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G R Mundy, ... , E M Canalis, L G Raisz

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Research Article

Although hypercalcemia, osteoporosis, and increased bone turnover are associated with thyrotoxicosis, no direct effects of thyroid hormones on bone metabolism have been reported previously in organ culture. We have now demonstrated that prolonged treatment with thyroxine (T4) or triiodothyronine (T3) can directly increase bone resorption in cultured fetal rat long bones as measured by the release of previously incorporated ⁴⁵Ca. T4 and T3 at 1 μM to 10 nM increased ⁴⁵Ca release by 10-60% of total bone ⁴⁵Ca during 5 days of culture. The medium contained 4 mg/ml of bovine serum albumin to which 90% of T4 and T3 were bound, so that free concentrations were less than 0.1 μM. The response to T4 and T3 was inhibited by cortisol (1 μM) and calcitonin (100 mU/ml). Indomethacin did not inhibit T4 response suggesting that T4 stimulation of bone resorption was not mediated by increased prostaglandin synthesis by the cultured bone. Matrix resorption was demonstrated by a decrease in extracted dry weight and hydroxyproline concentration of treated bones and by histologic examination which also showed increased osteoclast activity. The effects of thyroid hormones were not only slower than those of other potent stimulators of osteoclastic bone resorption (parathyroid hormone, vitamin D metabolites, osteoclast activating factor, and prostaglandins), but the maximum response was not as great. We conclude that T4 and T3 [...]

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Direct Stimulation of Bone Resorption by Thyroid Hormones

GREGORY R. MUNDY, JAMES L. SHAPIRO, JANET G. BANDELIN,
ERNESTO M. CANALIS, and LAWRENCE G. RAISZ

*From the Division of Endocrinology and Metabolism, Department of Medicine,
University of Connecticut School of Medicine, Farmington, Connecticut 06032*

ABSTRACT Although hypercalcemia, osteoporosis, and increased bone turnover are associated with thyrotoxicosis, no direct effects of thyroid hormones on bone metabolism have been reported previously in organ culture. We have now demonstrated that prolonged treatment with thyroxine (T_4) or triiodothyronine (T_3) can directly increase bone resorption in cultured fetal rat long bones as measured by the release of previously incorporated ^{45}Ca . T_4 and T_3 at $1\ \mu\text{M}$ to $10\ \text{nM}$ increased ^{45}Ca release by 10–60% of total bone ^{45}Ca during 5 days of culture. The medium contained 4 mg/ml of bovine serum albumin to which 90% of T_4 and T_3 were bound, so that free concentrations were less than $0.1\ \mu\text{M}$. The response to T_4 and T_3 was inhibited by cortisol ($1\ \mu\text{M}$) and calcitonin ($100\ \text{mU/ml}$). Indomethacin did not inhibit T_4 response suggesting that T_4 stimulation of bone resorption was not mediated by increased prostaglandin synthesis by the cultured bone. Matrix resorption was demonstrated by a decrease in extracted dry weight and hydroxyproline concentration of treated bones and by histologic examination which also showed increased osteoclast activity. The effects of thyroid hormones were not only slower than those of other potent stimulators of osteoclastic bone resorption (parathyroid hormone, vitamin D metabolites, osteoclast activating factor, and prostaglandins), but the maximum response was not as great. We conclude that T_4 and T_3 can directly stimulate bone resorption in vitro at concentrations approaching those which occur in thyrotoxicosis. This effect may explain the disturbances of calcium metabolism seen in hyperthyroidism.

INTRODUCTION

There is abundant evidence for altered bone metabolism in states of thyroid hormone excess (1, 2). Thyrotoxi-

cosis is occasionally accompanied by hypercalcemia, and the mean serum calcium concentration of hyperthyroid patients before therapy is greater than the mean serum calcium concentrations of matched controls. Thyrotoxicosis is often associated with negative calcium balance, with increased urine calcium, increased sweat calcium, and increased fecal calcium (1). Thyrotoxic patients with negative calcium balance may have radiologic evidence of osteoporosis, and bone biopsy may show the histologic appearance of osteitis fibrosa, with marked osteoclast activity (3–7). Increased urine hydroxyproline in thyrotoxicosis supports the evidence that bone resorption is increased although some of the urinary hydroxyproline is also of skin origin (8). Isotope studies in thyrotoxic patients and animals with experimental thyrotoxicosis using stable and radioactive calcium and strontium have shown that specific activity of the label in bone, urine, and blood declines more rapidly and to lower levels than controls, indicating that bone turnover is increased in states of thyroid hormone excess (9).

