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HORMONAL STIMULATION OF BONE CELL PROLIFERATION

A. M. KAYE,* Y. WEISMAN,[1] A. HARELL[1] and D. SÖMJEN[2]

Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, and [1]Bone Disease and [2]Endocrinology Units, Ichilov Hospital, Tel Aviv 64239, Israel

Summary—The recent demonstration of estrogen receptors in bone derived cells has stimulated the study of direct effects of sex steroids on bone. We have shown direct stimulation of proliferation by 17β-estradiol (E_2) of ROS 17/2.8 rat osteogenic osteosarcoma cells, and other bone-derived cells in culture, as well as sex-specific stimulation of diaphyseal bone *in vivo* by estrogen and testosterone, using [^{3}H]thymidine incorporation into DNA and stimulation of the specific activity of creatine kinase as markers.

ROS 17/2.8 cells were used as models of osteoblast-like cells to study the reciprocal modulation of stimulation of bone cell proliferation by sequential treatment by sex steroid and calciotrophic hormones. Pretreatment with $1,25(OH)_2D_3$ and PTH augmented stimulation by E_2, while pretreatment with PGE_2 followed by E_2 resulted in no additional stimulation. Reciprocally, pretreatment with E_2 significantly reduced the response to PGE_2 while showing an insignificant effect on the response to the other hormones.

Gonadectomized Wistar-derived rats provided a useful model system for study of postmenopausal osteoporosis. In diaphyseal bone, [^{3}H]thymidine incorporation and creatine kinase activity decreased 4 weeks after gonadectomy. At that time, a single i.p. injection of E_2 in females, and testosterone in males, resulted in a highly significant increase in both these parameters within 24 h.

INTRODUCTION

The recent demonstration of estrogen receptors in bone derived cells [1, 2] has stimulated the study of direct effects of sex steroids on bone. Our previous work has shown direct stimulation of proliferation by estrogen of several bone derived cells in culture including ROS 17/2.8 cells [3]. *In vivo* studies revealed sex-specific stimulation of diaphyseal bone cells in prepuberal rats, using [^{3}H]thymidine incorporation into DNA and stimulation of the specific activity of creatine kinase BB as markers [3]. Investigations of the mutual interactions between sex steroids and vitamin D metabolites have shown that the full proliferative response of diaphyseal bone cells to 17β-estradiol depends on the presence of a physiological concentration of $1,25(OH)_2D_3$ [4].

Parathyroid hormone (PTH) and prostaglandin E_2 (PG) also have a trophic action on skeletal derived cells [5–9]. We, therefore, investigated the reciprocal interactions between the sex steroid hormones and other osteogenic hormones in ROS 17/2.8 rat osteogenic osteosarcoma cells, and compared the results with our parallel studies using primary cultures of rat embryonic calvaria cells and epiphyseal cartilage cells. In addition, in order to begin the translation of these data, obtained *in vitro*, to a rat model of postmenopausal osteoporosis, we demonstrated in this paper the suitability of using sexually mature gonadectomized rats to study the sex-specific stimulation of bone cell proliferation by 17β-estradiol and testosterone.

EXPERIMENTAL

Cell culture

ROS 17/2.8 osteoblastic osteosarcoma cells [10] were seeded in 35 mm dishes in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) plus F12 (1:1), supplemented with 10% fetal calf serum. Under these conditions, cells reached a subconfluent state in 4 days. Creatine kinase activity and/or [^{3}H]thymidine incorporation into DNA were determined 24 h after the last addition of either vehicle or hormones to the subconfluent cultures.

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*To whom correspondence should be addressed.

Table 1. Comparison of response of skeletal derived cells to 24 h exposure to hormones

	Response to hormone stimulation		
Hormone	ROS 17/2.8 osteosarcoma	Epiphyseal cartilage	Embryonic calvaria
$1,25(OH)_2D_3$	− [8]	0[9]	− [13]
$24,25(OH)_2D_3$	0[8]	+ [9]	+ [13]
PTH	+ [8]	+ [9]	+ [7]
PGE_2	0[8]	+ [9]	+ [7]
17β-Estradiol	+ [3]	+ [3]	+ [3]
Testosterone	0[3]	0[3]	0[3]

Parallel effects were seen on CK specific activity and [^{3}H]thymidine incorporation into DNA. +, stimulation; −, inhibition; 0, no change. Numbers in brackets are the references for the parallel data published elsewhere. Note that highly significant increases in response to E_2 were obtained [3] without the use of dextran-charcoal stripped serum or the elimination of the estrogenic component, Phenol red, from the medium.

