GH substitution reverses the growth phenotype but not the defective ossification in thyroid hormone receptor α1−/−β−/− mice

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(J M Kindblom and S Göthe contributed equally to this work)

Abstract

Thyroid hormone receptor α1, β1 and β2-deficient mice (TRα1−/−β−/− mice) demonstrate growth retardation and defective ossification in the epiphyses associated with an inhibition of the GH/IGF-I axis. There are differences between TRα1−/−β−/− mice (receptor deficient) and the hypothyroid animal model (ligand deficient). Such differences include possible repressive actions exerted by unliganded receptors in the ligand-deficient (hypo-thyroid) model but not in the receptor-deficient model. In the present study we have investigated whether or not GH substitution rescues the skeletal phenotype of TRα1−/−β−/− mice.

TRα1−/−β−/− and wild-type (WT) mice were treated with GH from day 18 until 10 weeks of age. GH substitution of mutant mice resulted in a significant and sustained stimulatory effect on the body weight that was not seen in WT mice. GH-treated mutant mice but not GH-treated WT mice demonstrated increased length and periosteal circumference of the femur. However, GH substitution did not reverse the defective ossification seen in TRα1−/−β−/− mice. TRα1−/−β−/− mice displayed increased width of the proximal tibial growth plate, which was caused by increased width of the proliferative but not the hypertrophic layer. GH substitution did not restore the disturbed morphology of the growth plate in TRα1−/−β−/− mice.

In summary, GH substitution reverses the growth phenotype but not the defective ossification in TRα1−/−β−/− mice. Our data suggest that TRs are of importance both for the regulation of the GH/IGF-I axis and for direct effects on cartilage.

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Introduction

Thyroid hormone, tri-iodothyronine (T3), is involved in a wide variety of physiological and developmental processes including regulation of basal metabolism, development of the central nervous system, and the growth and remodeling of bone. Childhood onset hypothyroidism results in growth retardation (Abbasi et al. 1980) and hyperthyroidism is associated with adult bone loss (Fraser et al. 1971).

Thyroid hormone signaling is mediated via thyroid hormone receptors TRα and TRβ (Sap et al. 1986). TRα1, TRα2 and TRβ1 are expressed by osteoblasts and chondrocytes, and TRα2 and TRβ1 are expressed by osteoclasts in human bone (Abu et al. 1997). Two mechanisms of action have been proposed: (i) a direct action of TRs in bone and cartilage, or (ii) an indirect effect via regulation of the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis (Ohlsson et al. 1993). To understand the role of thyroid hormones and their receptors, mice deficient in the expression of one or several TR isoforms have been developed. No overt skeletal phenotype or growth retardation has been reported for TRβ−/− or TRα1−/− mice (Forrest et al. 1996, Wikstrom et al. 1998). In contrast, mice lacking both TRα1 and α2 (TRα−/−) have an impaired epiphysial ossification and are growth arrested (Fraichard et al. 1997, Gauthier et al. 1999). These mice are reported to exhibit identical skeletal phenotype to the one observed in TRα−/−β−/− compound mice (Gauthier et al. 1999). These findings indicate that separate roles might exist for the different TR subtypes in the regulation of growth and development.

The effects of GH on the growth plate and bone involve both direct and indirect effects via local or systemic IGF-I (Ohlsson et al. 1998, Sjogren et al. 1999). GH and T3
interact in the stimulation of longitudinal bone growth and bone maturation. The crosstalk between the GH and T₃ pathways occurs at several levels, and includes both T₃-mediated regulation of GH gene transcription (Sap et al. 1990, Schaufele et al. 1992) and stimulation of IGF-I expression (Lewinson et al. 1994, Varga et al. 1994, Huang et al. 2000, Lakatos et al. 2000).

We have previously reported pre- and postnatal growth retardation and defective ossification in TRα1−/−β−/− mice. These findings were associated with decreased pituitary content of GH mRNA and protein as well as decreased serum levels of IGF-I (Gothe et al. 1999). Therefore the aim of the present study was to investigate whether or not GH substitution rescues the skeletal phenotype of TRα1−/−β−/− mice.

Materials and Methods

Mouse strains

Mice were generated and genotyped as previously described (Forrest et al. 1996, Wikstrom et al. 1998, Gothe et al. 1999). Mice were housed under 12 h light:12 h darkness cycles in a controlled environment with 40–50% relative humidity at 22 °C.

