Experimental Hypothyroidism Inhibits δ-Aminolevulinate Dehydratase Activity in Neonatal Rat Blood and Liver

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The aim of this study was to investigate the potential relationship between hypothyroidism and δ-aminolevulinate dehydratase (δ-ALA-D) activity in rat blood and liver. Experimental hypothyroidism was induced in weanling rats by exposing their mothers to propylthiouracil (PTU) diluted in tap water (0.05% w/v), *ad libitum*, during the lactational period (PTU group). Control (euthyroid) group included weanling rats whose mothers received just tap water, *ad libitum*, during the lactational period. Reverted-hypothyroid group (PTU + 3,3′,5-triiodo-L-thyronine [T_3]) included weanling rats whose mothers were exposed to PTU similarly to those in the hypothyroid group, but pups received daily subcutaneous injections of T_3 (20 μg/kg, from Postnatal Days 2–20). After the treatment, serum T_3 levels were drastically decreased (around 70%) in the PTU group, and this phenomenon was almost reverted by exogenous T_3. PTU decreased blood δ-ALA-D activity by 75%, and T_3 treatment prevented such phenomena. Erythrocytes and hemoglobin levels were increased by 10% in PTU-treated animals and higher increments (around 25%) were observed in these parameters when exogenous T_3 was coadministered. Dithiothreitol did not change blood δ-ALA-D activity of PTU-exposed animals when present in the reaction medium, suggesting no involvement of the enzyme's essential thiol groups in PTU-induced δ-ALA-D inhibition. PTU did not affect blood δ-ALA-D activity *in vitro*. These results are the first to show a correlation between hypothyroidism and decreased δ-ALA-D activity and point to this enzyme as a potential molecule involved with hypothyroidism-related hematological changes. Exp Biol Med 232:1021–1026, 2007

Key words: hypothyroidism; anemia; thyroid hormones; propylthiouracil; δ-aminolevulinate dehydratase

Introduction

Thyroid diseases, particularly hypothyroidism, have been associated with pathological alterations in the cardiovascular system. In fact, hypothyroidism has been reported to induce size and function abnormalities of the heart (1), increased vascular resistance (2), increased arterial wall thickness (3), and decreased blood volume (4). In addition, anemia has been reported as an important clinical feature of hypothyroidism (5). Aside from the well-known signs of anemia observed in adult hypothyroid patients, anemia is also a frequent finding in infants with congenital hypothyroidism (6). Though it is well known that thyroid hormones stimulate growth of erythroid colonies (erythrocyte burst-forming unit [BFU-E], erythrocyte colony forming unit [CFU-E]), literature data on the molecular mechanisms related to hypothyroidism-induced anemia are scarce.

It has been evidenced that the activity of some enzymes involved in the heme metabolism are modulated by thyroid hormones. In this regard, 3,3′,5-triiodo-L-thyronine (T_3) was found to stimulate δ-aminolevulinate synthase (the initial and rate-limiting enzyme in heme synthesis) activity in the liver of thyroidectomized rats (7). In addition, Kleiman *et al.* (8) showed that T_3 treatment increased

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porphobilinogen (PBG) deaminase-uroporphyrinogen III cosynthase activity in the liver of thyroidectomized rats, but did not affect heme oxygenase and δ-aminolevulinate synthase activity. Conversely, Smith *et al*. (9) showed that T_3, but not reverse T_3, is able to stimulate basal hepatic heme oxygenase activity in thyroidectomized rats. Hence, data on T_3 effects on enzymes involved in the heme metabolism appear to be controversial. In addition, to the best of our knowledge, there are no data in the literature concerning thyroid function and δ-aminolevulinate dehydratase (δ-ALA-D) activity.

δ-ALA-D is an essential enzyme in most organisms, catalyzing the condensation of two molecules of δ-aminolevulinic acid (δ-ALA) to form the monopyrrole PBG (10), which, in subsequent steps, is assembled into tetrapyrrole molecules that constitute the prosthetic groups of many proteins, such as hemoglobin (Hb), cytochromes, and enzymes such as catalase (11, 12). This enzyme is a sulfhydryl-containing protein, and its activity is very sensitive to the presence of elements and/or compounds that possess a strong affinity for –SH groups (13–20). δ-ALA-D activity is also decreased after chronic ethanol intake (21, 22) and in insulin and non–insulin-dependent diabetes mellitus patients (23). In addition, the relationship between oxidative stress and δ-ALA-D inhibition has been shown (24). This inhibition results in the accumulation of δ-ALA, which has pro-oxidant activity *per se* (25).