CK preparation and assay

Cells were scraped from the plates and homogenized by sonication in homogenization buffer containing isotonic sucrose [3]. Portions of femur and tibia were homogenized using a Polytron microprobe. Supernatant extracts were obtained by centrifugation of homogenates at 12000 *g* for 5 minutes at 4°C in an Eppendorf microcentrifuge. CK activity was measured in a Gilford 250 automatic recording spectrophotometer at 340 nm using a coupled assay as described previously [3]. Protein was determined by Coomassie brilliant blue dye binding [11] using bovine serum albumin as the standard.

[^{3}H]thymidine incorporation into DNA

22 h after i.p. injection of vehicle (0.1 or 1% ethanol) the rats were killed and the central portion of the diaphysis and the epiphyses of the femur and tibia were cut into small fragments and incubated for 24 h at 37°C in DMEM containing 5 μCi ml [^{3}H]thymidine (5 μCi/mmol; 1 Ci = 37 GBq; Amersham, Bucks, England) under an atmosphere of 5% CO_2:95% O_2. Thymidine uptake and incorporation into acid-insoluble material was measured as previously described [3, 4]. DNA was determined by the Burton method [12].

Statistical significance

The significance of differences between experimental and control values was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

Modulation of proliferation in cell cultures

Cultures of cells derived from skeletal tissues respond to a variety of calciotrophic hormones by changes in cell proliferation as measured by [^{3}H]thymidine incorporation and creatine kinase specific activity (Table 1). PTH and E_2 stimulate growth in osteoblast-like cells (ROS 17/2.8) in chondroblast-like cells (rat epiphyseal cartilage) and in cultures containing a high proportion of osteoblast-like cells as well as chondroblast-like cells (rat embryo calvaria). The only example of inhibition of growth in these experiments was the effect of $1,25(OH)_2D_3$ on osteoblast-like cells. Testosterone was ineffective in these cell cultures (Table 1).

These results exemplify the use of the osteoblastic clonal cell line ROS 17/2.8, derived from a rat osteosarcoma [10], as a convenient model system for establishing the direct action of estrogen on bone derived cells [1, 3] as well as for studying the response of an osteoblastic cell to other calciotropic hormones (Table 1). In order to establish suitable conditions for testing the reciprocal interactions of steroid and calciotropic hormones on ROS 17/2.8 cells, we measured the response of these cells to 17β-estradiol for the first 4 days after transfer (Fig. 1). The greatest stimulation of CK activity occurred on day 4, when the cells were subconfluent. This result agrees well with our previous finding of a comparable stimulation of confluent cell cultures [3], and led us to adopt a standard daily pretreatment from day 2–4 after transfer, before challenge with 17β-estradiol (Fig. 2). This regime revealed augmentation of the response to E_2 by $1,25(OH)_2D_3$ and PTH pretreatment with no significant effect of $24,25(OH)_2D_3$ and inhibition of any augmentation of the response to E_2 by PG. These results should be compared with those obtained using calvaria cells, or epiphyseal cartilage (Table 2). PTH and $1,25(OH)_2D_3$ augment the response to E_2 in both osteoblast-like and chondroblast-like cells while $24,25(OH)_2D_3$ augments the response only in epiphyseal cartilage. On the other hand,

