Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat

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Lea, C. K., and A. M. Flanagan. Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E328–E335, 1998.—The effect of androstenedione (ADIONE) slow-release pellets on cancellous bone volume (BV/TV) at the tibial metaphysis was investigated in ovariectomized (OVX) rats at various times from 21 to 180 days. Plasma levels of ADIONE and testosterone (T) in OVX rats were significantly reduced at 21 days and were restored close to levels in the sham rats with the 1.5-mg ADIONE pellet. OVX animals with and without ADIONE pellets resulted in close to a 50% reduction with the 1.5-mg ADIONE pellet. OVX animals with and without ADIONE pellets resulted in close to a 50% reduction with the 1.5-mg ADIONE pellet.

The sex steroid precursor, dehydroepiandrosterone, has been shown to protect the ovariectomized (OVX) rat skeleton against bone loss (24), but this study did not test whether androgens or estrogens mediated the effect. We have recently reported that ADIONE and T are significantly reduced in OVX rats and that ADIONE slow-release pellets protect against cancellous bone loss in a dose-responsive manner in the OVX rat (15). This was achieved using static and dynamic histomorphometric analyses of the tibial metaphyses. However, only supraphysiological plasma levels of ADIONE showed a convincing skeletal-protective effect in the previous 21-day experiment. We also found, in this study, that the ADIONE-mediated effects in response to pharmacological levels of ADIONE (100-mg pellets) were abrogated by Arimidex, the aromatase inhibitor (21).

In this study, we elected to use the mature OVX rat model (13) to determine whether physiological plasma levels of ADIONE could unequivocally reduce bone loss if the experiment was continued beyond 21 days for a further 159 days. Once we established that this was the case, we tested whether the skeletal-protective effect of ADIONE was mediated by androgens or estrogens in the aged rat model. In these experiments, OVX animals were treated with ADIONE with and without an androgen antagonist, Casodex (7), and an aromatase inhibitor, Arimidex.

MATERIALS AND METHODS

Animal Experimentation and Histomorphometry

Female Sprague-Dawley rats were purchased from Harlan Olac (Bicester, Oxon, UK), housed at 21°C with a 12:12-h light-dark cycle, and fed rat laboratory diet (Lillico, Betchworth, Surrey, UK) and water. The animals were pair fed. All pellets were purchased from Innovative Research of America (Toledo, OH).

Experiment I. Thirteen-week-old animals, with an average weight of 216 g (range 196–234 g), were subjected to ovariectomy or a sham operation under halothane anesthesia using a dorsal approach on day 1. The animals were divided into six treatment groups and analyzed at five time points. At each time point, each of the six groups contained either six or eight animals (a total of 210 animals in experiment). Group 1 (n = 8) was subjected to a sham operation. Group 2 (n = 6) had a bilateral ovariectomy. Groups 3 (n = 8) and 5 (n = 8) had a bilateral ovariectomy and ADIONE slow-release pellets of 1.5 and 5 mg, respectively, inserted at the back of the neck at the time of ovariectomy. Groups 4 (n = 6) and 6 (n = 6) had
bilateral ovariectomy and placebo pellets of 1.5 and 5 mg, respectively, inserted subcutaneously.

Experiment II. Six-month-old animals, with an average weight of 270 g (range 252–290 g), were subjected to ovariectomy or a sham operation as described above. At this age, the animals exhibit imperceptible growth (13). Ten animals were included in each of the following groups: group 1, sham OVX; group 2, OVX plus placebo pellet; group 3, OVX plus ADIONE (1.5-mg slow-release pellet); group 4, OVX plus Casodex (kindly provided by Dr. B. M. Vose, Zeneca Pharmaceuticals, Macclesfield, UK); group 5, OVX plus ADIONE (1.5-mg pellet) and Casodex; group 6, OVX plus Arimidex (kindly provided by Dr. Vose); and group 7, OVX plus ADIONE (1.5-mg pellet) and Arimidex. Casodex (5 mg·kg⁻¹·day⁻¹) and Arimidex (0.1 mg·kg⁻¹·day⁻¹) were both dissolved in water and administered orally.

