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Direct effects of 17β-estradiol on trabecular bone in ovariectomized rats

(bone histomorphometry)

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ABSTRACT **High-affinity nuclear binding sites for 17β-estradiol ($17\beta E_2$) were recently found in bone cells; however, the mechanism by which estrogen exerts its effect on bone *in vivo* is still unknown. To study if estrogen acts on bone directly, we used an experimental model in which test substances are infused locally into rat femur trabecular bone. Sprague–Dawley rats weighing 150–160 g were ovariectomized (OVX) and 14 days later a polyethylene tube (1 mm in diameter) connected to an Alzet osmotic minipump was implanted into the distal femur 9 mm from the joint. $17\beta E_2$ (24 μl/day at 0.01–1 nM), 17α-estradiol ($17\alpha E_2$) (24 μl/day at 1 nM), or phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter; KH_2PO_4, 0.2 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 2.16 g/liter) was infused for 8 days. The contralateral limb remained intact. Animals were sacrificed and bones were examined by histomorphometry. Ovariectomy caused a 50% loss in trabecular bone volume (TBV) in the secondary spongiosa (from 20.3% ± 1.7% to 9.6% ± 1.1%; mean ± SEM), a 2-fold increase in osteoclast number (to 4.0 ± 0.4 per mm), a 3-fold increase in relative resorption surfaces (to 24.8% ± 2.9%), a 9-fold increase in osteoblast number (to 11.3 ± 2.1 per mm), and an 8-fold increase in relative osteoid surface (to 9.6% ± 1.7%). The local infusion of $17\beta E_2$ for 8 days into OVX rats (*i*) restored the TBV dose dependently to 75% and 85% of control (non-OVX) levels, at 0.1 nM and 1 nM $17\beta E_2$, respectively; (*ii*) decreased osteoclast number and the relative resorption surface to control (non-OVX) levels; and (*iii*) further increased osteoblast number and the relative osteoid surface dose dependently (by 5-fold at 1 nM $17\beta E_2$). Phosphate-buffered saline infusion was without effect. Infusion of $17\alpha E_2$ had no effect on TBV, osteoclast number, or resorption surface but increased slightly the osteoblast number and the osteoid surface. Its potency was 1/100 that of $17\beta E_2$. The local infusion of $17\beta E_2$ or $17\alpha E_2$ had no effect on body or uterine weight. We conclude from these findings that estrogen delivered directly to the bone of OVX rats *in vivo* at 2.4 and 24 fmol/day acted locally to inhibit bone resorption and stimulate bone formation.**

About 50 years ago, Albright *et al.* (1) suggested a link between postmenopausal osteoporosis and estrogen deficiency. The lack of estrogen is associated with increased bone remodeling rates, accelerated bone loss, and a negative calcium balance (2). Estrogen replacement therapy decreases bone turnover and the rate of bone resorption and reduces the occurrence of fractures in postmenopausal osteoporosis. However, the mechanism by which estrogen exerts its effects on bone is still unknown (3, 4).

For many years attempts to demonstrate estrogen effects on bone explants or bone cells *in vitro* were unsuccessful, suggesting that the action of estrogen on bone is indirect (5–10). However, several recent reports have described small estrogen effects on bone cells *in vitro* (11–16) and high-affinity nuclear binding sites for 17β-estradiol ($17\beta E_2$) have been described in human (14, 15) and rat (15) osteoblast-like cells. Further, estrogen was found to regulate the synthesis/secretion of growth factors (14, 17), which are considered to be important in the local regulation for bone cell replication or differentiation (18–21) such as transforming growth factor β (TGFβ) and insulin-like growth factors I and II (IGF-I and IGF-II). Recent findings thus suggest that estrogen may act directly on bone, but this hypothesis is based so far on limited evidence from *in vitro* studies. To demonstrate the action of estrogen on bone *in vivo*, one would have to separate its local action from systemic effects.

We have recently developed an experimental model in which small amounts of test substances can be delivered directly into rat femur trabecular bone *in vivo* to investigate their local action in the absence of complicating systemic effects. In the present study, a very small amount of $17\beta E_2$, infused directly into the distal femur metaphysis of ovariectomized (OVX) rats, elicited a localized anabolic effect on the secondary spongiosa, which reversed the bone loss produced by ovariectomy.

