Dehydroepiandrosterone improves murine osteoblast growth and bone tissue morphometry via mitogen-activated protein kinase signaling pathway independent of either androgen receptor or estrogen receptor

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Abstract

Dehydroepiandrosterone (DHEA) may be a promising agent for postmenopausal osteoporosis (PMO), but its mechanism to modulate osteoblasts (OBs) is yet to be explained. To elucidate the effects of DHEA treatment on the ovariectomized (OVX) mice and its mechanisms, we evaluated the morphology of mice bone tissue and expression of proliferating cell nuclear antigen (PCNA) in the vertebrae-derived OB after having treated the OVX animals with DHEA. The results showed that DHEA administration increased the expression of PCNA in OB and changed the bone tissue morphometry of the PMO model. To further investigate this mechanism, the OB was isolated from neonatal mice calvariae by the enzyme-digested assay, exposed to DHEA, and then analyzed for ultrastructure, DNA content, early apoptotic cells, and phosphorylation of extracellular signal-regulated kinase 1/2. It was found that DHEA promoted proliferation and inhibited apoptosis of OB significantly, via mitogen-activated protein kinase signaling pathway independent of either androgen receptor or estrogen receptor, suggesting that it may exert roles via a DHEA-specific receptor directly, not by way of conversion to androgens or estrogens.

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Introduction

Postmenopausal osteoporosis (PMO) is a metabolic bone disease characterized by a decrease in bone mass after menopause. Normal bone remodeling depends upon a balance between the osteoblast (OB)-mediated bone formation and the osteoclast-mediated bone resorption. After surgical or natural menopause, bone resorption exceeds bone formation, which leads to an imbalance in favor of bone resorption and a decrease in bone mass. Skeletal system homeostasis is greatly influenced by endocrine factors (McLachlan *et al.* 1996, Ben-Nathan *et al.* 1999, Rho *et al.* 2004). Up to now, approaches to managing PMO have been directed toward prevention of bone resorption. Estrogen replacement therapy appears to act mainly through inhibition of resorption and has been widely used in postmenopausal women for several years. However, concerning safety, an observational study questions the suitability of long-term estrogen therapy (Enserink 2002). The OB, regulated by the local microenvironment, is a bone-forming cell that regulates bone metabolism, and its dysfunction plays a central role

in the pathogenesis of PMO. A number of investigations have demonstrated that regulation of the OB lifespan is critical for the maintenance of the skeleton; so a promising prophylactic and therapeutic regimen to PMO should be administered that stimulates OB bone formation in PMO.

Dehydroepiandrosterone (DHEA) is among the most plentiful circulating adrenal hormones and is one of the most abundant adrenal sex steroid hormone precursors in adult women. It exerts biological activities, not by its metabolites (Yen *et al.* 1995, Araghi-Niknam *et al.* 1998, Jiang *et al.* 1998, Flynn *et al.* 1999, Zhang *et al.* 1999). It has been demonstrated that DHEA, as an additional molecule modulating bone mineral density (BMD) in postmenopausal women, emphasizes the need for more information concerning potential clinical benefit for the treatment of PMO (Casson & Buster 1995, Labrie *et al.* 1997, Lamberts *et al.* 1997). However, both the molecular and biochemical mechanisms by which DHEA exerts its roles in bone metabolism remain shrouded in mystery. The steroid receptor for DHEA has not been identified, and DHEA

is generally considered to exert its roles via conversion to androgens or estrogens (Haning *et al.* 1991*a,b*). It has been demonstrated that murine and human T cells have a specific receptor for DHEA (Meikle *et al.* 1992, Okabe *et al.* 1995).

The aim of present study is to elucidate the mechanisms underlying the DHEA-mediated OB lifespan modulation *in vivo* and *in vitro*, using 17 β -estradiol (E2) as control. We have examined the morphology of bone tissue *in vivo* and expression of proliferating cell nuclear antigen (PCNA) in the *ex vivo* vertebrae-derived OB after having treated the OVX animals with DHEA or E2. *In vitro* OBs from neonatal murine calvariae were exposed to DHEA or E2, examined for the ultra-structure, DNA content, early apoptosis, and phosphorylation status of extracellular signal-regulated kinase 1/2 (ERK1/2). We used ICI 182 780 (ICI) as an estrogen receptor (ER) antagonist, flutamide (FLUT) as an androgen receptor (AR) antagonist, and U0126 as inhibitor of mitogen activated protein kinase (MAPK) pathway to investigate the mechanisms underlying the effects of DHEA on OB.

Materials and methods

Mice

Inbred strains of *BALB/c* mice (10–12 weeks of age) were obtained from the Jackson Laboratory (Bar Harbour, ME, USA) and subsequently maintained in the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). The housing and handling of the experimental animals were performed in accordance with the guidelines of the Chinese Council for Animal Care.

