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REGULATION OF PROLIFERATION OF RAT CARTILAGE AND BONE BY SEX STEROID HORMONES

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Summary—We have demonstrated previously that 17β-estradiol (E_2) stimulates proliferation of skeletal tissues, both *in vivo* and *in vitro*, as measured by increased DNA synthesis and creatine kinase (CK) specific activity. The effect of E_2 on bone is sex specific. E_2 is active only in females and androgens only in males. By contrast, in cartilage of both sexes, dihydrotestosterone (DHT) as well as E_2 stimulates CK specific activity and DNA synthesis. In bone, we find that sex steroids stimulate skeletal cell proliferation in gonadectomized as well as in immature rats. Ovariectomized (OVX) rats, between 1 and 4 weeks after surgery, show stimulation of CK by E_2. The basal activity and response of CK changes with the varying endogenous levels of E_2 in cycling rats, in which the highest basal activity is at proestrus and estrus and the highest response is in diestrus. In rats of all ages tested, both the basal and stimulated specific activity of CK is higher in diaphysis and epiphysis than in the uterus, or in the adipose tissue adjacent to the uterus, which has a response similar to that of the uterus itself. The effect of E_2 *in vivo*, and in chrondroblasts and osteoblasts *in vitro*, is inhibited by high levels of the antiestrogen tamoxifen which, by itself, in similar high concentrations, shows stimulatory effects. In addition to the sex steroids, skeletal cells are also stimulated by secosteroid and peptide calciotrophic hormones. The interactions of the sex steroids with these hormones modulate the response of cartilage and bone cells to both sex steroids and the other calciotrophic hormones. These results provide the first steps towards understanding the regulation of bone cell proliferation and growth by the concerted action of a variety of hormones and growth factors.

INTRODUCTION

Estrogens profoundly influence bone metabolism. Estrogen deficiency is the cause of postmenopausal osteoporosis [1–4] which is characterized by a decrease in bone mass and strength which leads to an increased risk of fractures. Low circulating estrogen in females is associated with increased bone remodeling rates, accelerated bone loss and negative calcium balance [5]. Estrogen replacement therapy [6, 7] or treatment with synthetic anabolic steroids [8] which are derivatives of androgens, decreases bone turnover and the rate of bone resorption, prevents or delays bone loss and reduces the incidence of fractures.

The failure to demonstrate estrogen responsiveness of bone in organ or cell culture led to the idea that estrogens act indirectly by mechanisms concerned mainly with decreased bone resorption [9]. Recently it has been reported that 17β-estradiol (E_2) stimulates proliferation of osteoblasts from primary calvaria [10, 11], long bones [10] and chondroblasts [12]. Moreover, it was shown that E_2 stimulates bone formation *in vivo* [11, 13]. In addition to several mechanisms for indirect action of E_2 which have been suggested [14–16], it has now been demonstrated that E_2 has a direct action in cartilage [12] and bone [17–19] presumably via the low concentrations of estrogen receptors [20–22] which were detected in skeletal derived cells. It was also shown that androgens stimulate bone proliferation *in vitro* [23].

We have demonstrated that E_2 stimulates proliferation (DNA synthesis) and energy metabolism [creatine kinase (CK) specific activity], in osteoblast models such as ROS 17/2.8 cells, and osteoblast cultures derived from rat embryo calvaria; in a chondroblast model, rat epiphyseal cartilage cell cultures, and in diaphyseal bone and epiphyseal cartilage in immature female rats [11]. In these studies, we have also shown that testosterone (T) can stimulate these parameters in bone from immature

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male rats [11]. In addition, we have demonstrated that vitamin D metabolites can modulate the response to E_2 both *in vivo* [24] and *in vitro* [24–26].

In these studies, we measured two parameters of bone growth, [^{3}H]thymidine incorporation into DNA as the classical marker for cell proliferation, and the activity of the brain type (BB) isozyme of creatine kinase (CK, EC 2.7, 3.2), as a rapid and convenient early enzymatic marker which precedes cell division. CK, which is involved in the regulation of the intracellular ratio of ATP:ADP, was identified as the estrogen induced protein of the uterus [27] and other organs containing estrogen receptors [28].