Taking into account the lack of molecular mechanisms involved with hypothyroidism-induced anemia, and the crucial role of δ-ALA-D in the biosynthesis of heme (the prosthetic groups of Hb), this study was aimed at investigating potential relationships between hypothyroidism and δ-ALA-D activity in rat blood and liver in an experimental model of neonatal hypothyroidism induced by maternal propylthiouracil (PTU) exposure. The potential reactivation of δ-ALA-D activity with the reducing agent, dithiothreitol (DTT), and the possible direct inhibitory effect of PTU toward blood δ-ALA-D were also investigated under *in vitro* conditions in order to elucidate potential molecular mechanisms related to the hypothyroidism-induced decrease in δ-ALA-D activity.

Materials and Methods

Animals and Treatment. Adult Wistar rats (male and female), 90 days old, from our own breeding colony, were maintained at 22 ± 2°C (mean ± SD), on a 12:12-hr light:dark cycle, with free access to food (Nuvilab CR-1 Nuvital, Curitiba, Paraná, Brazil) and water. The breeding regimen consisted of grouping 3 virgin females with 1 male for 5 days. Pregnant rats were selected and housed individually in opaque plastic cages. On the first day after parturition (Postnatal Day [PND] 1), dams were randomly assigned to one of three groups—control (euthyroid), hypothyroid (PTU), and reverted hypothyroid (PTU + T_3)—of eight animals each. Pups (eight per litter) were maintained with their mothers during the treatment period (PND 1–21, lactational period). Dams from the hypothyroid group received PTU diluted in tap water (0.05% w/v), *ad libitum*, during the lactational period (PTU group), and pups received a daily injection of vehicle (4 m*M* NaOH, 10 ml/kg). The control (euthyroid) group included weanling rats whose mothers received just tap water, *ad libitum*, during the lactational period, and pups received daily subcutaneous injections of vehicle (NaOH 4 m*M*, 10 ml/kg). Dams from the reverted hypothyroid (PTU + T_3) received PTU diluted in tap water (0.05% w/v), *ad libitum*, during the lactational period (PTU group), and pups received daily injections of T_3 (daily subcutaneous injections, 20 μg/kg, dissolved in 4 m*M* NaOH, 10 ml/kg). Liquid and solid ingestions by mothers were monitored daily. All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989, and all experiments were approved by our ethics committee for animal use at the Universidade Federal do Rio Grande do Sul.

Tissue Preparation. At 24 hrs after the last injection (at PND 21), rats were killed by decapitation. Only male pups were included in the study. Blood samples were colleted in tubes containing heparin, and livers were quickly removed, placed on ice, and homogenized (1:30) in 80 m*M* potassium phosphate buffer, pH 6.4.

δ-ALA-D Activity. Erythrocyte and hepatic δ-ALA-D activity were determined based on the method previously described by Sassa (26), by measuring the rate of product formation (PBG) in a medium containing δ-ALA (4 m*M*) and 80 m*M* potassium phosphate buffer, pH 6.4, at 35°C. After 1 hr of reaction, the product (PBG) was determined using Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1 \times 10^4\ M^{-1}$ for the Ehrlich-PBG salt.

***In Vitro* Studies.** The potential inhibitory effect of PTU toward blood δ-ALA-D was evaluated under *in vitro* conditions. The methodology was very similar to that used in *ex vivo* studies, except that different concentrations of PTU (0–4 m*M*, dissolved in ethanol) were added to the reaction medium during the enzymatic assay. Ethanol concentrations did not exceed 25 m*M* and did not affect δ-ALA-D activity *per se*.

Protein Determination. Protein was measured by the method of Lowry *et al*. (27) using serum albumin as standard.

