Type 3 Deiodinase Deficiency Results in Functional Abnormalities at Multiple Levels of the Thyroid Axis

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The type 3 deiodinase (D3) is a selenoenzyme that inactivates thyroid hormones and is highly expressed during development and in the adult central nervous system. We have recently observed that mice lacking D3 activity (D3KO mice) develop perinatal thyrotoxicosis followed in adulthood by a pattern of hormonal levels that is suggestive of central hypothyroidism. In this report we describe the results of additional studies designed to investigate the regulation of the thyroid axis in this unique animal model. Our results demonstrate that the thyroid and pituitary glands of D3KO mice do not respond appropriately to TSH and TRH stimulation, respectively. Furthermore, after induction of severe hypothyroidism by antithyroid treatment, the rise in serum TSH in D3KO mice is only 15% of that observed in wild-type mice. In addition, D3KO animals rendered severely hypothyroid fail to show the expected increase in prepro-TRH mRNA in the paraventricular nucleus of the hypothalamus. Finally, treatment with T₃ results in a serum T₃ level in D3KO mice that is much higher than that in wild-type mice. This is accompanied by significant weight loss and lethality in mutant animals. In conclusion, the absence of D3 activity results in impaired clearance of T₃ and significant defects in the mechanisms regulating the thyroid axis at all levels: hypothalamus, pituitary, and thyroid. (Endocrinology 148: 5680–5687, 2007)

IN VERTEBRATES, THYROID HORMONES (TH) are critical for normal development, growth, and metabolism (1, 2). Their effects are predominantly exerted through their nuclear receptors, which, upon binding of TH, function as transcription factors to regulate gene expression (3–5).

Two TH are secreted by the thyroid: T₄, which is considered to be a prohormone, and the more active hormone T₃. Their concentrations in the plasma are tightly regulated by the hypothalamic-pituitary-thyroid (HPT) axis. TRH generated in the hypothalamus induces the secretion from the pituitary of TSH, which in turn stimulates the thyroid gland to release TH into the circulation. Serum TH provide additional regulatory control by exerting negative feedback effects on the axis at both the pituitary and hypothalamic levels. Given the pleiotropic and profound biological effects of TH, the proper regulation of the HPT axis is of great importance to numerous biological processes.

Systemic and intracellular TH concentrations are also regulated at a prereceptor level by the three selenodeiodinases, whose actions result either in the activation or inactivation of TH (6–8). Whereas the type 1 and 2 deiodinases (D1 and D2, respectively) activate T₄ by converting it to T₃, the type 3 deiodinase (D3) can convert both T₁ and T₃ into inactive metabolites by removing an iodine atom from the inner ring of these molecules (6, 9). In mammals, the D3 is highly expressed in the placenta and pregnant uterus (10–14), fetal and neonatal tissues (15, 16) as well as in the developing and adult brain (17, 18).

Recently we generated a D3-deficient (D3KO) mouse by inactivation of the Dio3 through homologous recombination (19, 20). The lack of D3 activity in these mice results in perinatal thyrotoxicosis as indicated by the marked increase in the serum T₃ level observed during development. As adults, D3KO mice develop a moderate hypothyroidism characterized by a low serum T₄ level and a modest decrease in the serum T₃ level that persists throughout life (20). The hypothyroidism observed in D3KO mice appears to be due primarily to central impairment of the HPT axis, because the serum TSH level is only minimally elevated in the face of the low levels of serum TH. In the present study, we have analyzed the physiology of the HPT axis in the D3KO mouse and have identified functional defects at all levels of the axis. These findings help explain the altered thyroid parameters in this mouse model and highlight the important role of D3 in the establishment and function of the HPT axis.

Materials and Methods

Animals and treatments

All experiments described herein were performed in adult 129/Sv male mice 3–4 months of age. Animals were kept on a 12-h light, 12-h dark cycle and were given food and water ad libitum. Mice were euthanized by asphyxiation with carbon dioxide. All experiments were approved by the Dartmouth College Institutional Animal Care and Use Committee.

Wild-type (WT) and D3KO mice were generated by breeding animals heterozygous for the D3 mutation. Genotyping was performed by
genomic PCR on DNA samples isolated from tail snips as previously described (20).

