Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration

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Yarrow JF, Conover CF, Lipinska JA, Santillana CA, Wronska TJ, Borst SE. Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration. Am J Physiol Endocrinol Metab 299: E841–E847, 2010. First published August 24, 2010; doi:10.1152/ajpendo.00384.2010.—Bone may contain an intraskeletal reservoir of sex steroids that is capable of producing biological effects. The purposes of these experiments were to 1) establish and validate methods to extract and measure intraskeletal sex hormones, 2) compare serum and intraskeletal sex hormone abundance, and 3) determine the impact of testosterone-enanthate administration and orchietomy on intraskeletal sex hormone concentrations. Tibiae from male F344 rats were crushed, suspended in an aqueous buffer, disrupted mechanically and sonically, extracted with organic solvents, dried, and reconstituted in assay buffer appropriate for measurement of testosterone, dihydrotestosterone, and estradiol by immunoassay. Prior to extraction, bone homogenate was spiked with [3H]testosterone, [3H]dihydrotestosterone, or [1H]estradiol, and >80% of each [3H]-labeled sex hormone was recovered. Extracted bone samples were also assayed with and without known amounts of unlabeled sex hormones, and >97% of the expected hormone concentrations were measured. Administration of testosterone-enanthate increased intraskeletal testosterone 11-fold and intraskeletal dihydrotestosterone by 82% without altering intraskeletal estradiol (P < 0.01). Conversely, orchietomy did not alter intraskeletal testosterone or estradiol but increased intraskeletal dihydrotestosterone by 39% (P < 0.05). In intact rats, intraskeletal testosterone and dihydrotestosterone were directionally higher than in serum, whereas intraskeletal estradiol was directionally lower than serum. Serum androgens were positively correlated with intraskeletal androgens (r = 0.74–0.96, P < 0.001); however, neither serum nor intraskeletal androgens nor serum estradiol were correlated with intraskeletal estradiol. We report the validation of a novel method for measuring intraskeletal sex hormones. Our findings demonstrate that the intraskeletal sex steroid reservoirs are modifiable and only partially influenced by circulating sex hormones.

**ANDROGENS AND ESTROGENS promote skeletal development and maintenance in both males and females (7).** The effects of testosterone (T) on bone occur directly via activation of intraskeletal androgen receptors and indirectly following the 5α-reduction or aromatization of T to the more potent dihydrotestosterone (DHT) and estradiol (E2), respectively (25). Skeletal protection induced by E2 is well documented in humans of both sexes (7, 16) and in various animal models (12, 15, 17, 26, 27) and occurs primarily via the activation of intraskeletal estrogen receptors (25). Skeletal tissue of both sexes also expresses aromatase and 5α-reductase (25), along with several other enzymes (e.g., 17β-hydroxysteroid dehydrogenase, 16α-hydroxylase, and steroid sulfatases) that are capable of localized androgen and estrogen biotransformation (20, 21). The ability of bone to interconvert androgens and estrogens suggests that intracrine action occurs within bone (20, 21); however, the degree to which this interconversion occurs in bone and the roles that the circulating and intraskeletal reservoirs of sex steroids play in skeletal biology require further elucidation.

Direct measurement of the intraskeletal sex hormones provides a unique view of androgen and estrogen reservoirs within bone. This methodology may also be used to directly assess the intraskeletal interconversion of androgens and estrogens, which addresses the intracrine potential of bone. The primary purpose of this study was to validate our novel methods of extracting and measuring intraskeletal concentrations of T, DHT, and E2 from intact bone. Our secondary aim was to demonstrate proof of principle by determining 1) the influence of the circulating sex hormones on intraskeletal sex hormone abundance and 2) the ability of our methods to measure changes in intraskeletal sex hormones in a rodent model that experiences the loss of trabecular bone mineral density (BMD) following orchietomy (ORX) and the prevention of this loss with supraphysiological testosterone-enanthate (TE) administration (28).

