Thyroid Hormone Receptor α Mutation Causes a Severe and Thyroxine-Resistant Skeletal Dysplasia in Female Mice

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A new genetic disorder has been identified that results from mutation of THRA, encoding thyroid hormone receptor α1 (TRα1). Affected children have a high serum T3:T4 ratio and variable degrees of intellectual deficit and constipation but exhibit a consistently severe skeletal dysplasia. In an attempt to improve developmental delay and alleviate symptoms of hypothyroidism, patients are receiving varying doses and durations of T4 treatment, but responses have been inconsistent so far. Thra1PV/H11001 mice express a similar potent dominant-negative mutant TRα1 to affected individuals, and thus represent an excellent disease model. We hypothesized that Thra1PV/H11001 mice could be used to predict the skeletal outcome of human THRA mutations and determine whether prolonged treatment with a supraphysiological dose of T4 ameliorates the skeletal abnormalities. Adult female Thra1PV/H11001 mice had short stature, grossly abnormal bone morphology but normal bone strength despite high bone mass. Although T4 treatment suppressed TSH secretion, it had no effect on skeletal maturation, linear growth, or bone mineralization, thus demonstrating profound tissue resistance to thyroid hormone. Despite this, prolonged T4 treatment abnormally increased bone stiffness and strength, suggesting the potential for detrimental consequences in the long term. Our studies establish that TRα1 has an essential role in the developing and adult skeleton and predict that patients with different THRA mutations will display variable responses to T4 treatment, which depend on the severity of the causative mutation. (Endocrinology 155: 3699–3712, 2014)
A six year-old girl with skeletal dysplasia and growth retardation was found to have a heterozygous THRA nonsense mutation resulting in expression of a truncated TRα1E403X protein. She had normal serum TSH with low/normal T4 and high/normal T3 concentrations. Further investigations revealed macrocephaly with patent and abnormal skull sutures, delayed tooth eruption and bone age, disproportionate short stature, and epiphyseal dysgenesis with delayed mineralization of secondary ossification centers. Treatment with T4 for 9 months resulted in suppression of TSH and an increased basal metabolic rate but did not improve linear growth or skeletal development (5). A second girl with similar thyroid function and skeletal dysplasia was found to have a heterozygous frameshift mutation in THRA resulting in expression of a truncated TRα1P397fs406X protein (6). She presented at the age of 3 years with macrocephaly, delayed tooth eruption, absent secondary ossification centers, and congenital hip dislocation. Reducing growth velocity became evident between 3 and 6 years of age. T4 treatment between 6 and 11 years of age only resulted in a small increase in growth velocity for a 2-month period but ultimately had no effect on her height, which continued along the 20th centile and was accompanied by persistently delayed bone age. The girl’s 47-year-old father had the same THRA mutation and displayed short stature with a height 3.77 SDs below normal and acquired hearing loss due to otosclerosis (6, 8). Recently, a 45-year-old female with similar thyroid function, macrocephaly, and disproportionate short stature was identified and found to have a heterozygous frameshift mutation in THRA, resulting in expression of a truncated TRα1P382fs388X protein. She presented in infancy with developmental delay and was treated intermittently with T4, which resulted in some improvement in growth velocity although her final adult height remained 2.34 SDs below normal (7).

These recent reports define a new genetic disorder characterized by a severe developmental phenotype with profound skeletal abnormalities that are thought to result from impaired T3 action in bone and cartilage (5–8). In an attempt to ameliorate the phenotype, three children have already received intermittent T4 at different doses and for varying durations. However, responses to date have been limited, and it is unknown whether long-term T4 treatment will be beneficial or detrimental. Thus, it is now essential to define the adult skeletal consequences of THRA mutations and determine the long-term effects of T4 supplementation, because life-long therapy is likely to be required. Importantly, Van Mullem et al (8) showed that dominant-negative inhibition of TRβ by TRα1PV inhibited target gene transcription but acts as a potent dominant-negative inhibitor of wild-type (WT) TRα1 or TRβ (14, 18, 19). Thus, the functional characteristics of TRα1PV closely resemble those reported for TRα1E403X, TRα1P397fs406X, and TRα1P382fs388X (5–7). Importantly, PV and none of the described human mutations affect the sequence of the THRA locus but which cannot bind T3 and has no known physiological function. Consistent with this, juvenile Thra1PV/+ mice display the same characteristics as children with heterozygous THRA mutations. They have a reduced T4:T3 ratio (14), delayed closure of the skull sutures with enlarged fontanelles, and severe postnatal growth retardation with delayed bone age. These abnormalities result from impaired TRα1-mediated T3 action in bone and cartilage (20–22), indicating that Thra1PV mice represent an excellent disease model in which to investigate the consequences of prolonged T4 treatment.

