Estrogen and Tamoxifen Interplay with T₃ in Male Rats: Pharmacologically Distinct Classes of Estrogen Responses Affecting Growth, Bone, and Lipid Metabolism, and Their Relation to Serum GH and IGF-I

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Abstract

Estrogens (E) and T₃ regulate gene expression by receptor mechanisms that may enable hormonal interplay affecting growth and metabolism. Prior studies of E and tamoxifen (TM) interplay with T₃ in female rats identified a subset of E responses that required T₃ for expression and exhibited large agonist responses to TM. In contrast, TM acted more like an antagonist in most T₃-independent E responses. This study used male rats to further explore the role of T₃ in E effects on growth and metabolism, and the relation of such effects to changes in serum GH and IGF-I. Orchiectomized, hypothyroid rats were treated 6 wk with vehicle, E₂ benzoate (E₂B), or TM with or without T₃. The following parameters were measured: body weight change; tibia length and bone mineral density; heart and kidney weight; food intake and body temperature; serum levels of glucose, cholesterol, triglycerides, GH, and IGF-I; seminal vesicle weight; and anterior pituitary levels of GH, PRL, glandular kallikrein, and total protein. Interplay with T₃ contributed to multiple E effects on growth and metabolism, and some E responses involved both T₃-dependent and T₃-independent components. Both E₂B and TM increased serum GH, but the increases were poorly coupled to IGF-I. Correlation/regression analysis of individual rat data sets suggested distinct roles for GH and IGF-I in specific E effects. E₂B and TM effects on somatic growth exhibited positive correlations with IGF-I and negative correlations with GH; effects on bone mineral density and triglycerides exhibited positive correlations with GH and negative correlations with IGF-I. Three pharmacologically distinct classes of in vivo E responses were identified in this study, and TM displayed a profile of biological activity that may be useful for men undergoing androgen-deprivation therapy. (Endocrinology 142: 4223–4235, 2001)

Introduction

Estrogens (E) regulate the growth and function of female sex organs, but effects on bone have led to use for osteoporosis (1). Conversely, selective estrogen receptor modulators (SERMs) such as tamoxifen (TM) and raloxifene inhibit E effects on the breast but mimic E effects on bone (2, 3). This paradoxical pharmacology reflects the diversity of ER mechanisms, with SERMs interfering with mechanisms at some targets while activating those at others. The mechanisms involved in ER signaling are influenced by cell phenotype, the target gene, and the activity of other signaling networks (4–6). With regard to the latter, there is evidence that multiple ER mechanisms may enable interplay with other hormone signaling systems, including those for T₃ (7–13).

T₃ regulates growth as well as energy and lipid and bone metabolism; E interplay with T₃ may provide a versatile mechanism for metabolic adaptations related to reproductive biology. The physiological relevance of such interplay has been explored in ovariectomized, hypothyroid female rats (14–17). The results identified a subset of E responses that are T₃ dependent; this includes effects on growth, bone mass, and triglycerides. T₃-independent E responses include uterine growth and induction of pituitary PRL and kallikrein. Moreover, TM exhibited high agonist efficacy in T₃-dependent responses but acted more like an antagonist in most T₃-independent responses. These findings suggest that estrogens may mediate two distinct types of biological regulation. In T₃-independent responses, E can be viewed as a primary driver that fully activates the ER mechanisms mediating the response, and SERMs appear to poorly activate such mechanisms. In T₃-dependent responses, however, E can be viewed as a modulator whose responses rely on interplay with T₃ signaling mechanisms; such modulator actions are also activated by SERMs.

The concept of distinct primary driver and modulator actions may clarify E actions in complex physiological systems regulated by multiple hormones. Moreover, targeted activation of modulator responses by SERMs may benefit novel groups of patients. For example, androgen deprivation therapy used in men with disseminated prostate cancer can increase the risk of osteoporosis and bone fractures (18–21). Estrogens can induce a state of androgen deprivation that can avoid such risks (22), but doses needed for satisfactory tumor responses are often toxic (23), and even low doses evoke breast growth. On the other hand, use of SERMs during androgen deprivation therapy may well prevent osteoporosis with little risk of toxicity or breast growth. However, there is little information about SERM effects in men.

Animal studies of SERMs or their interplay with T₃ have also focused on females. Thus, it is unclear if males exhibit equivalent responses, and there are reasons why such equivalence should not be presumed. In particular, the GH-IGF-I

Abbreviations: BMD, Bone mineral density; E₂B, E₂ benzoate; SERMs, selective estrogen receptor modulators; TM, tamoxifen.
axis is targeted by both T₃ and estrogens, and some estrogen effects on growth and metabolism have been proposed to reflect changes in the GH-IGF-I axis (24–27). However, an irreversibly sex difference in GH secretory patterns develops soon after birth (24, 28), and this may influence the nature of E effects in males and females. In addition, the association between E effects on the GH-IGF-I axis and E effects on growth and metabolism has not been rigorously analyzed in either male or female rats. Specifically, correlation/regression analyses of data sets of individual rats have not been reported. Thus, it is unclear if measures of growth or metabolism in a given rat during E treatment are significantly related to the GH or IGF-I levels in that rat.

To address the above considerations, male rats were used 1) to study the role of T₃ in E and SERM effects and their relation to GH and IGF-I, and 2) to evaluate the therapeutic potential of SERMs in androgen-deprived males. The results show that interplay with T₃ contributes to multiple E actions in male rats and imply that SERMs may benefit men on androgen deprivation therapy. In addition, the correlation/regression analyses suggest novel roles for GH and IGF-I in E effects on specific parameters. Finally, the data revealed three pharmacologically distinct classes of E responses that presumably reflect the diversity of E receptor mechanisms in vivo.

