

The effects of hypothyroidism on nerve growth factor and norepinephrine concentrations in weight-bearing and non-weight-bearing bones of rats

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Abstract

Thyroid hormones affect bone remodelling directly via receptors in osteoblasts. Previously, however, we have shown that the euthyroid and hyperthyroid states significantly influence the concentrations of both nerve growth factor (NGF) and norepinephrine (NE) in particular bones. Both NGF and NE directly affect bone metabolism and therefore it is possible that thyroid hormone action on bone may also be indirect via its actions on these two neural-related substances. In light of previous studies, the current experiments aimed to investigate whether hypothyroidism also influenced NGF and NE concentrations in weight-bearing and non-weight-bearing rat bones. Hypothyroidism was induced by oral ingestion of propylthiouricil (PTU; 3.8 ± 0.2 mg/kg/day) for 21 days. Histological examination on distal femurs and microparticle enzyme immunoassayed plasma concentrations of T3 and T4 verified the hypothyroid status in treated rats. NGF concentrations were assayed via enzyme-linked immunosorbent assay (ELISA) and NE concentrations were measured via high performance liquid chromatography (HPLC) with electrochemical detection (ECD). NGF concentrations: Femoral NGF concentrations were 207% higher in hypothyroid rats (674.9 ± 88.3 ng/g) than in euthyroid rats (326.7 ± 63.6 ng/g; $p < 0.05$). Rib NGF concentrations in hypothyroid rats (3125.1 ± 450.2 ng/g) were increased by 342% compared to euthyroid ribs (914.5 ± 128.6 ng/g; $p < 0.01$). Rib NGF concentrations in hypothyroid rats were 463% higher than in femurs of hypothyroid rats ($p < 0.001$). NE concentrations: In hypothyroid rats, NE concentrations were reduced by approximately 50% in both ribs (38.9 ng/g) and calvaria (41.5 ng/g) compared to euthyroid rats (74.7 ng/g and 87.4 ng/g respectively; $p < 0.05$ for both). These findings on hypothyroid rats may be taken in conjunction with our companion work on hyperthyroid rats (Yao *et al.*, 2002, JMNI 2:327-334) and put in context with other reports, to indicate that (i) there are several sources of NGF in bone, some of which are stimulated by hypothyroidism and others by hyperthyroidism and (ii) the concentrations of both NGF and NE in bone are sensitive to weight-bearing and thyroid hormone status.

Keywords: Bone, Hypothyroidism, Nerve Growth Factor, Norepinephrine, Weight-bearing

Introduction

Bone is constantly remodelled and the maintenance of bone requires a coordinated equilibrium between bone formation and bone resorption. Remodelling is influenced by a multitude of local and systemic factors acting either directly or indirectly on bone cells¹. Rat osteoblastic-like cells

(osteosarcoma cells) possess receptors for thyroid hormone², nerve growth factor (NGF)³ and norepinephrine (NE)⁴, which suggests that these factors are capable of acting directly on osteoblastic cells and could therefore play a role in bone remodelling. The involvement of NGF in bone metabolism is implied from the localization of NGF and its receptors in osteoblasts and other cells of unfractured and fractured bones^{5,6}. Additionally, the topical application of NGF increased the rate of fracture repair and also improved the biomechanical strength of calluses in fractured rat ribs⁷. The load borne by bone is also known to influence bone remodeling⁸, and load-bearing femurs from euthyroid rats have substantially lower concentrations of NGF and NE than rib⁹.

There are complex interactions between hyperthyroidism and load-bearing. Hyperthyroidism significantly decreased

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bone mineral density and increased osteoblastic and osteoclastic gene expression markers in weight-bearing femur, but had no effect on non-weight-bearing lumbar vertebrae in adult rats¹⁰. Previous studies in this laboratory reported that hyperthyroidism increased the NGF concentration in ribs but not in femurs while NE concentrations decreased in ribs and femur⁹. Our results suggested that the skeletal condition associated with hyperthyroidism might not be solely due to thyroid hormone, but may also be as a result of an indirect effect of osseous NGF and NE on weight-bearing and non-weight-bearing bones.