MATERIALS AND METHODS

Surgical Procedures. Thirty female Sprague–Dawley rats weighing 150–160 g were anesthetized with ketamine hydrochloride and acepromazine malate 3:1 (vol/vol). Twenty-five rats were subjected to bilateral ovariectomy and five rats were sham-operated. Food (Purina, Labcheckers) and water were provided ad libitum. Fourteen days after ovariectomy, a catheter for the direct infusion of estrogen was implanted into the femur trabecular bone using the following procedure. A small skin and muscle incision was made over the right femur. A 1-mm (diameter) hole, 1 mm deep, was drilled with a dental bur on the lateral aspect of the distal cortex of the rat femur 9 mm above the joint. A 2-mm piece of Intramedic PE-50 tube (Clay Adams) was inserted 1 mm deep and glued with cyanoacrylate on the outside aspect of the bone. This tube was connected to an Alzet osmotic minipump designed to deliver 1 μl/hr (Na 2001, Alza) that was implanted subcutaneously in the right hip area of the rats. The contralateral left limb remained intact.

Treatments. The rats were divided into six groups: (*i*) sham-operated; (*ii*) OVX; (*iii*) OVX receiving 0.01 nM $17\beta E_2$; (*iv*) OVX receiving 0.1 nM $17\beta E_2$; (*v*) OVX receiving 1 nM $17\beta E_2$; and (*vi*) OVX receiving 1 nM 17α-estradiol ($17\alpha E_2$). $17\beta E_2$ and $17\alpha E_2$ (Sigma) were dissolved in 100% ethanol at the concentration of 10 mM and were diluted to 1 nM, 0.1 nM, and 0.01 nM in phosphate-buffered saline (PBS) (NaCl, 8 g/liter; KCl, 0.2 g/liter; KH_2PO_4, 0.2 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 2.16 g/liter). The mean filling volume of the osmotic

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Abbreviations: $17\beta E_2$, 17β-estradiol; $17\alpha E_2$, 17α-estradiol; OVX, ovariectomized; TBV, trabecular bone volume; IGF, insulin-like growth factor; TGF, transforming growth factor.

minipumps specified by the manufacturer was 213 ± 6 μl and the nominal mean pumping rate was 1.03 ± 0.05 μl/hr. The daily dose of estradiol infused in the treated groups was thus close to 0.24, 2.4, and 24 fmol (0.07, 0.67, and 6.73 pg) per rat femur, respectively. PBS alone was infused into the femurs of sham-operated and OVX animals of groups *i* and *ii*, respectively. Rats from each treatment group were sacrificed 8 days after implantation of osmotic minipumps, 22 days after ovariectomy. The success of ovariectomy was confirmed by failure to detect ovarian tissue, by observation of marked atrophy of the uterine horns, and by the reduction in the wet weight of the uterine tissue.

Histomorphometry. The femora from each animal were removed and fixed in 70% ethanol. The bones were dehydrated in an ethanol series and embedded in methyl methacrylate. Coronal sections (6 μm thick) parallel to the long axis of the bones extending from one-third to one-half the thickness of each bone were prepared from femora and were stained with Masson's trichrome stain.

Bone histomorphometric parameters were evaluated in the secondary spongiosa of the distal femur metaphysis with a semiautomatic real-color Magiscan image analyzer (Joyce–Loebl) and a Nikon Microphot-Fx microscope at ×488 using the "Bone" software package developed for bone histomorphometry (Joyce–Loebl). The Sony model DXC-M3AP color video camera mounted to a microscope was used to display the image of the specimen on the video screen. The structure of interest is recognized based on its color. The region analyzed in each distal femur metaphysis extended between 1.0 and 1.9 mm from the epiphyseal growth plate and was 3.7 mm wide, centered on the long axis of the bone for a total metaphyseal area of 1.7 mm^2 (Fig. 1). For each condition, slides from five animals were examined and counted in eight fields. The following parameters were quantitated at this standard sampling site: trabecular bone volume (TBV, percentage of bone tissue); osteoclast number per mm of bone surface; relative bone resorption surface (percentage of bone surface with characteristic features of resorption, including surfaces with and without osteoclasts); osteoblast number per mm of bone surface; and relative osteoid surface (percentage of bone surface with osteoid, with and without osteoblasts). Osteoclasts were identified as multinucleated cells containing round nuclei and a large cytoplasm that stained light red with Masson's trichrome stain and were located immediately adjacent to the bone surface or in a resorption cavity. All of the osteoblasts in the section identified by their size, cuboidal shape, staining, and position were counted. These cells were either adjacent to osteoid or extended for several layers between the osteoid and the bone marrow, probably due to the plane of the sections, which may not have been perpendicular to the trabeculae. Random samples tested in a blinded fashion by an independent investigator yielded values that were within 5–15% of initial readings, confirming the accuracy of the measurements.