The present paper reports our studies on: (1) the maintenance of sex specificity in cell cultures *in vitro*; (2) the inhibition of E_2 response *in vivo* and *in vitro* by an antiestrogen, tamoxifen; (3) the use of *in vivo* models such as gonadectomized rats as a response model for the study of osteoporosis; (4) the correlation of E_2 response to the endogenous levels of E_2 in cycling rats. In addition we summarize the interactions of other calciotrophic hormones with sex steroids in stimulation of bone proliferation.

EXPERIMENTAL

Cell cultures

Rat epiphyseal cartilage cell cultures [29], rat embryo calvaria cell cultures [24], and ROS 17/2.8 cell cultures [25] were prepared as previously described.

Animals

Wistar-derived rats from the Hormone Research Departmental Colony were used either when immature, at 8 weeks after birth on different days of the estrous cycle, or 1–4 weeks post gonadectomy performed at 24 days.

Hormone treatment

E_2 was tested in cultures at 30 nM for 24 h, DHT or T at 300 nM. Immature rats were injected with 5 μg E_2 or 50 μg DHT or T and mature rats were given 10 μg E_2 or 100 μg DHT or T. Hormonal treatment was either for 4 or 24 h.

Enzyme preparation and assay

Creatine kinase was extracted from cells or organs and assayed as described previously [11]. A unit of enzyme activity was defined as the amount yielding 1 μmol of ATP/min at 30°C and specific activity as μmol/min/mg protein. Protein was determined by Coomassie Brilliant Blue dye binding [30] using bovine serum albumin as the standard.

[^{3}H]thymidine incorporation into DNA

Cells or rats were treated with hormones for 22 h and then DNA synthesis was measured by pulse labeling with [^{3}H]thymidine as described previously [11]. DNA was determined by the method of Burton [31].

Statistical significance

The significance of differences between experimental and control values was evaluated using Student's t-test in which n = number of culture dishes or animals per group.

RESULTS

Sex specificity of cell cultures

We have shown previously [11] that diaphyseal bone of immature female rats responds

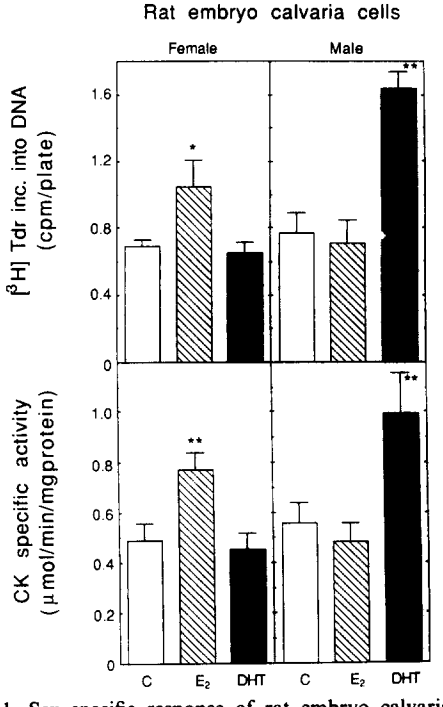

Fig. 1. Sex specific response of rat embryo calvaria cell cultures to sex steroid hormones. Cells were prepared from either male or female fetal rat calvaria as described previously [24]. Hormone treatment prior to CK determination was for 24 h with either 30 nM E_2 or 300 nM dihydrotestosterone (DHT). CK was extracted and analyzed as described in Methods. DNA synthesis was measured 22 h after hormonal treatment by 2 h labeling with [^{3}H]Tdr, as described in Methods. Results are means ± SE for n = 10–15, in 3 experiments. Statistical analysis by Student's t-test for differences between hormone treated and vehicle injected control groups. *$P \leq 0.05$; **$P \leq 0.01$.