Hypothyroidism reduces both osteoblastic and osteoclastic activity and eventually results in an increased bone mass^{11,12}. NGF and NE may be involved in the reduced turnover of bone in hypothyroidism. NGF maintains both sympathetic and sensory nerves that innervate bone^{13,14}. These nerves are predominantly found in regions of high osteogenic activity^{15,16} and are suggested to influence skeletal remodelling¹⁷. Norepinephrine released from sympathetic nerves appears to indirectly stimulate osteoclastic bone resorption by acting through beta-adrenergic receptors on osteoblasts⁴. Since hyperthyroidism decreased the NE concentrations in bone⁹, it was anticipated that hypothyroidism would also alter NE concentrations in bone and therefore possibly affect remodelling.

Therefore, in view of our previous study on hyperthyroidism, it was relevant to carry out the complimentary study, namely to investigate the effect of hypothyroidism on NGF and NE osseous concentrations in weight-bearing and non-weight-bearing bone, in order to compare the effects of hypothyroidism with those of hyperthyroidism.

Materials and methods

Animals

Approval was granted by La Trobe University Animal Ethics Committee for all procedures undertaken in this experiment. Sixteen male Sprague Dawley rats aged 12 to 13 weeks (300 to 400 g) were randomly allocated into two groups, hypothyroid ($n = 8$) or euthyroid groups ($n = 8$). Animals were caged in pairs and held at 21°C under controlled 12 h light-dark cycles. Weight and water consumption of each animal was recorded daily in order to estimate drug intake and to also observe changes in body weight. Animals were allowed unlimited access to food (laboratory rat and mouse feed) and drinking water.

Treatment

Hypothyroidism was induced for 21 days by the addition of propylthiouracil (PTU) to the drinking water. PTU (500 mg) was added to 500 ml of water containing 1% BSA and 2 ml of 4 M NH_4OH in methanol. The solution was heated gently with continuous stirring until PTU dissolved. Aliquots of this solution were then added to the drinking water on alternate days, resulting in a final PTU concentration of 0.4

mg/ml¹⁸. Euthyroid rats received a vehicle solution (500 ml of water containing 1% BSA and 2 ml of 4 M NH_4OH in methanol) every alternate day.

On completion of treatment, animals were killed via an overdose of carbon dioxide. Blood samples were then collected and bone samples were excised, cleaned and weighed wet.

Measurement of T3 and T4 Concentrations

Hypothyroidism was verified by measuring free T3 (T3) and free T4 (T4) concentrations in blood plasma of euthyroid and hypothyroid groups, using microparticle enzyme immunoassay (MEIA; Abbott AxSYM System Kit, Abbott Laboratories).

Bone Samples

The left distal femur was cut longitudinally and processed for histology, while the proximal femur and the left sixth rib from each rat were analysed for NGF concentrations via enzyme-linked immunosorbent assay (ELISA) (see below). The calvarium, the right sixth rib, the right proximal femur and the tibia from each rat were analysed for NE concentrations using high performance liquid chromatography (HPLC) and electrochemical detection (ECD) (see below).

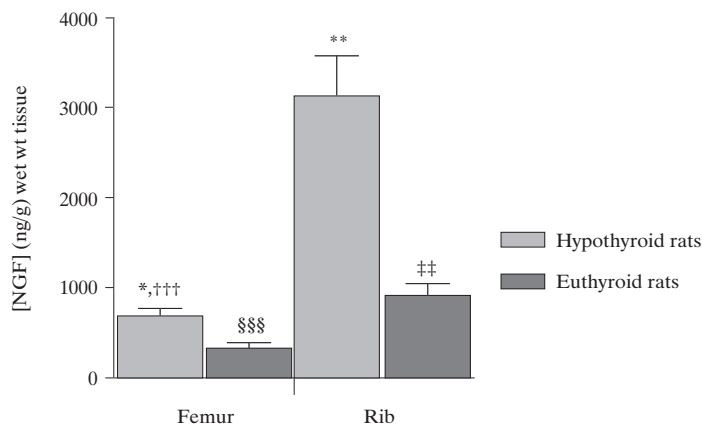
Histological and histomorphometric measurements of femoral trabecular bone