BALB/c mice were habituated to the housing conditions for 2 days. Mice were housed five per cage on a reversed 12 h light:12 h darkness cycle. Food and water were available *ad libitum* at room temperature. Mice that were ovariectomized were used as PMO model; newborn *BALB/c* mice were used as OBs.

Reagents

Fetal bovine serum (FBS), minimum essential medium (MEM), and MEM without phenol red were purchased from GIBCO Co. 17 β -E2, DHEA, U0126, flutamide, alkaline phosphatase (ALP) staining kit, trypsin, and collagenase type 2 were purchased from Sigma-Aldrich Co. ICI 182 780 was purchased from Tocris Cookson (Bristol, UK) SDS was obtained from Serva Co. (Heidelberg, Germany) ACTIVE DHEA enzyme immunoassay (EIA) kit was purchased from Diagnostic System Laboratories (DSL, Webster, TX, USA). DHEA sulfate (DHEAS) EIA kit was supplied by IBL. E2 EIA kit was supplied by BioCheck Inc (Burlingame, CA, USA).

Fluorescein isothiocyanate (FITC) – conjugated anti-PCNA antibody (IgG2a) was purchased from eBioscience (San Diego, CA, USA). Cycle TEST & DNA Reagent Kit was obtained from BD Biosciences (San Jose, CA, USA) Annexin V-FITC was supplied by Bender Med Systems (Vienna, Austria). Monoclonal antibody against phospho-pERK1/2 MAPK (Thr202/Tyr204) and rabbit polyclonal antibody against pERK1/2 MAP kinase were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and the secondary antibody conjugated with horseradish peroxidase was from Rockland Co (Gilbertsville, PA, USA).

Experimental protocols *in vivo*

Four groups of mice were studied using the following protocols. The animals were anesthetized with ether. Ovariectomy was performed through bilateral incisions. Sham group underwent the surgical procedure but without ovariectomy, and 1 week after surgical procedure the mice were treated daily with saline containing 0.1% ethanol. One week after gonadectomy, the mice were divided into three groups ($n=12$). Ovariectomy (OVX) group was subjected to ovariectomy at the beginning of the experiment and treated with saline containing 0.1% ethanol daily. Estrogen replacement treatment (OVX+E2) group was subjected to ovariectomy at the beginning of the experiment and received E2 treatment (100 μ g/kg per day orally). DHEA treatment (OVX+DHEA) group was subjected to ovariectomy at the beginning of the experiment and received DHEA treatment (5 mg/kg per day orally). All four groups received equal quantities of fluid at the same time during treatment. All mice were intragastrically administrated daily for 90 consecutive days at 8 a.m.

All mice were killed after 3 months treatment; blood was collected from the hearts, and serum was stored at -20°C for the determination of DHEA, DHEAS, and E2, which was performed using the EIA kits (data not shown).

Bone histomorphometry

Femur and lumbar vertebrae were removed at the time of necropsy, cleaned of soft tissue and fixed in 4% paraformaldehyde in PBS for ~ 12 h at 4°C . The specimens were then decalcified in 0.5 M EDTA in PBS (pH 7.4) and dehydrated with increasing concentrations of ethanol before being embedded in paraffin. The specimens were sectioned at 6 μ m and hematoxylin–eosin stained using standard techniques. Bone histomorphometry was performed in a blinded manner, by a board-certified veterinary pathologist. A histomorphometric analysis of the trabecular bone was performed using a Nikon microscope and digitizers equipped with a computerized semi-automatic image

analysis system (Cosmozone II; Nikon, Tokyo, Japan), as described previously (Zhioua *et al.* 1994, Thomsen *et al.* 2000, Morony *et al.* 2001, Kostenuik *et al.* 2004, Elefteriou *et al.* 2005). Six to nine fields were measured per section. We obtained the trabecular bone volume/total volume (BV/TV[B.Ar/T.Ar]) by counting the number of pixels representing the bone and marrow phases respectively. The number of pixels representing the bone area was divided by the sum of the number of pixels representing the bone and marrow area. The bone area (B.Ar), marrow area (Ma.Ar), and tissue area (T.Ar) could then all be computed.

Flow cytometry (FCM) analysis of PCNA in OB

Murine OBs were isolated from vertebrae using a technique as described previously (Granchi *et al.* 2004). Briefly, lumbar vertebrae (LV1–LV4) were obtained at the time of necropsy; trabecular bone was dissected into tiny fragments, washed twice with PBS, and incubated in MEM supplemented with 20% FBS and antibiotics. The bone fragments were fed regularly and left until many cells had spread out from the fragments to semi-confluent layer; OBs were characterized by the measurement of biochemical and cytochemical ALP with nitroblue tetrazolium salt/5 bromo-4-chloro-3-indolylphosphate (NBT/BCIP). The cells were >95% homogeneous and displayed a mature (normal) osteoblastic phenotype.

