Influence of clenbuterol on bone metabolism in exercised or sedentary rats

H. CAVALIÉ, G. LAC, P. LEBECQUE, B. CHANTERANNE, M.-J. DAVICCO, AND J.-P. BARLET

1Laboratoire de la Performance Motrice, Université Blaise Pascal, Clermont-Ferrand; and 2Unité Maladies Métaboliques et Micronutriments, INRA Clermont-Theix, 63177 Aubière, France

Received 28 May 2002; accepted in final form 12 August 2002

Clenbuterol is a selective $\beta_2$-adrenergic receptor agonist known to stimulate muscle hypertrophy and reduce body fat content (22). Clenbuterol treatment improves muscular functional capacity by increasing muscular strength (6, 24). Thus clenbuterol treatment is frequently used by athletes, especially those involved in strength- and power-related sports (15). In a recent study, Duncan et al. (7) showed that chronic clenbuterol administration deleteriously affected endurance and sprint exercise performance in rats. However, there are very few studies dealing with clenbuterol effects on bone metabolism and some controversy exists concerning the influence of adrenergic $\beta_2$-receptor agonist on the skeleton. Togari et al. (20) report that $\beta_2$ receptors are located in the osteoblast. In vivo in rats, clenbuterol reduces net bone loss in denervated (23) or suspended hindlimbs (5). The opposite was found in another study (10), in which clenbuterol inhibited longitudinal bone growth and decreased bone mineral content (BMC) in growing rats. In vitro, clenbuterol stimulates osteoclastogenesis (19). In the work presented here, we observed the influence of clenbuterol on bone metabolism in exercised (E) or untrained (U) Wistar 3-mo-old male rats.

MATERIALS AND METHODS

Animals and Treatments

This experiment was made in accordance with current legislation on animal experiments in France. Thirty-two male 12-wk-old Wistar rats weighing 234 ± 2 g were randomly divided into two groups of 16 E or U animals. Each rat was housed in a 22 × 22 × 18-cm plastic cage, which allowed for separation and collection of urine and feces, at 22 ± 1°C, with a 12:12-h light-dark photoperiod. Animals were fed ad libitum a laboratory chow (UAR, Villemoisson sur Orge, France) containing 16% protein, 3% fat, 0.8% calcium, and 0.6% phosphorus. Urine of each rat was collected during a 24-h period on day 53. Among the 16 E animals submitted to progressive isometric force training for 8 wk and among the 16 U animals, 8 animals (ECL and UCL, respectively) were simultaneously given clenbuterol (Sigma Chemical, St. Louis, MO) per os (2 mg·kg body wt$^{-1}$·day$^{-1}$) 5 days/wk. Control rats (E and U) received in the same way the same volume (1 ml) of NaCl 0.9%.

Each E rat was trained every morning, 5 days/wk for 8 wk, according to an already described protocol (12). Briefly, each rat was set on the horizontal floor of a box, and then the box was put in a vertical position. Because the floor was made with wire netting, the animal gripped with it claws and remained in a climbing position. This occurred 4–8 times during 2 × 30 s. Each animal was allowed to rest during 20 s.
between each 30-s period and 3 min between each set. The intensity of training program progressively increased by adding a load to the tail from 0 g the first day to 200 g during the eighth week.

Each rat was weighed each Wednesday before the training period.

On day 58, rats were killed by cervical dislocation. Blood was collected by cardiac puncture. After centrifugation, plasma was harvested and frozen until analysis. Femurs were separated from adjacent tissue, cleaned, and used for physical measurements.

Physical Measurements

Body composition and femoral bone density. On day 54 under light chloral anesthesia, lean, fat, and total BMC were measured on each animal by DEXA (4) with the use of a Hologic QDR 4500A X-ray densitometer (Hologic, Massy, France).

On day 59, total right femoral bone mineral density (BMD) also was determined by DEXA. Furthermore, the BMDs of two subregions, one corresponding to the distal metaphyseal zone, which is rich in cancellous bone, and the other to the diaphyseal zone, which is mainly cortical bone, were assessed (14).

Femoral mechanical testing. Immediately after collection, each left femoral bone was placed in 0.9% NaCl at 4°C. Mechanical femoral resistance was determined 24 h later by a three-point bending test. Each bone was secured on the two lower supports of the anvil of a universal testing machine (Instron 4501; Instron, Canton, MA). The upper roller diameter was 6 mm. The crosshead speed for all tests was 0.5 mm/min. The load at rupture was determined automatically by the Instron 4501 software. To ensure comparable test sites, the femur was always mounted so that the crosshead was applied in the middle of the shaft of the bone. With the use of the 450-g rat, the span of the specimen that was loaded was 20 mm to guarantee that 85%--90% flexure of the bone was caused by bending. This test had been previously validated by using Plexiglas standard probes (21). Results are expressed in Newtons.

Biochemical Analysis

Marker for osteoblastic activity. Plasma osteocalcin (OC) concentration was measured by homologous radioimmunoassay by using rat OC standard, goat anti-rat OC antibody, 

\[ \text{125I-labeled rat OC, and donkey anti-goat second antibody} \]

(Biochemical Technologies kit, Stoughton, MA); the lowest limit of detection for this assay was 55 pg/ml, and the intra- and interassay variations were 7 and 9%, respectively.