Hyperthyroidism was induced by providing the animals with T3 in the drinking water for a period of 1 month. The concentrations of T3 used were 0.1 and 0.25 μg/ml. During these treatments, no significant difference in the amount of water consumed was observed between WT and D3KO mice. Anti-thyroid serum was induced in the same manner in mice injected ip with T3. In these cases, the serum T3 levels were suppressed by more than 90%.

TRH and TSH response tests

To measure pituitary responsiveness to TRH, a blood sample was taken from the orbital sinus of the animals just before TRH injection (time = 0); 100 ng TRH (Sigma Chemical Co., St. Louis, MO) was then injected ip into each, and 20 min later, a second blood sample was taken from the opposite orbital sinus. Two hours and 20 min after TRH injection, the mice were euthanized, and blood samples were taken from the inferior vena cava. To analyze the thyroidal response to TSH, mice were provided with T3 in the drinking water (0.1 μg/ml) for 2 wk. A blood sample was then taken from the orbital sinus to confirm that the T3 level was suppressed. Animals were then injected ip with either 2 μl or 10 μl bovine TSH (Sigma). Blood samples were taken 3 h after TSH injection using the same procedure.

In situ hybridization of TRH mRNA

After harvesting, whole brains were placed in ice-cold PBS (pH 7.4) containing 4% paraformaldehyde and incubated at 4°C overnight, followed by incubation for 16 h at 4°C in the same solution plus sucrose (30% w/v). Brains were then embedded in OCT compound (Triangle Biomedical Science, Durham, NC), frozen in dry ice, and stored at −70°C for subsequent sectioning and analysis. Tissue sections (25 μm) were cut at −20°C in a cryostat, thaw-mounted onto Superfrost slides (Fisher Scientific Co., Pittsburgh, PA), and dried at 37°C overnight. To improve the accessibility of the probes to the mRNA, the thaw-mounted sections (25 μm thick) were stained with hematoxylin and eosin using a coverslip applied. Slides were incubated in humid chambers at 55°C for 2 h and then in 0.1 M captoethanol at 55°C for 2 h.

The samples were then fixed with 0.25% acetic anhydride for 10 min, postfixed in 4% paraformaldehyde (Sigma) containing 0.25% acetic anhydride for 10 min, postfixed in 4% paraformaldehyde for 10 min, and dehydrated in a series of ethanol solutions (40, 60, 80, and 90%) containing 0.25% acetic anhydride for 10 min, postfixed in 4% paraformaldehyde for 10 min, and dehydrated in a series of ethanol solutions (30, 50, 70, and 90%) for 2 min each, followed by 100% ethanol for 10 sec. Between each treatment, slides were washed in PBS for 5 min. Slides were air dried at room temperature. TRH sense and antisense RNA probes were labeled in vitro transcription using the T3 and T7 polymerases (Maxi-Script kit from Ambion, Austin, TX). In each case, an appropriately linearized p Bluescript plasmid (Strategene, La Jolla, CA) containing 0.8 kb mouse TRH cDNA was used as a template. The probes were purified using the ammonium acetate/ethanol precipitation protocol described by Ambion (Maxi-Script kit from Ambion, Austin, TX). In each case, a coverslip was applied. Slides were incubated in humid chambers at 37°C overnight.

Histological analysis

Thyroid glands with the trachea attached were collected from adult mice, placed in formalin, and embedded in paraffin. Mid-thyroid sections (4 μm thick) were stained with hematoxylin and eosin using standard procedures. Estimates of thyroid gland size and the area of individual follicular size were obtained using Image (NIH image software) in several tissue sections through the mid-portion of the gland.

Statistical analysis

Statistical significance between two groups was determined by the Student’s t test. Statistical significance among multiple groups was determined by ANOVA followed by Fisher’s least significant difference analysis using the GB STAT computer software on a Macintosh computer.

