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5′-Deiodinase type 1 activity in liver and brain of the thyroxine-treated dystrophic hamster

Yadhu N. Singh, Allison L. Gleysteen and Stephanie L. Ganschow

Abstract

Dystrophic hamsters (DH), as well as dystrophic patients, exhibit alveolar hypoventilation (AH) and low plasma thyroid hormone levels. Thyroxine (T_4) treatment of young DH retards AH development, and improves respiratory function and contractility of skeletal muscles. However, the mechanism responsible for the hypothyroidism in DH is not known. One possible cause of the hypothyroidism is reduced activity of the 5′-deiodinase enzyme system, which converts T_4 to the more active triiodothyronine (T_3). This study tested the above hypothesis by measuring the serum T_3 and T_4 levels and the activity of the enzyme type 1 5′-deiodinase (D1) in the liver and brain of normal and dystrophic hamsters before, and 8 weeks after, placebo or T_4 treatment. There was no significant difference in T_4 level between normal and dystrophic hamsters before or after treatment. However, the T_3 level was lower in DH before treatment and 8 weeks after placebo and T_4 treatment. Both in the liver and brain, D1 activity in DH was depressed compared with normal hamsters. In the liver, T_4 supplementation restored enzyme activity to normal level, while in the brain there was no significant difference. The data indicate that the hypothyroidism in DH may be, in part, due to reduced activity of D1 enzyme, which could be partially reversed by T_4 treatment.

Introduction

Cardiac, respiratory and skeletal muscle dysfunctions are observed in both the BIO 14.6 dystrophic hamster and in patients with limb girdle muscular dystrophy arising from sarcoglycan gene mutation (Sakamoto et al 1997; Politano et al 2001). Consequently, the dystrophic hamster is considered to be a potential animal model for this form of human muscular dystrophy. In the BIO 14.6 hamster, alveolar hypoventilation develops by about 2 months of age followed by development of heart failure (for review, see Schlenker & Burbach 1991). In these hamsters and the dystrophic patients, the alveolar hypoventilation is associated with thyroid hormone dysfunction, resulting in depressed plasma levels of 3,5,3′-triiodothyronine (T_3).

T_3 is the more active thyroid hormone that is derived in the periphery from thyroxine (T_4) by the action of type 1 5′-deiodinase (D1) enzyme (Anderson et al 2000). Although present in most tissues in the body, the highest levels of D1 are found in the thyroid, liver and kidney, and to a much lesser extent in euthyroid central nervous system (CNS). It has been estimated that about 20% of daily production of T_3 is derived from the thyroid gland and the rest originates from extrathyroidal deiodination of T_4 secreted by the thyroid. The type 2 isoform of the enzyme (D2) shows a significantly lower activity than D1, generating T_3 for local use primarily in the CNS, pituitary, and brown adipose issue (BAT) (Leonard & Koehrle 2000). It accounts for about 50% of the extrathyroidally generated plasma T_3 (DiStefano et al 1982; Salvatore et al 1996). A third isoform, type 3 (D3), is the major T_4- and T_3-inactivating enzyme, and is found predominantly in the CNS, skin and placenta. In contrast to D1 and D2, which are primarily outer-ring deiodinases, D3 exerts its action through inner-ring deiodinase activity and catalyses the conversion of T_4 to 3,3′,5′-triiodothyronine (reverse or rT_3), and of T_3 to 3,3′,-diiodothyronine (3,3′-T_2), both of which are biologically inactive (Bianco et al 2002).

Previous studies have shown that treatment of young dystrophic hamsters with T_4 retards progression of alveolar hypoventilation and normalizes respiratory function

College of Pharmacy, South Dakota State University, Box 2202C, Brookings, SD 57007-0099, USA

Yadhu N. Singh, Allison L. Gleysteen, Stephanie L. Ganschow

Correspondence: Y. N. Singh, College of Pharmacy, South Dakota State University, Box 2202C, Brookings, SD 57007-0099, USA.
E-mail: yadhu_singh@sdstate.edu

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(Burbach et al 1987). Levels of T_3 have been shown to be less in dystrophic hamsters than in normal ones (Schlenker & Burbach 1991; Singh & Hanson 1998). T_4 application also improves the contractile characteristics of the skeletal muscle of the diaphragm, extensor digitorum longus and the soleus (Singh et al 1998). Presently, the mechanism responsible for the hypothyroidism in dystrophic hamsters is unknown.