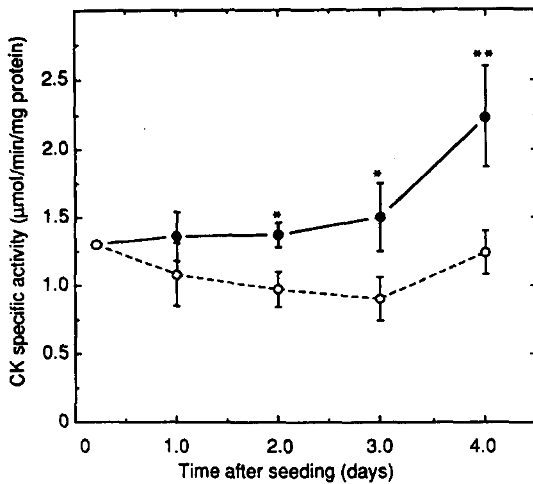

Fig. 1. Stimulation of CK activity in ROS 17/2.8 cells by E_2 on successive days after seeding. Cells were grown as described in the Experimental section, treated with vehicle (0.02% ethanol in saline) or E_2 (30 nM) for 24 h, beginning on the day indicated, and analysed for CK activity by a spectrophotometric method [3]. The first point was taken after cells adhered to the dishes at 4 h after seeding, before any E_2 treatment. Results are means ± SE for $n = 5$. $^*P \leq 0.05$; $^{**}P \leq 0.05$ for E_2-treated (●—●) vs vehicle-treated (○—○) cells.

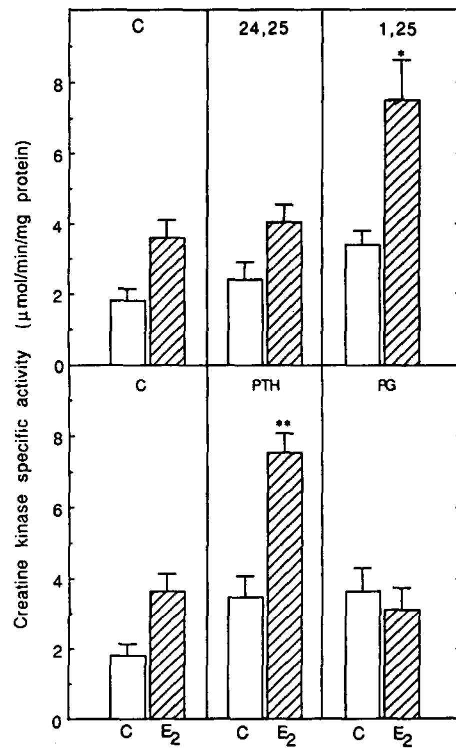

Fig. 2. Modulation of the stimulation of CK activity by E_2 caused by pretreatment of ROS 17/2.8 cell cultures with calciotrophic hormones. Cells were cultured as described in the Experimental section and treated daily, from day 2 to 4 after seeding, with either vehicle (C, 0.02% ethanol in saline); $24{,}25(OH)_2D_3$ (24,25; 5 ng/mg); $1{,}25(OH)_2D_3$ (1,25; 0.5 ng/ml), PTH (10 nM) or PGE_2 (PG; 1.5×10^{-6} M). Cultures in each group were then treated for 24 h with vehicle (C) or with 30 nM E_2. CK activity was measured spectrophotometrically [3]. Results are means ± SE for $n = 5$. $^*P \leq 0.05$; $^{**}P \leq 0.01$ for E_2 stimulated values for experimental vs control pretreatment. Values for control pretreatment are repeated in the bottom row for ease of comparison.

PG inhibits any augmented response to E_2 in all cases.

The reciprocal interaction, the effect of steroid hormone pretreatment of ROS 17/2.8 cells on their response to estrogenic hormones, is shown in Fig. 3. Pretreatment with E_2 significantly reduces the response to PG but does not have any considerable effect on the stimulation of CK activity by PTH and E_2 itself nor on the decrease in CK specific activity caused by $1{,}25(OH)_2D_3$. On the other hand, pretreatment of rat epiphyseal cartilage cell cultures with E_2 caused an increased response to PTH, a loss of the response to PGE_2 and $24{,}25(OH)_2D_3$ and the acquisition of inhibition by $1{,}25(OH)_2D_3$ of both [^{3}H]thymidine incorporation and CK specific activity [14]. These E_2-induced properties are characteristic of osteoblasts, suggesting E_2 modulated differentiation of cartilage cells in the direction of osteoblast-like cells.