The histological and histomorphometric measurements which enabled verification of increased femoral trabecular bone associated with hypothyroidism has been previously described in detail⁹. Briefly, distal femurs were fixed for two days in paraformaldehyde-glutaraldehyde immediately after excision. Bones were subsequently processed to LR Gold resin and sectioned (4 μm) midline longitudinally. Sections were stained with Goldner trichrome and viewed using a Leica DMRBE microscope. Trabecular bone volume measurements were obtained from a 4 mm² field, positioned 1 mm anterior and distal to the lowest point of the epiphyseal growth plate in the metaphysis. Measurements were assessed blindly before comparisons were made between euthyroid and hypothyroid groups.

Nerve Growth Factor Measurement

Details of NGF extraction from bone have been previously described⁹. Briefly, femurs and ribs were homogenized in 1 ml cold extraction buffer, sonicated and centrifuged, the supernatant was removed and stored at -80°C.

An NGF ELISA was used to measure NGF concentration in samples (Promega Corporation, Madison, WI, USA). Absorbance was measured using a Labsystems Multiskan MS spectrophotometer at wavelength of 450 nm. A standard curve was constructed for each plate using a GraphPad Prism™ software (GraphPad Software, Inc. San Diego, CA,



- * $p < 0.05$ (hypothyroid femurs vs. euthyroid femurs)
 ** $p < 0.01$ (hypothyroid ribs vs. euthyroid ribs)
 ††† $p < 0.001$ (hypothyroid ribs vs. hypothyroid femurs)
 ‡ $p < 0.01$ (euthyroid ribs vs. euthyroid femurs)
 §§§ $p < 0.001$ (hypothyroid ribs vs. euthyroid femurs)

Figure 1. Nerve growth factor (NGF) concentrations in femurs and ribs of hypothyroid ($n = 8$) and euthyroid ($n = 8$) rats. Mean \pm SEM.

USA). Analyses of the concentrations of NGF were performed in duplicate. In our laboratory the sensitivity of the assay was approximately 20 pg, intra-assay variation was less than 5% and cross-reactivity with other neurotrophins (at 10 ng/ml) was less than 3%.

NE Measurement

HPLC with ECD was employed to measure NE concentrations in bone samples¹⁹. Initially, catecholamines were extracted from bone samples which were homogenized and centrifuged (Beckman TJ6) in 1 ml extraction buffer containing DHBA as an internal standard. The supernatant was removed and transferred into test tubes containing acid-washed alumina and 10^{-7} M $\text{Na}_2\text{S}_2\text{O}_5$, with a subsequent addition of 1 M Trizma Base containing 2% EDTA. Samples were vortexed and the supernatant was discarded. The alumina was then washed with deionised water before the catecholamines were desorbed with 1 M perchloric acid containing 10^{-7} M $\text{Na}_2\text{S}_2\text{O}_5$ buffer. Samples were stored at -80°C .

The HPLC-ECD flow rate was set at 0.7 ml/min, with the electrochemical detector sensitivity at 20 nA and an applied potential between 0.65 V - 0.72 V. Fifty μl of standard or sample were injected using an automatic injector (WISP; Waters Corporation, Milford, MA, USA), at a filter mode of 5 s/reduction potential. Analyses of the concentrations of NE were performed in duplicate. The sensitivity of this assay in our laboratory was 500 pg. Intra-assay variation was eliminated by the use of internal standards.

Statistical Analysis

T3, T4, NE and NGF values were analyzed using two-tailed, unpaired t-tests, while bone histomorphometric measurements were analysed by a one-tailed t-test. Differences between control and treatment groups were accepted when $p < 0.05$. Results are presented as mean \pm SEM.