We hypothesized that the adult phenotype of Thra1PV/+ mice would predict the skeletal outcome of human THRA mutations and determine whether affected individuals may be susceptible to fracture or osteoarthritis, both of which are associated with altered thyroid hormone action in bone (23–26). We also hypothesized that prolonged treatment of Thra1PV/+ mice with a supraphysiological dose of T4 would ameliorate the developmental skeletal phenotype and improve bone structure and strength in adulthood.
The current studies demonstrate that adult \(Thra1^{PV+}\) mice have short stature but normal bone strength despite high bone mass, suggesting that patients with \(THRA\) mutations are unlikely to have an increased risk of fracture. By contrast, gross morphologic abnormalities of the bones and joints predict that individuals with \(THRA\) mutations may be predisposed to osteoarthritis (27, 28). Although treatment with a supraphysiological dose of \(T_4\) completely suppressed TSH secretion, it had no effect on skeletal maturation, linear growth, or bone mineralization, thus demonstrating profound tissue resistance to thyroid hormone in \(Thra1^{PV+}\) mice. However, prolonged \(T_4\) treatment increased bone stiffness and strength abnormally due to progressive enlargement of cortical bone diameter and thickness. Overall, the findings suggest that \(T_4\) treatment of individuals with dominant-negative \(THRA\) mutations is unlikely to improve their skeletal abnormalities substantially and may even be detrimental in the long term. Nevertheless, \(Thra1^{PV+}\) mice represent an important disease model in which to identify and evaluate new therapeutic approaches.

Materials and Methods

\(Thra1^{PV}\) mice

WT and heterozygous \(Thra1^{PV+}\) mice have a mixed C57BL/6J and NIH Black Swiss genetic background and were bred and genotyped as described elsewhere (14, 21). Detailed characterization of the adult skeleton in \(Thra1^{PV+}\) mice was performed in 14-week-old female mice after cessation of growth, and in fully mature 20-week-old female mice that had been treated with vehicle or \(T_4\) from weaning at 4 weeks of age until death. All mice were given ip injections of calcein (10 mg/kg in 100 \(\mu\)L PBS) 14 and 7 days before tissue collection (29).

Ethics

Animal studies were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the National Cancer Institute Animal Care and Use Committee granted ethical approval for all experiments.

Manipulation and measurement of thyroid status

TSH, \(T_4\), and \(T_3\) levels were determined in serum from mice \((n = 5–13\) per group) treated with vehicle or \(T_4\) \((1.2 \mug/mL\) in the drinking water) between 4–20 weeks of age. \(T_4\)-supplemented water was changed every 3 days, with the \(T_4\) concentration adjusted to intake in 2-week cycles to ensure all animals received the same amount of \(T_4\) and did not become markedly thyrotoxic (14, 30–32).

Histology

Tibias were fixed in 10% neutral buffered formalin and decalcified in 10% EDTA, embedded in paraffin wax. Sections (5 \(\mu\)m) were stained with alcian blue and van Gieson (29, 33). Measurements from at least 4 separate positions across the growth plate were obtained to calculate the mean height using a Leica DM LB2 microscope and DFC320 digital camera (Leica Microsystems). Results from 2 levels of sectioning were compared.

Faxitron digital x-ray microradiography

Femurs were imaged at 10 \(\mu\)m resolution using a Faxitron MX20 (Qados). Bone mineral content was determined relative to steel, aluminum, and polyester standards. Images were calibrated with a digital micrometer, and bone length, cortical bone diameter, and thickness were determined (33, 34).