Despite this compelling evidence suggesting that bone resorption is increased in vivo in states of thyroid hormone excess, no direct effects of thyroxine (T_4)¹ and triiodothyronine (T_3) on bone resorption at physiological concentrations have been reported previously (11–13). For this reason many investigators have looked for indirect effects of thyroid hormone on bone metabolism, including examination of the effects of increased basal metabolic rate and mediation through increased parathyroid hormone (PTH) or decreased calcitonin effects in patients with thyroid disease. None of these investigations have been able to account for the observed effects of thyroid hormone on bone metabolism. Using an in vitro

¹ Abbreviations used in this paper: T_4 , thyroxine; T_3 , triiodothyronine; PTH, parathyroid hormone; OAF, osteoclast activating factor.

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organ culture system we have found in this study that osteoclastic bone resorption is directly stimulated by prolonged exposure to thyroid hormones, and this effect can be distinguished by bioassay characteristics from the other known stimulators of osteoclasts such as PTH, active vitamin D metabolites, prostaglandins, and osteoclast activating factor (OAF).

METHODS

Bone resorption assay. The quantitative bioassay for measuring bone resorption has been described in detail previously (14, 15). Bone resorption was measured by the release of previously incorporated ^{45}Ca from fetal rat long bones in organ culture. Pregnant rats at the 18th day of gestation were injected with 0.2 mCi of ^{45}Ca and sacrificed the following day. Paired radii and ulnae from each fetus were removed, and the cartilaginous ends of the mineralized long bone shafts were removed. The bones were cultured for 18–24 h in "BGJ" medium² to allow for exchange of loosely complexed ^{45}Ca . The bones were then transferred to test or control media for assay. The bones were cultured in an atmosphere of 5% CO_2 and air at 37°C. The duration of culture varied from 2 to 8 days, but the culture medium was changed at 2 and 5 days for those bones that were cultured for the longer periods. At the completion of the culture period, the bones were removed and dissolved in 5% TCA, and the ^{45}Ca content of the bone and the culture medium were quantitated in a liquid scintillation counter. In most experiments bone resorption was measured as percent of total radioactivity in the medium at the end of culture. In some experiments where paired test and control bones were examined, medium ^{45}Ca concentrations only were measured, and the results were expressed as paired treated-to-control ratios. In those experiments where nonpaired bones were cultured and percent of total radioactivity released into the medium was measured, the final results were expressed as nonpaired treated-to-control ratios. 4–24 bones were tested in each treatment group, and statistical differences were analyzed using Student's *t* test. Bone resorbing activity was considered to be present when the treated-to-control ratio was greater than 1.0 ($P < 0.05$).

Preparation of test compounds. L-thyroxine, *d*-thyroxine, monoiodotyrosine, diiodotyrosine, di-iodothyronine, *l*-triiodothyronine, and triiodothyroacetic acid were all supplied by the Sigma Chemical Co., St. Louis, Mo. Reverse T_3 was kindly provided by Dr. R. I. Meltzer of Warner-Lambert Research Institute, Morris Plains, N. J. Tetraiodothyroacetic acid was supplied by K & K Laboratories, Inc., Plainview, N. Y. Thyroid hormones and thyroid hormone analogues were dissolved in 1 ml of absolute ethanol and 2 drops of 2 N NaOH to make a stock solution of 1 M. This stock solution was then diluted in BGJ culture medium to make the required concentration of the hormone to be tested. Control media were prepared the same way (except for the addition of the hormone). Propranolol, which was tested as an inhibitor of thyroid hormone-stimulated bone resorption, was kindly provided by Dr. A. Pappano of the University of Connecticut.

Determination of percent-free hormone. The percent of bound T_4 and T_3 was determined by adding radiolabeled T_3 and T_4 to BGJ medium containing 4 mg/ml bovine serum

albumin. Aliquots were placed separately in dialysis bags (boiled Spectrapor no. 3 dialysis tubing, Spectrum Medical Industries, Inc., Los Angeles, Calif.) with known amounts of nonlabeled T_4 and T_3 at different concentrations and dialyzed against BGJ medium. Samples from the dialysis bag and bath were counted in a liquid scintillation counter daily until the radioactivity on each side of the dialysis membrane was constant on successive days. The percent of unbound thyroid hormone in the original culture medium at each concentration before dialysis was then calculated from the percentage of radioactivity that had crossed the dialysis membrane (i.e., the dialyzable or free fraction).