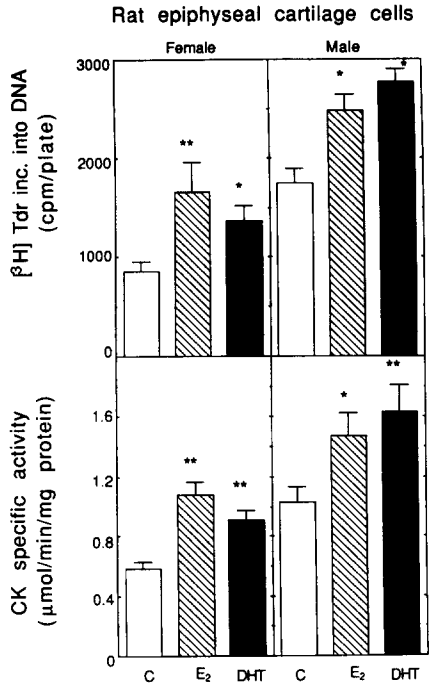

Fig. 2. Response of rat embryo epiphyseal cell cultures to sex specific hormones. The experimental details and symbols are the same as in the legend to Fig. 1. Results are means ± SE for $n = 10$–15, in 3 experiments.

only to E_2 whereas bone from male rats responds to DHT or T only. By contrast, epiphyseal cartilage from either female or male rats responds to both sex steroids. To determine if cells in culture continue to behave like the bone from which they were isolated, rat calvaria cell cultures, which contain a high proportion of osteoblasts [11], were tested and found to respond only to E_2 (Fig. 1) when derived from female rats and only to DHT when derived from

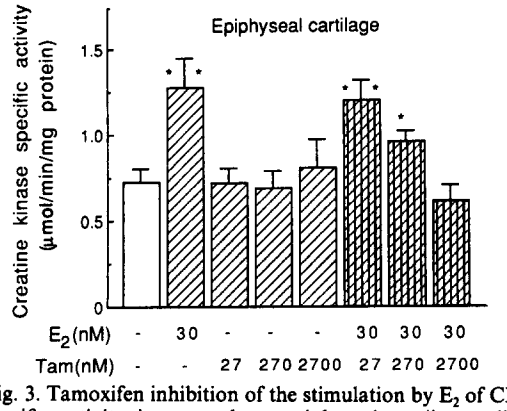

Fig. 3. Tamoxifen inhibition of the stimulation by E_2 of CK specific activity in rat embryo epiphyseal cartilage cells. Cells were prepared and cultured as described in Methods and incubated for 24 h with E_2, or tamoxifen (Tam), or the two simultaneously. CK was extracted and assayed as described in Methods. Results are means ± SE for $n = 6$, in 2 experiments. Statistical analysis was as in the legend to Fig. 1.

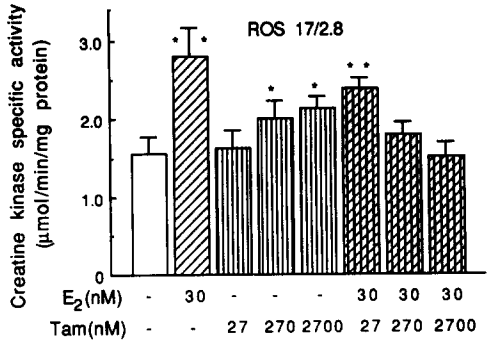

Fig. 4. Tamoxifen inhibition of the stimulation by E_2 of CK specific activity in ROS 17/2.8 osteoblast-like cells. Experimental details and symbols are described in the legend to Fig. 3. Results are means ± SE for $n = 5$.

male rats (Fig. 1). The same phenomenon of sex specificity was shown using primary cultures from human biopsies [32]. However, as *in vivo*, rat epiphyseal cartilage cells in culture respond to either hormone (Fig. 2). These results show that cells in culture continue to behave as they did in the organ from which they were isolated, and thus the culture conditions do not change their response pattern.

