

Estradiol and resveratrol stimulating effect on osteocalcin, but not osteonectin and collagen-1 α gene expression in primary culture of rat calvarial osteoblast-like cells

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Received April 7, 2006; Accepted June 3, 2006

Abstract. Evidence is available that some endocrine disruptors, acting as selective estrogen receptor modulators (SERMs), interfere with osteoblast differentiation and function. Therefore, we investigated whether 17 β -estradiol, bisphenol-A (BSP), silymarin, genistein, resveratrol, procymidone, linurone and benzophenone-3 (BP3) modulate differentiation of rat calvarial osteoblast-like (ROB) cells in primary *in vitro* culture. Disruptors were added at day 18 of culture and cells were harvested 48 h later. Real time-PCR revealed that estradiol and resveratrol enhanced osteocalcin mRNA expression in ROB cells, while other disruptors were ineffective. The expression of osteonectin and collagen-1 α was not affected by any disruptor. Estradiol, resveratrol, genistein and BSP stimulated the proliferative activity of ROB cells. In contrast, procymidone and linurone inhibited the proliferative activity, and silymarin and BP3 were ineffective. The conclusion is drawn that i) only resveratrol is able, like estradiol, to stimulate the specialized functions of ROB cells, and ii) the proliferative activity of ROB cells is more sensitive to endocrine disruptors, some of which could probably act via a mechanism independent of their SERM activity.

Introduction

Endocrine disruptors are both naturally occurring and synthetic chemical substances that exert adverse effects on an organism or its progeny acting through the endocrine system (1). The best recognized endocrine disrupting properties of chemicals

are those connected with their estrogenic activities, and several disruptors act as selective estrogen receptor (ER) modulators (SERMs) (1-3).

The presence of ERs in osteoblasts is well documented. Estrogens are known to affect differentiation and function of osteoblasts, including their proliferative activity (4-8). However, the results of studies dealing with the effects of SERMs on osteoblasts are rather conflicting, probably because estrogens act on osteoblasts not only directly, but also indirectly via their interaction with other cell types (6,9,10).

One of the most useful *in vitro* models for investigating the specific gene events associated with osteoblast proliferation, differentiation and mineralization of extracellular matrix is the primary culture of rat calvarial osteoblast-like (ROB) cells (11-18). ROB cells in primary culture spontaneously differentiate into osteoblasts, and this process is associated with the increased expression of both collagen and non-collagenous bone proteins, including osteocalcin, osteopontin and osteonectin (11,19,20).

The aim of the present study was to examine the direct effects of estradiol and several SERMs on the level of expression of collagen-1 α , osteonectin and osteocalcin genes and the proliferative activity of ROB cells in primary culture.

Materials and methods

Animals and reagents. Two-day-old Wistar rats, born in our laboratory facilities, were used, and the study protocol was approved by the local Ethics Committee for Animal Studies. Diphenylolpropane (bisphenol-A; BSP), benzophenone-3 (Eusolex-4360; BP3) and 17 β -estradiol were purchased from Merck & Co. (Whitehouse Station, NJ). Resveratrol was obtained from Nabio Biotech Co. (Shanghai, China). Other endocrine disruptors (genistein, procymidone, linurone and silymarin), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and all other reagents were provided by Sigma-Aldrich Corp. (St. Louis, MO).

Primary ROB cell culture. The technique used was described by Boden *et al* (13) with few modifications. Briefly, calvarias of 8 rats were immediately placed in DMEM, and the

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Key words: rat calvarial osteoblast-like cells, 17 β -estradiol, resveratrol, endocrine disruptors, osteocalcin, osteonectin, collagen-1 α , proliferative activity

Table I. PCR primers and PCR products.

Primer	Sequence (5'-3')	Product size (bp)	Accession number
Osteocalcin			
Sense (328-347)	CAAAGCCAGCGACTCTG	231	NM013414
Antisense (117-134)	AAACGGTGGTGCCATAGATG		
Osteonectin			
Sense (646-666)	CTGCCACTTCTTTGCGACCA	256	NM012656
Antisense (411-430)	CTCCAGGCGCTTCTCGTTCTC		
Collagen-1α			
Sense (3271-3292)	CAAGCTGACAGAGGCATAAAGG	174	Z78279
Antisense (3425-3444)	AGGGAGACCGTTGAGTCCAT		
GAPDH			
Sense (18-37)	TTCTAGAGACAGCCGCATCT	106	X02231
Antisense (104-123)	TGGTAACCAGGTGTCCGATA		

connective tissue was removed. Calvarias were cut into small fragments that were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I for 30 min at 37°C. ROB cells were harvested by centrifugation and resuspended in DMEM supplemented with NaHCO₃, 6% FCS and antibiotic-antimycotic solution. Cells were then plated in culture dishes (10⁴ cells/dish), and cultured for 20 days at 37°C in a humidified atmosphere of 95% air-5%CO₂, medium being changed every 24 h.