Total medium T_4 and T_3 concentrations were determined in BGJ medium containing 4 mg/ml bovine serum albumin before and after culture with fetal long bones for 4 days. These thyroid hormone radioimmunoassays were kindly performed by Dr. P. Sullivan using methods described previously (16).

Histology. The cellular mechanism of bone resorption in the cultured fetal rat long bones was assessed by histologic examination of paired test and control bones after 2 and 5 days of culture. The bones were fixed in modified Bouin's fixative, decalcified, and stained with hematoxylin and eosin.

Dry weights. Bones from which mineral was extracted by washing in 5% TCA, acetone, and ether were weighed on a Perkin Elmer electro-balance (Perkin-Elmer Corp., Norwalk, Conn.) after culture to confirm that matrix resorption had occurred.

Hydroxyproline was also determined in bones treated with T_4 (0.1 μM) and paired control bones after 5 days of culture. The bones were first dried with acetone and extracted with 5% TCA. The hydroxyproline concentrations were determined by the method of Cheng (17).

RESULTS

Both T_4 and T_3 directly stimulated bone resorption as measured by release of ^{45}Ca from cultured fetal rat long bones.

Dose-response curves. The dose-response curves for T_4 and T_3 were indistinguishable (Fig. 1). Maximal effects were obtained with concentrations of each in the culture medium of 1 μM after 5 days of treatment. With concentrations of 10 nM, although the treated-to-control ratio was still significant, the response was small. Increasing the concentration greater than 1 μM did not increase the effect.

Time-course. T_4 and T_3 were found to be slow stimulators of bone resorption in this system. The effects were usually small during the first 2 days of treatment but then increased substantially during the next 3–6 days (Fig. 2). This effect is probably even slower than that of prostaglandins which are also relatively slow bone resorbers (18).

Dry weights. Dry weights of extracted bones were measured to confirm that matrix resorption had accompanied mineral release. Bones cultured with T_4 (0.1 μM) at the end of 5 days of treatment weighed less than paired bones cultured in control medium (Table I).

The mean hydroxyproline concentration of bones treated with T_4 0.1 μM was 0.07 $\mu\text{g}/\text{bone}$ on 12 pooled

² BGJ, a chemically defined medium developed by Biggers et al. (10).

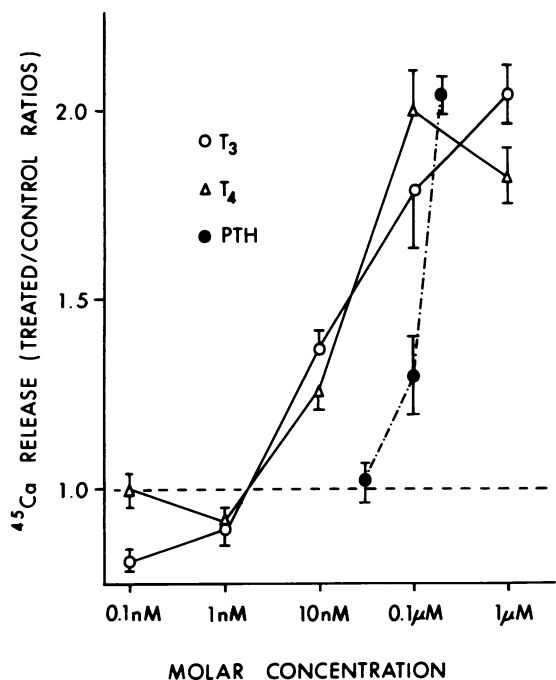


FIGURE 1 Dose-response curves for T_3 , T_4 , and PTH. The bones were cultured in BGJ medium containing 4 mg/ml bovine serum albumin. The bones were transferred to fresh media after 2 days, and then cultured for an additional 3 days. Values are mean \pm SEM for four bones per point for 5 days in culture.

bones. The mean hydroxyproline concentration in paired control bones cultured for the same period was 0.21 μ g/bone.