Materials and Methods

Animals

All protocols followed NIH guidelines on the use and care of animals and were approved by the institutional Animal Care and Use Committee. Forty-eight young adult male Sprague Dawley rats were used (Tac- onic Farms, Inc., Germantown, PA); rats were 75–80 d old and weighed 350–375 g upon arrival. Forty-four of the rats were given 0.03% methimazole (a thioamide inhibitor of thyroid hormone synthesis) in drinking water throughout the study to induce hypothyroidism and allow experimental control of T₄ levels. Calcium chloride (1%) was included with methimazole in the water to ensure adequate dietary calcium intake because hypothyroidism decreases food intake by up to 40% (14). Methimazole evoked functional hypothyroidism in all rats within 14 d because hypothyroidism decreases food intake by up to 40% (14). Measurement of T₄ levels confirmed this finding. 

Experimental design and drug treatments

A gonadectomized, hypothyroid rat model was used which allows TM, E₂B, and T₃ effects to be separately identified and hormonal interactions studied. The model was designed to selectively detect E₂B and TM effects on the pharmacodynamic actions of T₃ and avoid other types of hormonal interactions. Thus, use of methimazole eliminates changes in the synthesis or release of T₄, or T₃ as a mechanism of E₂B or TM effects. Use of T₃ eliminates interactions related to 5'-deiodination of T₄ to T₃. Use of T₃ also avoids interactions due to E₂B or TM effects on plasma T₂-binding proteins (transhyretin and T₂-binding globulin). T₃ has only 1/10 the affinity of T₄ for such proteins, and unlike T₄, the distribution of T₃ in the rat is not restricted by plasma protein binding (volume of distribution = 1.65 liter/kg for T₄ vs. 0.16 liter/kg for T₃) (31).

Six groups of orchidectomized rats received the following drug and hormone treatments starting 3 d after surgery: 1) vehicle solutions alone (n = 8); 2) TM (trans isomer, free base) at 0.5 mg/kg via sc injection, once daily (n = 7); 3) 17β-E₂–3-benzoate (E₂B) at 30 μg/kg via sc injection three times weekly (n = 6); 4) T₄ (sodium salt) at 10 μg/kg via ip injection once daily (n = 7); 5) T₃, plus TM (n = 7); and 6) T₃, plus E₂B (n = 8). The group of four euthyroid, testis-intact rats received daily injections of vehicle solutions. During the treatment phase of the study, body weight was measured three times per week and food intake was measured once a week. Rats were gang-caged (3–4 per cage) to decrease risk of hypothermia due to hypothyroidism; thus, total food intake per group was measured during a 24-h period, and average intake per rat interposed.

Body temperature was measured weekly with a rectal thermocouple probe and digital thermometer to provide an index of T₃ effects on thermogenesis.

The doses of E₂B, TM, and T₃ used in this study have been found to yield maximal target organ and growth responses in dose-response studies in ovariectomized or ovariectomized, hypothyroid female rats (15, 32). E₂B and TM were dissolved in sesame oil with 2% benzy alcohol; T₃ was dissolved in 0.9% NaCl with 5 mM NaOH.

Tissue processing

After 6 wk of treatment, rats were euthanized in random order with 100 mg/kg sodium pentobarbital (ip) between 1030 and 1530 h. Blood, anterior pituitaries, and right tibias were collected within 5 min of pentobarbital injection as previously described (14, 15). Blood samples were allowed to clot for 5 min at room temperature, cooled on ice, and then refrigerated at 5°C. Serum was collected the next day, aliquoted, and stored at −80°C until assay. The right tibia was stripped of most muscle and connective tissue and stored in 70% ethanol for subsequent measurement of tibia length (an index of longitudinal growth) and bone mineral density (BMD). Anterior pituitaries were sonicated in 400 μl of 10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl and 0.1% Triton X-100; samples were then aliquoted and stored at −20°C until assay. Seminal vesicles were dissected and weighed. The ventral prostate lobes were visually inspected for any gross differences in size. The left kidney and heart were dissected and weighed to examine the effect of TM or E₂B on T₃ actions that yield increases in heart and kidney mass that exceed changes in overall somatic growth (33, 34).

Analysis of BMD

Tibia BMD was measured by dual-energy x-ray absorptiometry with a QDR-1000 (Hologic, Inc., Waltham, MA) as previously described (14, 15). Image analysis software (Hologic, Inc.) automatically calculated bone mineral content (g), cross-sectional area (cm²), and BMD (g/cm²) in two regions: the proximal tibia and the diaphysis. The proximal tibia (upper 1/3 of tibia length) is relatively enriched in cancellous bone and has a higher ratio of cancellous to cortical bone than the diaphysis (middle 1/3 of tibia length) (35).

Metabolic analyses

Lipid metabolism was assessed by measuring total cholesterol, triglycerides, and β-hydroxybutyrate (an index of fatty acid β-oxidation) in serum using colorimetric kits from Sigma (St. Louis, MO). Serum glucose was measured as an index of carbohydrate metabolism using a colorimetric kit from Stanbio Laboratories (San Antonio, TX). In the rat, 70–80% of serum cholesterol is bound to high-density lipoprotein, and estrogens and SERMs have equivalent effects on high density lipoprotein- or low density lipoprotein-cholesterol (36).