Micro-computed tomography (CT)

Femurs were analyzed by micro-CT (Skyscan 1172a) at 50 kV and 200 \(\mu\)A with a detection pixel size of 4.3 \(\mu\)m\(^2\), and images were reconstructed using Skyscan NRecon software. A 1-mm\(^3\) region of interest was selected 0.2 mm from the growth plate, and trabecular bone volume as proportion of tissue volume (BV/TV), trabecular number, and trabecular thickness were determined (29, 33). Representative femurs from each treatment group were reconstructed with a SCANCO \(\mu\)CT 40 (SCANCO Medical AG) operating at 55 kVp peak energy detection, 6 \(\mu\)m resolution to obtain approximately 2300 cross-sections per specimen in 766 \times 763 pixel 16 bit DICOM files. Raw data were imported using 32-bit Drishti v2.0.221 (Australian National University Supercomputer Facility, http://anusf.anu.edu.au/Vizlab/drishti/) and rendered using 64-bit Drishti v2.0.000 to generate high-resolution images.

Back scattered electron-scanning electron microscopy (EM) (BSE-SEM)

Femurs were fixed in 70% ethanol and opened longitudinally (33). Carbon-coated samples were imaged using backscattered electrons with a Zeiss DSM962 digital scanning electron microscope (EM) at 20-kV beam potential (KE Electronics). High-resolution images were quantified using ImageJ to determine the fraction of trabecular and endosteal bone surfaces displaying osteoclastic resorption (33).

Quantitative BSE-SEM

Bone mineralization was determined by quantitative BSE-SEM at 1-\(\mu\)m\(^3\) resolution. Specimens were embedded in methacrylate and block faces polished to an optical finish for scanning electron microscopy (EM) analysis at 20 kV, 0.5nA with a working distance of 11 mm (33). Gradations of micromineralization density were represented in 8 equal intervals by a pseudocolor scheme (33, 35).

Osteoclasts

Sections from decalcified tibias were stained for tartrate-resistant acid phosphatase, counterstained with aniline blue, and imaged using a Leica DM LB2 microscope and DFC320 digital camera (29, 33). A montage of 9 overlapping fields covering an area of 1 mm\(^2\) located 0.2 mm below the growth plate was constructed for each bone. BV/TV was measured, and osteoclast numbers and surface were determined in trabecular bone normalized to total bone surface (BS) (29, 33).

Osteoblasts

Methacrylate-embedded specimens were imaged with a Leica SP2 reflection confocal microscope at 488-nm excitation to determine the fraction of BS undergoing active bone formation (33, 36). Mineral apposition rate was calculated by determining the...
separation between calcine labels at 20 locations per specimen beginning 0.2 mm below the growth plate. BS and mineralizing surface were measured using ImageJ, and the bone formation rate was calculated by multiplying mineralizing surface and mineral apposition rate.

**Bone strength**

Three-point bend tests were performed on tibias, with a constant rate of displacement of 0.03 mm/s until fracture, using an Instron 5543 load frame and 100N load cell (Instron Limited). Biomechanical variables reflecting cortical bone strength were derived from load displacement curves (33, 37).

**Statistics**

Data were analyzed by unpaired two-tailed Student’s t test; P < .05 was considered significant. Frequency distributions of mineralization densities obtained by Faxitron and quantitative BSE were compared using the Kolmogorov-Smirnov test (29, 33, 34).

**Results**

**Thyroid status and response to T4 administration in Thra1PV/+ mice**

The thyroid status of adult WT and Thra1PV/+ mice was determined following treatment with vehicle or a supraphysiological dose of T4 from weaning until 14 weeks of age (Figure 1). The basal T4 concentration did not differ between WT and Thra1PV/+ mice, whereas T3 and TSH levels were increased in Thra1PV/+ mice by 1.5-fold (P < .01) and 6-fold (P < .001), respectively. Thus, the characteristically reduced T4:T3 ratio identified in individuals with THRA mutations (5–7) was also present in Thra1PV/+ mice (T4:T3 ratio: Thra1PV/+ 23 vs WT 39). Supraphysiological T4 treatment completely suppressed TSH in both WT and Thra1PV/+ mice. Despite profound and similar suppression of TSH, the increases in circulating T4 and T3 concentrations were attenuated in Thra1PV/+ mice (T4, 3.5-fold increase; T3, 1.5-fold) compared with WT (T4, 6-fold increase, P < .001; T3, 4-fold, P < .01) indicating that they are resistant to T4 administration.