Calcine (30 mg/kg, Sigma Chemicals, Dorset, UK) and tetracycline hydrochloride (25 mg/kg, Lederle Laboratory, Gosport, Hants, UK) were injected intraperitoneally 14 and 7 days before each group of animals was killed. Cardiac puncture was performed under anesthesia, and plasma samples were stored at −70°C until required. The animals were then killed by cervical dislocation after periods of 21, 60, 90, 120, and 180 days in experiment I and after 90 days in experiment II.

The uteri were removed and weighed, and ovariectomy was confirmed by the absence of ovarian tissue. The tibiae were cleaned of soft tissue, fixed in 70% alcohol for 24 h, dehydrated through graded alcohols, and embedded without decalcification in London Resin (London Resin, Basingstoke, Hants, UK). Longitudinal sections of the proximal metaphysis were cut using a Reichert-Jung microtome (Leica, Germany). Sections (5 µm) were stained with toluidine blue, and 12-µm unstained sections were cut for fluorescent microscopy. Bone histomorphometry was performed using transmitted and epifluorescent microscopy linked to a computer-assisted image analyzer (Seescan, Cambs, UK). Bone volume and surface parameters were measured by tracing the relevant features with a cursor on the video screen. Cancellous bone volume (BV/TV) measurements were performed at ×40 magnification, and the surface parameters were measured at ×400 magnification. All sections were analyzed without knowledge of the group from which they came.

BV/TV at the proximal metaphysial cancellous bone from animals killed on days 21 and 60 was measured on two nonconsecutive sections, and four nonconsecutive sections were analyzed from animals killed on days 90, 120, and 180. The latter was done because of the relative lack of bone spicules in OVX rats. A standard area of 2 mm² (at least 2 mm from growth plate to exclude primary spongiosa) was measured. Trabecular number and thickness were calculated as previously described (20). Static parameters were measured in the same way as that described for BV/TV and included osteoblast surface, osteoclast surface, and osteoclast number. Longitudinal growth rate (LGR) was derived by measuring the distance between the tetracycline and calcein fluorescent bands that parallel the growth plate at four equally placed sites per section and dividing by the time interval between the two injections. The bone formation rate (BFR; tissue level, total surface referent) was calculated from the product of the percentage of the trabecular bone surface with a double fluorochrome label and the mineral apposition rate (MAR): the former was obtained by measuring the percentage of the trabecular bone surface, covered by two fluorochrome labels, and the latter by dividing the interlabel distance by the time interval between the injections of the labels in the corresponding area. The BFR values were not corrected for label escape errors. MAR values were not corrected for the obliquity of the plane of section of cancellous bone.

Radioimmunoassays for Plasma Hormone Levels

Plasma levels of ADIONE, estrone (E₁), and T (Diagnostics Systems Laboratories, TX) and estradiol (E₂; IncStar, Berks, UK) were measured in the animals from experiment I, which were killed on day 21, and in animals from experiment II on day 90 by radioimmunoassay (RIA) as instructed in the manufacturer’s guidelines. The RIAs had an intra-assay coefficient of variation of <5% and an interassay coefficient of variation of <6%. Each assay was validated by spiking serum samples with known amounts of the four hormones being tested.

Statistics

The results were analyzed using Fisher’s least significant difference method for multiple comparisons in a one-way analysis of variance and expressed as means ± SE. Significance was considered when P < 0.05. Statview 4.0 (Abacus Concepts, Cupertino, CA) was used to analyze the results.

RESULTS

Experiment I

OVX rats were significantly heavier than sham-operated rats at all time points. There was no difference in the weight gained by any of the OVX groups at any time point. All the animals were significantly heavier by day 21 compared with day 1. They continued to increase their weight until day 180 (day 180: sham, 299.80 ± 5.30 vs. OVX, 336.56 ± 4.53; P < 0.05 vs. relevant day 0). The uterus of OVX animals was markedly atrophic compared with the sham animals; the average weight of the former on day 21 was 107 mg (range 96–110 mg) and on day 180 was 102 mg (range: 89–108 mg). The average weight of the sham-operated animals on day 21 was 444 mg (range 349–588 mg) and on day 180 was 672 mg (range 579–822 mg), and that of ADIONE-treated OVX rats on day 21 was 116 mg (range 111–124 mg) and on day 180 was 120 mg (range 113–129 mg).