Assay of serum GH and IGF-I

Serum levels of GH and IGF-I were used to assess changes in the GH-IGF-I axis. Total serum levels of IGF-I were determined using a RIA kit from Nichols Institute Diagnostics (San Juan Capistrano, CA) after extraction of IGF-I from serum. IGF-I in rat serum was separated from
IGF-binding proteins using the acid-ethanol extraction method described by Crawford et al. (37). Serum samples (100 μl) were mixed with 900 μl acid-ethanol solution (12.5% 2 M HCl: 87.5% ethanol, vol/vol) and incubated 30 min at room temperature. Following centrifugation at 1,500 × g for 30 min at 5 C, 200 μl of the supernate was mixed with 100 μl 0.855 M Tris base (pH 11) and incubated 30 min at room temperature. After centrifugation at 1,500 × g for 30 min (5 C), 100 μl of the final supernate was mixed with 1.4 ml phosphate buffer (pH 7.5), and used for RIA. This acid-ethanol extraction method has previously been validated for RIA of total IGF-I in male and female rat serum by comparison with results using HPLC methodology (37). Serum GH was determined using a rat GH RIA kit from Amersham Pharmacia Biotech (Piscataway, NJ).

Anterior pituitary analyses

Induction of anterior pituitary PRL and GH by E and T3, respectively, was qualitatively analyzed using denaturing PAGE as previously described (15, 16). Such analyses have previously been validated with GH and PRL RIAs (15, 16). Anterior pituitary levels of glandular kallikrein (an E-induced protease in lactotrophs) was assayed using α-Val-Leu-Arg-p-nitroanilide as was previously described (38). Total protein provided an index of E-induced anterior pituitary hyperplasia (38).

Statistical analysis of grouped data

Data were subjected to ANOVA followed by a posthoc analysis with Duncan’s new multiple range test; P < 0.05 was the criterion of significance. Serum GH and triglyceride data values were log-transformed to normalize variances.

The change in a parameter due to T3 (Δ due to T3) was calculated by subtracting the mean of the vehicle, TM, or E2B group without T3 from each data point in the matching group with T3; transformed data were then analyzed as noted above. This facilitated comparison of E2B and TM interplay with T3 in diverse parameters and was used to calculate the % efficacy of TM relative to E2B (100%) in responses involving interplay with T3.

Correlation/regression analysis

Pearson’s product-moment correlation (r) and multiple linear regression analysis was used to identify significant associations of serum GH or IGF-I to other parameters. Correlations/regressions were calculated using sets of individual rat data (single units of expression) that captured the effects of the four distinct hormonal manipulations studied (T3 manipulation with and without an estrogenic background, and estrogenic manipulation with and without a T3 background). Correlations capturing the effect of T3 manipulation without an estrogenic background were calculated using individual rat data from groups 1 (vehicle alone) and 4 (T3 alone) (n = 15 data pairs per parameter). Correlations during T3 manipulation with an estrogenic background used data from groups 2 (TM alone), 3 (E2B alone), 5 (T3 plus TM) and 6 (T3 plus E2B) (n = 27). Correlations during estrogenic manipulation without T3 used data from groups 1 (vehicle alone), 2 (TM alone), and 3 (E2B alone) (n = 21). Correlations during estrogenic manipulation in the presence of T3 used data from groups 4 (T3 alone), 5 (TM plus T3) and 6 (E2B plus T3) (n = 21).

Results

TM, E2B, and T3 effects on tibia BMD

BMD of the proximal tibia (enriched in cancellous bone) was 9% lower in orchidectomized, hypothyroid controls than in euthyroid, testis-intact rats (Fig. 1). This probably reflects the lack of testicular androgens (39–42). T3 alone had no effect on BMD but potentiated TM and E2B effects; TM evoked 4.2% increases in rats lacking T3, and 9.8% increases in rats given T3. E2B evoked 12.8% and 17.6% increases in BMD in the absence or presence of T3, respectively. In T3-treated rats, TM prevented bone loss associated with orchidectomy, while E2B yielded BMD values exceeding euthyroid, testis-intact rats (Fig. 1).

TM had 1/3 the efficacy of E2B in rats lacking T3, but BMD increases due to T3 interplay with TM and E2B were equivalent (see inset, Fig. 1). This suggests that E effects on BMD in male rats involved a T3-independent component in which TM had low efficacy, and a T3-dependent component in which TM and E2B had equivalent efficacy.

It is of note that rats lacking T3 displayed no net growth (see Fig. 3), and E2B increased proximal tibia BMD in such rats without altering tibia length (see Fig. 4) or cross-sectional area (not shown). E2B effects on BMD were thus largely independent of changes in bone growth.

Tibia diaphysis BMD did not significantly differ among groups (Fig. 1). The diaphysis has a lower ratio of cancellous bone to cortical bone, and the data are consistent with differ-
ferences in the turnover rates and E sensitivity of cancellous bone and cortical bone (29, 30, 35).

**TM, E2B, and T₃ effects on serum levels of cholesterol and triglycerides**

TM lowered cholesterol by 38 mg/dl in orchidectomized, hypothyroid rats lacking T₃ (Fig. 2). Cholesterol was also reduced by T₃ (−24 mg/dl), and T₃ and TM effects were additive. Thus, TM and T₃ affect cholesterol independently of one another. Remarkably, E2B had no effect on cholesterol, even at doses as high as 250 µg/kg (unpublished data). Euthyroid, testis-intact male rats had slightly lower cholesterol levels (−18 mg/dl) than orchidectomized, hypothyroid rats treated with T₃, but the difference was not significant (Fig. 2).

Serum triglycerides in intact rats were over twice that of orchidectomized, hypothyroid controls (Fig. 2). T₃, TM, or E2B alone did not alter triglycerides in hypothyroid rats, although TM tended to lower levels. However, TM and E2B increased triglycerides in T₃-treated rats (Fig. 2); the TM increase was 65% that evoked by E2B (inset, Fig. 2).