Serum T_3 and Thyroxine (T_4) Assay. Serum-free T_4 and T_3 were measured by electrochemiluminescent method, using commercial kits (Roche, Mannheim, Germany), and its concentration determined in automated analyzer system (ELECSYS 2010, Roche), following the manufacturer's procedures, with an internal and external quality control program (PELM; Control-Lab, Rio de Janeiro, Brazil). Assay sensitivities for T_3 and T_4 were 0.300 nmol/liter (0.195 ng/ml) and 5.40 nmol/liter (0.42 μg/dl), respectively. For T_3 measurements, inter- and intraassay variations presented a percent variation coefficient of 5.4%

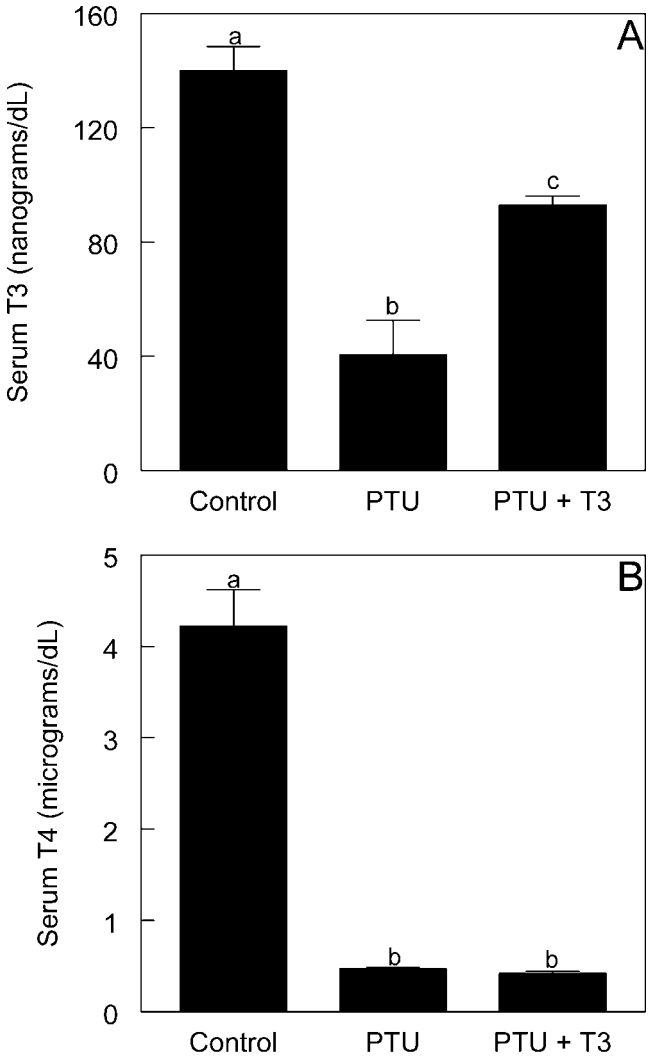

Figure 1. Serum T_3 (A) and T_4 (B) levels in weanling rats at PND 21. For treatment details, see Materials and Methods section: euthyroid (control), hypothyroid (PTU), and reverted-hypothyroid (PTU + T_3). Data are expressed as ng/dl (T_3) and μg/dl (T_4), and are presented as mean ± SEM ($n = 8$ per group). Different letters indicate significant difference ($P < 0.05$) by one-way analysis of variance followed by Duncan's multiple range test.