As a first step towards determining the mechanism(s) involved in the multihormonal modulation of CK activity in ROS 17/2.8 cells, we demonstrated that both cycloheximide and actinomycin D inhibited the stimulation of CK activity by E_2 (Fig. 4). This result parallels our previous finding that both these inhibitors prevent the increase in CK activity after PTH treatment of rat embryo calvaria cells [7], and

Table 2. Comparison of responsiveness to estrogen of skeletal derived cell after 3 days pretreatment with calciotrophic hormones

Hormonal pretreatment	Responsiveness to E_2 after hormone pretreatment: ROS 17/2.8 osteoblasts	Epiphyseal cartilage	Embryonic calvaria
$1{,}25(OH)_2D_3$	Increased	Increased [4]	Increased [14]
$24{,}25(OH)_2D_3$	Unchanged	Increased [4]	Unchanged [14]
PTH	Increased	Increased [14]	Increased [15]
PGE_2	Decreased	Decreased [14]	Decreased [15]
17β-Estradiol	Unchanged	Unchanged [14]	Not determined
Testosterone	Decreased	Decreased [14]	Not determined

Responsiveness = ratio of increase in response with pretreatment to increase in response without pretreatment. ROS 17/2.8 data are from Figs 2 and 3.

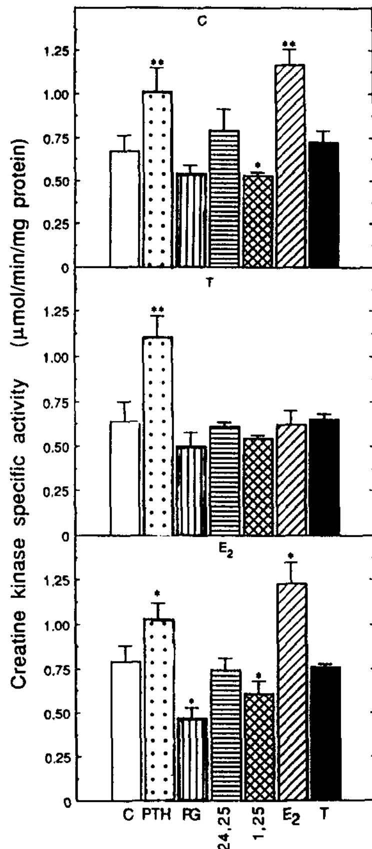

Fig. 3. Modulation of the CK response to calciotropic and sex steroid hormones caused by pretreatment of ROS 17/2.8 cell cultures with E_2 or T. Cells were cultured as described in the Experimental section and treated daily from day 2 to 4 after seeding, with either vehicle (C; 0.02% ethanol in saline) or E_2 (3 nM), or testosterone (T; 30 nM). Cultures in each of these 3 groups were then treated for 24 h with vehicle (C); PTH (10 nM); PGE_2 (PG; 500 ng/ml); $24{,}25(OH)_2D_3$ (24,25; 5 ng/ml); $1{,}25(OH)_2D_3$ (1,25; 0.5 ng/ml); E_2 (30 nM) or testosterone (T; 300 nM). CK activity was measured spectrophotometrically [3]. Results are means ± SE for $n = 5$. *$P \leq 0.05$; **$P \leq 0.01$ for experimental vs the control value for the same pretreatment.

suggests that a rise in the steady-state level of mRNA, as found in E_2 stimulated immature rat uterus [16] and in skeletal-derived cell cultures stimulated by PTH and $24{,}25(OH)_2D_3$ [17], may be required for the stimulation of CK activity.

Sex steroid effect in gonadectomized rats

E_2 stimulates thymidine incorporation and CK specific activity in diaphysis of Wistar-derived rats (Fig. 5). This stimulation is increased and becomes more highly significant in rats 4 weeks after ovariectomy, since there is a reduction in thymidine incorporation and CK activity by this time. The effect of ovariectomy is even more striking in epiphysis, in which no effect of E_2 on thymidine incorporation is seen in normal rats compared to a highly significant

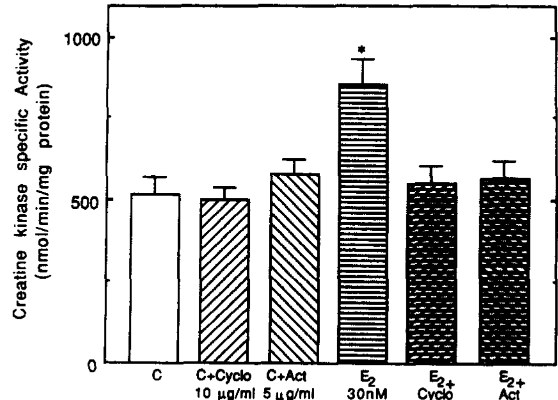

Fig. 4. Inhibition of E_2 induced CK activity by cycloheximide (Cyclo) or actinomycin D (Act). Subconfluent cells (grown as described in the Experimental section) were treated for 24 h with vehicle (C; 0.02% ethanol in saline), E_2 (30 nM), Cyclo (10 µg/ml), Act (5 µg/ml) or the combination of E_2 + Cyclo or E_2 + Act. CK activity was measured spectrophotometrically [3]. Results are means ± SE for $n = 5$. *$P \leq 0.05$; **$P \leq 0.01$ for experimental vs the control (C) value.

increase (80%) in ovariectomized rats (Fig. 5).