**TM, E2B, and T₃ effects on male sex organs**

TM had no effect on seminal vesicle weight in orchidectomized, hypothyroid rats with or without T₃ (Fig. 2); this was also the case with the ventral prostate (based on visual inspection). E2B in combination with T₃ evoked a small rise in seminal vesicle weight (Fig. 2), but seminal vesicles in rats given E2B plus T₃ were still minuscule compared with testis-intact rats.

**TM, E2B, and T₃ effects on somatic growth**

In the first 10 d, orchidectomized, hypothyroid controls lost 3% of body weight while E2B- or TM-treated rats lost 9% (Fig. 3). Weight then stabilized in control and TM-treated rats, but a slow weight loss continued in E2B-treated rats (−4% in 32 d) (Fig. 3). T₃ increased weight, and this gain was inhibited 78% by TM and 65% by E2B (inset, Fig. 3).

In rats lacking T₃, TM or E2B did not alter tibia length. However, increases in tibia length evoked by T₃ were similarly inhibited by TM (−69%) and E2B (−81%) (inset, Fig. 4).

During the 6-wk treatment phase of the study, euthyroid, testis-intact rats gained 70% more weight than orchidectomized, hypothyroid rats treated with T₃ alone (not shown). This presumably reflects a stimulation of somatic growth by testicular androgens (43), which may also partly account for the greater tibia lengths of testis-intact rats (Fig. 4).

**TM, E2B, and T₃ effects on serum and pituitary GH**

Serum GH in orchidectomized, hypothyroid rats was only 8% that of euthyroid, testis-intact rats (Fig. 4), and this was associated with a loss of pituitary GH content (Fig. 5). Although T₃ alone increased serum GH, it yielded only 25% the level of euthyroid, testis-intact males even though pituitary GH content was almost fully restored. In rats lacking T₃, TM and E2B evoked 4.0-fold and 6.9-fold increases in serum GH, respectively, whereas T₃ alone yielded a 3.2-fold increase (Fig. 4). However, TM and E2B evoked only weak T₃-like effects on pituitary GH content (Fig. 5). Thus, TM and E2B effects on serum GH do not simply reflect changes in pituitary GH content; alterations in neuroendocrine regulation are evident.

TM and E2B effects on serum GH were enhanced by T₃ and yielded increases greater than expected from an additive interaction with T₃ (inset, Fig. 4). In either the presence or absence of T₃, TM displayed half the efficacy of E2B on serum GH. On the other hand, T₃ induction of pituitary GH content was clearly inhibited by TM, whereas E2B had little effect (Fig. 5). Thus, although TM mimicked E2B

![Fig. 2](https://academic.oup.com/endo/article-abstract/142/10/4223/2988571/4226)
effects on serum GH in T₃-treated rats, it displayed a distinct effect on pituitary GH. The pituitary GH data of male rats match the results of similar studies in female rats (14–17). TM or E2B effects to increase serum GH, however, were much smaller in female rats, and were not enhanced by T₃ (15).

Only orchidectomized, hypothyroid rats given T₃ in combination with TM or E2B had serum GH levels approaching those of euthyroid, testis-intact males. However, serum GH increases evoked by E2B and TM did not yield corresponding increases in somatic growth.

**TM, E2B, and T₃ effects on serum IGF-I**

Orchidectomized, hypothyroid rats had serum IGF-I levels that were half that of testis-intact, euthyroid rats (Fig. 4). T₃ alone fully restored IGF-I to the levels of euthyroid, testis-intact rats despite its relatively modest effect on serum GH. In contrast, E2B or TM did not yield IGF-I increases similar to T₃ even though they caused greater increases in serum GH. Interestingly, E2B and TM had differing effects on IGF-I in the absence of T₃, TM modestly increased IGF-I (+20%), whereas E2B markedly lowered IGF-I (−40%) (Fig. 4).
Rats treated with E2B plus T₃ also had much lower IGF-I levels than those given T₃ alone, due in large part to a smaller IGF-I response to T₃. TM also displayed a tendency to lower IGF-I in T₃-treated rats (−12%) that did not reach significance. However, unlike E2B, TM alone modestly increased IGF-I (+20%), and this partly masked inhibition of T₃ effects. When IGF-I increases due to T₃ were calculated, it was evident that this T₃ effect was significantly inhibited by TM (−42%) as well as by E2B (−73%) (inset, Fig. 4). TM effects on IGF-I in male rats resemble results in female rats (14): this includes both TM’s tendency to increase IGF-I in the absence of T₃ and the inhibition of T₃ effects on IGF-I.

Effects on food intake, body temperature, serum glucose, and serum β-hydroxybutyrate

Orchidectomized, hypothyroid male rats lacking T₃ consumed 35% less food per kg body weight than euthyroid, testis-intact males (Fig. 7). T₃ increased food intake to that of euthyroid, testis-intact rats. E2B and TM did not alter food intake in rats with or without T₃ (inset, Fig. 7). TM and E2B effects on growth and metabolism are unlikely to reflect changes in food intake. The data further document that not all T₃ effects are sensitive to E2B or TM.

Body temperature was unaffected by TM or E2B in the absence of T₃ (Fig. 7). T₃ produced a 1.5°C increase in temperatures of orchidectomized, hypothyroid rats. T₃ effects on body temperature were slightly inhibited by TM and E2B (−11% and −33%, respectively), but only E2B had a significant effect. Only a small fraction of the thermogenic effects of T₃ are sensitive to E2B, and TM has only 1/3 the efficacy of E2B.