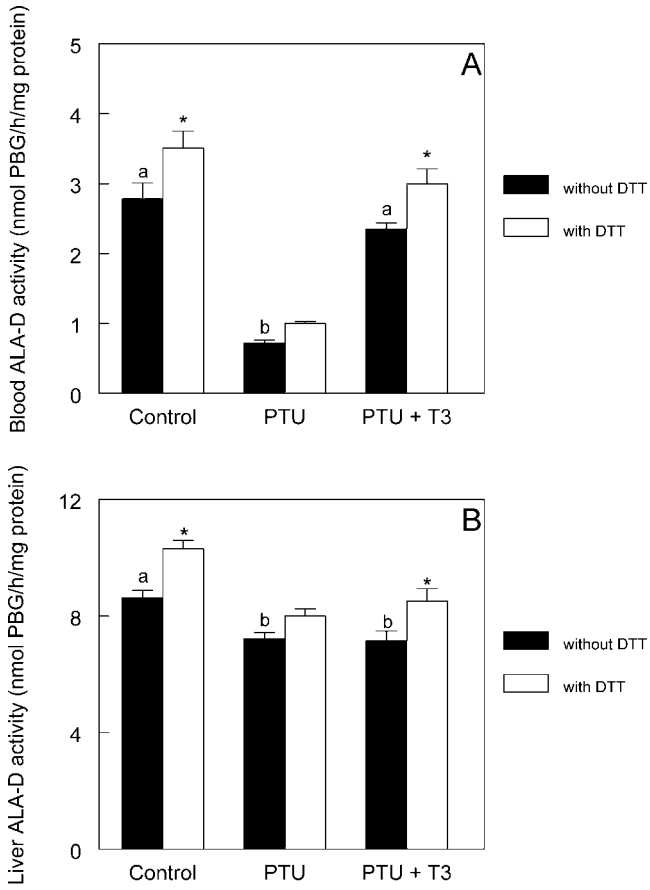

Figure 2. Blood (A) and hepatic (B) δ-ALA-D activity in weanling rats at PND 21. For treatment details, see Materials and Methods Section: euthyroid (control), hypothyroid (PTU), and reverted hypothyroid (PTU + T_3). Data are expressed as nmol of PBG/mg protein/hr and presented as mean ± SEM ($n = 8$ per group). Parallel experiments with the reducing agent, DTT (2 m*M*), were carried out. Different letters indicate significant difference ($P < 0.05$) by one-way analysis of variance followed by Duncan's multiple range test (treatment effect). * Significant difference ($P < 0.05$) when compared with the respective control without DTT in the reaction medium.

and 3.6%, respectively. For T_4 measurements, inter- and intraassay variations presented a percent variation coefficient of 6.9% and 4.7%, respectively.

Hematological Parameters. Red blood cell (RBC) and Hb levels were analyzed by automated parameter hematology analyzer (MICROS 60, Abx Diagnostics, Montpellier, France).

Statistical Analysis. All data are presented as mean ± standard error of mean (SEM) ($n = 8$ animals per group, one pup per litter). Differences between groups were performed by one-way analysis of variance, followed by Duncan's *post hoc* test ($P \leq 0.05$ was considered significant). Pearson's correlation analyses (two-tail) were performed in order to correlate T_3 levels and δ-ALA-D activity.

Results

Liquid and solid ingestions by dams during lactation were not different between groups. Signs of overt toxicity were not observed in lactating dams. In addition, the body weight of dams and pups at the end of treatments were not statistically different between groups (data not shown).

Figure 1 depicts T_3 (Fig. 1A) and T_4 (Fig. 1B) levels in the serum of weanling rats. The lactational exposure to PTU induced a significant decrease (about 70%) in the serum levels of T_3 (Fig. 1A). The coadministration of exogenous T_3 partially abolished this phenomenon: serum T_3 levels in the PTU + T_3 group were more than twice as high as those in the PTU group. PTU exposure during the lactational period also decreased (about 90%) serum T_4 levels when compared with control animals, and this phenomenon was not prevented by exogenous T_3 administration (Fig. 1B).

Blood δ-ALA-D activity was significantly decreased (around 75%) after PTU treatment, and this phenomenon