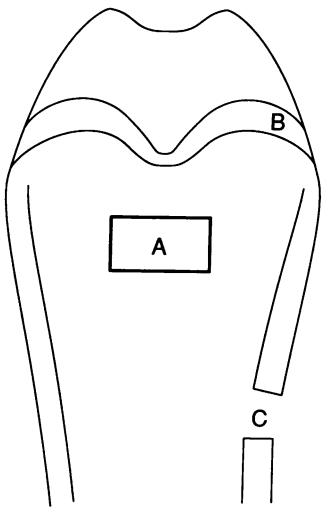

FIG. 1. Diagram of the selected sampling site for histomorphometric analysis. The distal femur metaphyseal region analyzed by histomorphometry (A) was located in the secondary spongiosa extending between 1.0 and 1.9 mm from the epiphyseal growth plate (B), 3.7 mm wide, centered on the long axis of the bone. For each group of rats, slides from five animals were examined and eight fields were analyzed for a total metaphyseal area of 1.7 mm^2. A, a sampling site; B, epiphyseal growth plate; C, 1-mm (diameter) hole on right femur of rat.

Means were compared using Student's *t* test for groups or paired values and *P* values of <0.05 were considered statistically significant.

RESULTS

The effects of ovariectomy and of locally infused $17\beta E_2$ or $17\alpha E_2$ on body and uterine weights were examined in all animals. Ovariectomy resulted in a substantial reduction in uterine weight: 114 mg in OVX vs. 396 mg in sham-operated animals. The initial mean body weight was the same in all groups. The mean body weight at sacrifice was on the average 8% higher in OVX rats than in the sham-operated animals (the difference was statistically significant only for some of the groups). The local infusion of $17\beta E_2$ or $17\alpha E_2$ into the distal femur metaphasis had no significant effect on the uterine or body weight of the OVX rats.

The bone histomorphometric parameters are presented in Fig. 2. The OVX rats were markedly osteopenic relative to sham-operated rats, ovariectomy causing a 50% loss in TBV (9.6% ± 1.1% vs. 20.3% ± 1.7%; mean ± SEM) 22 days after ovariectomy (Fig. 2*A*). The local infusion of $17\beta E_2$ for 8 days, 24 μl/day at 0.1 and 1 nM $17\beta E_2$, restored most of the bone loss, increasing TBV to 75% and 85% of non-OVX control levels, respectively, without affecting the TBV of the contralateral femur. PBS infusion had no effect on TBV at the same sampling site in sham-operated or in OVX rats nor did $17\alpha E_2$ infused at 1 nM into bones of OVX rats.

Assessment of bone resorption parameters showed that ovariectomy caused a 2-fold increase in osteoclast number, to 4.0 ± 0.4 per mm vs. 1.7 ± 0.3 per mm in sham-operated rats, and a 3-fold increase in relative resorption surface to 24.8% ± 2.9% vs. 7.1% ± 2.0%, 22 days after ovariectomy (Fig. 2 *B* and *C*). PBS infusion had no effect on these parameters. The infusion of $17\beta E_2$ to OVX rats reduced osteoclast number and the relative resorption surface dose dependently to nearly control (sham-operated) levels and had no effect on the contralateral femurs, where osteoclast number and resorption surfaces were similar to those in OVX animals with or without PBS infusion. $17\alpha E_2$ infusion had no effect on bone resorption-related parameters. Thus, local treatment with $17\beta E_2$ specifically suppressed the increased bone resorption in OVX rats to control levels.

Evaluation of bone formation parameters showed that ovariectomy caused in the secondary spongiosa a 9-fold increase in osteoblast number to 11.3 ± 2.1 per mm and an 8-fold increase in relative osteoid surface to 9.6% ± 1.7% relative to sham-operated rats (Fig. 2 *D* and *E*). PBS infusion was without effect. $17\beta E_2$ infusion to OVX rats further increased osteoblast number and relative osteoid surface dose dependently up to 5-fold at 1 nM $17\beta E_2$. Infusion of $17\alpha E_2$ had a small stimulatory effect on osteoblast number and relative osteoid surface, being 50- to 100-fold less potent than $17\beta E_2$ (estimated from the dose–response curve). Local administration of $17\beta E_2$ to the metaphyseal trabecular bone of OVX rats thus promotes bone formation in addition to inhibiting bone resorption.