When OBs reached confluence, they were detached using trypsin; the cells were then passaged and maintained until use. The primary OBs were seeded onto 24-well flat-bottomed culture plates at a density of approximately 1×10^5 cells/cm². Within 24 h, the supernatant was removed and the fresh medium containing 20% FBS was added. This cell analysis requires the establishment of culture conditions that permit the rapid expansion of these cells while retaining their potential for further differentiation. Several culture models have been proposed, using fetal calf serum (FCS) as a source of growth factors (Schecroun & Delloye 2004). Within 24 h of culture, the cells were harvested and resuspended in PBS at a density of 5×10^6 /ml and then distributed (100 µl per tube) to the Falcon 2054 polystyrene round-bottomed tubes (Becton Dickinson) for immunolabeling. The cells were fixed, permeabilized, and stained for PCNA using FITC-labeled antibodies. Thereafter, the cells were washed twice and resuspended in PBS for FCM analysis. The samples were analyzed in a FACS Calibur flow cytometer (Becton Dickinson, Palo Alto, CA, USA) using Cellquest software (Becton Dickinson). Statistical analysis was done using isotype-matched controls as a reference. The experiments were repeated five times.

Osteoblastic cell isolation by enzymatic digestion and culture

Murine osteoblastic cells were isolated from calvariae of neonatal *BALB/c* littermates by enzymatic digestion as described previously (Robey & Termine 1985, Inoue *et al.* 1999). In brief, aseptically removed frontal and parietal bones were digested with the enzyme mixture (0.1% collagenase type 2, 0.05% trypsin, and 0.5 mM EDTA). The osteoblastic cells released were collected and seeded onto a 60 mm dish in MEM supplemented with 10% FBS. Half million OBs at the second passage were cultured in 25 cm² culture flask or seeded onto a 24-well plate and cultured at 37 °C in 95% humidified air plus 5% CO₂. The osteoblastic phenotype derived from calvariae was confirmed by staining for the presence of ALP with NBT/BCIP. The cells were >95% homogeneous and displayed a mature osteoblastic phenotype. The cell density was similar in all samples. The OBs were grown in MEM without phenol red, and supplemented with the 5% charcoal–dextran-treated FCS (5% CD-FCS) for at least 4 days before the start of DHEA or E2 treatment.

The primary OBs were treated *in vitro* with DHEA or E2

The OBs were grown in 5% CD-FCS MEM medium without phenol red for at least 4 days until 70–80% confluence (2×10^5 cells/cm², 24-well plates). After deprivation of serum for a further 12–24 h, the OBs were pretreated with the solvent control, 1 µM ICI 182 780, 10 µM flutamide, or 100 µM U0126 for 1 h, and then treated with 10^{-5} – 10^{-10} M DHEA, 10^{-9} – 10^{-10} M E2, or the solvent control for 72 h in serum-free medium.

Transmission electron microscopy (TEM)

The primary OBs were cultured until approximately 80% confluent and then treated with 10^{-7} M DHEA or the solvent control for 72 h. After washing with PBS, the OBs were fixed in 2.5% glutaraldehyde postfixed in 1% osmium tetroxide. They were rinsed with water, dehydrated, and embedded in epoxy resin. Ultrathin sections (70–90 nm) were cut and stained with 2% uranyl acetate and Reynold's lead citrate for TEM observation with a JEM-1200 EX microscope (JOEL Co., Tokyo, Japan). Ten fields were randomly chosen at each section and the images were stored, and morphological quantitative analysis was performed using a computer-imaging analysis system. Estimations of ER area per unit volume of OB cytoplasm were performed according to the method described previously (Itani *et al.* 1992). The mitochondrial density was calculated as the ratio of the total area of all mitochondria to the entire area of the OB cytoplasm (Mei *et al.* 1999).

FCM for cell cycle of OBs

For the determination of the cell cycle progression, the cells were harvested and washed with PBS, and then incubated in 70% ethanol for at least 12 h at 4 °C. The fixed and permeabilized cells were collected by centrifugation, washed with PBS, stained with propidium iodide (10 µg/ml), and analyzed with FACS. The percentage of the cells in the G0/G1, S, G2/M phases, and proliferation index (PI, $PI = (S + G2/M) / (G0/G1 + S + G2/M) 100\%$) were evaluated.

Apoptosis analysis by FCM

After incubation for 72 h, the OBs were released from the culture dish using trypsin-EDTA, resuspended in the medium, and collected by centrifugation. The cells were washed with PBS, and the apoptotic cells stained with Annexin V-FITC/PI dual-labeling technique and analyzed by FCM. The quantity of Annexin V-FITC⁺/PI⁻ cells corresponded to early apoptosis, while that of Annexin V-FITC⁺/PI⁺ cells corresponded to late apoptosis or secondary necrosis. In every sample, 1×10^5 cells were counted. The experiment was repeated five times.