**MATERIALS AND METHODS**

**Animal care.** Barrier-raised and viral pathogen-free Fischer F344 male rats, aged 3 mo, were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed individually in a temperature- and light-controlled room on a 12:12-h light-dark cycle. Rats were fed an ad libitum diet of Purina rodent chow containing 3.3 kcal/g, distributed at 58.9% carbohydrate, 12.4% fat, and 28.7% protein (no. 5001; Purina Mills, St. Louis, MO), and tap water. All experimental procedures conformed to the Institute for Laboratory Animal Research Guide to the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Gainesville VA Medical Center.

**Surgery.** Rats were assigned to one of the following groups: 1) sham surgery plus vehicle (sham), 2) ORX plus vehicle, or 3) ORX plus supraphysiological TE (ORX + TE) (n = 6/group). Animals received sham surgery or bilateral closed ORX, which involved removal of the testes, epididymis, and epididymal fat. Surgery was performed aseptically under isoflurane anesthesia. After surgery, rats received a nutritional supplement (Jello-O plus protein and fat) daily for 2 days to promote weight maintenance. Additionally, rats received a once weekly injection of TE (Savient Pharmaceutical, East Brunswick, NJ) (7.0 mg/wk) or vehicle (sesame oil) into the quadriceps musculature. We have shown previously that this route and dose of TE administration is bone protective following ORX (28). On day 29, rats were euthanized via pentobarbital sodium (120 mg/kg ip), blood was...
Table 1. Intraskelatal sex steroid concentrations in intact tibiae and femora and in the contralateral bones with marrow removed

<table>
<thead>
<tr>
<th></th>
<th>Marrow</th>
<th>No Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>T, ng/g</td>
<td>4.0 ± 0.9</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Tiba</td>
<td>3.8 ± 0.5</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>DHT, pg/g</td>
<td>4,649 ± 312</td>
<td>5,256 ± 413</td>
</tr>
<tr>
<td>Tiba</td>
<td>4,940 ± 368</td>
<td>4,842 ± 312</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 3/group. T, testosterone; DHT, dihydrotestosterone. No differences were present between bones and conditions.

Table 2. Relative recovery of $^3$H-labeled sex hormones in each extraction layer

<table>
<thead>
<tr>
<th></th>
<th>Organic Layer Counts</th>
<th>%Organic Recovery</th>
<th>Inorganic Layer Counts</th>
<th>%Inorganic Recovery</th>
<th>Interphase Layer Counts</th>
<th>%Interphase Recovery</th>
<th>%Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>102,381 ± 2,137</td>
<td>85,709 ± 1,932</td>
<td>84 ± 2</td>
<td>704 ± 51</td>
<td>0.7 ± 0.1</td>
<td>1,375 ± 152</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>DHT</td>
<td>107,168 ± 3,256</td>
<td>83,300 ± 2,415</td>
<td>78 ± 2</td>
<td>1,164 ± 57</td>
<td>1.1 ± 0.1</td>
<td>2,113 ± 185</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>E$_2$</td>
<td>86,475 ± 2,385</td>
<td>69,159 ± 1,159</td>
<td>80 ± 1</td>
<td>3,388 ± 123</td>
<td>3.9 ± 0.1</td>
<td>1,454 ± 129</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 2 separate runs (n = 12/group). E$_2$, estradiol.