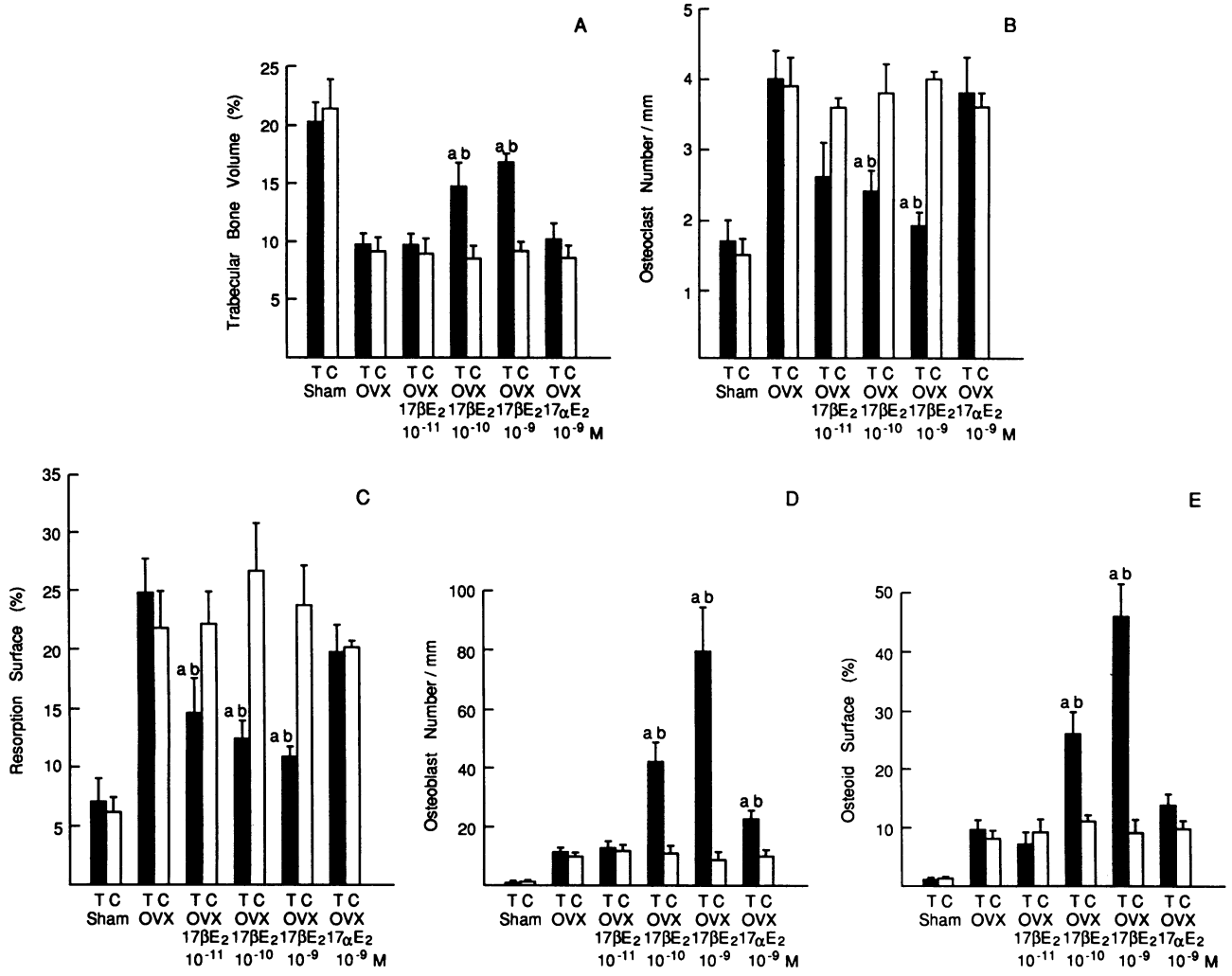

FIG. 2. Effects of ovariectomy and the local infusion of estradiol into the rat femur metaphysis on TBV (*A*), osteoclast number per mm (*B*), relative resorption surface (*C*), osteoblast number per mm (*D*), and relative osteoid surface (*E*). Fourteen days after ovariectomy, rats were infused with PBS, at the indicated concentrations of $17\beta E_2$ or $17\alpha E_2$, directly into the right femoral metaphysis (T) for 8 days, 24 μl/day. The contralateral left limb (C) remained intact. Rats were sacrificed at 22 days after ovariectomy and femurs were subjected to bone histomorphometry. Results are presented as the mean ± SEM (five rats per group). Similar results were obtained in two additional experiments of similar design. a, $P < 0.05$ compared to the contralateral left femur according to paired *t* test. b, $P \leq 0.05$ compared to the right femur of OVX rat infused with PBS according to Student's *t* test. All remaining comparisons were statistically nonsignificant.

The histological appearance of the metaphyses in sham-operated, OVX, and $17\beta E_2$-infused OVX rats is shown in Fig. 3. The increase in the number of osteoblasts is clearly apparent. Several prominent osteoclasts are also seen in Fig. 3*F*; however, when averaged over multiple sections their number was lower than in nontreated OVX animals (Fig. 2*B*).