Ovariectomy caused the expected reduction in the plasma levels of E₂ and also resulted in a significant reduction in plasma levels of ADIONE, T, and E₁. The 1.5- and 5-mg ADIONE pellets raised the plasma concentrations of ADIONE and T significantly above OVX levels. The androgens levels, however, remained below sham levels in the presence of the 1.5-mg pellets, whereas they were raised close to male levels in response to the 5-mg pellet. The E₂ level in the ADIONE-treated rats was only marginally increased above those in OVX rats. The level of E₁ in ADIONE-treated rats was elevated above sham levels; the reason for this was not clear. No effect on hormone levels were seen in placebo-treated animals (Fig. 1).

The LGR of OVX rats was found to be significantly increased above that of the sham rats until day 90 but returned to control levels at subsequent times. The increased LGR in OVX animals on day 21 partially returned to sham levels after administration of the 1.5-mg ADIONE pellet, and the larger 5-mg pellet...
completely reverted the measurements to those in the sham-operated animals. After day 21, the LGR of the 1.5- and 5-mg ADIONE-treated rats was similar to that of the sham animals (Fig. 2).

Cancellous bone volume was largely maintained throughout the experiment in the sham animals, whereas it was reduced by ~50% in OVX rats as early as day 21 (Fig. 3). This was followed by a slower decline in bone loss over the next 100 days, resulting in a further ~25% loss of cancellous bone. After this time, the bone volume appeared to be maintained at ~5%.

There was a minor but significant difference in the measurements between the ADIONE and placebo groups.
values of OVX and ADIONE-treated rats as early as day 21, but the difference became more pronounced with time. In fact, there was no further reduction in the cancellous bone volume in ADIONE-treated animals after day 21. Cancellous bone loss in ADIONE-treated OVX rats was reduced by 35%, measured 180 days post-OVX. By the end of the experiment, ADIONE-treated animals had 130% greater cancellous bone volume than did OVX rats (Fig. 3). This greater cancellous bone volume was largely the result of an increase in the thickness of the metaphyseal trabeculae (Table 1), whereas there was no increase in trabecular number (data not shown).

The loss in cancellous bone in OVX rats was associated with an increase in osteoclast number that was most marked on day 21 but remained significantly different from the controls even at day 180. A reduction in osteoclast numbers and the bone surface covered with osteoclasts were significantly reduced in ADIONE-treated animals compared with OVX animals, and by day 180, both of these parameters were similar to those in the sham-operated animals (Fig. 4).

BFR was increased in OVX animals compared with controls. Again, the greatest difference was seen early in the experiment on days 21 and 60. Thereafter it declined gradually but always remained significantly different from the controls (Fig. 5). The increase in BFR was the result of an increase in the double-labeled bone surface and in the MAR. ADIONE reduced the increase in the fluorochrome-based indexes of bone turnover to values midway between those of sham and OVX animals (data not shown). Likewise, osteoblast numbers were significantly increased in OVX animals compared with controls and this parameter was reduced in the presence of ADIONE. The effects brought about by administration of ADIONE occurred in the presence of both sizes of pellets, and although the larger pellet was always found to exert a marginally greater effect, there was no significant difference between the results.
(Fig. 5). The results obtained from the placebo-treated animals were not significantly different from those from OVX animals (Figs. 2–5).

Experiment II

Both sham and OVX rats gained weight in the 90-day experiment. There was no significant difference in the weight gained by any of the OVX groups. The sham animals gained $22.70 \pm 2.17$ g, and the latter gained $70.46 \pm 3.86$ g.

To determine whether the results in experiment I were accounted for by conversion of ADIONE to androgens and/or estrogens, 1.5-mg ADIONE pellets were administered in the presence and absence of Casodex and Arimidex. As shown in experiment I, the 1.5-mg ADIONE pellet protected cancellous bone loss significantly in OVX rats (Table 2). As in experiment I, this was achieved by reducing bone turnover (Figs. 6 and 7). The ADIONE-induced protective effect was completely abrogated by antiandrogen therapy, whereas it was maintained in the presence of Arimidex (Figs. 6 and 7). The finding that neither the antiandrogen nor the aromatase inhibitor treatment in OVX animals reduced parameters below the OVX placebo group is consistent with previous reports (8, 15) (Figs. 6 and 7, Table 2).