Western blot for phosphor-ERK1/2 vs total ERK1/2 in OB

Western blot was carried out to evaluate protein levels of phosphor-ERK1/2 and total ERK1/2 in the OBs. The OBs were lysed in 1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 µg/ml phenylmethanesulfonyl fluoride (PMSF), 1 µg/ml aprotinin, and 0.1% SDS for 30 min at 4 °C. Nuclei were removed by centrifugation at 12 000 g at 4 °C for 15 min, and cell lysates were assayed for protein contents using the Bradford protein assay. The proteins (50 µg) were resuspended in sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.1% bromophenol blue, 2.5% 2-mercaptoethanol, and 10% glycerol), separated on 10% SDS-polyacrylamide gel. The proteins were electrotransferred to a polyvinylidene difluoride membrane for 1.5 h. The membrane was blocked with 5% non-fat milk in TBS-T (150 mM NaCl, 20 mM Tris, pH 7.4, and 0.1% Tween 20) and then incubated with anti-phospho-ERK1/2 (1:1000) or anti-ERK1/2 (total; 1:1000) antibodies overnight at 4 °C, followed by incubation with secondary horseradish peroxidase-conjugated antibody (1:2000) for 1 h at 37 °C. The membrane was developed using dimethylaminoazobenzene according to the manufacturer's recommendations. Data were presented as a ratio of phosphor-ERK1/2 vs total ERK1/2 (Fitzpatrick *et al.* 2002).

Statistical analysis

All values were expressed as the mean $\pm$ S.E.M. Data were analyzed with the aid of SPSS database, and variance was

evaluated by one-way ANOVA. Differences were accepted as significant at $P < 0.05$.

Results

Effects *in vivo* of DHEA administration on bone morphology and proliferation of OB

DHEA reversed bone volume loss in OVX mouse model

The vertebrae and femur cancellous (BV/TV) of the OVX mice without DHEA treatment were significantly lower than that of the sham-operated mice ($P < 0.01$, Fig. 1). After 3 months of administration, OVX mice treated with DHEA or E2 appeared to have an increased trabecular bone volume of vertebrae and femur compared with that of OVX mice given vehicle ($P < 0.01$, Fig. 1), which suggests that DHEA treatment can change the bone tissue morphometry of the PMO model.

DHEA increased expression of PCNA in the vertebrae-derived OB

The flow cytometric analysis showed that the average fluorescence intensity (5.676 ± 0.562) and positive cell percentage (3.232 ± 0.852) of PCNA in the vertebrae-derived OB of OVX group increased significantly compared with that of the sham group (4.85 ± 0.401 , 2.264 ± 0.219 , $P < 0.05$). Compared with the OVX group, the average fluorescence intensity (6.97 ± 0.784) and positive cell percentage (5.712 ± 0.704) of PCNA in OB of the OVX group after treatment with DHEA increased significantly ($P < 0.05$, $P < 0.01$), suggesting that DHEA treatment could promote proliferation of OB. The average fluorescence intensity and positive cell percentage of PCNA in OB also tended to increase in OVX mice treated with E2, but the difference did not achieve statistical significance ($P > 0.05$, Fig. 2). Moreover, we examined the effect of DHEA administration on mineralized nodule formation of the primary cultured OB and found that this formation is remarkably improved (data not shown). Therefore, we propose that DHEA administration may promote the proliferation of OB.

Effects *in vitro* of DHEA on OB growth

TEM analysis of ultrastructure in OBs

Within 24 h of culture, the expression of ALP with NBT/BCIP was characterized in murine osteoblastic cells isolated from calvariae of neonatal BALB/c littermates by enzymatic digestion. The isolated cells were then stained for ALP. Generally, these cells had

Figure 1 Effects of DHEA on bone histomorphometry of vertebra and femur in OVX mouse. One week after operation, Sham group mice were treated daily with saline containing 0.1% ethanol. One week after gonadectomy, the mice were divided into 3 groups ($n=12$). OVX group were treated with saline containing 0.1% ethanol daily. Estrogen replacement treatment (OVX+E2) group received E2 treatment (100 $\mu\text{g/kg}$ per day orally). DHEA treatment group (OVX + DHEA) received DHEA treatment (5 mg/kg per day orally). OVX and sham mice were treated with either vehicle, DHEA (10^{-7} M), or E2 (10^{-7} M). These mice were necropsied in 3 months, and histomorphometry was performed on cancellous bone in the lumbar vertebrae (A) and proximal femur metaphysis (B). The cancellous bone volume was determined as a percentage of the total bone tissue (bone volume/total volume, BV/TV). Data are expressed as means $\pm$ S.E.M. ($n=5$ mice/group). * $P<0.01$.