DISCUSSION

Ovariectomy causes osteopenia in rats (22–27) and has been used as a model for postmenopausal bone loss (25–27). Wronski *et al.* (27) reported osteopenia and increased bone resorption and formation, estimated by histomorphometry, as early as 14 days after ovariectomy. Turner *et al.* (28) reported that TBV in the proximal tibia metaphysis of OVX rats was reduced by >60% 28 days after ovariectomy. Similar changes were observed in this study where ovariectomy for 22 days caused in the secondary spongiosa of rat femora a 50% loss in TBV, a 2-fold increase in osteoclast number, a 3-fold increase in the relative resorption surface, a 9-fold increase in osteoblast number, and an 8-fold increase in the relative osteoid surface. The mechanism for the loss of trabecular bone in OVX rats is unclear. The increase in bone resorption and osteoclastic activity has to be larger than the increase in osteoblastic activity and bone formation to account for the net loss of bone. In the present study, the change in osteoblast number produced by ovariectomy is actually not so great on an absolute basis but is large on a percentage basis since the starting levels are so low.

The effects of estrogen on bone and mineral metabolism have been extensively documented in rodents, quail, and humans, but its mechanism of action is still uncertain. A major unanswered question is whether estrogen action on bone *in vivo* is direct or indirect (4, 28–41). It has been suggested that *in vivo* estrogen promotes the systemic production of growth factors, so-called estromedins, or the removal of growth inhibitors, estrocolyons, elsewhere in the body (42). However, the findings presented here strongly suggest that estrogen acts directly on bone. Similar to $17\beta E_2$ administered systemically (22, 24, 28), local treatment reverted skeletal changes produced by ovariectomy.

The local infusion of $17\beta E_2$ decreased osteoclast number and the relative resorption surface dose dependently to nearly non-OVX control levels. This observation supports the general concept that estrogen inhibits bone resorption *in vivo* (2, 3, 22, 24, 28, 35–38), although *in vitro* studies showed