Euthyroid, testis-intact rats had significantly lower body temperatures (−1.0°C) than orchidectomized, hypothyroid rats given T₃ (Fig. 7). This temperature difference is due to testicular androgens; it does not reflect use of an excessive T₃ dose (our manuscript in preparation).

TM and E2B tended to lower serum glucose in orchidectomized, hypothyroid rats lacking T₃, but the changes were insignificant (Fig. 7). T₃ alone had no effect on glucose but enhanced TM and E2B effects over 2-fold. Decreases in glucose evoked by E2B (−28%) were significant in T₃-treated rats, but not those for TM (−13%). Serum β-hydroxybutyrate levels were less than 2.5 mg/dl in all groups (data not shown); thus, no major treatment effects on fatty acid oxidation were evident. The glucose and β-hydroxybutyrate data did not reveal alterations in energy metabolism likely to explain E2B and TM effects on growth.

**TM, E2B, and T₃ effects on relative heart and kidney weights**

Calculation of organ weight relative to body weight (relative weight, g/kg) can detect changes in organ weight that are disproportionate from changes in body weight. T₃ significantly increased relative heart weight in hypothyroid male rats (Fig. 8); this may reflect direct actions on the heart as well as effects secondary to changes in vascular tone and autonomic nervous system function (33). TM and E2B did not alter relative heart weight in rats lacking T₃, and increases evoked by T₃ were insensitive to E2B or TM (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8). Relative kidney weight was slightly increased by TM or E2B in rats lacking T₃ (inset, Fig. 8).

**TM, E2B, and T₃ effects on the anterior pituitary**

Total pituitary protein content is well correlated with pituitary weight and provides a useful index of E-evoked pituitary hyperplasia in the rat (38, 44). E2B or T₃ alone caused only modest protein increases, but synergized with one another to evoke striking increases (Fig. 6). Unlike E2B, TM slightly decreased pituitary protein in the presence or absence of T₃.

E2B selectively induced pituitary PRL in rats lacking T₃ (Fig. 5). T₃ alone evoked only small PRL increases, but markedly enhanced E2B actions. However, PRL increases with E2B plus T₃ were coupled to nonspecific increases pituitary proteins characteristic of tissue hyperplasia (Figs. 5 and 6). TM did not alter PRL either with or without T₃.

T₃ alone slightly decreased glandular kallikrein (Fig. 6), an E-induced lactotropin protease (32). E2B induced total kallikrein activity (nmol/min-pituitary) by 6-fold in the absence of T₃, and 18-fold in the presence of T₃. However, E2B induction of kallikrein was unaffected by T₃ when expressed as specific activity (nmol/min-mg protein) to adjust for
changes in total protein due to tissue hyperplasia (Fig. 6). TM lacked effect on glandular kallikrein.

Overall, the data indicate that E2B induction of PRL and kallikrein was T3 independent, whereas E2B actions to evoke pituitary hyperplasia were T3 dependent. The hyperplastic effect of E is due to a selective induction of lactotroph proliferation (45, 46), and this amplifies E2B effects on PRL and kallikrein by increasing lactotroph numbers.

Correlation of serum GH and serum IGF-I with other physiological parameters

Group means suggested that E2B and TM effects on GH and IGF-I may be related to changes in four other parameters: Δ body weight, tibia length, proximal tibia BMD and serum triglycerides. Correlation/regression analysis was used to quantitatively assess these associations.

E2B and TM effects on a parameter might reflect changes in T3 regulation of GH or IGF-I, changes in GH or IGF-I independent of T3, effects unrelated to GH or IGF-I, or combinations thereof. To separately evaluate these alternatives, correlation analysis was performed on four unstructured sets of individual rat data capturing the relationships associated with T3 manipulations with or without an estrogenic background (E2B or TM treatment), and estrogenic manipulations with or without a T3 background (see Materials and Methods for details).

Relative heart weight illustrates results for a parameter that was unaffected by E2B or TM and was unlikely to be related to GH or IGF-I. Relative heart weights during T3 manipulation in the absence of E2B/TM were strongly correlated with GH and IGF-I, but these correlations were absent from T3 manipulations with E2B/TM (Table 1). Because E2B or TM altered GH and IGF-I without affecting relative heart weight (Figs. 4 and 7), the correlations during T3 manipu-

### Table 1. Correlation of serum GH and IGF-I with other parameters in hypothyroid, orchidectomized rats during distinct hormonal manipulations under different hormonal backgrounds

<table>
<thead>
<tr>
<th>Other Parameters</th>
<th>Correlation coefficients (r) for GH</th>
<th>Correlation coefficients (r) for IGF-I</th>
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<tr>
<td></td>
<td>T3 manipulation</td>
<td>E2B/TM manipulation</td>
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<td></td>
<td>−E2B/TM (n = 15)</td>
<td>+E2B/TM (n = 27)</td>
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<tr>
<td>IGF-I</td>
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<tr>
<td></td>
<td>0.709&lt;sup&gt;b&lt;/sup&gt;</td>
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Pearson’s correlation coefficients (r) were calculated using data pairs from individual rats in the groups relevant to the indicated hormonal manipulation and hormonal background (see text for details); r values needed for significance vary depending on the number of data pairs (n) analyzed.

<sup>a</sup> P < 0.05.

<sup>b</sup> P < 0.01.

FIG. 6. Effect of TM, E2B, and T3 on anterior pituitary levels of glandular kallikrein and total protein in hypothyroid, orchidectomized rats. Left panel, Total glandular kallikrein activity. Middle panel, Glandular kallikrein specific activity. Right panel, Total anterior pituitary protein. Panel insets. Changes due to T3. V, Vehicle; I, euthyroid, testis-intact. Values represent the mean ± SEM. * P < 0.05 vs. V. ** P < 0.05 vs. all other groups.
lation without E2B/TM must be coincidental. Indeed, heart weight was not significantly correlated with GH or IGF-I in estrogenic manipulations (Table 1).