Figure 2 Modulation of DHEA on PCNA expression in OBs. One week after operation, the OVX and sham mice were treated with either vehicle, DHEA (10^{-7} M; 5 mg/kg/day), or E2 (10^{-7} M; 100 $\mu\text{g/kg/day}$). Mice were necropsied 3 months after the treatment, and the OBs isolated from vertebrae were analyzed by flow cytometry for the average fluorescence intensity (A) and positive cell percentage (B) of PCNA. The results shown were from five separate experiments. Data are expressed as means $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$.

Figure 3 The ultrastructure of OBs after treatment with DHEA ($\times 10\,000$). Primary OBs were cultured until approximately 80% confluent, and then treated with solvent (A) or 10^{-7} M DHEA (B) for 72 h, and the ultrastructure of OBs was observed by TEM. The OBs induced by DHEA showed more organelles, expanded endoplasmic reticulum (ER), and abundant mitochondria (M) compared with the control. (Bars=500 nm).

diverse appearance, every cell appeared to have three to four synapses, which may connect each other, with cytoplasm staining positive for ALP, containing one or two nucleoli; no nuclear staining was observed. The purity of the isolated OBs was above 95%.

After the OBs were treated *in vitro* with 10^{-7} M DHEA or solvent for 72 h, the ultrastructure of OBs was evaluated by TEM. The results showed that the OBs had organelles of active cells (numerous rough endoplasmic reticulum and well-developed mitochondria; nuclei were large with one or two nucleoli; Fig. 3A); the OBs treated with DHEA were found to have more organelles, expanded endoplasmic reticulum, and abundant mitochondria compared with the control (Fig. 3B). Endoplasmic reticulum area per unit cytoplasmic volume ($3.128 \pm 0.852 \mu\text{m}^2/\mu\text{m}^3$) in OB treated with DHEA increased significantly compared with the control ($0.385 \pm 0.201 \mu\text{m}^2/\mu\text{m}^3$, $P < 0.01$). The augmentation of ER area in OB after DHEA treatment suggests an increase in the protein synthesis of OB. In addition, the mitochondrial density in OB increased significantly in the DHEA group ($0.785 \pm 0.351\%$) compared with the control ($0.328 \pm 0.102\%$, $P < 0.01$). The increase in mitochondrial density in OB after DHEA administration suggests improvement in the energy metabolism of OB.

Modulation of DHEA on cell cycle of the primary OB

To determine modulation of DHEA on cell cycle of OB, the DHEA-treated OBs were evaluated for the percentage of G₀/G₁, S, and G₂/M phases by FCM. It was shown in Fig. 4 that 10^{-7} and 10^{-8} mol/l DHEA increased the S-phase ($P < 0.01$) and G₂/M phase ($P < 0.01$), decreased the G₀/G₁ phase ($P < 0.01$) of OBs, and improved the PI significantly ($P < 0.01$), while E₂ had no effect on PI of OB compared with the control ($P > 0.05$). Moreover, there was no significant difference in the percentage of G₀/G₁, S, G₂/M phases, and PI between the DHEA-treated OB pretreated with and without ICI 182 780

(antagonist of estrogen receptor) and flutamide (antagonist of androgen receptor). The promoting effect of DHEA on cell cycle of the primary OB was neither blocked by the estrogen receptor antagonist, ICI 182 780, nor by the androgen receptor antagonist, flutamide, but could be blocked by the specific inhibitor of MAPK pathway, U0126.

Modulation of DHEA on the early apoptosis of the primary OB

In order to investigate the inhibiting effects *in vitro* of DHEA on the OB apoptosis, we analyzed the percentage of the early apoptotic cells by FCM with the Annexin V-FITC/PI dual-labeling technique and found that 10^{-6} – 10^{-9} M DHEA and 10^{-9} – 10^{-10} M E₂ were all capable of inhibiting the serum deprivation-induced early apoptosis of OB ($P < 0.05$, $P < 0.01$ respectively). Moreover, similar to the proliferation promoting effect, the inhibiting effect of DHEA was neither blocked by the estrogen receptor antagonist nor by the androgen receptor antagonist, but could be blocked by the specific inhibitor of MAPK pathway, U0126 (Fig. 5A). Differently, the inhibiting effect of E₂ on the OB apoptosis could be blocked by both ICI 182 780 and U0126, but not by flutamide (Fig. 5B).