All animal experiments were performed under approved protocols.

GH injections and body weights

Male TRα1−/−β−/− (hereafter referred to as knock out (KO)) and wild-type (WT) pups were divided into four groups receiving either saline or GH (human GH; Pharmacia & Upjohn, Stockholm, Sweden): n=10 for the WT and saline group; n=9 for the WT and GH group; n=9 for the KO and saline group; and n=8 for the KO and GH group. From day 18 postnatally (P18; P13 to P20) until P40 (P36 to P43), mice were given a daily s.c. dose of 1·5 mg/kg GH or saline. Serum levels of IGF-I were measured to determine whether or not the dose given reversed the GH deficiency. From day P41 (P37 to P44) to day P67 (P63 to 70), the mice were given a daily dose of 3·8 mg/kg GH s.c.. The mice were thereafter anesthetized and killed by heart puncture.

Serum parameters

Serum IGF-I levels were measured by double-antibody IGF-binding protein–blocked RIA (Blum & Breier 1994).

Dual X-ray absorptiometry (DXA)

Areal bone mineral content (BMC) and areal bone mineral density (BMD) (BMC/cm²) were measured using the Norland pDEXA Sabre (Norland Medical Systems, Fort Atkinson, WI, USA) with Sabre Research 3.6 software. In vivo measurements were performed on 10-week-old anesthetized animals to measure total body BMC. A maximum of three mice could be analyzed in the same scan. To avoid interscan variations, a WT mouse was included as an internal control in each scan. Medium resolution scans were performed (line-spacing 0·05 cm). Ex vivo measurements of the left femur were performed on excised bones placed on a 1 cm thick Plexiglas table (line spacing: tibia and femur 0·02 cm, vertebrae 0·01 cm).

Peripheral quantitative computerized tomography (pQCT)

Tomographic measurements were performed using the STRATEC pQCT XCT (software version 5·4B; Norland Medical Systems) operating at a resolution of 70 µm as previously described (Windahl et al. 1999). Mid-diaphyseal pQCT scans of the left femora were performed to determine cortical BMD, cortical area, cortical thickness, periosteal circumference and endosteal circumference. The mid-diaphyseal region of femora in mice contains only cortical bone. Metaphyseal pQCT scans of the left femora were performed to measure trabecular volumetric BMD. The metaphyseal scan was positioned 4% of the total length of the femur proximal to the distal growth plate (an area consisting of a central portion of trabecular bone). The trabecular bone was defined by setting an inner threshold to 45% of the total area. The interassay coefficients of variation for the pQCT measurements were <2%.

Histological staining and growth plate measurements

Right femora were excised and fixed in 4% buffered paraformaldehyde and subsequently decalcified, embedded in paraffin and sectioned. Sections were stained with Alcian Blue/Van Gieson stain. The width of growth plates were measured using an image processing system (Easy Image; Bergströms Instruments, Stockholm, Sweden) coupled to a microscope. For measurements of total growth plate and the hypertrophic layer the average of 30 measurements was calculated. The width of the proliferative layer was calculated by subtracting the width of the hypertrophic layer from the width of the total growth plate. The observer was blinded to the treatment groups during these measurements.

Fat measurements

We have previously developed a combined DXA image analysis procedure for the in vivo prediction of fat content in mice (Ohlsson et al. 2000). The interassay coefficient of variation for the measurements of percent fat area was less than 3%.

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Results

GH substitution normalizes the GH/IGF-I axis in KO mice

In the present study the objective was to give KO mice full substitution of GH in order to reverse the GH deficiency. In accord with our previous findings, serum levels of IGF-I were decreased in KO compared with WT mice (−18%) (Gothe et al. 1999). To confirm that an adequate replacement dose of GH was used, serum IGF-I levels were measured. The initial dose of GH (1.5 mg/kg per day) did not significantly increase serum IGF-I levels in the mutant mice and therefore the dose of GH was increased (3.8 mg/kg per day). During the high-dose period the serum IGF-I levels were increased by 25% (P<0.05) in the mutant mice, which represents a complete normalization as compared with WT mice (WT and saline, 421±13·1 ng/ml; KO and GH, 433±34·6 ng/ml) (Fig. 1).