Results

The response of the thyroid axis to TRH was assessed in WT and D3KO mice. As we have described recently (20), the
serum TSH level in D3KO animals at baseline is approximately 50% higher compared with WT mice, although this difference is not clearly appreciated in Fig. 1A given the scale of the y-axis (baseline values were 61.0 ± 5.6 and 95.2 ± 8.9 mU/liter in WT and D3KO mice, respectively, \( P < 0.01 \)). Twenty minutes after TRH injection, a marked elevation (15-fold) in the serum TSH level was observed in WT mice (Fig. 1A). In D3KO mice, the serum TSH level was also stimulated by TRH but significantly less (only a 5-fold elevation), and the absolute level of TSH reached was considerably lower (D3KO, 475 ± 43 vs. WT, 742 ± 82 mU/liter, \( P < 0.01 \)). In both groups of mice, the serum TSH level had returned to baseline 140 min after the injection.

In WT mice, the rise in the serum TSH level induced by TRH injection led, as expected, to a significant increase in serum TH levels. Thus, at 140 min after TRH injection, serum T3 and T4 levels were increased 50 and 25%, respectively, in WT animals when compared with initial levels before TRH injection (bars on the left side of Fig. 1, B and C). In contrast, T3 and T4 levels were not increased in the D3KO mice (Fig. 1, B and C, bars on the right), despite the observed 5-fold increase in the serum TSH level. In fact, the serum T3 level was slightly and significantly reduced.

These results indicate an impaired responsiveness of the pituitary of D3KO mice to TRH. In addition, the D3KO thyroid gland responds poorly to endogenously secreted TSH.

**TSH bioactivity**

To investigate the possibility of altered TSH bioactivity in the D3KO mice, we collected serum from both WT and D3KO mice under basal and hypothyroid conditions and examined these samples for TSH bioactivity in a cultured TSH-responsive CHO cell line (23). As shown in Fig. 2, TSH bioactivity expressed as cAMP production relative to TSH immunoreactivity was not significantly different in the serum samples taken from WT and D3KO mice. The similarity in TSH bioactivity was observed both under control conditions and in animals treated with MMI/ClO4\(^-\), where the TSH level was elevated. These results indicate that there is no significant abnormality in the bioactivity of the TSH produced by D3KO mice.

**Thyroid stimulation test and thyroid size**

Because the TSH secreted by the D3KO mouse is fully bioactive, our previous observation of slight elevation in serum TSH in the presence of low serum levels of both T3 and T4 in the basal state and the blunted response of the D3KO thyroid to the rise in endogenous TSH resulting from TRH administration (Fig. 1) suggested that the thyroid does not respond appropriately to TSH. To explore this possibility directly, we performed a TSH stimulation test. WT and D3KO mice were treated with 0.1 \( \mu \text{g/ml} \) T3 in the drinking water for 2 wk to suppress TSH and serum T4 levels. With this treatment, the serum T4 level was just above the limits of detectability (Fig. 3A). The mice were then injected with 2 mU bovine TSH. Three hours later, the serum T4 level was approximately 3-fold higher in WT mice than in D3KO mice after TSH injection (Fig. 3A). Similar results were observed with a higher dose of TSH (10 mU).

Histological analysis revealed a significant reduction in thyroid gland size (Fig. 3, C vs. B), and in the average follicular size (Fig. 3, E vs. D) in the D3KO animal. Using computer-assisted quantification of midsections of the thyroid, we estimate that the D3KO thyroid is only one third the size of the WT gland and half the normal size when adjusted for body weight (Fig. 3F). In addition, average thyroid follicular size was reduced 50% in D3KO mice.

**Response to induced hyper- and hypothyroidism**

To evaluate how the HPT axis of D3KO mice adjusts to altered TH levels, we measured serum T3, T4, and TSH in WT and D3KO mice after induction of hyper- and hypothyroidism. In WT animals, the addition of T3 to the drinking water at two different concentrations (0.1 and 0.25 \( \mu \text{g/ml} \)) resulted, respectively, in 2- and 6-fold increases in the serum T3 level (Fig. 4A, white bars). In D3KO mice, these treatments produced much larger increases (16- and 34-fold elevation) in this parameter (Fig. 4A, black bars). These results suggest that the absence of D3 activity significantly compromises T3 clearance.