Prevention of E_2 stimulation by an antiestrogen

Tamoxifen, a clinically important antiestrogen [33] inhibits, at high concentrations

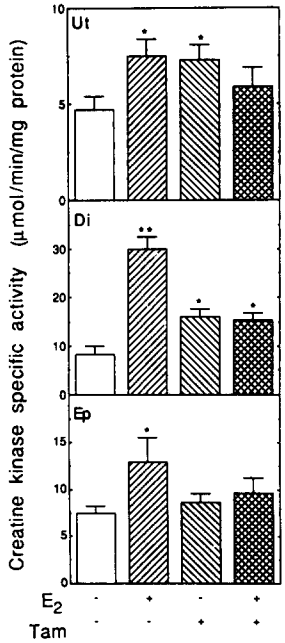

Fig. 5. Tamoxifen inhibition of the stimulation by E_2 of CK specific activity in rat organs. Female rats (24 days old) were injected with E_2 (5 μg) or tamoxifen (Tam 500 μg), or both. CK from uterus (Ut), diaphysis (Di), and epiphysis (Ep) was extracted and assayed as described in Methods. Results are means ± SE for $n = 5$. Statistical analysis was as described in the legend to Fig. 1.

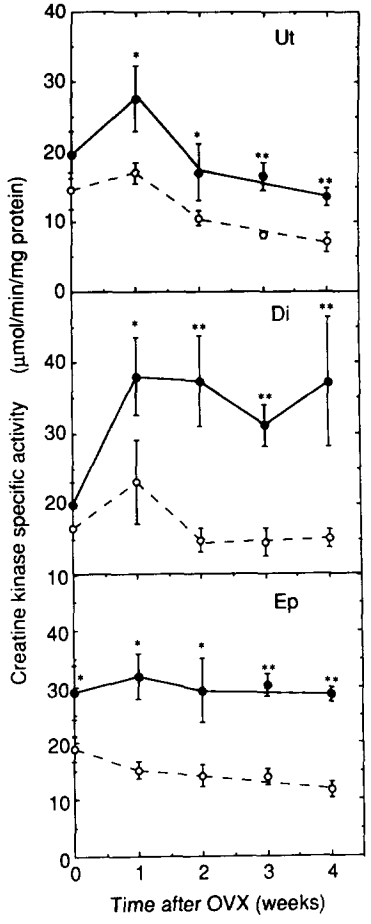

Fig. 6. The effect of the time after ovariectomy on CK activity and its stimulation by E_2. Female rats (24 days old) were ovariectomized, and 1, 2, 3 or 4 weeks post surgery they were injected with 5 μg E_2 and killed 24 h later. CK from uterus (Ut), diaphysis (Di), and epiphysis (Ep) was extracted and analyzed, as described in Methods. Age matched intact rats were treated in parallel with each group. Results are means ± SE. Statistical analysis was as described in the legend to Fig. 1.

(100 × E_2), E_2 stimulation of CK in epiphyseal cartilage cultures (Fig. 3). In this system, tamoxifen by itself did not have an agonistic effect. In ROS 17/2.8 cells, tamoxifen at 10 × and 100 × the E_2 concentrations, both inhibits E_2 stimulation (Fig. 4) and by itself shows agonistic effects. This same combination of agonistic and antagonistic effects seen in ROS 17/2.8 cells (Fig. 4) was found *in vivo* in diaphysis and uterus of immature rats (Fig. 5) while in the epiphysis, as in epiphyseal cartilage cells (Fig. 3) only antagonist action was seen.

Gonadectomized rats as a model for sex steroid deficiency osteoporosis

Not only immature rats (Fig. 5) but also adult gonadectomized rats can be used as a response model for E_2 or T action. When 24-day-old animals were ovariectomized and tested for E_2 responsiveness, a decrease was seen in the basal CK activity during the 4 weeks after surgery (Fig. 6); stimulation by E_2 takes place throughout this period, compared to groups of intact rats used at random days of their cycle which are not always consistently responsive (e.g. Fig. 6, 0 time).

Moreover, even aging animals, 24-month-old females, respond to E_2, 4 weeks after ovariectomy (Fig. 7). The same retention of responsiveness is also seen in 30-month-old adult males, 4 weeks post-castration, which respond to T injection (Fig. 8).

In these experiments, we also found that the adipose cells in the mesentery bordering the uterus, are able to respond to E_2 to the same extent as the uterus in ovariectomized rats (Fig. 7).