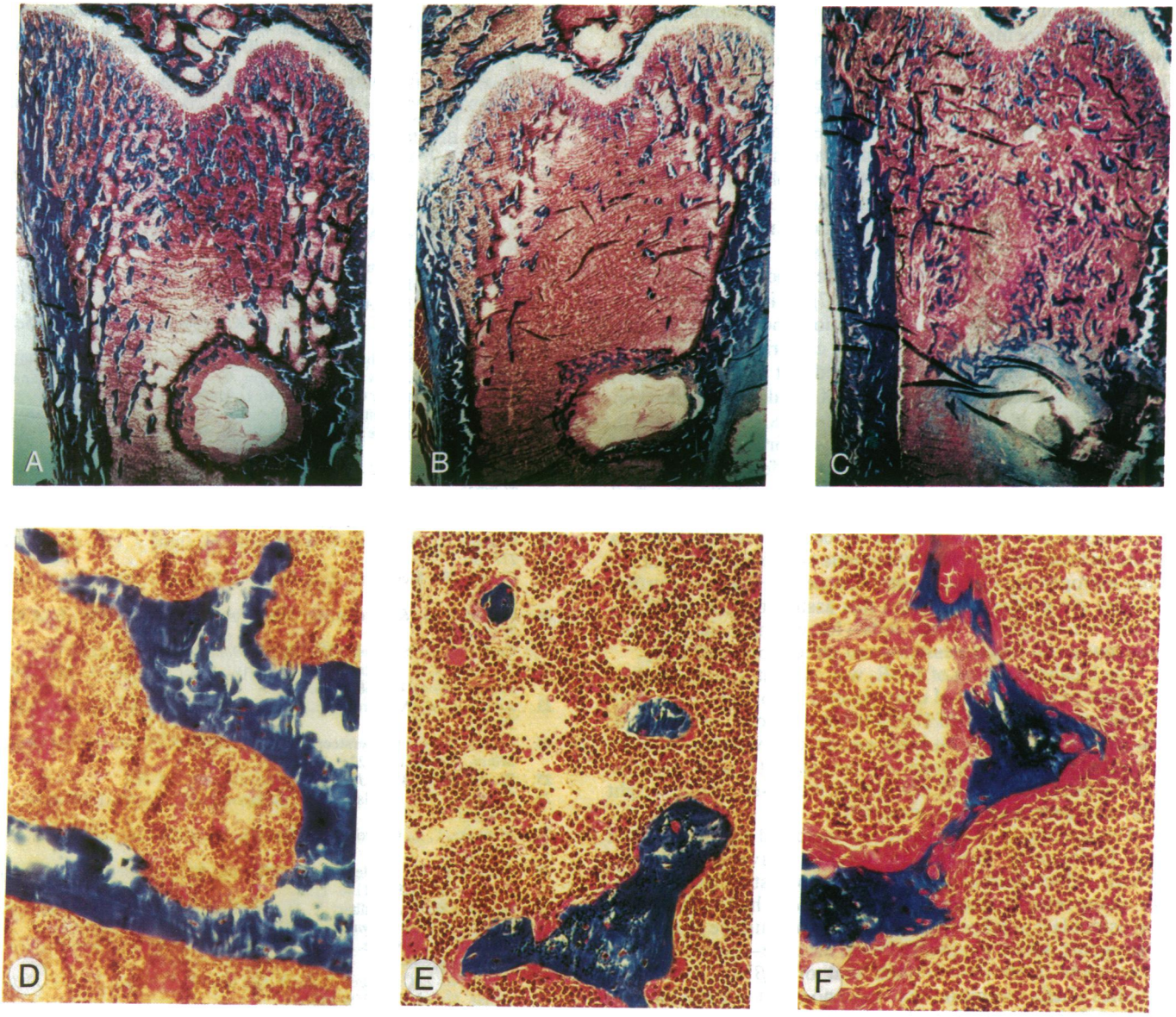

FIG. 3. Photomicrographs of distal femur metaphyses from sham-operated (*A* and *D*) and OVX (*B* and *E*) rats treated with PBS and from OVX rats that were infused with 17βE$_2$ (*C* and *F*). Fourteen days after ovariectomy, rats were infused with PBS or 1 nM 17βE$_2$ into the metaphyseal region of the femur for 8 days, 24 μl/day. Rats were sacrificed at 22 days after ovariectomy. Note the reduced amount of trabecular bone stained blue beneath the epiphyseal growth plate in the PBS-infused OVX rat (*B*) compared to the PBS-infused sham-operated rat (*A*). Increased TBV in the OVX rat infused with 17βE$_2$ (*C*) is apparent. Higher magnification photomicrographs illustrate that ovariectomy (*E*) increases the number of osteoblasts and osteoclasts compared to sham-operation (*D*). The local infusion of 17βE$_2$ to OVX rats resulted in abundant cuboidal osteoblasts on steroid surfaces (*F*), whereas infusion of PBS failed to elicit this response (*E*). (Masson's trichrome stain; *A*, *B*, and *D*: ×5.4; *D*, *E*, and *F*: ×225.)

no effect or effects at very high concentrations, which may not be receptor-mediated and may not relate to the *in vivo* action of estrogen (6, 7, 43–45). The mechanism for estrogen inhibition of bone resorption is still unknown, but recent cell culture experiments have shown that estrogen treatment suppresses parathyroid hormone (PTH) stimulation of adenylate cyclase in osteoblastic cells (13, 16). The bone resorptive action of PTH is probably mediated, at least in part, by cyclic AMP. Therefore, if this phenomenon occurs *in vivo*, it could explain the estrogen suppression of bone resorption, but additional studies are needed to clarify the effect of estrogen on bone resorption.

17βE$_2$ not only reduced bone resorption but also promoted new bone formation, restoring in 1 week 85% of the bone lost during 3 weeks of ovariectomy. The local infusion of 17βE$_2$ to OVX rats increased osteoblast number and relative osteoid surface dose dependently up to 5-fold at 1 nM 17βE$_2$ compared to the contralateral limb or PBS-infused OVX rats. The response to estradiol was obtained at a concentration of infused 17βE$_2$ of 0.1 nM, which is probably around the circulating physiological concentration of estradiol in rats (46, 47). However, since the infused 17βE$_2$ is further diluted and the total daily effective local dose was 2.4–24 fmol, the osteoblastic response to 17βE$_2$ seems to be exquisitely sensitive in this system.

In vivo studies have yielded controversial findings regarding estradiol stimulation of bone formation (4, 22, 24, 29, 41). Although some studies in women and mice suggest that estrogen increased bone formation and bone mineral content (4, 29, 41), Wronski *et al.* (22) and Turner *et al.* (24) reported that estrogen treatment of OVX rats decreased the histomorphometric indices of bone formation as well as bone resorp-

tion but restored bone mass. We have obtained similar results in pilot experiments with systemic infusion of 1.2 μg of 17βE_2 per day to 200-g OVX rats (data not shown). The differences between the effects of local and systemic infusion of 17βE_2 on bone formation could be due to dose, which was higher for systemic administration. Biphasic effects of 17βE_2 (lower or inhibitory effects being produced by higher doses) were also reported by Shull and Gorski (48) for prolactin production, Gray *et al.* (11) for alkaline phosphatase stimulation in rat osteogenic sarcoma UMR106 cells, and Ernst *et al.* (12) for mitogenic effects on osteoblastic cells.