The serum T4 level is lower in D3KO mice than in WT mice (20), and it was markedly suppressed in both WT and D3KO animals after T3 treatments (Fig. 4B). As shown previously (20), the serum TSH level was elevated 50% in untreated D3KO mice (Fig. 4C). Treatment with T3 (0.1 \( \mu \text{g/ml} \)) resulted in an undetectable level of TSH in the serum of both WT and
D3KO mice (dotted line in Fig. 4C represents the sensitivity of the assay).

As reported previously (20), the baseline serum T₃ level in D3KO mice was 20% lower than that in WT animals (Fig. 5A, bars on the left). Hypothyroidism induced by MMI/ClO₄⁻ treatment decreased the serum T₃ level to approximately 60% of baseline level in both WT and D3KO animals (Fig. 5A, bars on the right) and markedly suppressed serum T₄ to a level that was less than 5% of the normal value (Fig. 5B). The same level of T₄ suppression appeared to be achieved in both WT and D3KO mice. It is notable that MMI/ClO₄⁻ treatment resulted in a marked increase (180-fold) of the serum TSH level in WT mice (Fig. 5C), whereas this rise was significantly blunted in D3KO animals (30-fold increase) and reached an absolute value that was less than 20% of that observed in WT mice. This marked discrepancy in TSH responsiveness occurred despite serum levels of T₄ and T₃ that were comparably low in both groups. These data demonstrate a defect in the HPT axis of the D3KO mouse that results in a blunting of the TSH response to hypothyroidism.

Treatment with T₃ and antithyroid drugs resulted in significant differences in the changes in body weight between WT and D3KO mice. Control animals of both genotypes
showed a similar small weight gain during the 1-month period, whereas animals receiving antithyroid drugs did not show such weight gain (Fig. 6A). After T3 treatment, WT mice given the lower T3 dose had the same weight as the WT control animals, whereas D3KO mice given the same treatment showed significant weight loss. This weight change in response to T3 treatment was greater with the higher T3 dose and more pronounced in the D3KO animals. Thus, after high-dose T3 treatment, WT mice exhibited a 10% increase in weight, whereas D3KO mice lost 20% of body weight.

A significant number of deaths occurred in D3KO mice subjected to these treatments, particularly those receiving T3 (Fig. 6B). In contrast to WT mice, where no deaths were observed in 34 animals treated with either dose of T3, 45% of D3KO mice (29 of 64) died during the month of treatment (P<0.001). Lethality was 39% (11 deaths in 28 mice) and 50% (18 deaths in 36 mice) after treatment with, respectively, 0.1 and 0.25 μg/ml T3, suggesting a correlation between morbidity and the T3 concentration achieved. No deaths were observed in the 16 WT animals treated with MMI/ClO4, but five D3KO mice of 27 (18.5%) died during the antithyroid treatment. Regarding untreated animals, no deaths were observed during this period in 20 WT animals, but two D3KO mice of 12 (17%) died.

Hypothalamic TRH expression

An integral part of the regulation of the thyroid axis is the TH-dependent expression of TRH in the hypothalamus. Studies by other investigators (25) have demonstrated that TRH expression in the hypothalamus is significantly influenced by TH status only in the paraventricular nucleus (PVN). We performed in situ hybridization in coronal brain...
sections of WT and D3KO mice under control conditions and in mice that were rendered hypothyroid with MMI/ClO$_4$ treatment. The expression of prepro-TRH mRNA was largely confined to the hypothalamus. The hybridization signal was specific, because no signal was detected when using a sense riboprobe (Fig. 7). We analyzed in detail the brain sections encompassing the PVN. The autoradiographs revealed that in this area, TRH expression is very similar in WT and D3KO mice (Fig. 7, B vs. C). As expected, antithyroid treatment resulted in a marked increase in TRH expression in WT mice (Fig. 7, B vs. F); however, no increase was observed in D3KO mice (Fig. 7, C vs. G). None of the hypothalamic sections from MMI/ClO$_4$−-treated D3KO mice showed any significant increase in TRH expression relative to control animals, whereas several consecutive sections of the corresponding WT animals showed a level of expression as high as the one shown in Fig. 7F. We obtained similar results when in situ hybridization of prepro-TRH mRNA was performed in sections from another set of animals and the signal was detected using photographic emulsion (data not shown). These results indicate that the normal TH-dependent regulation of TRH expression in the hypothalamic PVN is impaired in the D3KO mouse.