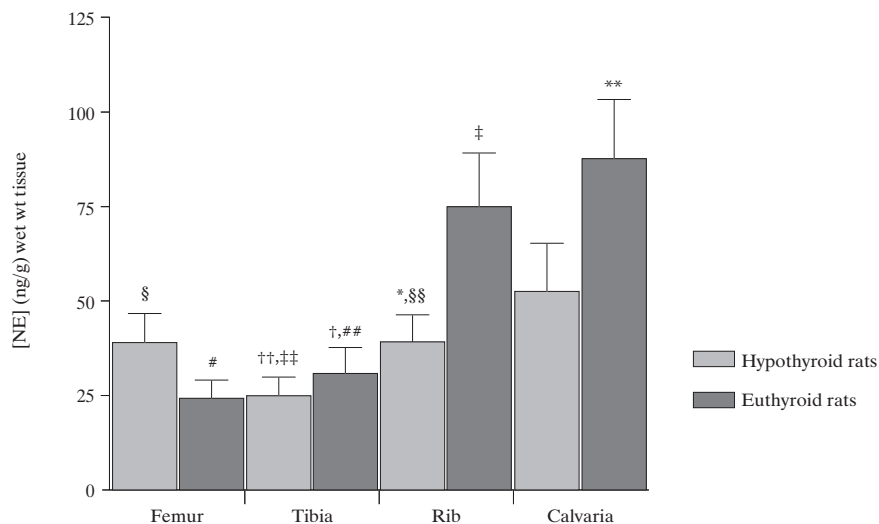
Results

Plasma T3 and T4 Concentrations

Mean daily PTU consumption by hypothyroid animals over 21 days of treatment was 3.8 ± 0.2 mg/day/kg of body weight. After 21 days, plasma T3 and T4 concentrations in hypothyroid animals were significantly less ($p < 0.001$) than in euthyroid animals, confirming the hypothyroid status in the PTU-treated rats. The mean plasma T3 concentrations in hypothyroid animals (1.1 pmol/l) were 74% less than that of euthyroid animals (4.3 pmol/l) and plasma T4 concentrations in hypothyroid rats (3.7 pmol/l) were approximately 80% less than that of euthyroid rats (20.2 pmol/l) (Table 1).

Body Weight

Euthyroid rats gained 13% in body weight over the treatment period, from 406 ± 8 g at Day 0 to 466 ± 8 g at Day 21. Hypothyroid rats weighed 430 ± 12 g at Day 0, then gained weight to 451 ± 12 g at Day 8. Thereafter, animals lost weight such that at Day 21 they weighed 439 ± 10 g, which was 2% greater than their weight at Day 0.



*p < 0.05 (hypothyroid ribs vs. euthyroid ribs)
 #p < 0.05 (euthyroid femurs vs. euthyroid ribs)
 **p < 0.01 (euthyroid femurs vs. euthyroid calvaria)
 †p < 0.05 (euthyroid tibiae vs. euthyroid ribs)
 ##p < 0.01 (euthyroid tibiae vs. euthyroid calvaria)
 ‡p < 0.05 (hypothyroid ribs vs. euthyroid calvaria)
 §p < 0.05 (hypothyroid femurs vs. euthyroid calvaria)
 ††p < 0.01 (hypothyroid tibiae vs. euthyroid ribs)
 ‡‡p < 0.01 (hypothyroid tibiae vs. euthyroid calvaria)
 §§p < 0.05 (hypothyroid ribs vs. euthyroid calvaria)

Figure 2. Norepinephrine (NE) concentrations in femurs, tibiae, ribs and calvaria of hypothyroid and euthyroid rats. Mean ± SEM.

Histomorphometric Measurements

Histomorphometric measurements showed that distal femoral metaphyseal trabecular bone volume in hypothyroid rats expressed as a percentage of total bone volume ($36.5 \pm 1.8 \%$) was 16% greater than in euthyroid animals ($30.7 \pm 1.2 \%$; $p < 0.05$).