Innovative Methodology

E842 INTRASKELATAL SEX HORMONE EVALUATION

Bone homogenization and steroid extraction. Tibiae were cut into small pieces, pulverized with a liquid nitrogen cooled Spex Certiprep freezer mill (Edison, NJ) for 2 min, and stored at −80°C. Bone powder (200 mg) was homogenized at 4°C in 4 ml of Krebs-Ringer phosphate buffer (116 mM NaCl, 10 mM phosphate, 4.5 mM KCl, 2.5 mM MgCl$_2$, 1.3 mM CaCl$_2$, 5% glycerol, 2 mM EDTA and 4 mM DTT, pH = 6.9; all chemicals were purchased from Sigma-Aldrich, St. Louis, MO) via high speed polytron (15 s) and probe sonication (30 s). Homogenates were stored frozen at −80°C. Homogenates (1.0 ml) were extracted with 2.0 ml of chloroform-methanol (2:1 vol/vol), vortexed for 45 s, and centrifuged at 1,500 rpm for 10 min, facilitating separation into three distinct layers: an organic layer, an aqueous layer, and a semisolid interphase layer. The aqueous layer was removed, reextracted with 2.0 ml of chloroform, vortexed, and centrifuged as described above. The organic layers from both extractions were then combined, dried under a gentle stream of nitrogen gas, and stored at −20°C until hormone analysis.

Bone selection and removal of marrow. A pilot experiment was performed to determine whether bone marrow influences the sex steroid concentrations contained in intact bone. Left and right tibiae and femora were obtained from intact male rats (n = 3) because these bones have a large and readily accessible marrow cavity and because these bones are highly sensitive to endogenous sex hormone status (28). The left tibia and femur remained intact, whereas the right bones were split longitudinally, and the marrow was removed by flushing with saline and by scraping the marrow cavity with gauze. Following removal of the marrow, sex steroids were extracted. T and DHT were evaluated as described above because measureable differences of these androgens are present in sham-, ORX-, and ORX + TE-treated male rodents (28). No differences in T or DHT concentrations were observed between intact or marrowless tibiae or femora (Table 1). The tibia was chosen for all subsequent procedures for purposes of consistency.

Measurement of bone by pQCT. Left femora were scanned by pQCT at a distance of 5 mm proximal to the distal end of the femur for 48 h. Following demineralization, sex steroids were extracted as described above. Samples were then evaluated for DHT because measurable differences in this androgen exist in sham-, ORX-, and ORX + TE-treated male rodents (28). The no-demineralization technique and the 48-h demineralization technique from this pilot experiment resulted in similar intraskelatal DHT concentrations for these groups, whereas DHT was significantly lower with 10-day demineralization (see Supplemental Table S1; Supplemental Material for this article is available at the AJP-Endocrinology and Metabolism web site). Thus, for all subsequent experiments, extraction was performed without prior demineralization.

Verification of hormone recovery during extraction. [3H]T, [3H]DHT, or [3H]E$_2$ were added to separate bone homogenate samples prior to solvent extraction to verify that the sex hormones were fully recoverable during the extraction process. The samples were extracted as described above, and the presence of the counts in each of the extraction phases was assessed using a LS6500 β-radiation scintillation counter (Beckman Coulter, Fullerton, CA). The combined organic layer of each extract contained the majority of each $^3$H label, with 84, 78, and 80% present for [3H]T, [3H]DHT, or [3H]E$_2$, respectively. Only a minor amount of label was measured in the aqueous layer (<4%) and the semisolid interphase layer (<2%) (Table 2). The same procedure was repeated on an additional set of samples, and similar results were obtained.

Verification of the absence of interfering substances. To determine whether hormone extracts contained any substances that might interfere with hormone assays, we assayed bone extracts with and without the addition of unlabeled T, DHT, or E$_2$. The hormone concentrations measured in the spiked samples were between 105 and 112% of expected values for T, 104 and 115% of expected values for DHT, and 97 and 99% of the expected values for E$_2$ (Table 3).

Measurement of bone by pQCT. Left femora were scanned by peripheral pQCT with a Stratec XCT Research M Instrument (Nordland Medical Systems, Fort Atkinson, WI). Scans were performed at a distance of 5 mm proximal to the distal end of the femur for...
measurements of cancellous and cortical bone structure. This site is at the level of the secondary spongiosa of the distal femoral metaphysis, where major bone structural changes are known to occur in rats after gonadectomy. The structural variables that were measured include total, trabecular, and cortical bone area (mm²), mineral content (mg/mm), and mineral density (mg/cm³).