DHEA improved OB growth via activation of MAPK signal pathway

To probe into the intracellular signal pathway mediating the OBs proliferation/apoptosis by DHEA, the activation of mitogen-activated protein kinases (MAPKs), which convert extracellular stimuli to intracellular signals that control gene expression, cell growth, differentiation, survival, proliferation, and apoptosis was investigated. The cell cycle and apoptosis analysis showed that U0126 completely inhibited the up-regulated proliferation and the down-regulated apoptosis in OBs induced by DHEA

Figure 4 Effect of DHEA on cell cycle of primary OB. The primary OB were grown in 5% CD-FCS MEM medium without phenol red for at least 4 days until 70–80% confluent. After deprivation of serum for a further 12 h, the OBs were pretreated with solvent, 1 μ M ICI 182 780, 10 μ M flutamide, or 100 μ M U0126. Then the OBs were incubated for 72 h in serum-free medium containing DHEA or E2 at the concentrations indicated. The cells were released by trypsin–EDTA, and the cell cycle of OBs was analyzed by FCM with the propidium iodide (10 μ g/ml) labeling technique. The percentage of the cells in the G0/G1, S, and G2/M phases were determined. The results are expressed as a percentage of total cells (mean $\pm$ S.E.M.; $n=5$). * $P<0.05$; ** $P<0.01$ compared with the control.

(Figs 4 and 5). Recent evidence indicates that DHEA administration results in rapid phosphorylation of ERK in endothelial cells (Simoncini *et al.* 2003). Therefore, we wondered what the action of DHEA or E2 was on the modulation of ERK activation in OB. We analyzed ERK1/2 activation in OBs treated with DHEA or E2 by western blot. As shown in Fig. 6A, a significant increase in the rapid phosphorylation of ERKs was observed in OBs after 10^{-7} – 10^{-8} M DHEA treatment, showing that DHEA activates the Raf/MEK1/2/ERK1/2 kinase module in OB. To assess further the involvement of the ERK signaling pathway of DHEA in OB, pharmacological agents U0126 (which inhibits ERK), ICI 182 780 (ER antagonists), and flutamide (AR antagonists) were used. Phosphorylation of ERK was blocked efficiently by the specific inhibitor of MAPK pathway, U0126; neither ICI 182 780 nor flutamide could block this effect of DHEA (Fig. 6B). Moreover, although 10^{-9} – 10^{-10} M E2 were

capable of inducing ERK phosphorylation in OBs, the effects of E2 on the ERK phosphorylation of the OBs was not blocked by flutamide, but was blocked by both ICI 182 780 and U0126 (Fig. 6C).

Discussion

Skeletal system homeostasis is greatly influenced by endocrine systems (McLachlan *et al.* 1996, Ben-Nathan *et al.* 1999, Rho *et al.* 2004). PMO has been known to be associated with a variety of endocrine alterations. Recent research presented an association between these pathogenic changes and disturbances of the endocrine system. DHEA is among the most plentiful circulating adrenal hormones. For many years, scientists assumed that DHEA merely functioned as an inert precursor of the sex steroids, E2 and testosterone. However, following the

Figure 5 Effect of DHEA on the early apoptosis of primary OBs. The calvariae OBs were allowed to adhere and grow for 24 h in 5% CD-FCS MEM medium without phenol red, and then deprived of serum for a further 24 h (serum-free medium). The OBs were pretreated with solvent, 1 μ M ICI 182 780, 10 μ M flutamide, or 100 μ M U0126, and then treated with solvent, DHEA, or E2 at the concentrations indicated in the serum-free medium. After incubation for 72 h, the cells were released by trypsin-EDTA, and the apoptotic cells were stained with Annexin V-FITC/PI dual-labeling technique and analyzed by FCM. The results are expressed as a percentage of total cells (mean $\pm$ S.E.M.; $n=5$). * $P<0.05$; ** $P<0.01$ compared with the control; $\square$ $P<0.05$; $\square$ $P<0.01$ compared with the other groups.

work of Kalimi and Regelson, the identification of DHEA receptors in the liver, kidney and testes of rats suggests that DHEA has specific physiological actions via its own receptor (Kalimi & Regelson 1988). Since then, a series of studies has shown that DHEA indeed has a variety of physiological effects, for example, normalizing blood sugar (Bates *et al.* 1995) and anticarcinogenic (Nyce *et al.* 1984, Schwartz *et al.* 1988). Moreover, it has beneficial effects on atherosclerosis (Gordon *et al.* 1988), dementia

(Flood & Roberts 1988), and neoplasia (Schwartz & Pashko 1993). Although DHEA has been found to present many different effects on various types of cells, tissues, and organs, its concentration in serum declines in a striking age-related fashion in humans (Hakkinen & Pakarinen 1993, Short & Nair 1999, Kostka *et al.* 2000, Miller 2001), which may be strongly associated with multiple age-related clinical disease including PMO.

Figure 6 Effect of DHEA on activation of MAPK signal pathway in OBs. The primary OBs were starved in MEM (serum-free medium) for 24 h, pretreated with solvent, 1 μ M ICI 182 780, 10 μ M flutamide, or 100 μ M U0126 for 25 min, and then treated with DHEA (10^{-7} – 10^{-8} M) (A, B) or E2 (10^{-9} – 10^{-10} M) (A, C) for 10 min. Western blot analysis was performed to analyze ERK1/2 phosphorylation. Data were presented as a ratio of phosphor-ERK1/2 to total ERK1/2 (mean $\pm$ S.E.M.; $n=5$). * $P<0.01$ compared with the control.