Marker for bone resorption. Deoxypyridinoline (DPD) in urine was measured by radioimmunoassay (Pyrlinks D kit; Metra Biosystems, Moutain View, CA). The assay required the addition of a 50-μl urine sample (or DPD standard or control) to each well of the DPD-coated microplate. The monoclonal antibody against DPD was added to the plate, and the free DPD in urine competed with the DPD coated on the plate for the antibody. A second antibody conjugated to alkaline phosphatase was added to the plate to bind with antibody against DPD. A substrate p-nitrophenol phosphate was added to produce a yellow color. Optical density was measured at 405 nm. The lowest limit of detection for the assay was 2 nmol. The intra- and interassay variation was 5 and 7%, respectively. Results are expressed as nanomolar DPD/mM creatinine to avoid the possible influence of glomerular filtration rate (16). The creatinine assay was a modified Jaffe’s method in which picric acid forms a yellow compound with creatinine.

Statistics

Results are means ± SE. All data were analyzed by using a two-way ANOVA to detect effects of each treatment and to determine whether there is interaction among the treatments. Post hoc test (Bonferonni) was used to detect differences among means. Differences were considered significant at P < 0.05.

RESULTS

Body weight of each rat significantly increased during the experimental period (Fig. 1). From the sixth to the eighth week, body weight was higher in resting than in E rats. Nevertheless, any significant difference concerning body weight between ECL and E or between UCL and U was never observed.

Body composition (Table 1) was affected by strength training exercise and/or clenbuterol treatment. Fat mass (% from body weight), which was lower in E (34.1% lower) than in U animals, was decreased by clenbuterol treatment. The lowest fat mass was measured in ECL animals. Oppositely, the lean mass was higher in clenbuterol-treated rats.

Whole body mineral content (% from body weight) was lower in UCL (7.4% lower) than in U and in ECL (7.4% lower) than in E.

Both femoral length and diameter were lower in ECL than in any other group. Exercise alone had no signif-

Table 1. Body composition analyses of ECL, UCL, E, and U rats

<table>
<thead>
<tr>
<th></th>
<th>U</th>
<th>UCL</th>
<th>E</th>
<th>ECL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat mass, %</td>
<td>12.6 ± 1.6a</td>
<td>7.9 ± 1.9b</td>
<td>8.3 ± 1.1b</td>
<td>5.5 ± 0.7bc</td>
</tr>
<tr>
<td>Lean mass, %</td>
<td>84.7 ± 1.6a</td>
<td>89.6 ± 1.6b</td>
<td>89.0 ± 1.1b</td>
<td>92.0 ± 0.7bc</td>
</tr>
<tr>
<td>BMC, %</td>
<td>2.7 ± 0.1a</td>
<td>2.5 ± 0.1b</td>
<td>2.7 ± 0.0b</td>
<td>2.5 ± 0.0b</td>
</tr>
</tbody>
</table>

Values are means ± SE. Fat mass and body mass are % from body weight. U, untrained rats; E, exercised rats; UCL, U + clenbuterol-treated rats; ECL, E + clenbuterol-treated rats; BMC, whole body mineral content. a vs. b, P < 0.05; b vs. c, P < 0.05; c vs. a, P < 0.05 within each line.
icant effect on total right femoral BMD, metaphyseal zone BMD, and diaphyseal zone BMD. Moreover, total right femoral BMD, metaphyseal zone BMD, and diaphyseal zone BMD were lower in ECL than in E animals and in UCL than in U animals. Changes in femoral mechanical resistance paralleled those for BMD (Table 2).

No difference concerning plasma OC concentration was observed between groups. Urinary DPD excretion was lower in U than in UCL animals and E than in ECL animals, respectively (Table 3).

**DISCUSSION**

Mechanical loading plays a major role in the development and maintenance of bone mass. Clinical and experimental studies demonstrate that moderate and repeated physical activity is able to increase bone mass in both animals and humans (1). Mechanical loading influences bone mass through the strains it engenders into bone tissue either as a result from the strain itself or due to changes in the streaming potentials, intralacunar pressure, and fluid flow, or through deformations in the extracellular matrix (17). However, in our experimental conditions, progressive isometric force training had no significant effect on femoral bone metabolism, as evidenced by a lack of any significant change in BMD, plasma OC concentration, and urinary DPD excretion (Tables 2 and 3). This lack of effect might result from a training period that is too short (8 wk) to induce an increase in lean mass. Nevertheless in 13-wk-old Sprague-Dawley rats assigned either to low-intensity exercise (walking) alone or combined with sudden impact loading (2.5-cm upward or downward vertical movements) for 9 wk, the later exercise increased femoral mechanical resistance without any significant change in femoral mineral content (8).