Statistics. One-Way ANOVAs with Tukey’s post hoc tests (for normally distributed data) or the Kruskal-Wallis test with a Mann-Whitney U-test (for nonnormally distributed data) were used to analyze serum and intraskeletal hormone concentrations and pQCT measurements. Paired t-tests were performed to evaluate differences between accompanying serum and intraskeletal sex hormone concentrations. Linear dependence between serum and intraskeletal sex hormones was evaluated with Pearson’s correlations, which were calculated only for specific variables of interest. Results are reported as means ± SE, and P < 0.05 was defined as the threshold of significance. All data were analyzed with SPSS version 15.0.0 statistical software.

RESULTS

Serum sex hormone concentrations. Serum T was 1.9 ± 0.1 ng/ml (shams), below detectable limits (<0.04 ng/ml; ORX), and 34.7 ± 2.5 ng/ml (ORX + TE) (Fig. 1A). ORX appeared to completely ablate serum T, whereas supraphysiological TE administration increased serum T 18-fold over shams (P < 0.01).

Serum DHT was 318 ± 55 (shams), 89 ± 9 (ORX), and 10,362 ± 643 pg/ml (ORX + TE) (Fig. 1B). ORX reduced

Table 3. %Recovery of unlabeled sex hormone spike from bone homogenates of male F344 rats

<table>
<thead>
<tr>
<th></th>
<th>Initial Concentration, ng/ml</th>
<th>Added Spike</th>
<th>Expected Concentration, ng/ml</th>
<th>Measured Concentration, ng/ml</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2.52 ± 0.20</td>
<td>2.25 ng</td>
<td>4.81 ± 0.20</td>
<td>4.98 ± 0.31</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>ORX</td>
<td>2.46 ± 0.15</td>
<td>2.25 ng</td>
<td>4.75 ± 0.15</td>
<td>5.02 ± 0.44</td>
<td>106 ± 9</td>
</tr>
<tr>
<td>ORX + TE</td>
<td>27.36 ± 1.73</td>
<td>15.75 ng</td>
<td>43.40 ± 1.73</td>
<td>48.7 ± 2.05</td>
<td>112 ± 4</td>
</tr>
<tr>
<td>DHT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>6.353 ± 413</td>
<td>4,274 pg</td>
<td>10,627 ± 413</td>
<td>11,010 ± 614</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>ORX</td>
<td>8.823 ± 662</td>
<td>4,274 pg</td>
<td>13,097 ± 662</td>
<td>14,043 ± 967</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>ORX + TE</td>
<td>11,554 ± 286</td>
<td>14,960 pg</td>
<td>26,513 ± 286</td>
<td>30,520 ± 1,596</td>
<td>115 ± 5</td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.87 ± 0.11</td>
<td>0.72 pg</td>
<td>1.59 ± 0.11</td>
<td>1.23 ± 0.35</td>
<td>99 ± 14</td>
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<tr>
<td>ORX</td>
<td>0.96 ± 0.20</td>
<td>0.72 pg</td>
<td>1.68 ± 0.20</td>
<td>1.63 ± 0.40</td>
<td>98 ± 22</td>
</tr>
<tr>
<td>ORX + TE</td>
<td>0.91 ± 0.05</td>
<td>0.72 pg</td>
<td>1.64 ± 0.05</td>
<td>1.58 ± 0.11</td>
<td>97 ± 8</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 5–6/group. ORX, orchiectomy; TE, testosterone-enanthate.

Fig. 1. A–F: accompanying serum and intraskeletal sex steroid hormone concentrations for sham-operated, orchiectomized (ORX), and ORX + testosterone-enanthate-treated (ORX + TE) 3-mo-old male F344 rats. T, testosterone; DHT, dihydrotestosterone; E2, estradiol. Values represent means ± SE; n = 5–6/group. *P < 0.01 vs. sham; †P < 0.01 vs. ORX.
serum DHT by 72% \((P < 0.01)\), whereas TE administration increased serum DHT 32-fold compared with shams \((P < 0.01)\) and 116-fold compared with ORX \((P < 0.01)\). Across groups, serum DHT was highly and positively correlated with serum T \((r = 0.964, P < 0.001; \text{Fig. 2A})\).