GH correlations with IGF-I illustrate results obtained with parameters known to be functionally related in vivo. During T₃ manipulation, GH exhibited a significant positive correlation with IGF-I in the absence of E2B/TM, but not in the presence of E2B/TM (Table 1). Thus, an estrogenic background impaired GH induction of IGF-I during T₃ manipulation, as expected from the grouped results (Fig. 4, inset). GH and IGF-I were poorly related during estrogenic manipulations without T₃, but a surprising negative correlation was seen during estrogenic manipulation of T₃-treated rats (Table 1). This inverse relation suggests the operation of an IGF-I negative feedback loop that inhibits GH release and is T₃ dependent. These results suggest that decreases in IGF-I (or reduced sensitivity of the feedback loop to IGF-I) may contribute to E2B and TM effects on GH in T₃-treated rats.

Body weight change during T₃ manipulation without E2B or TM was positively correlated with GH and IGF-I (Table 1). E2B and TM degraded such correlations during T₃ manipulation; this suggests that a portion of E2B/TM effects on body weight involve T₃ actions unrelated to changes in GH and IGF-I levels per se (see below). On the other hand, weight change in estrogenic manipulations was positively correlated with IGF-I and negatively correlated with GH regardless of T₃ background. Given the essential role of IGF-I in growth (25), the results suggest that IGF-I decreases also partly contribute to E effects on weight.

Tibia length was not correlated with GH during T₃ manipulation without E2B/TM but displayed a strong positive correlation with IGF-I (Table 1). This IGF-I correlation was
obiterated in the presence of E2B/TM. Thus, changes in serum IGF-I poorly explain E2B and TM effects on T3-evoked bone growth; effects on other T3 actions that promote bone growth seem likely. This might include T3 actions to enhance tissue sensitivity to IGF-I because hypothyroidism eliminated growth but lowered IGF-I only 50% (see Figs. 3 and 4).

On the other hand, tibia length was positively correlated with IGF-I and negatively correlated with GH during estrogenic manipulations in the presence of T3, but not in its absence. Given the role of IGF-I in longitudinal bone growth (25), this suggests that decreases in IGF-I may also contribute to E2B and TM effects on tibia length. It is also noteworthy that, unlike body weight change, tibia length displayed an association with IGF-I and GH only in the presence of T3.

Proximal tibia BMD was positively correlated with serum GH during T3 manipulations in the presence of E2B or TM, but not in their absence. The BMD and GH correlation was even stronger in estrogenic manipulations, and T3 had little influence on the association. Unlike body weight or tibia length, BMD was not significantly correlated with IGF-I during T3 manipulations, and BMD was negatively correlated with IGF-I during estrogenic manipulations regardless of T3.

In view of evidence that GH can increase BMD (47), the correlation analyses suggest that E2B and TM effects on BMD may partly reflect their effects on serum GH.

Triglycerides displayed a significant positive correlation with GH only during T3 manipulations in the presence of E2B/TM. Triglycerides and IGF-I were poorly associated during T3 manipulations. Triglycerides were also significantly correlated with GH (positive) and IGF-I (negative) during estrogenic manipulations with T3, but not in its absence. In view of evidence that GH can increase triglycerides (48, 49), the results suggest that GH may contribute to E2B and TM effects on triglycerides. Unlike BMD, the triglyceride response to E2B and TM displayed a T3 requirement that appears unrelated to effects on GH and IGF-I.

Multiple linear regression analysis

During estrogenic manipulations with T3, serum GH and IGF-I were inversely related, and it wasn’t surprising that GH and IGF-I correlations with a parameter were often the inverse of one another. However, GH and IGF-I were not correlated during estrogenic manipulations without T3, thus, it was notable that in such manipulations BMD was positively correlated with GH and negatively correlated with IGF-I, whereas the opposite occurred with body weight change (Table 1). This suggested that GH and IGF-I may independently contribute to E effects on BMD and body weight. Multiple linear regression analysis was used to evaluate this possibility.

Multiple linear regression analysis can be used to test hypotheses that two or more independent variables (GH and IGF-I in this case) make significant, independent contributions to regressions predicting the observed levels of a dependent variable (BMD, etc.). This involves fitting a regression equation to the data using the method of least-squares. The residual sum squares (total, regression, and error) are then used to determine the significance of the regression coefficients contributed by each independent variable (null hypothesis: coefficients = 0), and the regression correlation coefficient (r). For the present analysis, the significance of the regression coefficient of each independent variable was of primary interest.

Proximal tibia BMD was the only parameter where GH and IGF-I both made significant, independent contributions to regressions predicting observed values. In estrogenic manipulations without T3, BMD was well correlated (r = 0.78; P < 0.01) with regression predictions [BMD = 0.258 + 0.0242(logGH) − 0.000064(IGF-I)], and the regression coefficients for GH and IGF-I were each significant (P < 0.01). In estrogenic manipulations with T3, BMD displayed an r = 0.71 (P < 0.01) with regression predictions [BMD = 0.270 + 0.0172(logGH) − 0.0000373(IGF-I)]; the IGF-I coefficient was significant (P = 0.04) and the GH coefficient neared significance (P = 0.067). Regression predictions from analysis of all orchidectomized rat data [BMD = 0.239 + 0.0271(logGH) − (0.0000234 × IGF-I)] yielded an r = 0.72 (P < 0.01), with significant regression coefficients for both GH (P < 0.01) and IGF-I (P = 0.02).