The increase in osteoblast number and osteoid surface produced by the local infusion of 17βE_2 is consistent with 17βE_2 stimulation of cell proliferation and the increase in collagen and IGF-I expression in rat calvarial osteoblast-like cells (12, 16). Gray *et al.* (17) reported that in UMR106 cells, 17βE_2, in addition to stimulation of alkaline phosphatase, enhanced the secretion of growth factors, such as TGFβ, IGF-I, and IGF-II, which may be important in the local regulation of bone cell proliferation and differentiation. However, it should be stressed that the *in vitro* effects are small and seem to be dependent on carefully chosen experimental conditions. The demonstration of local effects of 17βE_2 on bone *in vivo* thus offers considerable support to the concept that estrogen acts directly on bone cells. However, further studies are needed to confirm this assumption, since 17βE_2 could act locally on other cells—for example, in the bone marrow or on blood vessels—and affect bone indirectly.

The localized action of 17βE_2 was evident from the lack of changes in the untreated contralateral femora and especially in the proximal region of 17βE_2-infused femora. As expected, no estrogenic effects on body and uterine weights could be detected in these experiments, whereas systemic administration of estradiol to OVX rats clearly increases uterine weight (49) and decreases body weight (22).

The specificity of estrogen was indicated by the action of the biologically inactive or weakly active stereoisomer 17αE_2, which had no effect on TBV, on osteoclast number, or on the relative resorption surface. 17αE_2 had a small effect on osteoblast number and relative osteoid surface at 1 nM; its estrogenic potency estimated from the dose–response curves was about 1/50th to 1/100th that of 17βE_2. In previous studies 17αE_2 was reported to have no effect in UMR106 cells (11) and the human Saos-2 cells (13) but showed biological activity in calvarial bone and uterine tissue *in vivo* (49) as well as in the human monocytic cell line U937 (50) and in human breast cancer cells in culture (51).

In conclusion, the findings presented here show that 17βE_2 delivered directly to trabecular bone of OVX rats *in vivo* at 2.4–24 fmol/day inhibited bone resorption, stimulated bone formation, and restored the bone loss produced by ovariectomy. It remains to be shown whether the effects of estrogen on the secondary spongiosa of growing rats apply to other bones, to older animals, and to postmenopausal bone changes in women. Finally, the experimental system described here could be useful for further investigation of physiological and pharmacological effects of estrogen and other agents on bone *in vivo*.