Thus, some scholars have proposed treating PMO with DHEA (Lamberts *et al.* 1997). Some experimental and clinical evidence suggests that DHEA plays an important role in the regulation of bone metabolism, for example, Nozaki *et al.* (2004) reported that the adrenal sex steroids and their derivatives, such as DHEA, might affect bone resorption to some extent in oophorectomized women. DHEA as an additional factor modulating BMD in postmenopausal women (Labrie *et al.* 1997) emphasizes the need for more information concerning the potential clinical benefit of DHEA for the treatment of PMO (Casson & Buster 1995, Lamberts *et al.* 1997). However, both the molecular and biochemical mechanisms by which DHEA exerts its effect on bone metabolism remain shrouded in mystery. Our finding indicates that DHEA has multiple effects on bone metabolism, especially OBs.

DHEA improves the bone tissue morphometry

After surgical or natural menopause, estrogen deficiency facilitates bone resorption. The bone resorption augmentation promotes bone formation by feedback. Therefore, after estrogen exhaustion, bone turnover increased, but bone resorption exceeds bone formation, which leads to a decrease in bone mass. The bone tissue morphometry analysis of the ovariectomized mice showed that the cancellous (BV/TV) from the OVX mice was significantly lower than that of the sham-operated mice. The OVX mice treated with DHEA had significantly increased BV/TV compared with that of the OVX mice given by solvent, which suggests that DHEA can improve the bone tissue morphometry of the PMO model. Furthermore, we isolated OB from calvariae of neonatal *BALB/c* littermates by enzymatic digestion and then treated OB with DHEA; the ultrastructure of the OBs was evaluated by TEM. The results indicate an augmentation of the ER area and increase in mitochondrial density in OB after DHEA administration; these changes are consistent with the cells involved in anabolic metabolism. Therefore, we propose that DHEA increased the anabolic metabolism-related organelle content in OBs significantly, thus improving the bone milieu. The changes in bone tissue morphometry by DHEA may improve mechanical strength in bone, but this is still untested, which has considerable clinical significance in the prevention and treatment of PMO.

DHEA promotes proliferation and inhibits apoptosis of OB through MAPK signaling pathway independent of either ARs or ERs

The number of OBs in bone tissue is dependent not only on their proliferation but also on their apoptosis.

The balance between proliferation and apoptosis affects the pool of OBs available to maintain bone homeostasis. The recent observations have demonstrated that regulation of OB lifespan is critical for the maintenance of the skeleton. The decrease of apoptosis and increase of proliferation in OBs is significantly related to the number of OBs, bone formation rate, and BMD (Manolagas 1999).

In various murine and rat models, the effects of some agents on OB proliferation have been described. These studies have, however, been mainly performed in OVX animals that were supplemented with E2 and are limited to a few reports. In sexually immature rats, OVX caused an increase in cell number and PCNA protein expression compared with sham group; in contrast to OVX, E2 supplementation caused a reduced number of PCNA-positive cells (not significant; Tajima *et al.* 1998, van der Eerden *et al.* 2002). Likewise, our results in the present study indicate that PCNA expression in OBs from the OVX mice increases significantly.

Estrogen is believed to play an important role in the regulation of bone remodeling. It is reported that administration of estrogen into OVX rats not only inhibited bone resorption but also stimulated bone formation (Chow *et al.* 1992). In contrast, it is shown that administration of estrogen into OVX rats inhibits the accelerated bone formation induced by OVX (Yokose *et al.* 1996). Similarly, we found no difference in the expression of PCNA in OBs between OVX mice and OVX mice treated with E2. This suggests that the effects of E2 on bone metabolism cannot be explained by a direct effect of E2, but it may be a secondary effect on OB proliferation. We also found that PCNA expression of OB after DHEA administration increased significantly compared with the OVX group. The findings show that DHEA can promote OB proliferation by a direct effect.

The DNA content analysis showed that DHEA increased the S-phase and G₂/M phase, decreased the G₀/G₁ phase of OB cell cycle, and increased PI significantly, while E2 had no effect on PI of OBs compared with the control. The results of apoptosis analysis showed that DHEA could inhibit the serum deprivation-induced early apoptosis of the primary murine OBs in a dose-dependent manner. Normal circulating levels of free E2 in mice have been estimated to range between 40 and 80 pg/ml (Edwards *et al.* 1992, Nelson *et al.* 1992, Walmer *et al.* 1992, Bi *et al.* 2001, Fata *et al.* 2001). Normal plasma concentration of DHEA is 0.001–0.03 µmol/l. Our investigation confirmed that 10^{−9}–10^{−6} mol/l DHEA were capable of decreasing the apoptosis cell population significantly, and the optimal concentration is 10^{−8}–10^{−7} mol/l, which is higher than the normal plasma concentration. It means high plasma level of DHEA may be beneficial to PMO.