Discussion

Selective metabolic effects of tamoxifen in male rats

Elderly men are often intolerant or poorly responsive to drugs commonly used to prevent osteoporosis or lower cholesterol, and may benefit from alternative therapies. SERMs selectively mimic metabolic effects of estrogens on bone and low density lipoprotein cholesterol in postmenopausal women (50–54) and might evoke such metabolic benefits in men without risk of feminization. In orchidectomized-hypothyroid rats treated with T3, in this study, TM prevented BMD decreases associated with orchidectomy, and lowered blood cholesterol. Moreover, TM lacked androgenic effects on seminal vesicle or prostate growth. Euthyroid male rats have yielded equivalent findings (our manuscript in preparation). Ovariectomized female rats similar in age to the males of this study have usefully modeled E effects on bone in postmenopausal women (29, 30), and the present results may have corresponding relevance for androgen-deprived men. Studies of TM in androgen-deprived men should soon address this issue.

Influence of T3 on E2B and tamoxifen effects, and relation to the GH-IGF-I axis

The hypothyroid, gonadectomized rat model enables the detection of target-selective T3 and E interplay arising from pharmacodynamic (receptor-mediated) mechanisms (14–17). The present data further indicate that some actions of E2B and TM involve interplay with T3 and also imply some sex differences. Moreover, correlation analysis provided a rigorous test of the association of serum GH and IGF-I with specific E responses in individual rats. The significant correlations found in data sets capturing the effects of estrogenic manipulation do not prove a cause and effect linkage. Nonetheless, the significant correlations satisfy an essential requirement of hypotheses that changes in GH and IGF-I contribute to E effects on growth and metabolism, and also imply distinct roles for GH and IGF-I in different E responses.
Interactions related to the GH-IGF-I axis and somatic growth

The GH-IGF-I axis has a major role in growth and metabolism (25, 27, 47, 55), and is a likely target of E and T3 interplay. T3 promotes GH synthesis and secretion, enhances GH induction of hepatic IGF-I synthesis, and induces IGF-I receptors and binding proteins (56–58). Estrogens and TM also increase GH secretion but lower serum IGF-I as well as hepatic expression of IGF-I mRNA (26, 59–65).

In the present study, increases in serum GH evoked by T3 were coupled to significant increases in IGF-I. In contrast, TM or E2B failed to increase IGF-I despite striking effects to increase GH. Interestingly, estrogen effects on serum IGF-I appear to involve two components. A T3-dependent component was evident in E2B effects to lower IGF-I by 40% in rats lacking T3; TM lacked agonist efficacy in this component and slightly raised IGF-I. A T3-dependent component was evident in E2B and TM inhibition of T3 induction of IGF-I; TM had 58% the efficacy of E2B in this component. These data are consistent with findings in hypothyroid female rats (14) in which TM slightly elevated IGF-I in rats lacking T3, but inhibited T3 effects on IGF-I. It should be noted that TM did not inhibit ovine GH effects on somatic growth or IGF-I in hypothyroid rats (14), nor do estrogens consistently inhibit GH effects on serum IGF-I or hepatic IGF-I mRNA in hypophysectomized rats (61, 62). Overall, E effects on serum IGF-I are complex and seem likely to target T3 actions that enhance GH effects, as well as mechanisms independent of T3 or GH.

Multiple mechanisms also seem to mediate E effects on T3-evoked growth. Weight gain and tibia length were correlated with IGF-I during estrogenic manipulations, suggesting that decreases in IGF-I contribute to E2B and TM effects to inhibit growth. In T3-treated rats, the net decrease in IGF-I caused by E2B (~55%) seemed large enough to impair growth, but the 12% decrease with TM was unlikely to do so. This might indicate that IGF-I production in targets such as bone is more relevant to E2B and TM effects on growth (66, 67). Alternatively, decreases in tissue sensitivity to IGF-I due to E effects on IGF-I receptors or binding proteins may contribute to the inhibition of somatic growth (63–65). Indeed, IGF-I feedback inhibits GH release (68, 69), and TM effects on serum GH in T3-treated rats seem consistent with reduced sensitivity of the feedback mechanism to IGF-I. Decreases in IGF-I sensitivity may also be relevant to E2B and TM effects to degrade the correlation of body weight change and tibia length with serum GH and IGF-I during T3 manipulations.

Interactions related to BMD

In ovariectomized-hypothyroid female rats, T3 decreased proximal tibia BMD, and E2B and TM effects seemed to reflect inhibition of T3 actions (14, 15). This appeared consistent with T3 actions directly on bone to stimulate resorption (70, 71), the increased risk of osteoporosis due to thyrotoxicosis (72, 73), and E prevention of T3-evoked bone loss in postmenopausal women (74, 75). Nonetheless, T3 alone lacked effect on BMD in the present male rats, and E2B increased BMD in the absence of T3. This suggests a sex difference in T3 effects on BMD; direct comparisons of the sexes are needed to clarify this issue.

TM had less effect on BMD than E2B in the present male rats, but TM has been shown to fully mimic E effects on BMD in female rats (76, 77), again hinting at a sex difference. In this regard, it is of note that E effects on BMD seemed to involve two components; TM had efficacy equal to E2B in a T3-dependent component but only 33% efficacy in a T3-independent component. Given the T3-dependence of E effects on BMD in female rats (14), the T3-independent component may be most relevant to any sex differences.