Incubation with SERMs. Two days before exposure to SERMs, ROB cells were cultured in DMEM deprived of FCS. SERMs were dissolved in ethanol or DMEM and added to the cultures (at concentrations from 10⁻¹² to 10⁻⁶ M) at day 18, and cells were harvested 48 h later. Final concentrations of solvent were as follows: BSP, 0.001% ethanol; resveratrol, 0.01% ethanol; silymarin and BP3, 0.1% ethanol; and estradiol and other SERMs, 0.01% DMSO. The same concentrations of solvent were added to the appropriate control cultures.

Real time-polymerase chain reaction (PCR). Total RNA was extracted from ROB cells, as previously detailed (21,22), and contaminating DNA was eliminated by DNase-I treatment (RNase-free DNase set; Promega, Madison, WI). The amount of total RNA was determined (23), and reverse transcription was performed using AMV reverse transcriptase (Promega) with oligo dT (PE Biosystems, Warrington, UK) as primers. Real-time PCR was carried out in a Roche Light-Cycler 2.0 with software version 4.0 (24,25), using the primers shown in Table I. Briefly, reactions were performed in 20 μ l final volume solution, containing 4 μ l template cDNA, 0.5 μ M specific primers, 3.5 μ M MgCl₂ and 12.5 μ l Light-Cycler Fast Start DNA Master SYBR-Green-I mix (Roche Molecular Biochemicals, Mannheim, Germany). The following PCR program was used: denaturation step (95°C for 10 min), and 45 cycles of three-steps of amplification (denaturation, 95°C for 10 sec; annealing, 58°C for 5 sec; and extension, 72°C for 10 sec). Subsequently, a melting curve (60-90°C with a heating

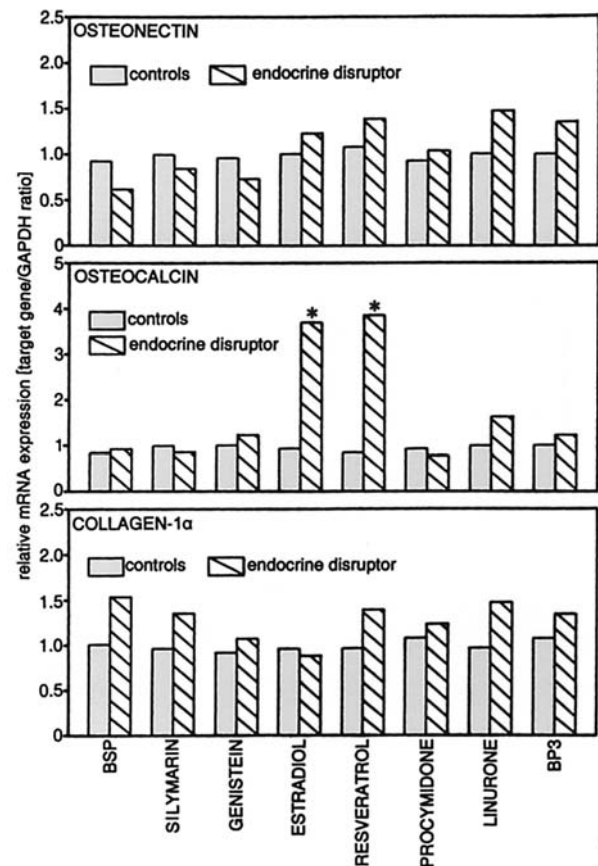


Figure 1. Effects of endocrine disruptors on osteonectin, osteocalcin and collagen-1 α gene expression in ROB cells in primary *in vitro* culture. Bars are means of three independent experiments. *P<0.05 from controls.

rate of 0.1°C/sec) was carried out to check the specificity of amplification and the presence of byproducts. All samples were amplified in duplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference to normalize data.