GH substitution reverses the growth phenotype in KO mice

Similar to a previous report (Gothe et al. 1999), KO mice in the present study exhibited postnatal growth retardation which was reflected by a reduction in body weight (−27%, P<0.001) and a decrease in bone lengths, most pronounced in the femur (−16%, P<0.001). GH-substituted mutant mice demonstrated an 18% (P<0.01) increase in body weight but were smaller than saline-treated WT mice (Fig. 2a). A detailed analysis of the effect of GH on body growth demonstrated that a significant and sustained stimulatory effect of GH was seen after 14 days of high-dose treatment in the mutant mice (Fig. 2b). WT mice did not exhibit significantly increased body growth in response to the given GH dose over the whole period, but showed a transient increase in body weight at day 41 of GH substitution (Fig. 2b). Two-way ANOVA of the whole period of GH substitution demonstrated that the GH effect was significantly larger in the mutant mice than in WT mice (P<0.05; Fig. 2b). On a high dose of GH the mutant mice outgrew the saline-treated WT mice by 49%. These findings show that GH substitution of mutant mice restores the normal increase in body weight. Furthermore, they also indicate that GH-treated mutant mice exhibit a catch-up growth.

DXA scans showed that GH substitution also increased the length of the femur by 6% (P<0.01) and of total femoral area by 10% (P<0.05) in the mutant mice, but failed to significantly alter these parameters in WT mice (Fig. 3a). Mid-diaphyseal pQCT scans of femora revealed increased periosteal cortical circumference in response to GH substitution in mutant mice, but not in WT mice (Table 1). Furthermore, the cross-sectional cortical area was significantly reduced in saline- but not in GH-treated mutant mice (Table 1).

Thus, our results demonstrate that GH substitution induces compensatory body growth and leads to a reversal of the growth phenotype in terms of body weight and dimensions of long bones in KO mice.

GH does not reverse the defective ossification in the epiphysis

In accord with our previous findings, KO mice in the present study have severe defects in epiphyseal ossification/mineralization (Gothe et al. 1999). GH

Figure 1 Serum IGF-I in 12-week-old KO mice. KO and WT mice were treated with GH for 10 weeks. (n = 10 for WT and saline; n = 9 for WT and GH; n = 9 for KO and saline; n = 8 for KO and GH.) Values are means ± S.E.M. Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05 vs saline, +P<0.01 vs WT.
substitution did not normalize the maturational status of the epiphyseal bone in the mutant mice (Fig. 4). DXA scans revealed that areal BMD and BMC were reduced in the mutant mice but none of these parameters were significantly altered by GH substitution (Fig. 3b). Cortical as well as trabecular volumetric density as measured with pQCT in the femur (Table 1) were reduced in the mutant mice, and again GH substitution did not reverse this phenotype. Thus, GH substitution neither reverses the defective ossification in the epiphysis nor the decreased areal BMD and BMC of the femur in KO mice.

**Disturbed relationship between hypertrophy and proliferation in the growth plate**

The present study revealed that KO mice exhibit a 10% wider epiphyseal growth plate of the distal femur compared with WT mice (Fig. 5). This finding is reflected by an increased width of the proliferative layer (28%). In contrast, the hypertrophic layer is diminished by 8% in the mutant mice (Fig. 5). GH substitution affected neither the total growth plate width nor the width of the individual layers in the mutant mice.

**Weight of other tissues and organs**

The liver, kidneys, spleen, heart and testes were weighed and correlated to body weight at the termination of the

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**Figure 2** Effect of GH substitution on body weight in KO and WT mice. (n=9 for KO and saline; n=8 for KO and GH; n=10 for WT and saline; n=9 for WT and GH.) (a) Weight gain over the whole period. Values are means ± S.E.M. (b) Weight gain on high-dose GH in WT (■) and KO (▲) mice. The effects are given as percent increase compared with saline-treated mice and values are given as means. The effect vs saline-treated mice at different times was calculated by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05, **P<0.01. To compare the GH effect in KO mice with that in WT mice a two-way ANOVA followed by a Student–Newman–Keuls multiple range test was performed for the high-dose period. The P value for KO vs WT mice is indicated.