**Delayed ossification and impaired bone modeling in Thra1PV/+ mice**

Delayed bone development in juvenile Thra1PV/+ mice (21) led to severe skeletal abnormalities in adults. Growth plates in 14- and 20-week-old Thra1PV/+ mice were 39% and 70% wider than in WT mice (Figure 2, A and B), demonstrating persistent delay of endochondral ossification. An increased degree of retention of mineralized cartilage within trabeculae revealed that bone modeling was also impaired (Figure 2C). T4 administration did not affect either of these abnormalities in mutant mice (Figure 2 and data not shown).

**Structural consequences of defective ossification, modeling, and remodeling in adult Thra1PV/+ mice**

Bones from 14- and 20-week-old Thra1PV/+ mice were grossly dysmorphic. They were 17% and 15% shorter than WT and had splayed metaphyses, an abnormal crossection throughout the diaphysis, and misshapen joint surfaces (Figure 3A). Micro-CT analysis indicated that trabecular bone volume, number, and thickness were increased in 20-week-old Thra1PV/+ mice (BV/TV, 2.1-fold; trabecular number, 1.9-fold; trabecular thickness, 1.1-fold greater) (Supplemental Figure 1), and these findings were confirmed by back-scattered electron-scanning EM (BSE-SEM) (Figure 3B). Similarly, cortical bone thickness (48% wider at 14 weeks, 43% at 20 weeks) and periosteal diameter (13% larger at 14 weeks, 20% at 20 weeks) were markedly increased in Thra1PV/+ mice (Supplemental Figure 1). T4 administration had no effect on these morphologic abnormalities (Figure 3A) but resulted in a gradual increase in cortical bone thickness and diameter in Thra1PV/+ mice (Supplemental Figure 1). Importantly, the endosteal diameter did not change in Thra1PV/+ mice following T4 treatment, whereas in WT mice it increased by 16% (P < .01). Thus, the increase in cortical bone thickness in Thra1PV/+ mice resulted from a failure of endosteal bone resorption combined with a likely increase in periosteal bone deposition.

**Increased bone mineral content but reduced mineralization in Thra1PV/+ mice**

X-ray microradiography revealed that 14-week-old Thra1PV/+ mice had lower bone mineral content than WT mice, consistent with reduced mineral accrual during postnatal growth (21). Thus, in Figure 4A, the pseudocolored images in 14-week-old mice show more yellow and fewer red pixels in Thra1PV/+ mice compared with WT, indicating reduced bone mineral content. These differences are
shown graphically in Figure 4B, in which the frequency distribution for
Thr1PV/+ mice is shifted to the left. By contrast, in 20-week-old mice there
was a small shift to the right in the pixel frequency distribution for Thr1PV/+,
mice indicating higher, rather than lower, bone mineral content in older
animals (Figure 4, A and B). Remark-
ably, supraphysiological T4 treatment
further increased bone mineral content
in Thr1PV/+ mice even though, as ex-
pected, it was reduced in WT mice fol-
lowing treatment (Figure 4, A and B).
Thus, Thr1PV/+ mice were resistant to
T4-induced bone loss and had a para-
dofoxical increase in bone mineral con-
tent following treatment. Despite this,
BSE-SEM revealed that cortical and
trabecular bone mineralization density
was reduced in 20 week-old Thr1PV/+ mice, the difference being greater in
cortical bone, and that T4 treatment did
not affect mineralization (Figure 5,
A–D). Thus, Thr1PV/+ mice have an
increase in bone mineral content (Fig-
ure 4) despite the reduction in tissue
mineralization density (Figure 5) be-
cause their trabecular and cortical bone
volume is substantially increased (Fig-
ure 2 and Supplemental Figure 1).
Overall, therefore, Thr1PV/+ mice have increased cortical and trabecular
bone volume compared with WT, but
their bone is less mineralized.