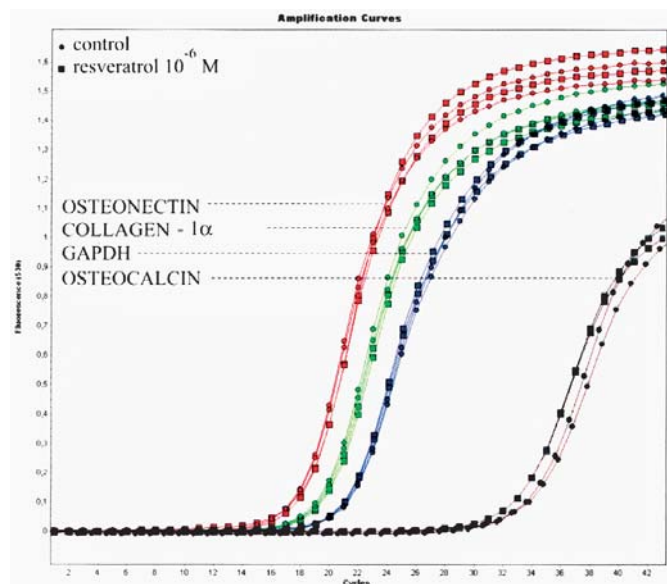


Figure 2. Exemplary results of real-time PCR amplification curves of osteonectin, osteocalcin, collagen-1 α and GAPDH from control and resveratrol-treated ROB cells in primary *in vitro* culture.

Cell proliferation. The proliferation rate of ROB cells was measured by the EZ4U nonradioactive cell proliferation and cytotoxic assay (Biomedica, Wien, Austria) (26,27). In this assay, cultured cells were incubated for 90 min with EZ4U, and formazan production, which is linearly related to the cell number, was measured at 490 nm wavelength in a microplate autoreader (EL-13; Bio-Tek Instruments, Winooski, VT).

Statistics. Data were expressed as means \pm SEM of 3 or 5 independent experiments, and their statistical comparison was made by the unpaired Student's t-test.

Results

Real-time PCR showed that neither estradiol nor other SERMs altered collagen-1 α and osteonectin gene expression in ROB cells. In contrast, estradiol and resveratrol increased osteocalcin mRNA, while other disruptors were ineffective (Fig. 1). Exemplary amplification curves of appropriate cDNA obtained from control and resveratrol exposed ROB cells are shown in Fig. 2.

EZ4U assay showed that BSP (10^{-12} and 10^{-6} M), genistein (10^{-8} and 10^{-6} M), and estradiol and resveratrol (10^{-10} M) enhanced the proliferative activity of ROB cells. Silymarin and BP3 were ineffective, while procymidone (10^{-6} M) and linurone (from 10^{-10} to 10^{-6} M) significantly decreased the proliferative activity of cultured ROB cells (Fig. 3).

Discussion

The multifactorial regulation of bone formation and resorption is very sensitive to estrogens and endocrine disruptors but, as pointed out in the Introduction, rather controversial findings have been reported. It is well established that the primary action of estrogens on bone metabolism is related to their indirect effects on osteoclasts: the anti-resorptive action of

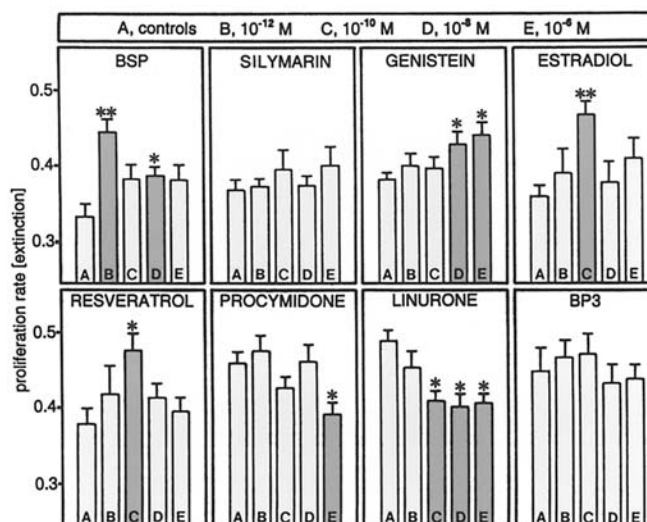


Figure 3. Effects of endocrine disruptors on the proliferative activity of ROB cells in primary *in vitro* culture. Bars are means \pm SEM of five independent experiments. *P<0.05 and **P<0.01 from controls.

estrogens is thought to be mediated by their modulating effect on the release from osteoblastic lineage of paracrine factors that act on the osteoclasts (6,28-30). Osteoblasts are known to express two functionally active ER isoforms, ER α and ER β . In primary cultures of ROB cells, the expression of ER β is rather constant throughout osteoblast differentiation, while the ER α level increases during matrix maturation and then declines during mineralization (31). Our present study shows a clearcut stimulating effect of estradiol on osteocalcin, but not osteonectin and collagen-1 α expression in ROB cells, thereby confirming earlier observations (6). However, using the same experimental model, Chen *et al* (32,33) were unable to evidence any effect of estradiol on osteocalcin gene expression. These discrepancies may be explained by taking into account the temporal sequence of expression of bone proteins in ROB cells: type I collagen and alkaline phosphatase are expressed early during the commitment to the osteoblastic phenotype, whereas osteopontin and osteocalcin are expressed later during osteoblastic differentiation (34-36). Hence, it may be reasonable to conceive that different results can be obtained depending on the stage of ROB cell culture. Estradiol (10^{-10} M) was also presently found to enhance the proliferative activity of ROB cells, an effect already observed in different models of osteoblastic cell culture (4-6,28,29,37).

