Nonhypercalcemic analogs of vitamin D stimulate creatine kinase B activity in osteoblast-like ROS 17/2.8 cells and up-regulate their responsiveness to estrogens

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We have reported that pretreatment with 1α, 25(OH)2D3 (1,25) up-regulates responsiveness and sensitivity to 17β estradiol (E2) in osteoblast-like cells, as measured by parallel stimulation of [3H]thymidine incorporation into DNA and the specific activity of creatine kinase BB (CK). Increased responsiveness was correlated with increased E2 receptor concentration. In this study, we have extended these observations to new nonhypercalcemic analogs of 1,25. We compared the analogs hexafluoro vitamin D3 (FL), and the side chain modified derivatives: EB 1089 (EB), CB 1093 (CB) and MC 1288 (MC) with 1,25 and 25 (OH)D3 (25 D3). Treatment with 30 nM E2 for 4 h stimulated CK activity in ROS 17/2-8 cells by 40%; there was no further increase after 3 daily additions of E2. Treatment by 3 daily additions, at 1 nM, of all analogs except 25 D3 caused a 2–3-fold increase in CK specific activity. This schedule of treatment also upregulated the response to 4 h exposure to 30 nM E2 by 30–70% above the response to vitamin D analogs alone, and by up to 2 fold compared to E2 without pretreatment. At 1 pM, the analogs doubled CK activity, and, except for 1,25, upregulated the response to E2 to levels characteristic of each analog. Pretreatment with vitamin D analogs also increased the sensitivity to E2 by lowering the dose for a comparable response to E2 by one or two orders of magnitude. Stimulation of specific activity of CK by the analogs was paralleled by increases in the steady state level of mRNA for CKB, but not in its half life. Whereas pretreatment by vehicle followed by E2 for 2 h was unable to increase CKB mRNA, pretreatment with the analogs made possible detection of mRNA responsiveness to E2. These results add to the evidence for the interaction of estrogens and antiestrogens with vitamin D metabolites in regulation of bone growth in vitro. They also strengthen the potential for treatment of bone loss, as occurs in postmenopausal osteoporosis, by a combination of nonhypercalcemic vitamin D analogs and estrogens. (Steroids 63:340–343, 1998) © 1998 by Elsevier Science Inc.

Keywords: vitamin D analogs; creatine kinase B activity; osteoblasts; estrogen

Introduction

There is increasing awareness of the interaction between steroid and secosteroid hormones. Our previous studies showed that vitamin D metabolites stimulated responses to 17β estradiol (E2) and to androgens (DHT) both in vivo and in cell culture in a cell specific manner, as measured by increased [3H]thymidine incorporation into DNA and increased specific activity of the brain type isozyme of creatine kinase (CK). ROS 17/2.8 cells contain receptors for both estrogens and vitamin D metabolites and respond to both hormones. Moreover, mutual interactions between these hormones have been demonstrated and an increased response to E2 after 1,25 pretreatment was correlated with an increase in estrogen receptors (ER). This enhancing effect of vitamin D metabolites raises the possibility that lower therapeutic doses of estrogen could be used in cases of postmenopausal osteoporosis, and thereby reduce the incidence of endometrial cancer and other hazardous side-effects, while preserving its protective effect on bone.

In aiming to overcome the hypercalcemic effect of 1,25, in the present study, we have investigated the en-
hancing activity of new 1,25 analogs that mimic its actions on regulation of cell proliferation and/or differentiation, but not on calcium transport. Several non-hypercalcemic analogs are promising candidates for treatment of diseases correlated with vitamin D status.\textsuperscript{12,13} We compared the ability of non-hypercalcemic analogs of vitamin D to stimulate the specific activity of CK and the steady state level of its mRNA. In previous studies of skeletal cells, which contain low concentrations of steroid hormone receptors,\textsuperscript{6,14} we used the stimulation of CK specific activity as a sensitive and rapid post-receptor response marker. The brain type (BB) isozyme of CK, the major component of the estrogen induced protein (IP) of rat uterus,\textsuperscript{15} is part of the energy buffer system which regenerates ATP from ADP, and has been a useful marker for the action of steroid, secosteroid and protein hormones.\textsuperscript{1–5,16}

\textbf{Experimental}

Osteoblast-like ROS 17/2.8 rat osteosarcoma cells\textsuperscript{17} were cultured in 24 well plates in 1 mL of DMEM + F12 (1:1) containing 10\% FCS, in a humidified 7.5\% CO\textsubscript{2} atmosphere. Starting Day 1 after seeding, cells were treated with 3 daily additions of either vehicle (0.02\% ethanol in medium, C) or 1 nM or 1 pM final concentrations of 1\alpha\textsubscript{(OH)}\textsubscript{2}D\textsubscript{3} (1,25), EB 1089 (EB), MC 1288 (MC), CB 1093 (CB), hexafluoro-vitamin D (FL), or 25 (OH)D\textsubscript{3}.\textsuperscript{25} On Day 4 after seeding, cells were challenged with increasing doses of E\textsubscript{2}, either for 4 h for assay of CK specific activity or for 2 h for assay of the steady state level of CKB mRNA. Cells were scraped from culture dishes, homogenized, extracted and assayed as described previously.\textsuperscript{4} Protein was determined by Coomassie brilliant blue dye binding\textsuperscript{18} with BSA as standard. CKB mRNA was extracted and analyzed by Northern hybridization as previously described.\textsuperscript{19}

The significance of differences between experimental and control values was evaluated by ANOVA in which \( n \) = number of culture dishes.