Reduced osteoclastic bone
resorption in Thr1PV/+ mice

Consistent with micro-CT and BSE-
SEM analysis, histomorphometry stud-
ies demonstrated increased bone vol-
ume and surface in Thr1PV/+ mice.
Furthermore, osteoclast surfaces were
reduced and fewer osteoclasts were
present in Thr1PV/+ mice compared
with WT (Figure 6, A–C). Thus, Thr1PV/+ mice had a smaller propor-
tion of their increased BS covered by
osteoclasts (see also Supplemental Fig-
ure 2). The differences in BS, BV/TV,
osteoclast surface/BS, and osteoclast
number/BS between WT and Thr1PV/+
mice were accentuated following T4 treatment (Figure 6, A–C). Consistent with these findings, bone resorption was generally lower in Thra1PV+/+H11001 mice (Supplemental Figure 2) but bone formation parameters were similar (Supplemental Figure 3). However, it is important to note that small differences in dynamic bone formation may not have been detected in these studies because only 3 mice were analyzed per group.

Abnormal bone stiffness and strength after prolonged T4 treatment of Thra1PV+ mice

Biomechanical testing revealed no difference in bone strength between untreated WT and Thra1PV+ mice (Figure 7, A and B). Nevertheless, T4 treatment resulted in gradual increases in yield load, maximum load, fracture load, and stiffness of bones from Thra1PV+/+ mice (Figure 7, A and B). Thus, prolonged T4 administration abnormally and progressively increased bone stiffness and strength in Thra1PV+ mice.

Discussion

Skeletal phenotype resulting from mutation of Thra

During development Thra1PV+/+ mice have delayed closure of the skull sutures, severe growth retardation, delayed bone age, and impaired bone mineral accrual (22). The delayed ossification persists into adulthood and is accompanied by impaired bone modeling and remodeling, resulting in short stature, increased bone mass, and gross morphologic abnormalities of the bones and joints, but normal bone strength. These findings suggest that, despite severe skeletal abnormalities, adults with THRA mutations are unlikely to have an increased risk of fracture. However morphologic abnormalities affecting the bones and joints predict that they may be at increased risk of osteoarthritis (27, 28).

Cellular and molecular mechanisms

The abnormalities in Thra1PV+ mice are consistent with effects of prolonged hypothyroidism on the growing and adult skeleton (38–42). Hypothyroidism disrupts growth plate chondrocyte differentiation leading to delayed endochondral ossification and linear growth, impairs bone modeling, and uncouples the processes of osteoclastic bone resorption and osteoblastic bone formation (43). In adults, even though it is well established that thyroid hormones increase bone resorption and promote bone loss, it is not known whether T3 acts directly in osteoclasts or whether effects on osteoclasts are secondary to the direct actions of T3 in osteoblasts (43). In Thra1PV+ mice, prolonged impairment of chondrocyte differentiation is manifest by growth retardation and short stature in adulthood. Similarly, defective osteoclastic bone resorption is evidenced by reduced metaphyseal in-wasting, ab-

Figure 3. Effect of T4 treatment on bone structure in Thra1PV+ mice. A, Micro-CT images of femurs from 14- and 20-week-old WT and Thra1PV+ mice following treatment with vehicle (no Rx) or T4. A longitudinal image of the BS, a midline section, and transverse sections at 4 levels are shown. Bars = 1000 μm. B, BSE-SEM views of distal femur trabecular bone from 14- and 20-week-old WT and Thra1PV+ mice. Bars = 500 μm. Rx, treatment; 4–20w, 4–20 week.
normal diaphyseal cross-section, and increased trabecular bone volume with retention of mineralized cartilage. Moreover, the grossly delayed formation of secondary ossification centers and reduced bone mineral accrual in Thra1PV/+ mice persisted throughout growth when mice were active and gaining weight. Thus, unmineralized epiphyses were exposed to abnormal and greater mechanical loads, resulting in compensatory enlargement of the epiphyses and metaphyses and culminating in adult joint deformity. Surprisingly, the strength of adult Thra1PV/+ mice decreased compared to wild-type, and T4 treatment failed to correct the reduced mineral density.

