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Original article

Effects of fenoterol on the skeletal system depend on the androgen level

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ABSTRACT

Background: The role of sympathetic nervous system in the osseous tissue remodeling is not clear enough.
Methods: The effects of fenoterol, a selective β_2-adrenomimetic drug, on the skeletal system of normal and androgen deficient (orchidectomized) rats were studied *in vivo*. Osteoclastogenesis and mRNA expression in osteoblasts were investigated *in vitro* in mouse cell cultures.
Results: Fenoterol administered to animals with physiological androgen level unfavorably affected the skeletal system, damaging the bone microarchitecture. Androgen deficiency induced osteoporotic changes, and fenoterol protected the osseous tissue from consequences of androgen deficiency. The results of *in vitro* studies correlated with the *in vivo* observations. A significantly increased number of osteoclasts in bone marrow cell cultures to which testosterone and fenoterol were added simultaneously was demonstrated. In cultures without the addition of testosterone, fenoterol significantly inhibited osteoclastogenesis in comparison with control cultures.
Conclusions: The results indicate the favorable action of fenoterol in conditions of testosterone deficiency, and its destructive influence upon the skeleton in the presence of androgens. The results confirm the key role of sympathetic nervous system in the regulation of bone remodeling.

Introduction

Bones are abundantly innervated by sympathetic neurons [1–3]. Noradrenaline promotes bone resorption *via* β-adrenergic receptors, which belong to the family of receptors coupled with G protein (GPCR), present on osteoblasts and osteoclasts. Of the three subtypes of the receptors: β_1, β_2, and β_3, of key importance in the osseous tissue is the stimulation of β_2 receptor, which inhibits the proliferation and differentiation of osteoblasts and favors osteoclastogenesis [4,5]. Also, a substantial input of the sympathetic system to mechano-adaptive regulation of bone remodeling has been demonstrated [2,3]. The stimulation of β_2 receptors activates adenyl cyclase, which leads to the increase of cAMP intracellular concentration and subsequently to stimulation of protein kinase A (PKA), responsible for genomic and non-genomic action of cAMP in the cell [6].

Multiple literature data indicate the preventive action of β-adrenolytic drugs in the development of osteoporosis resulting from estrogen deficiency [7–9]. Some reports also indicate that adrenomimetics cause skeleton damage in organisms with physiological level of sex hormones [10–13].

Our earlier studies demonstrated the destructive influence of fenoterol on the skeletal system in female rats, which occurred only in the presence of estrogens. In conditions of estrogen deficiency, fenoterol exerted protective influence on bones, counteracting the development of osteopenia [10]. The action of adrenomimetics on bones of males with androgen-deficiency has not been examined so far. The problem seems important because the osteoporosis risk in men increases with the increasing life expectancy.

The aim of the present study was to establish the effect of fenoterol, a selective β_2-adrenomimetic, on the skeletal system of rodents in the presence of androgens or in their deficiency *in vivo* and *in vitro*.

Materials and methods

In vivo *experiments*

3-Month-old male Wistar rats (Center of Experimental Medicine, Medical University of Silesia) were used. The initial rat body mass was 230–270 g. The rats were fed a standard laboratory diet

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(Labofeed B) *ad libitum*. All procedures were approved by the Local Ethics Commission in Katowice, Poland.

Orchidectomy and sham-operation were performed under general anesthesia induced by intraperitoneal injection of ketamine (80 mg/kg; Bioketan, Vetoquinol Biowet) with xylazine (12 mg/kg; Xylapan, Vetoquinol Biowet). After 7 days, which allowed for full recovery after the surgery, the rats were divided into 4 groups (n = 10): sham-operated (Sham) control rats, Sham rats receiving fenoterol, orchidectomized (ORX) control rats, and ORX rats receiving fenoterol. The animals were weighed every second day.

Fenoterol hydrobromide (Sigma-Aldrich, at a dose of 5 mg/kg daily) was administered by a gastric tube (*po*) once daily, for 7 weeks (6 days a week), in a volume of 2 ml/kg *po*. The control rats were administered the vehicle (tap water) in the same volume daily. Moreover, all rats were given intraperitoneal injections of 20 mg/kg of tetracycline hydrochloride (Sigma-Aldrich), to mark the calcification front [14], one day before the start of drug administration, and one day before sacrifice.

On the next day after the last fenoterol administration, after 24-h fasting, the animals were sacrificed by cardiac exsanguination, under ketamine-xylazine anaesthesia. The adrenal glands, the left and right tibia, and right femur were isolated and weighed. The left tibia were kept under −20 °C until the mechanical tests were performed [15].

Bone mineralization studies

The left tibias were mineralized at the temperature of 640 °C for 48 h in the muffle furnace, and weighed. The ratio of bone mineral mass to bone mass was treated as a substitute for bone mineral density measurements.