GH stimulates bone remodeling associated with longitudinal growth and also participates in the remodeling of mature bone (47). Although GH stimulates both bone resorption and formation, an anabolic effect eventually emerges due to greater bone formation. In the present study, BMD was positively correlated with serum GH during estrogenic manipulations in either the presence or absence of T3; such results suggest that GH increases contribute to E2B and TM effects on BMD. This association is unlikely to reflect changes in growth-related bone remodeling since somatic growth was absent in rats lacking T3. Given the sex difference in GH secretion patterns (see Introduction), GH might also be relevant to possible sex differences in T3 and E effects on BMD. Nonetheless, it is important to note that GH release evoked by T3 alone failed to increase BMD in this and other studies of hypothyroid, gonadectomized rats (14, 15). This may be relevant to evidence that E can act directly on bone to alter remodeling (1, 78); such actions may enhance the net anabolic effect of GH. Indeed, TM enhanced ovine GH effects on BMD in hypothyroid rats (14), and transgenic mice overexpressing human GH require ovarian estrogens to develop elevated BMD (79).

BMD was also negatively correlated with IGF-I during estrogenic manipulations. Bone growth and remodeling involves a complex interplay between T3, GH, IGF-I as well as other hormones and cytokines, and is not fully understood (27, 47, 78). Many GH effects result from IGF-I released from the liver or target tissue (the somatomedin theory of GH action). However, effector mechanisms unrelated to IGF-I also contribute in some tissues, including bone (the dual-effector theory of GH action) (47, 80, 81). An implication of the dual-effector theory is that GH and IGF-I may make distinct contributions to GH responses involving dual effectors. Indeed, multiple linear regression analysis showed that GH and IGF-I each made significant, independent contributions to regression equations predicting BMD during estrogenic manipulations; this was true of no other physiological parameters. This result appears to satisfy a major requirement of the hypothesis that GH actions via multiple effector mechanisms (IGF-I dependent and independent) may be relevant to E effects on BMD.

Interactions related to serum triglycerides and cholesterol

Estrogens and SERMs can increase serum triglycerides in rats and man (50, 54, 82, 83). In the male rats used in this study, such increases were T3 dependent, and TM had 65% the efficacy of E2B; female rats gave similar results (14, 15, 17). Moreover, triglycerides were positively correlated with...
GH during estrogenic manipulations in T₃-treated rats. This may be relevant to reports that GH can stimulate the hepatic synthesis or release of triglycerides (48, 49). Although T₃ alone had little effect on triglycerides, it is well known to stimulate multiple pathways involved in fatty acid and triglyceride synthesis, storage, mobilization, and oxidation. E interplay with a subset of such T₃ actions might alter the balance between opposing pathways to increase triglycerides.

The role of T₃ in E and SERM effects on serum cholesterol had not been previously studied. TM evoked cholesterol decreases that were T₃ independent, but E2B had no effect despite powerful actions on other parameters in the same rats. Others have reported similar findings. Thus, ethinyl E2 and SERMs lowered cholesterol at doses affecting the uterus, somatic growth and bone metabolism, but 17β-E2 had no effect on cholesterol at doses evoking maximal effects on other targets (36, 84). Moreover, this phenomenon is not dependent on the route of drug dosing (36). Classic estrogens such as 17β-E2, E2B, and ethinyl E2 have been thought to have equivalent pharmacodynamic actions despite differing pharmacokinetics, and act as potent agonists in most T₃-independent E responses. However, the cholesterol response reveals a more complex pharmacology and defines a novel T₃-independent E response. LH suppression in ovariectomized rats is another unusual T₃-independent response because E2B and TM exhibit similar agonist efficacy (15).

**Interactions related to lactotroph hyperplasia**

Pituitary hyperplasia evoked by E2B represents another distinct E response: it was T₃ dependent, but TM lacked agonist efficacy (unlike other T₃-dependent responses). E2B and T₃ interplay to evoke cell proliferation is tissue-specific since induction of uterine growth does not require T₃ (15). The interplay is also gene specific because PRL and kallikrein induction in lactotrophs was largely T₃-independent.

### TABLE 2. Pharmacological classes of estrogen responses in vivo

| Class A | Responses in which conventional estrogens act as potent agonists and tamoxifen predominantly acts as an antagonist with less than 33% the agonist efficacy of estrogens. Conventional estrogens include 17β-estradiol, estradiol benzoate (E2B) and ethinyl estradiol. |
| Class B | Responses in which tamoxifen predominantly acts as an agonist with ≥50% the agonist efficacy of conventional estrogens. |
| Class C | Responses in which tamoxifen and ethinyl estradiol are potent agonists, and E2B and 17β-estradiol are inactive at doses yielding maximal agonist effects in Class A responses. |

**Classification Examples in the Gonadectomized Rat**

| Class B | E2B or tamoxifen suppression of somatic growth. E2B or tamoxifen effects to increase serum triglycerides. E2B or tamoxifen induction of T₃, induction of serum IGF-I. E2B or tamoxifen suppression of LH secretion. |
| Class C | Tamoxifen-evoked decreases in serum cholesterol. |

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a. Pharmacological classes may be related to differences in the ER subtypes or ER effectors involved in the estrogen response, and interplay with other hormone signaling networks.

b. Estrogen effects on some parameters in vivo may reflect the integrated contributions of multiple classes of estrogen responses.
and B). Moreover, the various classes may partly reflect the ER subtype involved. For example, both ERα and ERβ exhibit Class A pharmacology on model genes regulated via E response elements (85–89). However, in models regulated by transcription factor AP-1, ERα exhibits Class B pharmacology while ERβ exhibits Class C pharmacology (88); ERβ also exhibits Class C pharmacology in certain other models (89).

Systematic characterization of in vivo E responses using a larger number of distinct ER ligands may further refine the pharmacological classification.

Acknowledgments

Received March 27, 2001. Accepted June 27, 2001.

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