Figure 4. Effect of T4 treatment on bone mineral content in Thra1PV/+ mice. A, Quantitative Faxitron x-ray microradiography images of femurs from 14- and 20-week-old WT and Thra1PV/+ mice following treatment with vehicle (no Rx) or T4. Gray-scale images were pseudocolored according to a 16-color palette in which low mineral content is blue-black and high mineral content is pink-white. Bars = 1000 μm. B, Relative frequency histograms of femur bone mineral content (n = 3 per genotype per group). Kolmogorov-Smirnov test, WT vs Thra1PV/+ or no Rx vs T4 treatment, **, P < .01, ***, P < .001. Rx, treatment; 4–20w, 4–20 week.
bones was normal despite these structural abnormalities and is accounted for by the increased cortical bone thickness and diameter (33, 44).

A series of studies in genetically modified mice have shown that TRα1 is the principal mediator of T3 action in bone and cartilage (12, 21, 45–48). The finding of an identical skeletal phenotype in patients with THRA mutations (5–7) now demonstrates that TRα1 has a similar essential role in human bone development. Analysis of the mechanisms underlying the skeletal phenotypes in Thra mutant mice revealed decreased expression of T3 target genes including GH receptor (Ghr), insulin like growth factor-1 (Igf1), Igf1 receptor (Igf1r), fibroblast growth factor receptor-1 (Fgfr1) and Fgfr3, and reduced downstream signaling responses mediated by the MAPK, signal transducer and activator of transcription 5, and AKT signaling pathways in chondrocytes and osteoblasts (12, 20, 21, 45, 49, 50). These data demonstrate impaired T3 action in cartilage and bone in Thra mutant mice despite a normal systemic T3 concentration and thus indicate the skeletal phenotype in individuals with THRA mutations is a consequence of local resistance to thyroid hormone.

The phenotypes in ThraPV mice and patients with THRA mutations result from the actions of potent dominant-negative mutant receptors. However, we have previously reported that mice harboring a less severe ThraR384C mutation have a milder phenotype with only transiently delayed ossification and growth retardation, although modeling and remodeling defects resulting in increased bone mass, cortical thickness, and diameter were present in adults (45, 47). Importantly, and in contrast to ThraPV mice, treatment of ThraR384C mice with a dose of T3 that overcomes the reduced ligand binding affinity and dominant-negative activity of the mutant receptor did ameliorate their skeletal abnormalities (45).

**Figure 5.** Effect of T4 treatment on bone mineralization density in ThraPV mice. A, Quantitative BSE-SEM images of femur mid-diaphysis cortical bone from 14- and 28-week-old WT and ThraPV mice following treatment with vehicle (no Rx) or T4. Gray-scale images were pseudocolored according to an 8-color palette in which low mineral content is blue and high mineral content is pink-gray. Bars = 200 μm. B, Relative frequency histograms of cortical bone micromineralization densities (n = 3 per genotype per group). C, Images of distal femur trabecular bone. Bars = 200 μm. D, Relative frequency histograms of trabecular bone micromineralization densities (n = 3 per genotype per group). Kolmogorov-Smirnov test, WT vs ThraPV or no Rx vs T4 treatment, **, P < .01, ***, P < .001. Rx, treatment; 4–20w, 4–20 week.