Among the various endocrine disruptors examined, resveratrol is the most frequently studied. Resveratrol, a phytoalexin found in red wine, belongs to the family of phytoestrogens, which are structurally similar to the synthetic estrogen diethylstilbestrol and exhibit selective SERM activity (38-41). As far as bone is concerned, resveratrol was found to prevent dioxin-induced inhibition of collagen type I, osteopontin, bone sialoprotein and alkaline phosphatase synthesis (42). Moreover, in human bone marrow-derived mesenchymal stem cells, resveratrol has been reported to concentration-dependently enhance osteocalcin and osteopontin gene expression (43). On these grounds, it is not surprising that resveratrol exerts estradiol-like effects on ROB cells in primary

culture: i.e. stimulation of osteocalcin gene expression and proliferative activity. Of interest, resveratrol has been shown to stimulate apoptosis in other cell systems, such as human breast cancer cells xenografted in nude mice (44).

The soy isoflavone genistein has structural characteristics similar to those of 17 β -estradiol (40,45,46), which enables it to exert estrogenic and antiestrogenic actions. It should be underlined that isoflavones can also exert biological effects independent of their phytoestrogenic activity (47,48), inasmuch as genistein acts as a potent inhibitor of tyrosine kinase (49,50), an enzyme which is known to play a key role in the activation of MAPK cascade (51,52). Available data suggest that diets rich in phytoestrogens have bone-sparing anabolic effects, although their mechanism(s) of action on the bone cells are largely unknown (53). In human cultured osteoblasts, genistein exerts a weak 17 β -estradiol-like effect via both ER α and ER β (7), and in the osteoblastic MC3T3 cell line it inhibits interleukin-6 production and enhances osteoprotegerin mRNA expression (33). Moreover, in cultured ROB cells genistein was found to raise osteocalcin release (54). Despite this last finding, we were unable to show any effect of this phytoestrogen on osteocalcin mRNA expression in ROB cells, which may again stress that the effects of SERMs on these cells do vary in relation to the stage of their *in vitro* culture. However, genistein, like estrogen and resveratrol, stimulates the proliferative activity of cultured ROB cells, an effect already observed in mouse bone marrow-derived mesenchymal stem cell cultures (55).

Like genistein, none of the other endocrine disruptors tested affect the expression of osteoblast marker genes in cultured ROB cells. However, some of them are able to variously modulate the proliferative activity of ROB cells. Briefly, BSP exerts estrogenic activity in some organs and tissue (56-59), but when orally administered has no effect on either bone mineral density and osteocalcin level in rats or osteocalcin and osteopontin secretion from cultured osteoblasts (58,61). In the present study, BSP was found to exert a strong proliferogenic action on ROB cells. BP3, commonly used in cosmetics and plastics, displays some structural relationships with steroid hormones (62), and has been reported to exert variable uterotrophic effects (63). Despite the fact that BP3 was found to enhance the proliferative activity of the MCF-7 breast cancer-derived cell line (64), it did not alter the proliferation rate of cultured ROB cells. The plant flavonoid silymarin (65), although exerting estrogen-like effects restricted to the metaphysis of the femur of ovariectomized rats (66), was unable to change the proliferative activity of cultured ROB cells. Procymidone and linurone are commonly used pesticides, possessing SERM activity (59,67) and reported to exert different effects on sex hormone-target organs and tissues (59,67-71). We observed a strong anti-proliferogenic action of both of these disruptors on cultured ROB cells.

In light of the present findings, we conclude that i) of the endocrine disruptors tested only resveratrol is able, like estradiol, to enhance the expression of osteocalcin in cultured ROB cells; and ii) in contrast with specialized osteoblast functions, ROB-cell proliferative activity is very sensitive to endocrine disruptors, some of which (procymidone and linurone) probably act via a mechanism independent of their SERM activity.

Acknowledgements

This study was supported by the European Community grant EURISKED (EVK1-CT 2002-00128).

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