Serum E\(_2\) was 4.0 \pm 0.3 \text{ng/mL} (shams), 3.1 \pm 0.8 \text{ng/mL} (ORX), and 6.0 \pm 0.4 \text{pg/mL} (ORX + TE) (Fig. 1C). ORX did not alter serum E\(_2\) in males. However, TE administration increased serum E\(_2\) by 50% compared with shams (trend, \(P = 0.061\)) and by 93% compared with ORX \((P < 0.01)\). Across groups, serum E\(_2\) was highly and positively correlated with serum T \((r = 0.650, P < 0.01; \text{Fig. 2B})\).

**Intraskeletal sex hormone concentrations.** Intraskeletal T was 2.5 \pm 0.2 \text{ng/mL} of bone mass (shams), 2.5 \pm 0.2 \text{ng/mL} (ORX), and 27.4 \pm 1.7 \text{ng/mL} (ORX + TE) (Fig. 1D). ORX did not alter intraskeletal T; however, TE administration increased intraskeletal T 11-fold above sham and ORX \((P < 0.01)\).

Intraskeletal DHT was 6,353 \pm 413 \text{pg/g} of bone mass (shams), 8,823 \pm 662 \text{pg/g} (ORX), and 11,554 \pm 286 \text{pg/g} (ORX + TE) (Fig. 1E). ORX increased intraskeletal DHT by 39% compared with shams; furthermore, TE administration elevated intraskeletal DHT by 31% compared with ORX and 82% compared with shams \((P < 0.01)\). Across groups, intraskeletal DHT was highly and positively correlated with intraskeletal T \((r = 0.779, P < 0.001; \text{Fig. 3A})\).

Intraskeletal E\(_2\) was 0.87 \pm 0.11 \text{pg/g} of bone mass (shams), 0.96 \pm 0.20 \text{pg/g} (ORX), and 0.91 \pm 0.05 \text{pg/g} (ORX + TE) (Fig. 1F). Neither ORX nor TE administration altered intraskeletal E\(_2\), and intraskeletal E\(_2\) was not significantly correlated with intraskeletal testosterone (Fig. 3B).

**Relationship between intraskeletal and serum sex hormones.** It is difficult to make direct comparisons between the concentrations of serum and intraskeletal sex hormones because of differences in the concentrations contained within serum (ml) vs. tissue (g). However, when compared directionally, it appears that intraskeletal T was greater than serum T in sham \((2.5 \pm 0.2 \text{ vs. } 1.9 \pm 0.1 \text{ ng/mL}, P < 0.05)\) and ORX \([2.5 \pm 0.2 \text{ ng/mL vs. below detectable limits (<0.04 ng/mL)}]) animals while being present in roughly similar supraphysiological concentrations in ORX + TE animals \((27.4 \pm 1.7 \text{ vs. } 34.7 \pm 2.5 \text{ ng/mL})\). Overall, intraskeletal T was highly and positively correlated with serum T \((r = 0.957, P < 0.001; \text{Fig. 4A})\).

Intraskeletal DHT appeared to be directionally higher than accompanying serum DHT in both sham \((6,553 \pm 413 \text{ pg/g vs. } 318 \pm 55 \text{ pg/mL}, P < 0.01)\) and ORX animals \((8,823 \pm 662 \text{ pg/g vs. } 89 \pm 9 \text{ pg/mL}, P < 0.01)\), whereas intraskeletal and serum DHT were present in similar concentrations in ORX + TE animals \((11,554 \pm 286 \text{ pg/g vs. } 10,362 \pm 643 \text{ pg/mL})\). Overall, intraskeletal DHT was highly and positively correlated with serum T \((r = 0.752, P < 0.001; \text{Fig. 4B})\) and serum DHT \((r = 0.742, P < 0.001; \text{Fig. 4C})\).