One possible aetiology of the hypothyroidism in the dystrophic hamster is, at least in part, an impairment of D1 activity leading to a reduction in the peripheral rate of the monodeiodination of T_4 to T_3. We have tested this hypothesis by treating normal and dystrophic hamsters with T_4 for eight weeks and measuring the peripheral thyroid hormone levels and D1 activity in the liver. The enzyme activity in the brain was also evaluated to assess whether there was a similar perturbation in that organ.

Materials and Methods

Chemicals

D,L-Dithiothreitol (DTT), 6-propyl-2-thiouracil (PTU), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), L-thyroxine (T_4), 3,3′,5′-triiodo-L-thyronine (rT_3), 3,3′,5-triiodo-L-thyronine (T_3) and Dowex 50WX2 (100–200 mesh) were obtained from Sigma Chemical Co. (St Louis, MO). ^{125}I-labelled T_3 and T_4 (^{125}I-T_3, ^{125}I-T_4) and ^{125}I-labelled reverse T_3 (^{125}I-rT_3) were purchased from NEN (Boston, MA). All other agents were obtained commercially and were of analytical grade.

Animals

Four-week-old weanling male hamsters of both normal (BIO F_1B) and dystrophic (BIO 14.6) strains were obtained from Biobreeders (Fitchburg, MA). They were housed in groups of 3 or 4 in plastic cages with wire tops at ~25°C, with controlled humidity and a 14–10-h light–dark cycle. They had free access to Purina 5001 chow (PMI Feeds, Inc., St Louis, MO) and tap water. The experimental procedures were approved by the Animal Care and Use Committee of South Dakota State University. After 5 days of acclimatization, baseline measurements were obtained using 6 hamsters each from normal (N0) and dystrophic (D0) groups, which were subjected to the experimental protocol that follows.

Treatment

The remaining hamsters were randomly divided into four separate groups of six each, as follows: normal placebo (NP), normal treated (NT), dystrophic placebo (DP) and dystrophic treated (DT). The NP and DP groups were implanted with placebo pellets containing the biodegradable carrier binder only. The NT and DT groups received 90-day-release pellets containing 22 mg L-thyroxine (T_4). According to the manufacturer's specifications, 0.24 mg of T_4 was released per day. Both placebo and T_4 pellets were obtained from Innovative Research of America (Sarasota, FL).

The pellets were implanted subcutaneously in the midscapular region while the hamsters were under isoflurane anaesthesia and the incision was closed with a staple. The hamsters were then housed individually until they were sacrificed. Body weights of all hamsters were recorded individually on a weekly basis at the same time of the day throughout the treatment period.

Tissue sampling and processing

After eight weeks, with the hamster under deep anaesthesia (pentobarbital sodium, 50 mg kg^{-1} and urethane, 250 mg kg^{-1}, i.p.), blood was collected by intracardiac puncture into cryogenic tubes. The samples were allowed to clot, centrifuged at 500 *g* for 5 min, and the serum decanted and stored at −80°C for thyroid hormone assays. The hamster was then euthanized by cervical dislocation and liver samples and brain were removed, trimmed of extraneous tissue on a Petri dish sitting on crushed ice, blotted and weighed. They were immediately processed to obtain the microsomal fractions based on methods previously described (Pazos-Moura et al 1991; Campos-Barros et al 1994). Briefly, the samples were individually homogenized on ice in 5–6 volumes of a buffer containing (mM): 320 sucrose, 10 HEPES (pH 7.0), 1 DTT, and 1 EDTA for liver, or 10 DTT and no EDTA for brain. The homogenates were centrifuged at 10 000 *g* for 10 min at 4°C, and the supernatants obtained further centrifuged at 100 000 *g* for 60 min to obtain microsomal pellets. The pellets were washed and resuspended in the original volume of a buffer containing 0.1 M K_2HPO_4, 1 mM EDTA (pH 7.0) and 2 mM DTT, and 1.0-mL samples of the resuspension were stored at −80°C for enzyme assay.