Adult cycling female rats

In the experiments on ovariectomized rats (Figs 6 and 7), we used intact rats as controls. The basal activity and the response levels

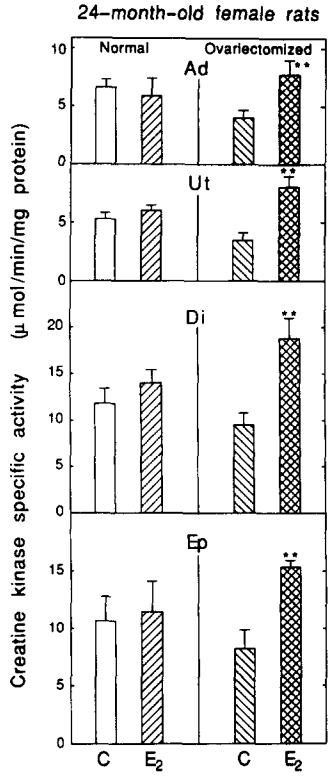

Fig. 7. The response of 24-month-old ovariectomized female rats to E_2. Rats were ovariectomized and 4 weeks later, along with intact control rats, they were injected with 10 μg E_2. Twenty-four hours afterwards, CK from adipose tissue (Ad), uterus (Ut), diaphysis (Di), and epiphysis (Ep), was extracted and analyzed as described in Methods. Results are means ± SE for $n = 3$–4.

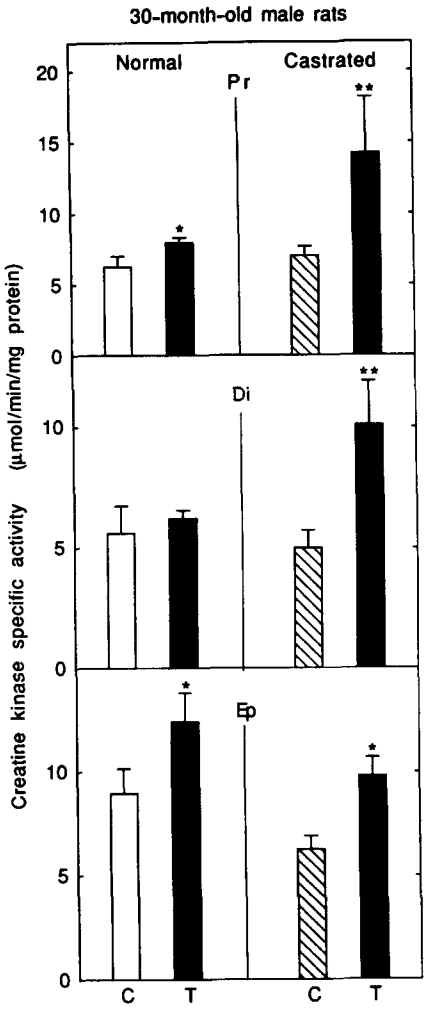

Fig. 8. The response of 30-month-old castrated male rats to T. Rats were castrated and 4 weeks later, along with intact controls, injected with 100 μg testosterone (T). Twenty-four hours later, CK was extracted from ventral prostate (Pr), diaphysis (Di), and epiphysis (Ep) and analyzed as described. Results are means ± SE for $n = 4$–5.

showed considerable variation. To correlate this variation with endogenous levels of circulating E_2, we studied 8-week-old female rats injected with 10 μg E_2/rat on different days of the estrus cycle and killed 4 h later. The basal activity was highest in estrus and proestrus, which have the highest circulating levels of E_2, while the response to exogenous estrogen (Fig. 9) is highest in diestrous rats which have the lowest E_2 concentration in their blood.

DISCUSSION

We have demonstrated previously that E_2 stimulated proliferation of skeletal tissues [11, 24, 26]. The effect on diaphyseal bone is sex specific and is maintained in cell culture (Fig. 1). It can be modified under different conditions, such as androgenization [32, 34] and testicular feminization [34] or as a result of gonadectomy [35]. We demonstrated here that, in addition to using immature rats, either young or old gonadectomized rats can be used as a model for studying these effects. Even normal cycling females can be reproducibly stimulated, provided that they are tested in diestrus when their endogenous level of E_2 is low. These findings add to the growing body of data showing that the rat is a good model for studies on osteoporosis [13, 36]. We could also demonstrate the use of adipose tissue as a test system for E_2, showing both basal levels of CK and response to E_2 stimulation comparable to the uterus.