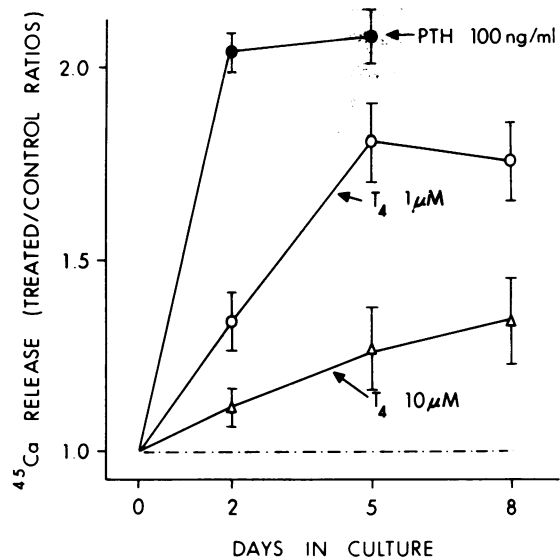


FIGURE 2 Time-course of action of T_4 and PTH. The bones were cultured in BGJ medium containing 4 mg/ml bovine serum albumin for 8 days, but were transferred to fresh media after 2 and 5 days. Values are mean \pm SEM for four bones.

TABLE I
Effect of T_4 Treatment on Extracted Dry Weight of Cultured Bones

	cpm released into medium	Dry weight of extracted bone at end of culture (μ g) for 5 days
T_4 0.1 μ M	2,377 \pm 656	6.8 \pm 1.1
Control medium	1,376 \pm 148	10.7 \pm 2.5

Values shown are \pm SEM for four bone cultures. Bones were weighed after extraction with 5% TCA, acetone, and ether.

Histology. The morphologic appearance of the cultured bones showed increased osteoclastic activity, appearances which cannot be distinguished from those seen with other humoral mediators of bone resorption such as PTH, active vitamin D metabolites, and prostaglandins.

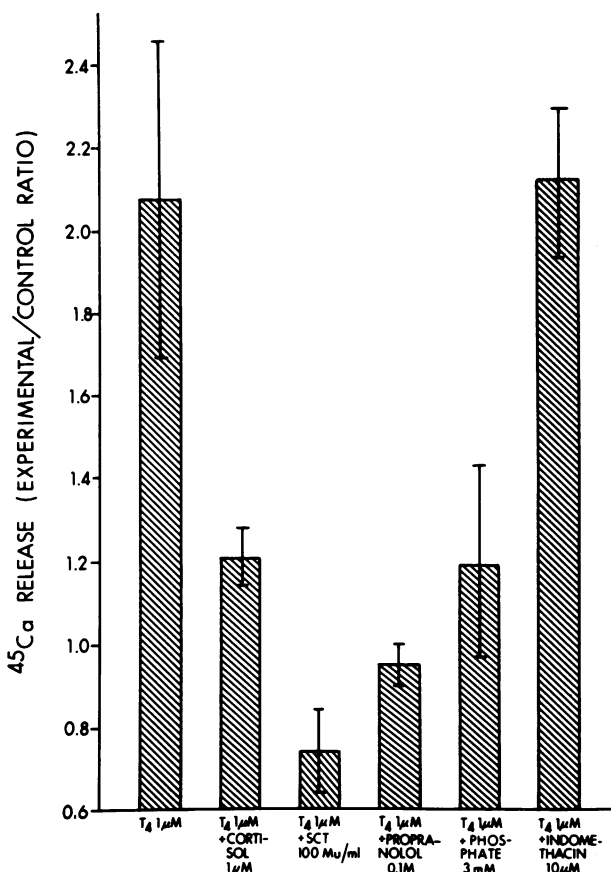


FIGURE 3 Effect of inhibitors on T_4 -stimulated bone resorption. The duration of the culture period was 5 days. For each group, one bone was cultured with T_4 and inhibitor and the paired bone was cultured with the appropriate inhibitor alone in control medium. Values are mean \pm SEM for four pairs of bone cultures.

TABLE II
Effect of Thyroid Hormone Analogues on Bone

Compound	Percent release of ⁴⁵ Ca
<i>l</i> -T ₄	85 ± 3*
<i>d</i> -T ₄	31 ± 3
3,5-Diiodo- <i>l</i> -thyronine	33 ± 2
3,3',5'-Triiodothyronine	36 ± 6
3',3,5-Triiodothyroacetic acid	56 ± 6*
3-Iodo- <i>l</i> -tyrosine	28 ± 2
3,5-Iodo- <i>l</i> -tyrosine	29 ± 2
Control media	28 ± 6

Values are ±SEM for four to eight bone cultures. All compounds were tested at 0.1 μM for 5 days.