Thyroid hormone assays

The total thyroid hormone levels were assayed using a coated tube radioimmunoassay (RIA) kit (Diagnostic Systems Labs, Webster, TX). For T_4 assays, 25 μL of standards, controls and samples were pipetted into the appropriate T_4 antibody-coated tubes, followed immediately by 200 μL of ^{125}I-T_4 reagent. The reagent was also put in a plain uncoated tube for total count. After gentle vortexing for 1–2 s, all tubes were incubated for 60 min at room temperature (~25°C) in a shaker set at 180 rev min^{-1}. The tube contents were then completely decanted by simultaneous inversion in a waste receptacle and striking and allowing them to drain for 2 min on an absorbent mat. Adhering droplets were blotted from the rim and outside of the tubes. Each tube was washed three times with 2.5 mL of a diluted wash solution and decanted as before. For T_3 assays, the following modifications were made. Goat anti-mouse monoclonal antibody (GAMG)-coated, rather than T_4 antibody-coated, tubes were used and 200 μL of ^{125}I-T_3 reagent, rather than ^{125}I-T_4 reagent, followed by 100 μL of anti-T_3 antibody were added to all except the total count tubes.

All tubes were then counted in a gamma counter (COMPAC, Ricker Corporation, CT). Samples for total counts, standards, controls and unknowns were assayed in duplicate. The concentrations of T_3 and T_4 were then determined using the mean counts per minute for the standards, controls and samples and the mean total count (DSL-3100 and DSL-3200 kit package inserts). The detection limits for the assays were $4.3\,ng\,dL^{-1}$ for T_3 and $0.4\,\mu g\,dL^{-1}$ for T_4.

Assay of D1

The activity of D1 in the liver samples was measured using a modification of the method of Pazos-Moura et al (1991). All reagents and samples were allowed to reach room temperature (~25°C) and then thoroughly mixed by gentle inversion. Fifty microlitres of the substrate solution containing $2\,\mu M$ ^{125}I-rT_3 were incubated in a shaking water bath at 37°C. The reaction was started by addition of $50\,\mu L$ of microsomal suspension corresponding to 15–$30\,\mu g$ of protein. Protein content of the samples was assayed by the method of Bradford (1976) using a diagnostic kit and bovine serum albumin (BSA) as standard from Bio-Rad Labs (Hercules, CA). A blank was run with each set of assays, containing $50\,\mu L$ of the substrate solution and $50\,\mu L$ of buffer. The reaction was stopped after 10 min of incubation by the addition of $33\,\mu L$ of 8% BSA–10 mM PTU (1:1), followed by $133\,\mu L$ of cold 20% trichloracetic acid (TCA). For the brain samples, the following modifications were made from the above procedure (Campos-Barros et al 1994). The reaction mixture contained 50–$100\,\mu g$ of brain protein, the incubation period was 60 min, and the reaction was stopped by adding $50\,\mu L$ of cold 5% BSA–10 mM PTU (1:1), followed by $400\,\mu L$ of cold 10% TCA.

After mixing the samples and incubating on ice for 10 min, they were centrifuged at $12\,000\,g$ for 3 min at 4°C. Two hundred microlitres of supernatant were applied to small chromatography columns containing Dowex resin (about 1 mL bed volume). Iodide was eluted from the cation exchange column with two 1-mL portions of 10% acetic acid and the ^{125}I in the eluate was measured in a gamma counter. The data were used to calculate the enzyme activity as nanomoles rT_3 deiodinated per mg protein per hour (Pazos-Moura et al 1991).

Statistical analysis

The statistical analysis of the data was performed using a two-way analysis of variance (GraphPad Prism, San Diego, CA) followed by Tukey's test. The differences were considered to be significant at $P < 0.05$.