Nerve Growth Factor Concentrations in Bone

Euthyroid rats: NGF concentrations in femurs (326.7 ± 63.6 ng/g) were significantly smaller ($p < 0.01$) than in ribs (914.5 ± 128.6 ng/g). As reported previously⁹, this suggests that weight-bearing decreases NGF concentrations in bone.

Hypothyroid rats: Hypothyroidism significantly increased NGF concentrations in femurs by 207% to 674.9 ± 88.3 ng/g compared to euthyroid animals ($p < 0.05$) and increased NGF concentrations in ribs by 342% to 3125.1 ± 450.2 ng/g (Fig. 1). NGF concentrations in ribs from hypothyroid rats were 463% greater than in the femurs from hypothyroid rats ($p < 0.001$).

These results show firstly that osseous NGF concentrations are sensitive to hypothyroidism and secondly, that non-weight-bearing bones are more sensitive to the effects of hypothyroidism than weight-bearing bones.

Norepinephrine Concentrations in Bone

Euthyroid rats: Weight-bearing femurs (24.0 ng/g) and tibiae (30.5 ng/g) had similar NE concentrations (Figure 2).

Non-weight-bearing ribs (74.7 ng/g) and calvaria (87.4 ng/g) had similar NE concentrations to each other. NE concentrations in both ribs and calvaria were significantly higher than the concentrations in both femurs and tibiae (see Figure 2 for p values). As reported previously⁹, this suggests that weight-bearing decreases NE concentrations in bone.

Hypothyroid rats: Hypothyroidism had no effect on NE concentrations in femurs and tibiae: in femurs concentrations rose slightly to 39.0 ng/g and fell slightly in tibiae to 24.7 ng/g. In contrast, hypothyroidism substantially decreased NE concentrations by approximately 50% in ribs to 38.9 ng/g ($p < 0.05$), and by a similar percentage in calvaria, to 41.5 ng/g ($p < 0.05$).

These results show the effects of hypothyroidism on NE concentrations of bone are strongly stimulated by weight-bearing and that weight-bearing largely cancels the effect of hypothyroidism observed on the NE concentrations in non-weight-bearing bones.

Discussion

In the present study, hypothyroidism was successfully induced in rats by oral administration of PTU as indicated by the significant reduction in plasma T3 concentrations compared to euthyroid animals (Table 1). Bone volume was measured by histomorphometric techniques and revealed that hypothyroidism increased distal femoral metaphyseal trabecular bone volume by 16%. Such an increase in bone volume confirmed that the mode of inducing hypothyroidism was effective in altering bone remodelling. Furthermore, this

	HO rats n=8	Euthyroid rats n=8
[T3] (pmol/l)	1.1±0.2***	4.3±0.4
[T4] (pmol/l)	3.7±0.2***	20.2±0.8

Table 1. Plasma concentrations of triiodothyronine (T3) and thyroxine (T4) after 21 days' treatment with PTU (hypothyroid), or vehicle (euthyroid). Mean ± SEM. ***p < 0.001, hypothyroid rats vs. euthyroid rats.

result was similar to that of previous workers using the same dose of PTU¹². Allain and colleagues (1995) showed that the increase in bone volume in PTU-induced hypothyroidism is attributed to a marked decrease in bone resorption, compared to bone formation (which is also reduced compared to euthyroid rats).

The first major finding from the present study is that hypothyroidism increased NGF concentrations in weight-bearing and non-weight-bearing bones. The response to hypothyroidism was modulated by weight-bearing, since the extent of the increase in NGF concentrations in ribs were substantially greater than that in femurs.

The increase in NGF concentrations in bone in response to hypothyroidism seem anomalous, since they are qualitatively similar to the effects of hyperthyroidism on NGF concentrations in femurs and ribs reported previously⁹. Clearly, an increase in the NGF concentration in bone cannot be used to identify an altered thyroid status.