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1. Albright, F., Smith, P. H. & Richardson, A. M. (1941) *J. Am. Med. Assoc.* **116**, 2465–2474.
2. Nordin, B. E. C., Peacock, M., Crilly, R. G., Francis, R. M., Speed, R. & Barkworth, S. (1981) in *Osteoporosis: Recent Advances in Pathogenesis and Treatment*, eds. DeLuca, H. F., Frost, H. M., Jee, W. S. S., Johnston, C. C., Jr., & Parfitt, A. M. (University Park Press, Baltimore), pp. 359–367.
3. Raisz, L. G. (1988) *N. Engl. J. Med.* **318**, 818–828.
4. Lindsay, R., Aitken, J. M., Anderson, J. B., Hart, D. M., MacDonald, E. B. & Clarke, A. C. (1976) *Lancet* **i**, 1038–1040.
5. Canalis, E. & Raisz, L. G. (1978) *Calcif. Tissue Res.* **25**, 105–110.
6. Stewart, P. J. & Stern, P. H. (1987) *Calcif. Tissue Int.* **40**, 21–26.
7. Caputo, C. B., Meadows, D. & Raisz, L. G. (1976) *Endocrinology* **98**, 1065–1068.
8. Langeland, N. (1977) *Acta Orthop. Scand.* **48**, 266–272.
9. Nutik, G. & Cruess, R. L. (1974) *Proc. Soc. Exp. Biol. Med.* **146**, 265–268.
10. Chen, T. L. & Feldman, D. (1978) *Endocrinology* **102**, 236–244.
11. Gray, T. K., Flynn, T. C., Gray, K. M. & Nabell, L. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6267–6271.
12. Ernst, M, Schmid, C. & Froesch, E. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2307–2310.
13. Fukayama, S. & Tashjian, A. H., Jr. (1989) *Endocrinology* **124**, 397–401.
14. Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C. & Riggs, B. L. (1988) *Science* **241**, 84–86.
15. Komm, B. S., Terpening, C. M., Benz, D. J., Graeme, K. A., Gallegos, A., Korc, M., Greene, G. L., O'Malley, B. W. & Haussler, M. R. (1988) *Science* **241**, 81–84.
16. Ernst, M., Heath, J. K. & Rodan, G. A. (1989) *Endocrinology* **125**, 825–833.
17. Gray, T. K., Mohan, S., Linkhart, T. A., Williams, M. E. & Baylink, D. J. (1989) *Biochem. Biophys. Res. Commun.* **158**, 407–412.
18. Canalis, E., McCarthy, T. & Centrella, M. (1988) *J. Clin. Invest.* **81**, 277–281.
19. Canalis, E., McCarthy, T. & Centrella, M. (1988) *Endocrinology* **122**, 22–27.
20. Centrella, M. & Canalis, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7335–7339.
21. Centrella, M. & Canalis, E. (1987) *J. Bone Miner. Res.* **2**, 29–36.
22. Wronski, T. J., Cintron, M., Doherty, A. L. & Dann, L. M. (1988) *Endocrinology* **123**, 681–686.
23. Aitken, J. M., Armstrong, E. & Anderson, J. B. (1972) *J. Endocrinol.* **55**, 79–87.
24. Turner, R. T., Vandersteenhoven, J. J. & Bell, N. H. (1987) *J. Bone Miner. Res.* **2**, 115–122.
25. Saville, P. D. (1969) *J. Am. Geriatr. Soc.* **17**, 155–166.
26. Wronski, T. J., Walsh, C. C. & Ignaszewski, L.-A. (1986) *Bone* **7**, 119–123.
27. Wronski, T. J., Cintron, M. & Dann, L. M. (1988) *Calcif. Tissue Int.* **43**, 179–183.
28. Turner, R. T., Wakley, G. K., Hannon, K. S. & Bell, N. H. (1988) *Endocrinology* **122**, 1146–1150.
29. Seaman, W. E., Gindhart, T. D., Greenspan, J. S., Blackman, M. A. & Talal, N. (1979) *J. Immunol.* **122**, 2541–2547.
30. Stock, J. L., Coderre, J. A. & Mallette, L. E. (1985) *J. Clin. Endocrinol. Metab.* **61**, 595–600.
31. Gallagher, J. C., Riggs, B. L. & DeLuca, H. F. (1980) *J. Clin. Endocrinol. Metab.* **51**, 1359–1364.
32. Pansini, F., Bergamini, C. M., Bellinazzi, A., Andreoli, M., Perri, G., Bagni, B. & Mollica, G. (1988) *J. Endocrinol.* **116**, 155–159.
33. Williams, G. A., Kukreja, S. C., Bowser, E. N., Hargis, G. K., Greenberg, C. P. & Henderson, W. J. (1986) *Bone Miner.* **1**, 415–420.
34. Imanaka, S., Morimoto, S., Onishi, T., Takamoto, S., Fukuo, K., Koh, E., Itoh, K., Hironaka, T., Shiraishi, T., Morita, R., Taniguchi, K. & Kumahara, Y. (1988) *Endocrinol. Jpn.* **35**, 593–599.
35. Riggs, B. L., Jowsey, J., Goldsmith, R. S., Kelly, P. J., Hoffman, D. L. & Arnaud, C. D. (1972) *J. Clin. Invest.* **51**, 1659–1663.
36. Gallagher, J. C. & Wilkinson, R. (1973) *Clin. Sci. Mol. Med.* **45**, 785–802.
37. Gordan, G. S. & Eisenberg, E. (1963) *Proc. R. Soc. Med.* **56**, 1027–1029.
38. Horsman, A., Gallagher, J. C., Simpson, M. & Nordin, B. E. C. (1977) *Br. Med. J.* **2**, 789–792.
39. Kusuhara, S. & Schraer, H. (1982) *Calcif. Tissue Int.* **34**, 352–358.
40. Miller, S. C. & Bowman, B. M. (1981) *Div. Biol.* **87**, 52–63.
41. Urist, M. R., Budy, A. M. & McLean, F. C. (1950) *J. Bone Jt. Surg. Am. Vol.* **32**, 143–162.
42. Soto, A. M. & Sonnenschein, C. (1987) *Endocr. Rev.* **8**, 44–52.
43. Atkins, D., Zanelli, J. M., Peacock, M. & Nordin, B. E. C. (1972) *J. Endocrinol.* **54**, 107–117.
44. Cruess, R. L. & Hong, K. C. (1976) *Calcif. Tissue Res.* **20**, 317–320.
45. Liskova, M. (1976) *Calcif. Tissue Res.* **22**, 207–218.
46. Ismail, F., Epstein, S., Fallon, M. D., Thomas, S. B. & Reinhardt, T. A. (1988) *Endocrinology* **122**, 624–630.
47. Butcher, R. L., Collins, W. E. & Fugo, N. W. (1974) *Endocrinology* **94**, 1704–1708.
48. Shull, J. D. & Gorski, J. (1989) *Endocrinology* **124**, 279–285.
49. Feyen, J. H. M. & Raisz, L. G. (1987) *Endocrinology* **121**, 819–821.
50. Sivam, G., Cohen, M. S., Dodd, C. & Gray, T. K. (1987) *Endocrinology* **121**, 853–857.
51. Edwards, D. P. & McGuire, W. L. (1980) *Endocrinology* **107**, 884–891.