Results and Discussion

Body weight of the hamsters in all four groups increased over the entire 8-week period with the weights being less for the corresponding dystrophic hamsters at all times (Figure 1). However, only the T_4-treated normal hamsters exhibited a significantly greater weight gain ($P < 0.05$) than those treated with placebo at all time points except in the first week.

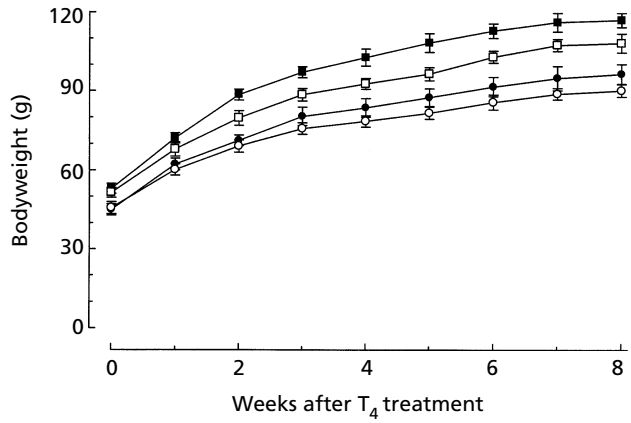

Figure 1 Body weight of normal (squares) and dystrophic (circles) hamsters treated with placebo-treated (open) and T4-treated (closed symbols) over an 8 week period. Data are mean ± s.e.m., n = 6 or 7 per group.

The observed T_4-induced increase in body weight is surprising because, in other rodents, thyroid hormone treatment normally leads to a reduced gain in body weight, presumably due to increased metabolism. For instance, Prange & Bakewell (1966) found that during a 14-day test period, young rats gained only 104.2% of their initial weight when treated with T_4 compared with a gain of 113.7% in placebo-treated counterparts (30 rats per group). Experimental hyperthyroidism in rats has been shown to increase proteolysis in various organs, including the soleus and extensor digitorum longus (EDL) rather than to decrease protein synthesis (Angerås & Hasselgren 1985). A possible reason for the disparity between the dystrophic hamsters and other rodents may be that, because of species difference, the anabolic effects of the hormones in the hamster may be greater than the catabolic effects, resulting in enhanced body-weight increase. In our laboratory, we have found that T_4 supplementation, besides the body weight change, also increased the mass of normal hamster diaphragms by 25%, and EDL by about 12%, in a period of 8 weeks (Singh et al 1998).

Serum T_4 levels of both normal (N0) and dystrophic (D0) hamsters before and 8 weeks after placebo treatment (NP, DP) were not significantly different at the same time points (Table 1). The values were comparable with those previously reported for the same, or similar, strains of hamsters (Kopecky et al 1986; Schlenker & Burbach 1995). As expected, the placebo-treated hamsters had a lower T_4 value than their age-matched T_4-treated counterparts; however, it was also less than pre-treatment levels. The reason for the latter observation is unknown but may be related to the growth and development that occur in the hormonal and enzyme systems during the neonatal and weanling stages of the animal's life (Bates et al 1999; Bianco et al 2002).

Table 1 Serum levels of thyroxine (T_4) and triiodothyronine (T_3) in normal and dystrophic hamsters before and after treatment with placebo and T_4.

Group	T_4 ($\mu g\,dL^{-1}$)	T_3 ($ng\,dL^{-1}$)
N0	8.23 ± 1.55	97.29 ± 7.45
D0	6.20 ± 0.62	$69.96 \pm 5.58^{\dagger}$
NP	$3.39 \pm 0.48^{\S}$	101.42 ± 3.67
DP	$3.22 \pm 0.23^{\S}$	$82.83 \pm 3.17^{\ddagger}$
NT	24.62 ± 7.22	229.42 ± 47.54*
DT	16.27 ± 3.37	$116.21 \pm 10.64^{\#}$

Each value represents the mean ± s.e.m., n = 6 or 7 per group. N = normal, D = dystrophic, 0 = pre-treatment, P = placebo-treated, T = T_4-treated. $^{\S}P < 0.05$ vs N0, D0, NT and DT. $^{\dagger}P < 0.05$ vs N0. $^{\ddagger}P < 0.05$ vs NP. *$P < 0.05$ vs NP and DP. $^{\#}P < 0.05$ vs DP and NT.