Conversely, intraskeletal E\(_2\) appeared to be directionally lower than accompanying serum E\(_2\) in all groups [sham: 0.87 \pm 0.11 \text{ pg/mL vs. } 4.0 \pm 0.3 \text{ pg/mL} (P < 0.01); ORX: 0.96 \pm 0.20 \text{ pg/mL vs. } 3.1 \pm 0.8 \text{ pg/mL} (P < 0.05); ORX + TE: 0.91 \pm 0.05 \text{ pg/mL vs. } 6.0 \pm 0.4 \text{ pg/mL} (P < 0.01)]. Overall, intraskeletal E\(_2\) was not significantly correlated with any serum androgen or estrogen (data not shown).

**pQCT measurements.** ORX resulted in a 12% reduction in total bone mineral content \((\text{BMC; } P < 0.05)\) and total BMD \((P < 0.01)\) and a concurrent 46% reduction in trabecular \((\text{tBMC and tBMD})\) at the distal femoral metaphysis \((P < 0.01; \text{Table 4})\). TE administration prevented the ORX-induced reductions at this skeletal site, ultimately increasing total BMD by 12% \((P < 0.01)\), tBMC by 54% \((P < 0.05)\), and tBMD by 58% \((P < 0.01)\). The total, trabecular, and cortical areas were unaltered by either treatment, and no changes in cortical bone mineral measurements were observed at this skeletal site (data not shown).
DISCUSSION

We describe and validate novel methods to extract and measure sex steroid hormones contained within intact bone. Historically, the circulation was thought to represent the primary reservoir of sex hormones that are capable of binding with androgen receptors and estrogen receptors and subsequently inducing biological effects within bone. However, our results indicate that bone also contains a large modifiable reservoir of androgens and a relatively smaller reservoir of E2. These reservoirs are only partially influenced by the circulation and may be capable of promoting skeletal development and maintenance. The measurement of the intraskeletal sex hormones provides a useful tool that may assist in further elucidating the androgen and/or estrogen-mediated mechanism(s) influencing skeletal biology and may also provide a means of directly assessing the intracrine potential of bone.

To establish the validity and reliability of our extraction procedures, we spiked male rat bone homogenates with 3H-labeled T, DHT, and E2 before subjecting them to extraction procedures. Approximately 80% of each hormone was recovered following extraction, whereas ~4% was lost to the aqueous layer and ~2% to the interphase layer. It is unclear why we could not account for 100% of each 3H label, although it is possible that the additional ~14% may have remained bound to the glass extraction tubes. We also assayed male rat bone extracts as follows: sample 1 = bone extract, sample 2 = a known amount of unlabeled hormone, and sample 3 = the same known hormone amount added to bone extract (i.e., sample 1 + sample 2). If sample 3 was less than the sum of samples 1 and 2, it would indicate the presence of an interfering substance in the bone extract. On average, we observed that sample 3 was >97% of the expected value for all three hormones, indicating a lack of interfering substances. Overall, our results clearly demonstrate that the majority of intraskeletal T, DHT, and E2 is preserved throughout the extraction procedures, that the intraskeletal steroid hormones are contained primarily within the reconstituted organic layer, and that we are capable of accurately and reliably detecting changes in intraskeletal sex steroids using immunoassays, which validates our methods.

Systemic alterations in androgens and/or estrogens influence bone mineral accretion (7). In the present study, ORX induced an abrupt loss of circulating androgens and a concomitant reduction in total and trabecular BMD, whereas supraphysiological TE administration elevated serum androgens and E2 concentrations and prevented the BMD loss. Androgens and estrogens may also undergo localized steroid interconversion within bone via the actions of 5α-reductase, aromatase, 17β-hydroxysteroid dehydrogenase, 16α-hydroxylase, and/or steroid sulfatase (20, 21), suggesting that localized enzyme expression influences the biological actions of sex hormones within bone. In our study, ORX did not lower the intraskeletal concentrations of any sex hormones, whereas TE administration elevated intraskeletal T and DHT, but not intraskeletal E2, indicating that the intraskeletal androgens represent a somewhat modifiable reservoir of sex hormones. Interestingly, the intraskeletal sex hormone concentrations were not reduced following ORX, whereas tBMD was reduced significantly, which suggests that the intraskeletal sex steroids are 1) sequestered in a bone compartment that is not capable of protecting against the loss of trabecular bone and/or 2) present in a nonbioavailable form.