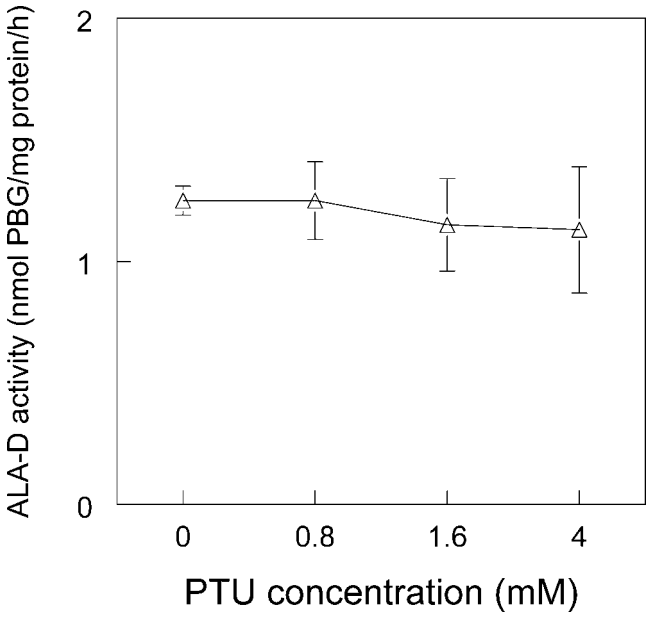

Figure 3. *In vitro* effect of PTU on blood δ-ALA-D activity. Different concentrations of PTU (0–4 m*M*) were added to a medium containing 80 m*M* potassium phosphate buffer, pH 6.4, and 1 mg protein samples. Only euthyroid rats were used. Enzyme assays were carried out at 35°C and started by adding 4 m*M* δ-ALA. After 1 hr of reaction, the product (PBG) was determined using Ehrlich's reagent at 555 nm (for details, see Materials and Methods). Data are expressed as nmol of PBG/mg protein/hr and presented as mean ± SEM for four independent assays. One-way analysis of variance showed no significant *in vitro* effects of PTU on blood δ-ALA-D.

was totally reverted by the coadministration of exogenous T_3 (Fig. 2A). Interestingly, the reducing agent, DTT, did not reverse PTU-induced decrease in δ-ALA-D activity. Hepatic δ-ALA-D activity was also significantly decreased after PTU treatment, but to a lesser extent when compared with blood enzyme (Fig. 2B). However, this phenomenon was not reverted by the coadministration of exogenous T_3.

The potential inhibitory effect of PTU toward blood δ-ALA-D was evaluated under *in vitro* conditions in an attempt to elucidate molecular mechanisms involved with PTU-induced decrease in δ-ALA-D activity. PTU, at concentrations ranged from 0 to 4 m*M*, did not change δ-ALA-D activity when present in the reaction medium (Fig. 3).

RBCs and Hb were the parameters selected to evaluate possible hematological changes induced by PTU treatment (Table 1). Both parameters were significantly increased after PTU treatment. Interestingly, a higher increase was observed for both parameters when exogenous T_3 was coadministrated (Table 1).

Discussion

PTU is an antithyroid drug widely used for the treatment of hyperthyroidism (28). Aside from the beneficial properties of PTU in the treatment of hyperthyroidism, this drug has also been reported to be a useful experimental tool for elucidating the physiologic roles of thyroid hormones

Table 1. Hematological Parameters in Weanling Rats at PND 21[a]

Group/Parameter	RBC ($\times 10^6/\mu l$)	Hb (g/dl)
Control	5.25 ± 0.08*	9.6 ± 0.25*
PTU	5.80 ± 0.12**	10.7 ± 0.24**
PTU + T_3	6.55 ± 0.12***	12.0 ± 0.15***

[a] Values are mean ± SEM ($n = 8$ per group). RBC, red blood cells; Hb, hemoglobin; control, euthyroid; PTU, propylthiouracil (hypothyroid); PTU + T_3, PTU + 3,3′,5-triiodo-L-thyronine (reverted hypothyroid). See Materials and Methods section for treatment details. Different numbers of asterisks indicate significant difference ($P < 0.05$) by one-way analysis of variance followed by Duncan's multiple range test (treatment effect).

(29–32). Here, we used the PTU-induced hypothyroidism protocol for studying the potential relationship between thyroid hormones, hematological changes, and δ-ALA-D activity. Measurements of the serum levels of thyroid hormones showed that our protocol of exposure, which was based on the lactational exposure to PTU, was able to induce significant changes in the levels of serum T_3 and T_4 in the weaning period. Indeed, serum T_3 and T_4 levels were around 70% and 90% lower in PTU-exposed animals, respectively, when compared with control animals. In addition, the exogenous administration of T_3 increased serum T_3 levels in PTU-treated animals, indicating that our experimental protocol was accurate for studying the effects of hypothyroidism and reverted hypothyroidism.

An important finding of our study was the decreased activity of blood δ-ALA-D in hypothyroid animals when compared with those of the control group. Even though changes in the activities of enzymes related to heme metabolism (δ-aminolevulinate synthase, PBG deaminase-uroporphyrinogen III cosynthase, and heme oxygenase) have been reported to be modulated by thyroid hormones (7–9), our study is the first to show that δ-ALA-D is a potential molecular target for the effects of thyroid hormones, particularly T_3. This enzyme catalyzes a crucial reaction of the heme biosynthetic pathway (10), allowing for the adequate synthesis of hemoproteins, such as Hb. In this regard, the relationship between decreased δ-ALA-D activity and signs of anemia in humans has been reported (33, 34). Regarding the potential linkage between T_3 function and δ-ALA-D activity, a significant positive correlation (Pearson's coefficient = 0.853; $P < 0.01$) was detected between serum T_3 levels and blood δ-ALA-D activity in our study. It is noteworthy that the PTU-induced decrease of blood δ-ALA-D activity was completely reverted by the exogenous administration of T_3, reinforcing the idea that this hormone modulates the activity of such an enzyme.