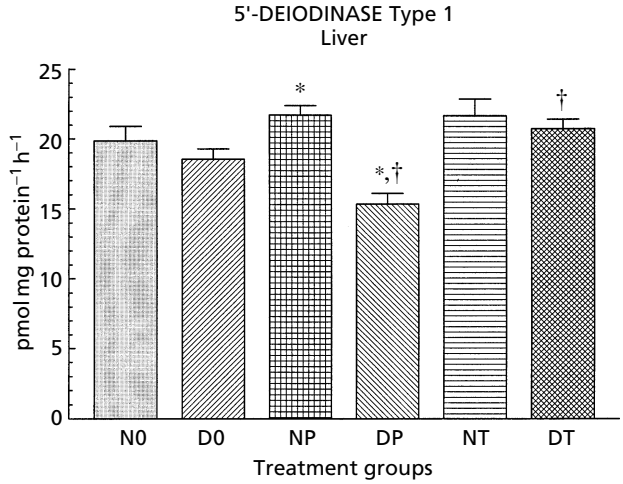

Figure 2 Effect of T_4 treatment on type 1 5′-deiodinase (D1) activity in the liver of normal and dystrophic hamsters. N, normal; 0, pretreatment; P, placebo-treated; T, T_4-treated. *$P < 0.05$, DP compared to NP; $^{\dagger}P < 0.05$, DP compared to DT. Data are mean ± s.e.m., n = 6 or 7 per group.

On the other hand, serum T_3 in dystrophic hamsters was significantly lower ($P < 0.05$) than in normal hamsters at both time points without T_4 intervention. T_4 application caused hyperthyroidism in normal as well as dystrophic hamsters with respect to T_4, but only in normal hamsters with respect to T_3. While the T_3 level was significantly greater ($P < 0.05$) in dystrophic hamsters compared with their age-matched placebo-treated counterparts, the hamsters, nonetheless, were only rendered euthyroid with respect to this hormone. The more modest increase in T_3 level in dystrophic compared with the normal hamsters following T_4 administration is addressed later. The failure of serum T_4 in dystrophic hamsters to rise to that in normal hamsters may be due to perturbations in pharmacokinetic factors (e.g., reduced absorption from the administration site or a more rapid clearance from the blood).

Before T_4 administration, the activity of the D1 enzyme in the liver was similar in the dystrophic and normal hamsters (Figure 2). After eight weeks, the level of activity in the DP hamsters was significantly less ($P < 0.05$) than the pre-treatment level in the same genotype (D0) and the normal counterparts (NP). While T_4 supplementation did not affect enzyme activity in normal hamsters, it was significantly ($P < 0.05$) elevated in the dystrophic group, so that it was comparable with the NT level.

Like in the liver, enzyme activity in the brain was significantly less ($P < 0.05$) in the DP than the NP hamsters, but not between the genotypes pre-treatment (Figure 3). After T_4 application, the activity in DT hamsters was higher than in the DP hamsters, but less than in the NP hamsters ($P < 0.05$). Interestingly, enzyme activity measured in the NT hamsters was less than for both the NP and the DT hamsters by a significant amount.

Although D1 activity in T_4-treated normal and dystrophic hamsters was similar, this was, as mentioned earlier, not reflected in the relative serum levels of T_3 following T_4 supplementation (Table 1). On the other hand, T_3 in DT and NP hamsters was comparable in amount, but the physiological consequences in the dystrophic hamsters were not always consistent with this hormonal status. For instance, there was no significant increase in body weight or some of the contractile and gravimetric characteristics of the diaphragm and EDL muscles (Singh et al 1998). However, Schlenker & Burbach (1995) reported that when the same strain of dystrophic hamsters received T_4, diaphragmatic necrosis was reduced, gas exchange surface area of the lung was increased and ventilation improved compared with placebo-treated counterparts.