The hormone plasma levels in the animals provide supportive evidence that the skeletal protective effect of ADIONE was mediated through androgens and not estrogens (Table 2). The finding that T plasma level was increased in ADIONE-Arimidex-treated OVX rats above estrogens (Fig. 8). The finding that T plasma level was maintained in the presence of Arimidex (Figs. 6 and 7). The ADIONE-induced reduction in the LGR was reversed by Arimidex, demonstrating that the effect was mediated by estrogens and not androgens (Table 2). Likewise, the ADIONE-induced reduction in the LGR was reversed by Arimidex, demonstrating that the effect was mediated by estrogens and not androgens (Table 2).

Table 2. Effect of ADIONE, placebo pellets, Casodex, and Arimidex on uterine weight, cancellous bone volume, and LGR of 6-mo-old rats 90 days postovariectomy

<table>
<thead>
<tr>
<th></th>
<th>Uterine Wt, mg</th>
<th>BV/TV, %</th>
<th>LGR, µm/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>627.73 ± 33.32</td>
<td>24.15 ± 0.81</td>
<td>6.43 ± 0.36</td>
</tr>
<tr>
<td>OVX + placebo (1.5 mg)</td>
<td>138.33 ± 4.18</td>
<td>7.88 ± 0.27</td>
<td>11.62 ± 0.48*</td>
</tr>
<tr>
<td>OVX + ADIONE (1.5 mg)</td>
<td>158.67 ± 4.83</td>
<td>14.07 ± 0.35</td>
<td>7.60 ± 0.29</td>
</tr>
<tr>
<td>OVX + Cas</td>
<td>140.70 ± 4.31</td>
<td>7.79 ± 0.15</td>
<td>11.24 ± 0.37*</td>
</tr>
<tr>
<td>OVX + ADIONE (1.5 mg) + Cas</td>
<td>164.89 ± 2.44</td>
<td>8.81 ± 0.26</td>
<td>7.34 ± 0.22</td>
</tr>
<tr>
<td>OVX + Arim</td>
<td>136.91 ± 5.57</td>
<td>7.85 ± 0.12</td>
<td>11.85 ± 0.25*</td>
</tr>
<tr>
<td>OVX + ADIONE (1.5 mg) + Arim</td>
<td>138.00 ± 6.26</td>
<td>13.1 ± 0.23</td>
<td>11.88 ± 0.53*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cas, Casodex; Arim, Arimidex; Uterine wet: *P < 0.0001 vs. all other groups; †P < 0.001 vs. all other groups except each other; Cancellous bone volume (BV/TV); *P < 0.001 vs. all other groups; †P < 0.001 vs. all other groups; $P < 0.0001$ vs. all other groups; LGR of 6-mo-old rats 90 days postovariectomy: $P < 0.0001$ vs. sham, OVX + ADIONE (1.5 mg), and OVX + ADIONE + Cas.

DISCUSSION

The role of ovarian-derived estrogen in the maintenance of the skeleton is well characterized (12, 16, 22, 28, 29), whereas the effect of ovarian androgens is less clear. We now report that cancellous bone loss in the OVX rat can be significantly reduced by restoring plasma levels of ADIONE and T to those found in sham-operated animals. Similar results were found in 13-wk- and 6-mo-old animals. The 6-mo-old animals were used in experiment II, since older rats are generally considered to provide a better model of postmenopausal bone loss in humans (12). However, similar results were observed in both experiments. Our results, in terms of static and dynamic bone histomorphometry in sham-operated and OVX rats of the serial histomorphometric analysis, are very similar to the effects seen in OVX rats in response to estrogen (28). Hence the skeletal-protective effect of estrogens (28) and androgens in OVX rats are both brought about by suppressing bone turnover.