A parallel situation was found in male castrated rats compared with intact males (Fig. 6). While T injection stimulated significantly both [^{3}H]thymidine incorporation into DNA and CK specific activity in both epiphysis and diaphysis

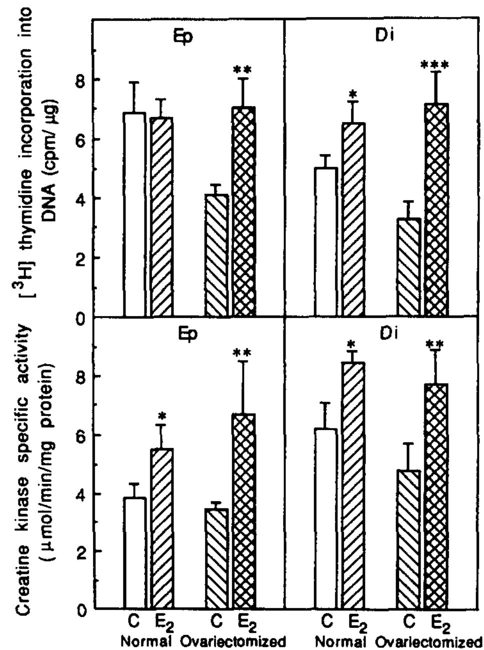

Fig. 5. Stimulation by E_2 of proliferation of skeletal tissues of intact and ovariectomized rats. Intact females and rats ovariectomized at 24 days after birth were injected i.p. with either vehicle (C; 0.1% ethanol) or 5 µg of E_2, at 52 days, and killed 24 h later. DNA synthesis and CK activity were measured as described previously [3]. Results are means ± SE for $n = 5$. *$P \leq 0.05$; **$P \leq 0.01$ for E_2-treated vs untreated rats.

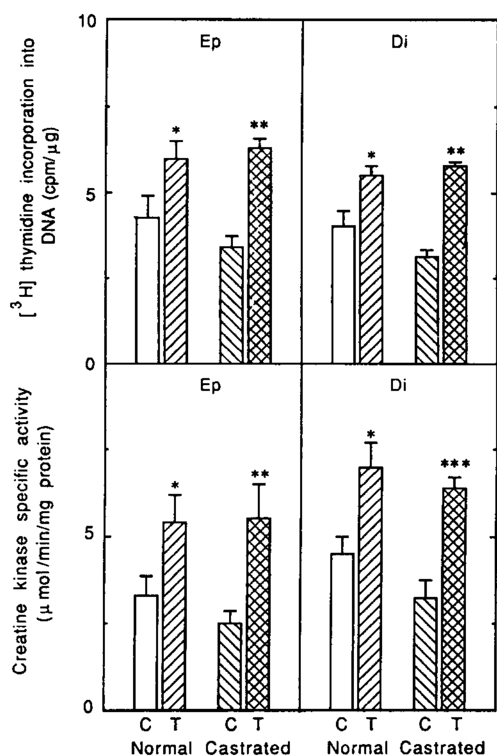

Fig. 6. Stimulation by T of proliferation of skeletal tissues of intact and castrated rats. Intact males and rats castrated at 24 days after birth were injected i.p. with either vehicle (0.5% ethanol) or 50 μg of T at 52 days and killed 24 h later. DNA synthesis and CK activity were measured as described previously [3]. Results are means ± SE for $n = 5$. *$P \leq 0.01$; **$P \leq 0.01$ for T-treated vs untreated rats.

of intact males, there was a greater response in castrated males, which reached higher levels of significance (Fig. 6).

This finding in gonadectomized rats, seen in Figs 5 and 6, and our previous results regarding sex specificity of E_2 and T stimulation of CK activity in bone [3], suggest that the gonadectomized rat is indeed a useful model for involutional osteoporosis in humans.

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