**Discussion**

We have recently reported that D3 deficiency in mice results in perinatal thyrotoxicosis, with the subsequent development of hypothyroidism in the adult that persists throughout the life of the animal (20). Thus, adult D3KO mice demonstrate low serum T$_4$ and T$_3$ levels along with a serum TSH level, which although mildly elevated, is low relative to the degree of hypothyroidism they exhibit. To gain further insights into the pathophysiological mechanisms underlying this phenotype, we examined in D3KO mice the response of the HPT axis and its individual components to a variety of challenges, including alterations in serum TH concentrations.

We speculated previously that central mechanisms might play a principal role in the hypothyroidism of the D3KO mouse (20). Thus, we suggested that the decreases in serum T$_3$ and T$_4$ levels in the face of a mild elevation in TSH resulted from the secretion of a TSH molecule with reduced bioactivity. Such a phenomenon has been observed in other mouse models of central hypothyroidism, where the TSH level is also minimally elevated (26, 27). However, the results reported herein demonstrate that this is not the case. As directly assessed using cultured cells expressing the TSH receptor, the TSH in the serum of D3KO mice, under both basal and hypothyroid conditions, manifests normal bioactivity.

This unexpected result suggests that a defect in the thyroid gland itself is responsible for the observed decreases in serum T$_4$ and T$_3$ levels in this animal model. This thesis was confirmed by the results of the TSH stimulation test, whereby the increase in the serum T$_4$ level in response to the administration of exogenous TSH was markedly blunted in the D3KO animal. Similarly, the secretory response of the thyroid to a 5-fold increase in endogenous TSH, as occurred during the TRH stimulation test, was also markedly blunted; indeed, no increases in serum T$_4$ and T$_3$ levels were noted in the D3KO mouse despite the reduced clearance of these compounds that occurs in these animals. Histological analysis of the thyroid gland suggests that the reduced size of the D3KO thyroid gland as well as the reduced follicular size contribute to the impaired thyroid gland function in these animals.

It could be argued that the interpretation of the results of these stimulation tests is complicated to some extent by the fact that serum T$_4$ and T$_3$ levels are decreased in the D3KO animal under basal conditions. However, the combination of low serum TH levels in the face of a mildly elevated level of TSH of normal bioactivity serves to reinforce the concept that the hypothryoid phenotype results from an impaired response of the thyroid to its trophic hormone. The profile of hormones in serum also renders irrelevant any concern that the impaired response to exogenous TSH results from an enhanced rate of clearance of the injected hormone.

Thus, regulatory or synthetic defects in the thyroid gland of the adult D3KO mouse appear to be a major consequence of D3 deficiency. Because D3 has not been detected in the adult rodent thyroid gland (28), this abnormality in thyroid function likely results from indirect effects of the D3 deficiency that alter the developmental or functional program of this organ.

This demonstration of defective thyroid function does not negate the possibility that central defects in the HPT axis also impact the thyroid status of the adult D3KO mouse. Indeed, our original observation that the TSH level, although mildly elevated, is relatively low for the degree of hypothyroidism as judged by serum T$_4$ and T$_3$ levels suggests that abnormalities are present in hypothalamic and/or pituitary function in this animal. This does indeed appear to be the case. The expected rise in the TRH mRNA level in the PVN as a result of hypothyroidism is significantly blunted in the D3KO mouse. We also demonstrated a very significant blunting of the TSH response to exogenously administered TRH, indicating abnormal responsiveness of the pituitary gland as well.