ADIONE is a precursor of estrogens and T, either or both of which could potentially protect the skeleton.
against bone loss. The results of experiment II show that the protective effect of ADIONE was maintained in the presence of Arimidex, when E1 and E2 plasma levels were returned to levels in OVX placebo-treated ani-
mals. This excludes the possibility that estrogens medi-
atrate the effect of ADIONE. The finding that there was complete abrogation of the ADIONE-induced protective effect in the presence of the antiandrogen demonstrates that androgens account for our findings. Androgens have previously been shown to exert an anabolic effect on the female rat skeleton at physiological levels (9, 14), but on this occasion, we were unable to detect such an effect. However, the anabolic effect of androgens may be masked by the resorption-induced changes in formation, i.e., where suppression of bone resorption sup-
presses bone formation.

It is not surprising that the ADIONE-induced reduc-
tion in the LGR of OVX rats was exerted by estrogens, because longitudinal bone growth increases as a result of ovariectomy (28) and is reversed by estrogen (25), but it is interesting that this occurred in the presence of very low levels of circulating E2, which were found to be without effect on the cancellous bone volume in OVX rats. It is therefore possible that these circulating

levels of E2 do not account for the reduction in LGR in OVX rats. The alternative explanation is that localized peripheral synthesis of E2 is responsible for the compart-
mentalized estrogenic effect that we have observed at the growth plate. If the appropriate enzymes were present at this site, conversion from T to E2 and ADIONE to E1 could be catalyzed by aromatase cyto-
chrome P-450, and conversion of the latter to E2 could be catalyzed by 17β-hydroxysteroid dehydrogenase.

Our results indicate that androgens inhibit bone resorption in the female, just as they do in the male (26, 27). Previous reports have shown that treatment of female rats with antiandrogens do not affect osteoclast parameters, but these experiments were carried out on estrogen-replete rats (9, 14). Our results are at odds with those showing that nonaromatizable dihydrotestos-
terone only suppresses histomorphometric indexes of resorption in OVX rats at nonphysiological levels (23). The reason for our different findings is not clear. However, the experimental designs were not the same.

Our data suggest that the antiresorptive effect of androgens in the female rat can only be detected in estrogen-deficient states, such as menopause. If this were the case, it might explain why ADIONE treatment in our study does not protect bone loss from day 1. There was only a minor difference between the bone lost in OVX and ADIONE-treated OVX animals on day 21, after which ADIONE prevented any further bone loss. This suggests that, after exposure to estrogen, the androgen-sensitive cells in the skeleton involved in the process of inhibiting bone resorption are resistant to the effects of androgens. The results also suggest that, after a period of estrogen depletion, the cellular target for androgens in bone becomes responsive to this hormone. At present, it is unclear whether androgens inhibit bone resorption by exerting their effect directly on the osteoclast or indirectly through other cells, including osteoblasts, since receptors have been identi-
fied on both (5, 19).

The absence of a significant difference in the results of the histomorphometric data between the 1.5- and 5-mg pellets, particularly in the presence of such a large difference in the plasma levels of ADIONE and T, suggests that a maximal inhibitory effect on the param-
eters of bone resorption is being exerted by the andro-
gens in response to the 1.5-mg pellet. However, this is not the case, since, in a separate series of experiments, supraphysiological levels of T were able to further reduce the resorptive parameters toward control levels (15).

Our findings that restoration of ADIONE and T plasma levels to those in sham-operated animals pro-
tects against cancellous bone loss and that this effect is exerted by androgens have not previously been re-
ported. The results suggest that ovarian androgens may be important in protecting against bone loss, particularly in the absence of estrogens. It will now be important to discover whether equivalent plasma lev-
els of androgens exert a similar effect on the human skeleton. If this proves to be the case, screening post-
menopausal women to identify those with low levels of
androgens may be a useful means of identifying individuals at risk of developing osteoporosis. Furthermore, administration of androgens to postmenopausal women who are deficient in these hormones should reduce bone turnover and consequently reduce bone loss. Because our findings indicate that physiological plasma levels of androgens protect against bone loss, the adverse effects generally associated with high levels of androgens should not be seen.

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REFERENCES


Fig. 8. Plasma levels of T, ADIONE, E2, and E1 in 6-mo-old rats after treatment with 1.5 mg ADIONE with and without Casodex or Arimidex for 90 days. ADIONE and T: * P < 0.0001 vs. all groups except each other; ** P < 0.0001 vs. all groups except each other. E2: * P < 0.005 vs. all groups except each other. E1: * P < 0.0001 vs. all groups except each other.