Mammalian δ-ALA-D is a sulfhydryl-containing enzyme, the activity of which is decreased during oxidative insults. In this regard, heavy metals (13, 14, 17–20), oxidant xenobiotics (35), and some additional pro-oxidant condi-

tions (36) have been reported to decrease δ-ALA-D activity, and these phenomena appear to be related to the oxidation of sulfhydryl groups located at the active center of the enzyme (37). It is important to state that reducing agents, such as DTT and reduced glutathione, can restore δ-ALA-D activity after oxidative challenges (38, 39). In fact, measurement data of restored δ-ALA-D enzyme activity after DTT addition have shown good correlation with those of the immunoenzymatic quantitation of the enzyme (40). Taking into account the recent evidence pointing to a link between thyroid diseases and oxidative stress (41, 42), one could suppose the oxidation of δ-ALA-D-sulfhydryl groups during PTU-induced hypothyroidism. However, in our study, blood δ-ALA-D activity was not restored in PTU-treated animals when DTT was present in the reaction medium, suggesting that a mechanism other than thiol oxidation is responsible for the decreased activity of this enzyme.

The occurrence of a direct inhibitory effect of PTU on blood δ-ALA-D activity was investigated under *in vitro* conditions: PTU (up to 4 m*M*) did not affect blood δ-ALA-D activity when present in the reaction medium, ruling out the possibility of a direct inhibitory effect of this thionamide on blood enzyme. The absence of a direct inhibitory effect of PTU on blood δ-ALA-D under *in vitro* conditions and the absence of DTT effects on the PTU-induced reduction of blood δ-ALA-D activity indicate that the enzyme's expression appears to be modulated by T_3.

Since mammalian liver is an important hematopoietic organ during the perinatal period, the potential inhibitory effect of PTU on hepatic δ-ALA-D was also evaluated. Similar to the blood enzyme activity, hepatic δ-ALA-D activity was also decreased in PTU-exposed rats, but to a lesser extent. Although the PTU-induced decrease in hepatic δ-ALA-D activity (around 17 %) was significant, it is impossible to confirm that such a modest effect could represent critical biological significance in PTU-treated animals. Interestingly, liver δ-ALA-D activity was similar in the reverted-hypothyroid and hypothyroid groups. The molecular mechanism related to this differential behavior of blood and liver δ-ALA-D activity after PTU + T_3 treatment is an unsolved topic of our study and represents a promising matter for further studies.

Hematological changes, particularly anemia, have been reported as an important clinical feature of hypothyroidism (5). Although a majority of patients with hypothyroidism have significant reduction in RBC mass (per kg of body weight), the presence of anemia may be not evident due to concomitant reduction of plasma volume (43). In addition, experimental evidence has shown that thiouracil (an antithyroid agent) increases hematocrit in rats (44). We observed that both RBCs and Hb were significantly increased in PTU-exposed animals. In addition, a greater increase was observed for both parameters when exogenous T_3 was coadministrated. These results suggest a reduction in the plasma volume after PTU treatment and stimulatory effects of T_3 on erythropoiesis, corroborating previous reports (43). Even though the actual molecular mechanism(s) related to the hematological changes observed in hypothyroidism is/are not completely understood, these findings indicate that T_3 has a crucial role in RBC homeostasis and point to δ-ALA-D as an important protein, the activity of which is modulated by T_3.

Conclusion. The present results are the first to show a correlation between hypothyroidism and decreased δ-ALA-D activity, pointing to this enzyme as a potential molecule involved in hypothyroidism-related hematological changes. The absence of DTT effects on the PTU-induced reduction of blood δ-ALA-D activity and the absence of a direct inhibitory effect of PTU on this enzyme under *in vitro* conditions suggest that T_3 modulates δ-ALA-D expression.

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