The response of the HPT axis to hypothyroidism in the D3KO mouse provides additional evidence of central defects in this animal. After treatment with MMI/ClO$_4$−, serum T$_3$ and T$_4$ were suppressed to similar levels in both WT and D3KO mice. However, the serum TSH response in the face of this hypothyroid challenge was markedly impaired in the D3KO mice; the serum level of TSH rose to only 15%
of the level observed in WT animals. Although it is likely that the blunted response of TRH in the hypothalamus and of TSH to TRH stimulation in the pituitary may be playing important roles in the impaired HPT axis response to hypothyroidism, other factors such as the effectiveness of TH feedback need to be examined in future studies. In this regard, we did administer T₃ at two dose levels on a chronic basis to WT and D3KO animals in the present studies. However, the TSH level was undetectable in all treated animals, precluding any assessment of differential sensitivity of the HPT axis in WT vs. D3KO mice to feedback inhibition by TH.

Important information was obtained from experiments in which mice were administered T₃ and rendered hyperthyroid. At both dosages of T₃, the serum level of this hormone was markedly higher in the D3KO animals than in WT mice. This observation demonstrates that in the setting of T₃ excess, D₃ plays a critical role in the clearance of this hormone and thus in protecting tissues from thyrotoxicosis. It is important to note that this conclusion applies to adult animals, in which D₃ expression is generally more limited than during development, and the brain and the skin are the only large adult tissues with high D₃ expression. As expected, D₃ expression in brain was markedly increased in WT mice treated with T₃ (data not shown). The lower serum T₃ levels in the D3KO mouse seem inconsistent with impairment in T₃ clearance. However, the latter may be due to the reduced serum T₃ and liver D₁ activity previously observed (20) as well as to the impairment in thyroid function reported here.

This protective effect of D₃ in adult hyperthyroidism is substantiated by the significant weight loss and notable lethality of D3KO mice during T₃ administration. Although chronic hyperthyroidism is known to result in weight loss, no such observation was made in T₃-treated WT animals compared with untreated controls. This could be explained by the fact that the T₃ was administered in the drinking water, which is a slow but steady method of inducing hyperthyroidism. During the treatment, both WT and D3KO mice initially gained weight before they started to lose it after onset of significant thyrotoxicosis (data not shown). Indeed, WT mice were losing weight at the end of the treatment, although their weight was still higher than that measured before the initiation of treatment. Our results likely reflect the fact that when using this specific protocol, D3KO mice have more difficulty clearing the excess T₃ and the onset of thyrotoxicosis and associated weight loss occur much sooner. Although food consumption is not a variable analyzed in this experiment, the initial weight gain in T₃-treated animals of both genotypes as well as in the untreated controls suggest that this is not likely an important factor.

Taken together, these results define for the first time a critical role for the D₃ in establishing the normal functioning of the HPT axis. Thus, the congenital absence of this enzyme results in marked abnormalities in the function of the hypothalamus, pituitary gland, and the thyroid gland. As a consequence, the D3KO animal exhibits a hypothyroid phenotype and impaired responsiveness to alterations in TH levels. At present, the molecular correlates of these abnormal physiological responses remain undefined.

These findings may have important clinical implications. They suggest that different degrees of TH exposure during development may be an important factor in determining the set-point of the HPT axis in adulthood. Indeed, perinatal thyrotoxicosis, such as occurs in the setting of poorly controlled maternal Graves’ disease, is known to result in central hypothyroidism (29, 30). Although most clinical reports suggest that this central hypothyroidism is transient, the long-term effects of perinatal thyrotoxicosis on the function and responsiveness of the thyroid axis are only now starting to be examined. Thus, consistent with our findings of significant abnormalities in the thyroid gland of the D3KO mouse, Kempers et al. (31) have recently reported that a significant proportion of children born to mothers with poorly controlled Graves’ disease manifest primary hypothyroidism in later childhood. This thyroidal dysfunction is characterized, at least in some cases, by a small thyroid gland as determined by ultrasound examination and low radioactive iodide uptake.

Our findings are also relevant to infants born to mothers with elevated serum TH levels due to resistance to TH. These infants have decreased birth weight (32) and as adults exhibit lower serum TSH concentrations relative to their serum TH levels (S. Refetoff, unpublished results). Finally, one might speculate that should genetic, medical, or environmental conditions be defined in the future whereby D₃ activity is impaired, these situations may be accompanied by significant alterations in the thyroid axis and an impaired ability of the individual to adapt to alterations in thyroid function.

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