Although 10^{-9} – 10^{-10} mol/l E2 also could inhibit the serum deprivation-induced early apoptosis of the primary murine OBs, the anti-apoptosis effect of 10^{-7} – 10^{-8} mol/l DHEA is stronger than that of 10^{-9} – 10^{-10} mol/l E2 in OBs.

The results above indicate that DHEA can effectively improve cell cycle, promote proliferation, and inhibit apoptosis of OBs, thus increasing the amount of OBs, which is for potent evidence for clinical application of DHEA in the prevention and treatment of PMO. Our results also showed that E2 could inhibit OB apoptosis too, but E2 treatment could not improve OB cell cycle and has no effect on PI of OB. Therefore, our investigation excluded the possibility of DHEA conversion to its downstream metabolic product (E2) and indicated a direct effect on OBs.

From invertebrates to humans, the ERK MAP kinase signaling plays a central role in cell proliferation and apoptosis (Sudoh *et al.* 2001, Chang *et al.* 2003). The gonadal hormone through classic ER or AR activates ERK to affect proliferation and apoptosis (Chambliss *et al.* 2002, Manolagas *et al.* 2002). To determine whether the effects of DHEA on proliferation, apoptosis, and ERK phosphorylation of OBs is via ER α , ER β , or AR, we used ICI 182 780 (ICI) as an ER antagonist, FLUT as an AR antagonist, and U0126 as an inhibitor of MAPK activity and examined whether these agents could block the above-mentioned effects of DHEA. It was shown that 10^{-7} – 10^{-8} M DHEA and 10^{-9} – 10^{-10} M E2 could induce ERK phosphorylation in OBs; ICI 182 780 or flutamide could not block efficiently the above-mentioned effects of DHEA, which suggests that modulation of OBs on DHEA is an ER- and AR-independent mechanism, while U0126 could block the above-mentioned effects of DHEA, which means that the effects of DHEA on OBs involve on MAPK signal pathway.

Specific intracellular DHEA-binding sites have been characterized in mouse and human T lymphocytes (Meikle *et al.* 1992, Okabe *et al.* 1995), mouse B16 melanoma cells (Kawai *et al.* 1995), human vascular smooth muscle (Williams *et al.* 2002), and rat liver (Kalimi & Regelson 1988). However, to date, no known cellular DHEA receptor has been isolated or characterized. There is increasing evidence of rapid, plasma membrane-dependent actions of other steroid hormones such as E2 (Caulin-Glaser *et al.* 1997, Lantin-Hermoso *et al.* 1997, Shaul 1999), glucocorticoid (Qiu *et al.* 1998), testosterone (Benten *et al.* 1997, 1999), growth hormone (GH; Gu *et al.* 2001), and insulin-like growth factor-I (Cheng *et al.* 2001, Pouyssegur & Lenormand 2003) in various cell types. These effects of steroids are mediated by specific plasma membrane receptors; ligand binding to these receptors activates intracellular signaling which modulates specific cellular processes. Liu & Dillon (2002) have reported that the

DHEA receptors may be expressed on endothelial cell plasma membranes and are coupled to eNOS activity through G α i2 and G α i3. These novel findings should allow us to isolate the putative receptor and reevaluate the physiological role of DHEA. It is important to determine whether the cell surface-initiated actions of DHEA exist in OBs. The direct effects of DHEA on OBs raise the possibility of developing non-metabolized DHEA receptor ligands to evaluate their effects on OBs. Our results support the presumption about plasma membrane-initiated, non-genomic activity of DHEA. This property has been demonstrated for many other steroids.

Rodents normally have much less DHEA than humans. In fact, humans, along with the other primates, are unique among animal species in having adrenals that secrete large amounts of DHEA and especially DHEA-S. Moreover, plasma DHEA-S levels in adult men and women are 100–500 times higher than those of testosterone and 1000–10 000 times higher than those of E2. Therefore, the situation may be very different between human and mice.

In conclusion, there is a bone remodeling imbalance in the ovariectomized mice. DHEA treatment significantly improved the bone tissue morphometry and increased the anabolic metabolism-related organelles contents in OBs; DHEA promotes proliferation and inhibits apoptosis of OBs by a mechanism independent of either AR or ER, suggesting that it may exert its effects via a DHEA-specific receptor directly, and not by way of conversion to androgens or estrogens, which involve phospho-pERK1/2. DHEA might be useful as a novel strategy for the treatment of the metabolism disease, especially PMO.

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