The interpretation of intraskeletal sex hormone content is considerably complicated because the relative distribution of hormones among the various bone compartments remains unknown. Using a male rodent model, we have demonstrated that the presence or absence of bone marrow did not influence the intraskeletal sex hormone concentrations, which suggests that the intraskeletal sex steroids are contained in roughly equal concentrations within the marrow and within cancellous and/or cortical bone compartments. Despite the difficulties in interpretation, some useful comparisons can be made, including the

Table 4. Peripheral pQCT of the distal femoral metaphysis of F344 male rats

<table>
<thead>
<tr>
<th></th>
<th>Total Area, mm²</th>
<th>Total BMC, mg/mm</th>
<th>Total BMD, mg/cm³</th>
<th>Trabecular Area, mm²</th>
<th>Trabecular BMC, mg/mm</th>
<th>Trabecular BMD, mg/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>19 ± 0.3</td>
<td>13 ± 0.3</td>
<td>669 ± 4</td>
<td>5.7 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>338 ± 12</td>
</tr>
<tr>
<td>ORX</td>
<td>19 ± 0.3</td>
<td>11 ± 0.4</td>
<td>586 ± 13ᵃ</td>
<td>5.7 ± 0.1</td>
<td>1.0 ± 0.1ᵃ</td>
<td>180 ± 19ᵃ</td>
</tr>
<tr>
<td>ORX + TE</td>
<td>18 ± 0.5</td>
<td>12 ± 0.5</td>
<td>658 ± 11ᵇ</td>
<td>5.5 ± 0.2</td>
<td>1.6 ± 0.1ᵇ</td>
<td>283 ± 18ᵇ</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 6/group. pQCT, quantitative computed tomography; BMC, bone mineral content; BMD, bone mineral density. ⁣ᵃP < 0.01 vs. sham; ⁣ᵇP < 0.01 vs. ORX; ⁣ᶜP < 0.05 vs. sham; ⁣ᵈP < 0.05 vs. ORX.
ratio of intraskeletal/serum hormone content. Specifically, serum and intraskeletal androgens were highly correlated, and the intraskeletal/serum hormone ratio was highest for DHT, nearly equal for T, and lowest for E2. 5α-reductase is expressed within bone (8), and DHT is more potent than T, having approximately three times greater affinity for androgen receptors than T (3). Considering that DHT is present in higher concentrations in bone than in serum, our results indicate that the 5α-reductase enzyme is capable of converting T to DHT within rodent bone. The systemic 5α-reduction of T is not essential for skeletal maintenance or bone accretion in men given that 1) long-term pharmacological inhibition of the type I and/or type II 5α-reductase enzymes does not alter BMD or markers of bone turnover in men (1, 13) despite a nearly complete ablation of circulating DHT, 2) high-dose T plus MK-434 (a type II 5α-reductase inhibitor) prevents orchectom- my-induced bone loss and maintains skeletal growth in rodents in the near absence of circulating DHT (4, 5), and 3) T augments BMD in elderly men when administered in combina- tion with finasteride (2). However, it remains unknown whether pharmacological 5α-reductase inhibitors are active in bone and whether these drugs alter the intraskeletal sex hor- mone milieu by preventing the intraskeletal 5α-reduction of T to DHT. As yet unexplainable is the finding that orchietomy appeared to increase intraskeletal DHT. In humans, dehydroepiandrosterone (DHEA) may be converted to DHT within bone via a series of intraskeletal intracrine reactions, as has recently been demonstrated in vitro using human bone marrow stromal cells (19); however, this pathway is not present in rodents. Regardless, we have provided what appears to be in vivo evidence demonstrating that 1) bone is an intracrine tissue capable of producing DHT and/or 2) bone selectively sequesters DHT from within the circulation.