**Therapeutic approaches in individuals with THRA mutations**

The response to thyroid hormone treatment in ThraR384C mice suggests that individuals with THRA mutations may benefit from similar treatment. Unfortunately, however, doses of T4 sufficient to normalize circulating hormone concentrations have been largely ineffective in the patients treated so far (5–8), presumably because the currently identified individuals have mutations that result in expression of mutant receptors with little or no T3 binding affinity. Despite this, Van Mullem et al (8) showed that dominant-negative inhibition of TRβ by TRα1F397fs406X in vitro could be overcome partially by increasing concentrations of thyroid hormones.
Figure 6. Effect of T4 treatment on osteoclastic bone resorption in Thra1PV/+ mice. A, Low-power views (bar = 100 μm) of tibia trabecular bone from 14- and 20-week-old WT and Thra1PV/+ mice following treatment with vehicle (no Rx) or T4, and stained for tartrate resistant acid phosphatase activity (pink) with aniline blue counterstain. The white boxes indicate the locations of the corresponding high-power images shown in panel B. B, High-power views (bar = 10 μm) of osteoclasts lining trabecular bone surfaces. C, Quantitative analysis of BS, BV/TV, osteoclast surface per bone surface (Oc.S/BS), and osteoclast number per BS (Oc.N/BS) (mean ± SEM) in 14- and 20-week-old mice (n = 3 per genotype per group). Statistical comparisons: 14- and 20-week-old mice, WT vs Thra1PV/+, Student’s t test, *, P < .05, **, P < .01, ***, P < .001. Rx, treatment; 4–20w, 4–20 week.
In this context, several studies have suggested that TRβ can mediate T₃ action in bone and cartilage (9–12), even though the principal physiological effects are mediated via TRα1. Thus, we hypothesized that treatment of Thra¹PV⁺ mice with a supraphysiological dose of T₄ might improve bone structure and strength.

Figure 7. Effect of T₄ treatment on cortical bone strength in Thra¹PV⁺ mice. A, Representative load-displacement curves from destructive 3-point bend testing of tibias from 14- and 20-week-old WT and Thra¹PV⁺ mice following treatment with vehicle (no Rx) or T₄. B, Quantitative analysis of yield load, maximum load, fracture load, and stiffness (mean ± SEM) in 14- and 20-week-old mice (n = 3 per genotype per group). Statistical comparisons: 1) 14- and 20-week-old mice, WT vs Thra¹PV⁺, Student’s t test, *, P < .05; 2) 20-week-old mice, no Rx vs T₄ treatment from 4–20 weeks, Student’s t test, #, P < .05, ##, P < .01, ###, P < .001. Rx, treatment.
However, such treatment of Thra<sup>PV/+</sup> mice had no beneficial effect on growth or skeletal deformity but did, nevertheless, increase cortical bone thickness and diameter. These responses were likely mediated by TRβ and resulted in abnormal increases in bone stiffness and strength that may adversely affect the optimal compromise between strength and flexibility that is essential to minimize fracture risk (51). Thus, prolonged treatment of individuals harboring THRA mutations with high doses of T4 may also have adverse consequences in other tissues where T3 action is predominantly mediated via TRβ.

GH therapy represents an alternative approach to improve linear growth and skeletal maturation in children with THRA mutations, but treatment in one individual so far was ineffective (6). The reduced expression of Ghr, Igf1, and Igf1r, together with impaired signal transducer and activator of transcription 5 and AKT signaling in growth plate chondrocytes in Thra mutant mice (12, 21, 50), suggests a mechanism to account for this lack of clinical response to GH.