There were quantitative differences in the response of NGF to hypothyroidism and hyperthyroidism: NGF concentrations in both femurs and ribs were higher in hyperthyroidism (ribs, p = 0.035; femurs, p = 0.056). This may be related to the relative changes in plasma T3 concentrations. Because T3 plasma concentrations in hypothyroid rats were approximately 75% smaller than in euthyroid rats, while T3 plasma concentrations were almost 10-fold higher in hyperthyroid rats. NGF in bone therefore appears to be more sensitive to hypothyroidism than to hyperthyroidism.

The task of reconciling a common effect of hypothyroidism and hyperthyroidism on osseous NGF concentrations is complicated by (a) the capacity of several cell types in bone to synthesize NGF. These cells include osteoblasts^{5,6,20}, vascular smooth muscle cells²¹, nerves²², Schwann cells²³ and mast cells²⁴ and (b) multiple factors that increase the activity or number of the above cells which synthesize NGF. Our results imply that in bone, hypothyroidism stimulates NGF formation in some cell types, and hyperthyroidism stimulates NGF formation in other cell types. There is evidence that supports this implication. For example, hypothyroidism in rats reduces osteoblastic activity while hyperthyroidism increases these cells' activity²⁵, and hypothyroidism in rats increases mast cell number in bone compared to both euthyroid and hyperthyroid counterparts²⁶.

Presently, the role of NGF in bone metabolism is unclear

and therefore it is difficult to suggest a physiological function for the increased osseous NGF concentrations in hypothyroidism or whether NGF is an end-product of remodelling. Raised osseous NGF concentrations may aid in the maintenance of osseous nerves¹³ or may protect bone from the effects of low thyroid hormone concentrations, since NGF has positive effects on bone formation^{7,27}.

The second major finding from the present study was that hypothyroidism depressed NE concentrations in non-weight-bearing calvaria and ribs but not in weight-bearing tibiae and femurs. This was another paradoxical result, as it was qualitatively similar to the effects of hyperthyroidism on osseous NE concentrations reported earlier⁹. The simplest explanation for these results lies with the effect of thyroid hormone on the nerve fibres that innervate bone cells: hypothyroidism inhibits the synthesis of NE in sympathetic nerves^{28,29}, which would result in reduced tissue NE concentrations; hyperthyroidism increases the enzymatic breakdown of NE in animal tissues³⁰ which could reduce the osseous concentrations of NE in hyperthyroid rats⁹.

The high concentrations of NGF in bone of both hypothyroid and hyperthyroid rats may have also contributed to the reduced concentrations of NE in non-weight-bearing bones of both hypothyroid and hyperthyroid rats, as NGF inhibits NE release from sympathetic nerve endings³¹.

The following summarizes the effects of hypothyroidism and hyperthyroidism⁹ on NGF and NE concentrations in bone:

(i) both hypothyroidism and hyperthyroidism substantially increase NGF concentrations in non-weight-bearing bones. In contrast, hypothyroidism and hyperthyroidism have smaller effects on NGF concentrations in weight-bearing bones.

(ii) both hypothyroidism and hyperthyroidism substantially decrease NE concentrations in non-weight-bearing bones but neither hypothyroidism nor hyperthyroidism affect NE concentrations in weight-bearing bones.

The functional implications of the effects of thyroid status on NGF and NE concentrations in bone remain obscure, and it is uncertain whether the changes to osseous concentrations of these peptides consequent to altered thyroid status are associated with bone remodelling, or a response to bone remodelling. Further studies of the involvement of NGF and NE in bone remodelling are needed before these uncertainties can be resolved.

These findings on hypothyroid rats may be taken in conjunction with other published reports and our companion work on hyperthyroid rats to suggest that (i) there are several sources of NGF in bone, some of which are stimulated by hypothyroidism and others by hyperthyroidism and (ii) the concentrations of both NGF and NE in bone are sensitive to weight-bearing and thyroid hormone status.

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