β2-Agonists are used in athletes as a substitute of anabolic steroids (15). Main expected results are muscular hypertrophy and decreased fat mass (22). In our animals, lean mass was increased by clenbuterol, whereas fat mass was decreased. Muscle hypertrophy induced by this treatment might result from an inhibition of proteolysis (3) and/or an increase in protein synthesis (2, 13). The lowest fat mass was observed in ECL, possibly through an additional effect of strength training and clenbuterol treatment.

Clenbuterol treatment also significantly decreased femoral BMD, BMC, and mechanical resistance in our animals (Table 2). Such results differ from other reports concerning the effects of β-adrenergic agonists on bone. Dobutamine is a synthetic catecholamine that attenuates the decrements in maximal oxygen consumption and skeletal muscle oxidative enzyme activity observed during bed rest in healthy men (18). In rats, dobutamine (2 mg·kg body wt·day⁻¹ for 14 days, ip) maintained femoral cortical bone area by attenuating the decrease in mineral apposition rate induced by simultaneous hindlimb suspension (5). In the same way, clenbuterol added to drinking water (8.5 mg/l) reduced net bone loss in murine-denervated hindlimbs. Nevertheless, such a treatment given for 1 yr had no significant effect on ovariectomy-induced osteopenia in 250- to 270-g female rats (23). In 9-mo-old Sprague-Dawley male rats, clenbuterol (2 mg·kg body wt·day⁻¹ for 4 wk, sc) inhibited both femoral and tibial growth. This inhibition was associated with a decrease in BMC but not in BMD (10). The lack of effect on BMD might be due to the short duration (4 wk) of the treatment.

This decrease in BMC observed both in this experiment and in our experimental conditions might be due to the adrenergic stimulation of osteoclastogenesis observed in vitro after treatment of MC3T3-E1 cells with epinephrine or isoproterenol, which was induced via an increase in cyclase activity or intracellular cAMP content (9, 11), or to the expression of not only IL-6, IL-11, and PGE2 but also an osteoclast differentiation factor (19). This might explain why, in our experimental condition, clenbuterol treatment for 8 wk was associated with decreased BMC and BMD in trabecular as

| Table 2. Femoral analyses of ECL, UCL, E, and U rats |
|----------|----------|----------|----------|
|          | U        | UCL      | E        | ECL      |
| Length, mm | 38.6 ± 0.4ᵃ | 38.0 ± 0.3ᵃ | 38.4 ± 0.3ᵃ | 37.0 ± 0.3ᵇ |
| Diameter, mm | 3.9 ± 0.1ᵃ | 3.9 ± 0.0ᵇ | 3.9 ± 0.1ᵃ | 3.7 ± 0.1ᵇ |
| FL, N      | 133 ± 5ᵃ   | 118 ± 7ᵇ  | 124 ± 4ᵃ  | 106 ± 5ᶜ  |
| BMC, mg    | 492.1 ± 14.8ᵃ | 451.3 ± 17.4ᵇ | 476 ± 14.3ᵃ | 425.3 ± 12.4ᶜ |
| T-BMD, mg/cm² | 242.6 ± 5ᵃ   | 227 ± 3ᵇ   | 240.8 ± 2.5ᵃ | 202.2 ± 3.4ᵇ |
| D-BMD, mg/cm² | 221.4 ± 5.6ᵃ | 208.5 ± 3.1ᵇ | 213.3 ± 2.7ᵃ | 202.2 ± 3.4ᵇ |
| M-BMD, mg/cm² | 245.1 ± 4.7ᵃ | 228.1 ± 4.4ᵇ | 247.3 ± 3.8ᵃ | 236.7 ± 4.4ᵇ |

Values are means ± SE. FL, femoral failure load; T-BMD, femoral bone density; M-BMD, femoral metaphyseal density; D-BMD, femoral diaphyseal density. α vs. β, P < 0.05; β vs. γ, P < 0.05; γ vs. α, P < 0.05 within each line.

| Table 3. Plasma OC concentration and urinary DPD excretion in ECL, UCL, E, and U rats |
|----------|----------|----------|----------|
|          | U        | UCL      | E        | ECL      |
| OC, ng/ml | 52.09 ± 4.5  | 49.94 ± 0.9  | 54.45 ± 2.9  | 58.40 ± 3.1  |
| DPD, nmol DPD/mmol creatinine | 110.90 ± 6.9ᵃ | 121.49 ± 10.2ᵇ | 110.88 ± 2.2ᵃ | 121.81 ± 8.7ᵇ |

Values are means ± SE. OC, osteocalcin; DPD, deoxypyridinoline. α vs. β: P < 0.05.
well as in compact bone (Table 2). Decreased BMC and BMD might result either from decreased osteoblastic activity and/or from increased resorption activity. Clenbuterol treatment had no significant effect on osteoblastic activity, as evidenced by plasma OC concentration that was not different in any group of rats (Table 3). Conversely, this treatment increased resorption: urinary DPD excretion was significantly higher in ECL than in E animals and in UCL than in U animals (Table 3).

In conclusion, the results of this investigation indicate that clenbuterol treatment decreased femoral BMC and BMD in both E and resting rats. This effect probably resulted from increased bone resorption, as evidenced by increased urinary DPD excretion in clenbuterol-treated animals.

REFERENCES