The role of the aromatase enzyme in mediating the effects of T on male bone development is well known (22). The aromatase-knockout mouse develops osteopenia in the absence of E2 (15, 17). Furthermore, in congenitally aromatase-deficient young men, osteopenia is reversed with E2 administration but not supraphysiological T (6, 18). Similarly, aromatase mediates the bone-protective effects of T in mature males, as evidenced by the reduction in BMD that occurs in aged male rats undergoing aromatase inhibition (23, 24) and by the increased bone resorption that occurs in men receiving lupon (a GnRH agonist that causes cessation of T production), which is best prevented by combined T and E2 administration (9). However, the extragonadal sites of aromatization that contribute to the circulating and intraskeletal E2 concentrations remain less clear, with adipose tissue (17) and bone (9, 10) appearing to be the leading candidates. In our study, it is unclear why intraskeletal E2 was not altered following ORX, especially considering that serum T (the sole source of endogenous E2 in males) was nearly completely ablated following ORX. Our results suggest that certain steroid precursors (e.g., DHEA or androstendione) or less potent estrogens (e.g., estrone (E1), E1-sulfate, or estriol (E3)) undergo interconversion within bone to maintain intraskeletal T and E2 concentrations (14), similar to the interconversions that occur within other peripheral tis- sues (11), or perhaps that sex hormones experience a longer presence in bone than in serum. Evidence supporting the hypotheses that less potent estrogens represent a major source of intraskeletal E2 exists, because the rate of E2 formation in freshly resected bone is 50 times higher from E1 sulfate than from the aromatization of T (14). It is also surprising that the intraskeletal E2 concentrations were unchanged following sus- praphysiological TE administration considering that the sys- temic aromatization of T clearly occurred, as evidenced by a near doubling of serum E2 concentrations following TE admin- istration. Overall, our results appear to at least partially conflict with a predominant theory in the literature suggesting that T undergoes localized aromatization to E2 within bone and then produces bone protection through subsequent ER activation (10). Thus, our results raise the distinct possibilities that 1) T does not undergo a significant degree of aromatization within bone or 2) T is aromatized to E2 within bone but subsequently undergoes intraskeletal interconversion to less potent estrogens (8, 20, 21). Ultimately, determining the intraskeletal concentrations of E1 and E2 may assist in elucidating the intraskeletal metabolism of T and E2 and may also assist in further defining the intracrine potential of bone.

Additionally, the results of this study raise several basic questions regarding the role that intraskeletal androgens and estrogens play in skeletal biology, including 1) what is the biological significance of a modifiable intraskeletal sex hor- mone reservoir, 2) what effects do sex and aging have on the intraskeletal sex hormone concentrations, 3) what is the sig- nificance of apparently differing concentrations of androgens and estrogens in bone compared with blood, 4) is E2 that is transported into bone or synthesized within bone rapidly con- verted to other estrogens, and 5) do the relationships that exist among the intraskeletal sex steroids in rats also exist in humans and other mammalian species?

In conclusion, we have developed and validated a reliable method to extract sex steroid hormones from intact bone for quantitation by immunoassay. Upon evaluation of our meth- ods, we have determined that the intraskeletal sex hormone reser voir is modifiable in nature, that it is not influenced by the presence of absence of marrow, and that it is only partially influenced by circulating androgens and estrogens. The biological significance of the intraskeletal sex hormone reservoir remains to be determined, with future research necessary to determine the bioavailability of the intraskeletal androgens and estrogen. Additionally, evaluating the intraskeletal androgen and estrogen concentrations across species, sex, and age is warranted and may assist in elucidating the biological actions associated with the intraskeletal sex steroid reservoir along with the intracrine potential of bone.

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DISCLOSURES
The authors have nothing to disclose.

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