* Significantly greater than control, *P* < 0.05.

Inhibitors. The effect of thyroid hormones on bone resorption was completely inhibited by salmon calcitonin (100 mU/ml), phosphate (3 mM), and propranolol (0.1 M). Cortisol (1 μM) partially inhibited the effect but had no effect at 10 nM (Fig. 3). Indomethacin at 10 μM was tested because the shallow dose-response curves and slow time-course of the thyroid hormone effects resemble the properties of prostaglandins in this system. If the effects of thyroid hormone on bone resorption were due to prostaglandin synthesis by the cultured bone cells, as can occur in this system when bones are cultured with complement-sufficient serum (19), then indomethacin should prevent this by inhibiting the activity of the enzyme prostaglandin synthetase. The observation that thyroid hormone-stimulated bone resorption was not inhibited by indomethacin demonstrates that thyroid hormone effects were not due to prostaglandin synthesis by the cultured bone.

Analogues. A series of thyroid hormone analogues were tested for bone resorbing activity at 1 μM, 0.1 μM, and 10 nM. None of the analogues tested caused bone resorption after 2 days culture (data not shown), although triiodothyroacetic acid had an effect after more prolonged culture (Table II). In particular, *d*-thyroxine and reverse T₃ (3',3',5'-diiodothyronine) were without effect. This is in contrast to the effects of some of these analogues on rat bone phosphodiesterase found by Marcus (20). He found using *l*-T₄ at 10 μM as a reference standard that tetraiodothyroacetic acid and triiodothyroacetic acid were more potent inhibitors of this enzyme in rat calvaria than T₄. When his results are compared with those reported here, it can be seen that there is no relationship between the effects of these analogues on phosphodiesterase inhibition and bone resorption.

Percent-free hormone. When labeled T₄ and T₃ were placed in the culture medium and dialyzed against BGJ medium without protein supplementation, the radioac-

tivity was constant on each side of the dialysis membrane after the 2nd day of dialysis. By this stage, 90% of the original radioactivity was still present within the dialysis bags indicating that 10% of T₄ and T₃ were not bound to the albumin in the medium. Assay of culture media for total T₄ and T₃ after 5 days of bone culture with T₄ 0.1 μM was performed. The total T₄ concentration fell by less than 5% during the culture period. Immunoassayable T₃ concentrations in the media containing T₄ (0.1 μM) before and after culture were the same as the concentrations in control media containing no thyroid hormone, and were less than 1 nM. The lowest concentration of T₃ which stimulated bone resorption in this system was 10 nM. Thus the effects of T₄ on bone resorption are unlikely to have been mediated by T₃ through peripheral deiodination of T₄ to T₃ by the culture system.

DISCUSSION

This study demonstrates that thyroid hormones can directly stimulate osteoclastic bone resorption in fetal rat long bones at moderate concentrations. Experiments with equilibrium dialysis using labeled T₄ and T₃ show that about 90% of each hormone was bound in the bone culture medium (BGJ with bovine serum albumin 4 mg/ml). T₄ and T₃ both stimulated bone resorption at concentrations of 10 nM in this culture medium. The minimum free concentrations (1 nM) of hormone which cause detectable bone resorption are within a 100-fold concentration of the free circulating concentrations of hormone found in thyrotoxicosis.

The concentrations of thyroid hormones required to cause bone resorption in vitro are greater than the circulating concentrations found in thyrotoxicosis. However, the local thyroid hormone concentration to which bone cells are exposed in vivo may not be equivalent to the circulating concentrations. It is also important to realize that in this in vitro system, thyroid hormones are more potent stimulators of osteoclastic bone resorption than PTH, which works only at 0.1 μM–10 nM (30).