The effects of E_2 on skeletal tissues both *in vivo* and *in vitro* can be inhibited by the antiestrogen tamoxifen. This drug, by itself, has agonistic effects in ROS 17/2.8 cells and *in vivo*. However, the response pattern appears to be cell type specific. Tamoxifen acts only as an antagonist of E_2 stimulation in epiphyseal cartilage (Figs 3 and 5) and UMR 106 cells [37].

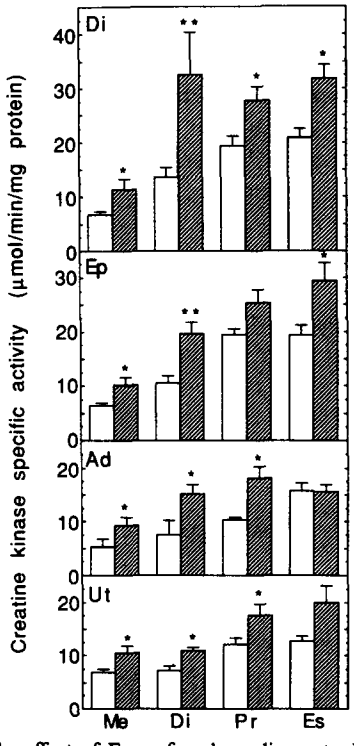

Fig. 9. The effect of E_2 on female cycling rats. Eight week old female rats, at different days of the estrous cycle, metestrus (Me), diestrus (Di), proestrus (Pr) and estrus (Es), were injected with ethanol vehicle (C) or 5 μg E_2. Four hours later, rats were killed and CK was extracted from diaphysis (Di), epiphysis (Ep), adipose tissue (Ad) and uterus (Ut) and analyzed as described in Methods. Results are means ± SE for $n = 5$.

Table 1. Hormonal effects on osteoblasts (ROS 17/2.8 or calvaria cells) or chondroblasts (epiphyseal cartilage in cell culture)

Osteoblasts	Chondroblasts
Hormonal responses	
Stimulated by:	Stimulated by:
E_2 [11]; PTH and PGE_2 [40, 41]	E_2 [11], PTH and PGE_2 [40, 41] $24,25(OH)_2D_3$ [39]
Inhibited by:	Inhibited by:
$1,25(OH)_2D_3$ [38, 41]	None
Unaffected by:	Unaffected by:
$24,25(OH)_2D_3$ [38, 39, 41]	$1,25(OH)_2D_3$ [39]
Modification of E_2 responses by pretreatment with calciotrophic hormones	
Increased by:	Increased by:
PTH and $1,25(OH)_2D_3$ [24–26]	PTH, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ [24, 26]
Inhibited by:	Inhibited by:
PGE_2 [25, 26]	PGE_2 [26]
Unaffected by:	Unaffected by:
$24,25(OH)_2D_3$ [24–26]	none
Modification of the response to calciotrophic hormones by pretreatment with E_2	
Increased response to:	Increased response to:
None	PTH [25, 26]
Inhibited response to:	Inhibited response to:
PGE_2 [26]	PGE_2, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ [26]
Unaffected response to:	Unaffected response to:
PTH, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ [25, 26]	None [26]

Results were obtained from changes in CK specific activity and DNA synthesis, and the following hormones: E_2, PTH, PGE_2, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$.

Skeletal cell proliferation is regulated by a variety of steroids [11, 24], secosteroids [38, 39], PTH [40, 41] and PGE_2 [40, 41]. Moreover, these hormones modulate each other's activity in a yet unknown fashion (Table 1). These interrelationships are potentially important in understanding the regulation of normal bone growth *in vivo* and in diseases such as osteoporosis. In order to understand the mechanism of these interactions, we will have to distinguish whether the changes are at the level of the receptor or via post-receptor phenomena such as control of mRNA synthesis for CK [42], c-*fos* [43] or for other regulatory factors.

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