Previous studies have indicated that D1 in rat liver increased in experimental hyperthyroidism and was low

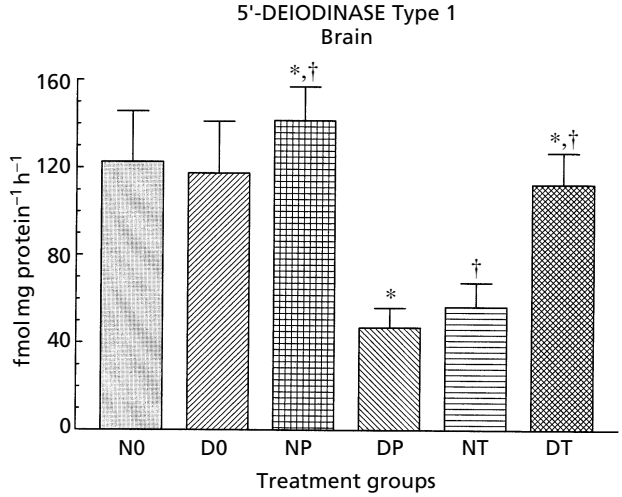

Figure 3 Effect of T_4 treatment on type 1 5′-deiodinase (D1) activity in the brain of normal and dystrophic hamsters. N, normal; 0, pretreatment; P, placebo-treated; T, T_4-treated. *$P < 0.05$, DP vs NP vs DT. $^{\dagger}P < 0.05$ NT vs NP vs DT. Data are mean ± s.e.m., n = 6 or 7 per group.

in experimental hypothyroidism (Kaplan & Utiger 1978; Jennings et al 1984; Silva et al 1984; Pazos-Moura et al 1991). Later it was found that changes in the enzyme's activity paralleled alterations in D1 mRNA levels (Berry et al 1990, 1991). However, T_3-induced increase in hepatic D1 mRNA was in the order of 10–50 fold, while increases in activity were only in the order of 3–4 fold (O'Mara et al 1993). In this study, D1 in the dystrophic hamster increased by about 40%, while the T_3 level was almost the same as for normal untreated hamsters.

In the brain, D1 in dystrophic hamsters showed an even greater increase (by about 150%) following T_4 treatment. However, an unexpected observation was the corresponding decrease in enzyme activity in normal hamsters. The reason for this is unclear but may be related to the involvement of D2. Since this enzyme is the primary deiodinase in the CNS, T_4 addition would lead to greatly increased formation of T_3. The highly elevated level of T_3 would then generate a negative feedback effect resulting in decreased D1 activity.

Neither the effects of T_4 addition on, nor the control levels of, D2, D3 or rT_3 were evaluated in the study. The low serum T_3 concentrations observed in the dystrophic hamster may not be due to a hypothyroid state but could relate to non-thyroidal illness or euthyroid sick syndrome which is associated with other malignancies or conditions, including stress, trauma, starvation, surgery, sepsis and, possibly, muscular dystrophy. An over-expression of D3 would lead to an accelerated inactivation of T_4 and T_3, leading to increased formation of rT_3 and 3,3′-T_2, respectively. Severe depression of thyroid hormones caused by high levels of D3 in hepatic infantile hemangiomas has been reported (Huang et al 2000). Thus, measurement of D3 and rT_3 might provide an explanation for our aberrant findings and experiments to test this possibility are underway in our laboratory.

Conclusion

This study found that T_3, but not T_4, serum levels are significantly reduced in dystrophic compared with normal hamsters and that T_4 application caused an increase in thyroid hormone levels in both groups. Activity of D1 in the liver was measurably lower in dystrophic hamsters before treatment, although T_4 administration did raise the level of activity close to that in the normal placebo-treated hamsters. Dystrophic brain levels of D1 activity were significantly lower than in normal brains, but after hormone treatment it was greatly elevated. Thus, the data indicate that the hypothyroidism in the dystrophic hamster may, in part, be due to a reduced activity of D1 enzyme, which may be partially reversed by T_4 treatment. This finding suggests that thyroid hormone treatment may be beneficial in reversing hypothyroidism in human muscular dystrophy, especially if administered early in life.

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