**Figure 3** DXA measurements in 12-week-old KO mice. KO and WT mice were treated with GH for 10 weeks. (n=10 for WT and saline; n=9 for WT and GH; n=9 for KO and saline; n=8 for KO and GH.) Values are expressed as percent of WT as means ± S.E.M. Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05, **P<0.01 vs saline; ++P<0.01 vs WT. (a) Dimensional parameters of the femur; (b) areal BMD and BMC of the femur.
Table 1  pQCT measurements (means ± S.E.M.) of left femora in 12-week-old mice. KO and WT mice were treated with GH for 10 weeks

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<tr>
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<th>WT</th>
<th>KO</th>
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<tr>
<td></td>
<td>Saline (n=10)</td>
<td>GH (n=9)</td>
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<tr>
<td>Trabecular density (mg/mm³)</td>
<td>335 ± 34</td>
<td>293 ± 28</td>
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<tr>
<td>Cortical content (g/mm)</td>
<td>1.71 ± 0.100</td>
<td>1.74 ± 0.081</td>
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<tr>
<td>Cortical density (mg/mm³)</td>
<td>1287 ± 9.3</td>
<td>1304 ± 4.9</td>
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<tr>
<td>Cortical area (mm²)</td>
<td>1.33 ± 0.074</td>
<td>1.34 ± 0.060</td>
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<tr>
<td>Cortical endosteal circumference (mm)</td>
<td>3.62 ± 0.069</td>
<td>3.68 ± 0.062</td>
</tr>
<tr>
<td>Cortical periosteal circumference (mm)</td>
<td>5.45 ± 0.120</td>
<td>5.50 ± 0.096</td>
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Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05 vs saline; †P<0.05, ‡P<0.01 vs WT.

Figure 4  Disturbed ossification in the epiphysis in 10-week-old saline- and GH-treated KO mice. Alcian Blue/Van Gieson staining of sections from the femur. Arrow indicates cartilaginous area within the epiphyseal region. GP = growth plate.
This measurement demonstrated a 60% reduction in DXA followed by a Student (mice. KO and WT mice were treated with GH for 10 weeks. Fat content was measured in age-matched 3- to 5-month-old mice (Table 2). The relative weight of the kidneys and spleen was reduced by 8 and 23% respectively in the mutant mice (Table 2). The relative weight of the kidneys, heart and testes decreased after GH substitution (Table 2).

**Fat content in the mutant mice**

Fat content was measured in age-matched 3- to 5-month-old mice (n=15 for KO, n=16 for WT) using DXA scans. This measurement demonstrated a 60% reduction in DXA fat content in KO mice (P<0.05, Student’s t-test; Fig. 6). The fat content was unaffected by GH substitution (data not shown).

**Discussion**

TRα1−/−β−/− mice display a severe skeletal phenotype with growth retardation and defective ossification of the epiphysis. Such KO mice are also GH deficient as evidenced by low serum IGF-I levels and low pituitary content of GH mRNA and protein (Gothe et al. 1999). In the current study we demonstrated that KO but not WT mice responded to GH substitution with significantly increased body weight as well as increased longitudinal bone growth. In contrast, the defective ossification in KO mice was unaffected by GH substitution. We believe that the difference in body weight that persisted in the mutant mice after GH substitution is likely to have arisen before GH substitution was initiated and during the first period of a low and insufficient dose of GH. On high doses of GH, KO mice outgrew both the saline- and GH-treated WT mice, indicating catch-up growth. GH also affected the dimensions of the long bones, such as the length and periosteal circumference in the mutant mice but not in WT mice. These findings are in accord with the known

![Figure 5](image51x540 to 278x671) Width of distal femoral growth plates in 12-week-old KO mice. KO and WT mice were treated with GH for 10 weeks. (n=10 for WT and saline; n=9 for WT and GH; n=9 for KO and saline; n=8 for KO and GH.) Values are expressed as percent of WT as means ± S.E.M. Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05, **P<0.01 vs WT.

![Figure 6](image297x481 to 523x671) DXA fat content in 12-week-old KO mice. KO and WT mice were treated with GH for 10 weeks. (n=10 for WT and saline; n=9 for WT and GH; n=9 for KO and saline; n=8 for KO and GH.) Values are expressed as percent of WT as means ± S.E.M. Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05 vs WT.

**Table 2** Weights of visceral organs in 12-week-old mice. KO and WT mice were treated with GH for 10 weeks. Values are expressed as percent body weight ± S.E.M.