The calcium and phosphorus content in the mineralized bones was determined colorimetrically, using kits produced by Pointe Scientific, Inc. [16].

Bone histomorphometric studies

Bone histomorphometric parameters were assessed on histological specimens, prepared as previously described [16,17]. Optiphot-2 microscope (Nikon), connected through an RGB camera to a computer using Lucia G 4.51 software (Laboratory Imaging); final magnifications of 200 and 500 times, or Osteomeasure XP v1.3.0.1 software (OsteoMetrics); final magnification of 70 times, were used. The width of trabeculae in the distal epiphysis and metaphysis was measured in the longitudinal preparation from the right femur (the medial part, the median plane). The area of the transverse cross-section of the cortical bone and the area of the transverse cross-section of the marrow cavity were determined in transverse cross-sections made from the right tibial diaphysis. The periosteal and endosteal transverse growth of the right tibia was also measured.

Studies of bone mechanical properties

Mechanical properties of the left tibial metaphysis were assessed using the Instron 3342 500N apparatus with Bluehill 2 software, version 2.14. Mechanical properties of the proximal metaphysis of the left tibia were studied using a three-point bending test, according to [18], as previously described [11,19]. The load was applied perpendicularly to the proximal tibial metaphysis. The displacement rate was 0.01 mm/s. The load-displacement curves, representing the relationships between load applied to the bone and displacement were analyzed. Maximum load and displacement, energy, and stress for the maximum load, as well as the load, displacement, energy, and stress for the fracture point were assessed. Young's modulus was also determined. The moment of inertia, necessary for the calculations of the intrinsic bone mechanical parameters, was determined as previously described [11,16].

Biochemical studies

Serum osteocalcin levels were determined using an enzyme immunoassay (Rat-MID Osteocalcin EIA, Immunodiagnostic Systems Ltd.). Serum levels of type I collagen fragments released during bone resorption were determined by an enzyme immunoassay (RatLaps EIA, Immunodiagnostic Systems Ltd.). Serum total cholesterol was assayed colorimetrically, using a Pointe Scientific reagent set.

Statistical analysis

The results are presented as arithmetical means ± SEM. One-way ANOVA followed by Duncan's *post hoc* test was used for statistical evaluation of the results. In case of lack of normality on homogeneity of variance, Kruskal-Wallis ANOVA followed by Mann-Whitney *U* test was carried out. The results obtained in each experimental group were compared with those of the sham-operated control rats. The results obtained in ORX rats treated with fenoterol were also compared with those of the ORX control rats.

In vitro **experiments**

Osteoclast formation from murine bone marrow cells

Bone marrow cells from long bones of 3–7-day-old BALB/c mice (Center of Experimental Medicine, Medical University of Silesia) were obtained and cultured as previously described [20,21]. In each of 3 independent experiments, bone marrow cells from 6 to 10 neonate mice were pooled and plated in 48-well culture plates (Nunc), in approximate proportion: the cells harvested from 1 neonate per 4 cm^2. The cells were incubated at 37°C in 5% CO_2 in humidified air in α-MEM with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution (final concentrations of 100 U/ml penicillin and 0.1 mg/ml streptomycin). The next day after plating, the medium was replaced with fresh medium with the addition of 1,25-dihydroxyvitamin D_3 (10^{-8} M), to which testosterone (10^{-10}, 10^{-9} and 10^{-8} M), fenoterol (10^{-8}, 10^{-7} and 10^{-6} M), or testosterone (10^{-9} M) with fenoterol (10^{-8} M, 10^{-7} and 10^{-6} M) were added.

The culture media were refreshed every second day. The cultures were ended 9 days after plating, fixed with 3% paraformaldehyde in the 0.1 M cacodylate buffer (pH 7.2) and stained for tartrate-resistant acid phosphatase (TRAP). TRAP staining was performed histochemically with Leucocyte acid phosphatase kit, Sigma Diagnostics (procedure 386).

Large, multinucleated, TRAP-positive cells were considered osteoclasts. The number of osteoclasts was counted. The observations and measurements were made using the Eclipse TE 300 microscope (Nikon), connected *via* a digital camera (Nikon) to personal computer (software: Nis-Elements 2.21, Nikon).

Osteoblast isolation and culture

Osteoblastic cells were obtained and cultured, as previously described, from 1 to 2-day-old BALB/c mice (Center of Experimental Medicine, Medical University of Silesia) [21]. In each of 3 independent experiments, osteoblasts were isolated from 10 to 12 calvariae by six sequential digestions (20 min each) with 0.1% collagenase and 0.05% trypsin. Osteoblastic cells collected from fractions 3–6 were combined and cultured in α-MEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin