the consequences for Ins3 transcription.

In a study using the goitrin methimazole (MMI), it was shown that a high dose of MMI resulted in a more severe hypothyroid status (plasma TSH levels ~71 ng/ml) compared with the present study. Leydig cell numbers in this study were decreased dramatically on 21 days postpartum, whereas a lower dose of MMI (TSH levels ~26 ng/ml) did not influence the development of the adult-type Leydig cell population (10). These experiments suggest that a severe hypothyroid condition may nearly completely inhibit differentiation and/or proliferation of stem Leydig cells and progenitor cells, whereas a milder form of hypothyroidism may have less influence on this process. Unfortunately, most previous experiments using either MMI or PTU as goitrogen did not accurately describe the thyroid status of the animals, making it difficult to extrapolate the thyroid status as a possible explanation for the observed discrepancies with the present study. TSH levels of the PTU-treated animals were only slightly higher than in our experiment and comparable to levels in the MMI study by Cristovão et al. (10) (3, 15, 27).

Whether thyroid hormones influence Leydig cell formation directly or indirectly is less clear. In vitro studies have shown that undifferentiated stem Leydig cells are unable to differentiate into 3β-HSD-positive androgen-producing Leydig cell progenitors if T_3, insulin-like growth factor I (IGF-I), and LH are not present in the culture media (11). Although thyroxine levels were decreased by at least 90%, total T_3 levels were less significantly affected in the hypothyroid rats of the present experiment. More importantly, the availability of the active thyroid hormone T_3 was greatly reduced, since free T_3 levels were decreased to 15–50% of the euthyroid control levels, depending on the age of the animals. This could very well have postponed the formation of Leydig cell progenitors from stem Leydig cells. Furthermore, several in vitro and in vivo studies have shown that the active thyroid hormone T_3 can directly influence Sertoli cell proliferation and function such as aromatase activity (34, 48), androgen metabolism (35), and IGF-I production (33). The Sertoli cells in their turn have been reported to influence the formation of the adult-type Leydig cell population (reviewed in Ref. 13).

In the early 1990s, the presence of thyroid hormone receptors in the interstitial compartment, presumably in the Leydig cells, was shown by Tagami et al. (44), although it was not clear at that stage which type of thyroid hormone receptor was expressed. Several years later, the presence of TRα1 and TRα2 mRNA as detected by Northern blot analysis in isolated highly purified progenitor-, immature-, and mature adult-type Leydig cells was demonstrated by Hardy et al. (15), observations that were confirmed by Buzzard et al. (7) using RT-PCR and immunohistochemistry. Hence, thyroid hormones may affect Leydig cell development and function directly through binding to the TRα1.

The hypothyroid animals showed a clear decrease in LH levels on days 21–35 postpartum, the age at which Leydig cell progenitors are thought to differentiate into immature-type Leydig cells under euthyroid conditions. LH is also known to regulate testosterone production. The high levels of testosterone at these ages in the hypothyroid animals might have reduced LH plasma levels via its negative feedback mechanism on gonadotropin-releasing hormone. Nevertheless, even very low LH levels have been shown to be sufficient to induce stem cell differentiation into Leydig cell progenitors (47). These observations suggest that low LH levels during days 21–35 might have no inhibitory effects or limited inhibitory effects on the formation of the adult-type Leydig cell population.

Taken together, the data demonstrate that hypothyroidism induced by iodide deficiency resulted in a combination of undetectable levels of thyroxine, a 30% reduction in total T_3 levels, and, more importantly, a 50–85% reduction in free T_3 levels. In contrast to studies using PTU up to the fourth week of life, these low thyroid hormone levels did not result in arrested Leydig cell development if hypothyroidism was continued up to adulthood. We demonstrated that stem Leydig cell differentiation and Leydig cell progenitor and immature Leydig cell proliferation were initially delayed, but that Leydig cell proliferation recovered and remained elevated for a prolonged period of time compared with the age-matched controls. Nevertheless, there are indications that, in adulthood, the adult-type Leydig cell population in hypothyroid animals is most likely decreased in size.

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