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<tr>
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<th>WT</th>
<th>KO</th>
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<tr>
<td></td>
<td>Saline (n=10)</td>
<td>GH (n=9)</td>
</tr>
<tr>
<td>Liver</td>
<td>5.09 ± 0.16</td>
<td>5.47 ± 0.16</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.62 ± 0.047</td>
<td>1.55 ± 0.041</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.44 ± 0.021</td>
<td>0.42 ± 0.020</td>
</tr>
<tr>
<td>Heart</td>
<td>0.51 ± 0.015</td>
<td>0.51 ± 0.018</td>
</tr>
<tr>
<td>Testis</td>
<td>0.56 ± 0.029</td>
<td>0.60 ± 0.041</td>
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</table>

Data were analyzed by one-way ANOVA followed by a Student–Newman–Keuls multiple range test. *P<0.05, **P<0.01 vs saline; *P<0.05, **P<0.01 vs WT.
effects of GH on bone, such as inducing longitudinal and periosteal bone growth (Andreassen et al. 1995, Ohlsson et al. 1998).

Studies on pubertal hypothyroid rats treated with human GH, have shown that GH substitution causes partial reversal of loss of body weight, while no effect of GH was seen on tibial length (Lewinson et al. 1989). The tibia has a different growth profile from the femur, with most of its longitudinal growth occurring before puberty. The femur, on the other hand, displays significant pubertal growth (Windahl et al. 1999). This difference between the tibia and the femur might explain why GH affected the growth of the femur in our study, but not tibial growth in the study using hypothyroid rats. Moreover, hypothyroid rats have decreased width of the growth plate due to a reduction in the number of proliferative cells and in the size of the hypertrophic cells (Lewinson et al. 1989). In contrast, total growth plate width in the distal femur was increased in KO mice as a result of a pronounced increased width of the proliferative layer but slightly reduced width of the hypertrophic layer. These findings indicate that loss of TR signaling leads to disturbed chondrocyte differentiation, resulting in a disruption in the balance between hypertrophy and proliferation in the growth plate. GH could not reverse the alterations in the growth plate seen in the mutant mice. It is important to consider the differences between the model for T3 deficiency and the model for receptor deficiency. The TRs have been reported to exert T_3-independent silencing of target genes in their unliganded state (Damm et al. 1989), and the relatively mild phenotype of KO mice as compared with hypothyroid WT mice has been explained by the transcriptional repressive effect of the unliganded receptor (Gothe et al. 1999). T3 has also been proposed to have non-genomic effects of GH on bone, such as inducing longitudinal and periosteal bone growth (Andreassen et al. 1995, Ohlsson et al. 1998).

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In contrast to the growth, no effect was seen on the skeletal maturation by GH substitution in adult KO mice. We therefore propose that T3 induces skeletal maturation via a direct effect on cartilage. This notion is supported by previous reports on the effects of T3 on cultured growth plate chondrocytes, where T3 has been shown to induce differentiation markers associated with ossification (Ohlsson et al. 1992, Alini et al. 1996, Robson et al. 2000).

The fat content, as measured with DXA, was decreased in KO mice. Thyroid hormone is known to affect the basal metabolism in almost all cells and pathological conditions in the thyroid axis often affect body weight. Hypothyroidism is frequently associated with increased fat mass and body weight, whereas weight loss is a pronounced sign in hyperthyroidism (Seppel et al. 1997, Miyakawa et al. 1999). As stated earlier there are important differences between the hypothyroid model and the receptor knock out model. However, it cannot be excluded that the diminished fat content in the mutant mice is secondary to the severe phenotype in the mutant mice.

The relative weight of the testes was dramatically increased in the mutant mice. Similarly, hypothyroidism has previously been associated with increased testicular weight, which was explained by increases in the number of Sertoli and Leydig cells (Hess et al. 1993, Cooke et al. 1996, Hardy et al. 1996). Further studies are necessary to elucidate the mechanisms for the enlargement of testes in KO mice. The relative weights of the kidney, heart and testis were reduced after GH substitution, presumably as a result of the rapid GH-induced body growth.

In conclusion, GH substitution reverses the growth phenotype but not the defective ossification in KO mice. Therefore we propose that TRs are of importance both for the regulation of the GH/IGF-I axis and for direct effects on cartilage.

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