\textbf{Results}

Cells treated daily for 3 days with vitamin D analogs showed a dose-dependent increase in CK activity the magnitude of which depended on the analog (Figure 1); maximal stimulation occurred at 10\textsuperscript{-12} M. Pretreatment with 1,25 or any of the analogs tested resulted in up-regulation of the response to 4 h challenge with E\textsubscript{2} on Day 4. While 1,25 enhanced the response to E\textsubscript{2} at 10\textsuperscript{-10} M and stimulated a maximal response at 10\textsuperscript{-9} M, FL showed biphasic enhancement with peaks at 10\textsuperscript{-12} M and at 10\textsuperscript{-8}–10\textsuperscript{-9} M. The new nonhypercalcemic analogs MC, EB, and CB enhanced the response to E\textsubscript{2} to an extent depending on the analog, with peaks at 10\textsuperscript{-12} and 10\textsuperscript{-9} M. In contrast, pretreatment with E\textsubscript{2} at 3 \times 10\textsuperscript{-8} M did not increase the response to E\textsubscript{2} on Day 4 (not shown).

All the compounds tested also increased the sensitivity to

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Stimulation of CK specific activity in ROS 17/2.8 cells after treatment with increasing doses of 1\alpha, 25(OH)\textsubscript{2} vitamin D\textsubscript{3} (1,25) or its analogs hexafluorovitamin D (FL), MC 1288 (MC), EB 1089 (EB) or CB 1093 (CB) for 3 days, or such pretreatment followed by 4 h incubation on Day 4 with 30 nM E\textsubscript{2}. CK was extracted and assayed as described in the Experimental section. Results are means \( \pm \) SEM of \( n \) = 8–20. Untreated control activity averaged 0.52 \( \pm \) 0.06 \( \mu \)mol/min/mg protein. ANOVA: *\( p \leq 0.05; **p \leq 0.01; ***p < 0.001 \) for experimental values compared to untreated control value.}
\end{figure}
stimulation by E_2 by lowering the dose of E_2 for effective stimulation to 0.3 or 3 nM, depending on the analog (Figure 2). Whereas vehicle pretreatment revealed a maximal response to E_2 at 30 nM and a slight response at 3 nM, treatment with 1.3 nM 1,25 lowered the concentration of E_2 for maximal response to 3 nM; FL at 1.3 nM caused a highly significant response at 0.3 nM, while the new analogs MC, EB and CB caused a highly significant response to E_2 at 3 nM.

All the analogs tested above increased the steady-state level of mRNA for CKB (Figure 3). This contrasted with E_2 alone (30 nM given daily for 3 days) which did not cause a detectable increase in mRNA for CKB (not shown). The schedule of pretreatment with EB, MC, and to a greater extent FL, for 3 days, followed by E_2 (30 nM) on Day 4, resulted in a greater stimulation than by the vitamin D analogs alone. The half life of CKB mRNA in these cells was approximately 3.5 h (not shown), when measured after pretreatment with 10 nM 1,25, which caused a 9-fold increase in CKB mRNA.

**Discussion**

The results of these studies demonstrate that treatment with the new nonhypercalcemic vitamin D analogs, as with 1,25, increases the specific activity of, and mRNA for, CK. When used as a pretreatment, the analogs up-regulate the maximal acute response to E_2 and lower the dose of E_2 needed for comparable stimulation. This up-regulation by vitamin D metabolites occurs not only in ROS 17/2.8 cells but also in rat embryo calvaria cells, and SaOS_2 cells, as well as in bone and cartilage in vivo. Up-regulation of the response to E_2 by increasing the number of estrogen receptors was shown by 1,25 in calvaria, SaOS_2 and MC3T3 cells and primary cultures of bone derived cells. Reciprocal stimulation of vitamin D receptor levels by E_2 in rat uterus as well as estrogenic modulation of vitamin D analog regulation of osteoblastic function in SaOS_2 cells has also been reported.

The low concentration of E_2 receptors in bone cells may be a limiting factor in determining their biological response to E_2. Vitamin D metabolites appear to be important in the hormonal milieu that determines the expression of ER in skeletal cells and in particular in augmenting the response to E_2 by increasing ER. However, due to the hypercalcemic effects of 1,25 it is impossible to use combined treatment in vivo. Therefore, use of nonhypercalcemic analogs may be advantageous. Indeed, in preliminary experiments on immature female rats (Sömjén et al., unpublished), while 3 daily injections of either 0.15 pg/gBW EB, 0.25 pg/gBW, CB or 250 pg/gBW of 1,25 or FL did not increase basal CK activity in diaphysis, epiphysis or uterus, this treatment significantly enhanced the response to 5 µg of E_2 in skeletal tissues (but not in the uterus), 24 h after injection. Such combined treatment with non-
Vitamin D analogs upregulate $E_2$ induction of CKB: Sömjen et al.

Figure 3 Stimulation of the steady state level of CK mRNA by pretreatment with $1\alpha$, 25(OH)$_2$ vitamin D$_3$ (1,25), CB 1093 (CB), EB1089 (EB), MC1288 (MC), hexafluoro vitamin D$_3$ (FL) or 25 (OH) D$_3$ (25) for 3 days, compared with their combined effect with 30 nM $E_2$ for 24 h on Day 4. RNA was extracted and 20 µg samples subjected to Northern blot hybridization with the plasmid CKB-1 and subsequently with an 18 S ribosomal RNA probe, for normalization of the amounts of total RNA applied, as described previously.19

Material by Harry S. Weisman and colleagues demonstrated increased sensitivity to calcimeter hormones in osteosarcoma cells. 20

References