**Thyroid hormone metabolism and response to T4 administration in Thra<sup>PV/+</sup> mice**

Thyroid hormone metabolism is mediated by 3 iodothyronine deiodinases. The type 1 enzyme (D1) catalyzes removal of an inner or outer ring iodine from T4 to generate T3 or 3,3′-diiodothyronine (T2). Acting via TRβ1, T3 increases D1 expression to complete a feed-forward loop. However, T3 also acts via TRα1 to increase D3 expression and thus limit feed-forward activation of D1. Thus, T4 excess results in a parallel increase in both D1 and D3 so that levels of T3, rT3, and T2 in the circulation rise to reflect increased T4 metabolism. The high levels of circulating thyroid hormones suppress TRH and TSH expression and inhibit endogenous T4 and T3 production. At steady state, most circulating T3 is derived from increased D1-mediated metabolism of T4. The TRα1-mediated actions of T3 in bone are increased. B. Abnormal response in Thra<sup>PV/+</sup> mice. High concentrations of T4 are metabolized in the liver. D1 converts T3 to rT3 or T3, and rT3 is metabolized to 3,3′-diiodothyronine (T2). Acting via TRβ1, T3 increases D1 expression to complete a feed-forward loop. However, in Thra<sup>PV/+</sup> mice the mutant TRα1<sup>PV</sup> prevents T3 stimulation of D3 expression, thus maintaining feed-forward activation of D1. Administration of T4 fuels this feed-forward activation and would result in enhanced metabolism of T4, and ultimately increased accumulation of T2. Thus, although circulating T3 and T4 levels rise to a lesser degree than in WT animals, they are still sufficient to suppress the hypothalamus-pituitary-thyroid axis. At steady state, the grossly increased D1 activity thus accounts for resistance of Thra<sup>PV/+</sup> mice to T4 administration. Despite exogenous thyroid hormone administration, T3 action in bone remains inhibited by dominant-negative TRα<sup>PV</sup> (21).
supply of T₃ and is subject to substrate-mediated inactivation (53). By contrast, the type 3 enzyme (D3) catalyzes removal of an inner ring iodine from T₄ or T₃ to generate the inactive metabolites rT₃ or 3,3'-diiodothyronine. D3 expression is induced by thyroid hormone, thus limiting the supply of T₃ in conditions of thyroid hormone excess (52).

Remarkably, and despite complete suppression of TSH, Thra₁PV+/+ mice had a blunted increase in circulating thyroid hormones following a supraphysiological dose of T₄. This discrepancy indicates that the hypothalamus-pituitary-thyroid axis is intact in Thra₁PV+/+ mice, but metabolism of thyroid hormones must be increased. Indeed, we previously showed that untreated Thra₁PV+/+ mice have a 9-fold increase in hepatic D1 mRNA expression (14) resulting in a 4.8-fold increase in enzyme activity (54). It is well established that T₃ acts via TRβ1 to stimulate D1 expression in the liver (55, 56) and, accordingly, hepatic D1 activity is increased further in Thra₁PV+/+ mice following treatment with T₃ (54, 57). By contrast, T₃ acts via TRα1 to stimulate expression of D3 (58) and we previously demonstrated that T₃ treatment of Thra₁PV+/+ mice fails to induce the normal increase in D3 activity observed in WT animals (54, 57).

We propose, therefore, that the resistance to T₄ administration observed in Thra₁PV+/+ mice results from the markedly increased D1 activity combined with this absent D3 response (Figure 8). Consistent with this model, TSH in individuals with THRA mutations was suppressed readily following T₄ treatment despite only small increases in T₄ and T₃ concentrations (7, 8). Detailed future metabolic studies will be required to confirm the precise underlying mechanisms responsible for these findings. For example, because defects in TRα1 action may result in intestinal problems, it is possible that absorption of orally administered T₄ could be impaired in Thra₁PV+/+ mice. However, it should also be noted that, following oral treatment with T₄, the TSH concentration was suppressed completely in both WT and Thra₁PV+/+ mice, indicating that intestinal absorption of T₄ was unlikely to be markedly impaired in Thra₁PV+/+ mice. Nevertheless, it would be instructive to investigate whether differences in serum T₄ and T₃ levels persist between WT and Thra₁PV+/+ mice following parenteral administration of T₄.

**Conclusions**

The overall resistance of the skeleton to T₄ treatment in Thra₁PV+/+ mice and the patients studied so far is likely to be a consequence of the potent dominant-negative activities of their mutant TRα1 proteins (5–7, 14, 18). It is inevitable, however, that individuals with less severe THRA mutations will be identified in the future and, in such cases, T₄ treatment is likely to be beneficial. Thus, treatment of Thra₁R¹³⁸₄C+/+ mice with doses of T₄ that overcome the reduced binding affinity of TRα₁R¹³⁸₄C rescued their skeletal phenotype by preventing delayed ossification and growth retardation, ultimately ameliorating adult bone structure and mineralization (45). Taken together, these studies predict that individuals with THRA mutations will display variable degrees of skeletal deformity and different responses to T₄ treatment that correlate with the functional consequences of the particular disease-causative mutation. Therefore, in patients with THRA mutations, it will be important to characterize the functional properties of their mutant TRα1 because this may predict their response to T₄ treatment and the optimal systemic T₄ concentration required.

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