The histologic appearance of fetal rat long bone cultures treated with T₄ and T₃ is similar to the appearance of histologic sections of bone in patients with thyrotoxicosis (3–7). Osteoclasts were increased both in number and activity. The appearances are also similar to those seen in cultured bones treated with PTH (12), and the bone morphology in hyperparathyroidism is similar to that seen in thyrotoxicosis. However, the effects of thyroid hormones and PTH on growing bones may be very different. Children with thyrotoxicosis often have accelerated linear growth; conversely, cretins have decreased bone growth, and previously normal linear growth may cease with the onset of juvenile myxedema (1). Whether this is a direct effect of thyroid hormone is unknown. PTH causes inhibition of collagen synthe-

sis and new bone formation in vitro (21). In preliminary studies we have not been able to show any effect of T_4 on collagen synthesis in fetal rat bones.

The demonstration that thyroid hormones can directly stimulate osteoclastic bone resorption in vitro does not preclude the possibility that there are other potential mechanisms of increased bone resorption in thyrotoxicosis. It has recently been found that peripheral blood leukocytes from patients with Graves' disease produce lymphokines such as macrophage migration inhibition factor when stimulated by crude particulate thyroid antigens (22). Leukocytes from patients with nodular goiter or normal controls do not produce lymphokines under these circumstances. Normal peripheral blood leukocytes can also secrete a lymphokine which we have called osteoclast activating factor (OAF) (23) when stimulated by an antigen to which they have been previously exposed or by a nonspecific mitogen. OAF has potent effects on bone metabolism similar to those of PTH. Leukocytes which have been activated to secrete migration inhibition factor may presumably also secrete OAF. It is possible that in thyrotoxicosis bone resorption is stimulated directly by increased T_4 and T_3 and enhanced by the alterations in immunologic function that occur in Graves' disease. It has also been suggested in the past that elevated circulating concentrations of T_4 may make bone cells more sensitive to the effect of PTH (24, 25). We have not been able to show that the effects of PTH and T_4 are synergistic or that responses to small doses of PTH were enhanced by prior T_4 treatment of the cultured bones.³

The recent observation that thyroid hormones inhibit phosphodiesterase in fetal rat calvaria has raised the possibility that thyroid hormones stimulate bone resorption by raising the intracellular concentrations of cyclic AMP (20). This hypothesis is supported by the observation that PTH increases intracellular cyclic AMP concentrations in bone cells (26), and that plasma and urine cyclic AMP concentrations are increased in both hyperparathyroidism and hyperthyroidism (27). Phosphodiesterase inhibition by dibutyl cyclic AMP (28) and theophylline is associated with increased cyclic AMP concentrations and bone resorption, and the possibility that thyroid hormones caused bone resorption via this mechanism was considered. However, we found that bone resorption could be stimulated by hormone concentrations 1,000-fold lower than those causing phosphodiesterase inhibition. Furthermore, a number of thyroid hormone analogues cause phosphodiesterase inhibition (20), whereas the only compounds with the thyronine or tyrosine structure we found to stimulate bone resorption were T_4 , T_3 , and triiodothyroacetic acid. Hyperthyroidism may be associated with increased cir-

³ Mundy and Raisz. Unpublished observations.

culating concentrations of cyclic AMP because of indirect mechanisms, for example increased sensitivity of beta-adrenergic receptors to catecholamines (27).

Thyroid hormones have different biological characteristics from other bone resorbers in organ culture. PTH has a steeper dose-response curve and a faster onset of action but is similarly partially inhibited by large doses of cortisol. OAF has a steep dose-response curve similar to that of PTH and also has long lasting effects after brief exposure with maximum doses (29). However, OAF differs from PTH and T_4 in being inhibited completely by cortisol at 0.1 μ M. Prostaglandins have more shallow dose-response curves than thyroid hormones but have a similar slow time-course (18, 30). It is likely that this slow onset of action was the reason thyroid hormones were not found to stimulate bone resorption when examined in this culture system previously (12, 13). The active vitamin D metabolites have a faster onset of action than the thyroid hormones but have very similar dose-response curves, more shallow than that of OAF or PTH but steeper than that of prostaglandins (30).

The inhibition of thyroid hormone-stimulated bone resorption by propranolol may have clinical implications. Patients with thyrotoxic hypercalcemia may become normocalcemic after propranolol therapy alone (31). This effect may be due to the ability of propranolol to inhibit bone resorption stimulated by T_4 , as we have shown here. Propranolol also inhibits bone resorption stimulated by PTH (32), active vitamin D metabolites, prostaglandins, and OAF.⁴

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⁴ Dietrich and Mundy. Unpublished observations.

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