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Treatment with non-hypercalcemic analogs of 1,25-dihydroxyvitamin D_3 increases responsiveness to 17β-estradiol, dihydrotestosterone or raloxifene in primary human osteoblasts

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Abstract

Pretreatment with 1 nM 1,25-dihydroxyvitamin D_3 (1,25), or non-hypercalcemic Vitamin D analogs, upregulated the response of creatine kinase (CK) to 17β-estradiol (30 nM E_2), raloxifene (3000 nM RAL) or dihydrotestosterone (300 nM DHT) in primary human bone cells. Previously, we reported that these osteoblast-like cells responded to gonadal steroids in a sex specific manner. Bone cells derived from pre-menopausal women showed greater stimulation of CK specific activity by E_2 than bone cells from post-menopausal women; in male-derived cells no age related difference was found. In this study, we treated cells derived from female or male bones, at different ages, with the side chain modified analogs of Vitamin D: CB 1093 (CB), EB 1089 (EB), MC 1288 (MC) and the demonstrably non-calcemic hybrid analog JK 1624 F2-2 (JKF), by daily addition of 1 nM, for 3 days. On day 4, cells were incubated with sex steroids for 4 h and cell extracts were prepared. Pretreatment with JKF or CB significantly upregulated the response to 30 nM E_2 in all female-derived cells and to 300 nM DHT in mature male-derived cells. In cells from older males, only JKF caused augmentation of DHT action. Bone cells from pre- or post-menopausal females responded to 3000 nM RAL by increased CK activity to the same extent as to 30 nM E_2; however, RAL and E_2, when applied together, resulted in mutual annihilation of their agonist activities. Vitamin D analogs prevented the antagonistic effect of RAL in the presence of E_2, possibly due to increased numbers of ERs. Both estrogen receptors, α (ERα) and β (ERβ), were expressed in male- as well as in female-derived cells. However, only in female-derived cells were ERα and ERβ upregulated by pretreatment with Vitamin D analogs. This study raises the possibility of testing combined Vitamin D analog and estrogen replacement treatment for post-menopausal women to prevent osteoporosis.

Keywords: Human primary osteoblasts; Estrogen receptors α and β; Non-hypercalcemic Vitamin D analogs; Sex hormones; Raloxifene; Sex and age specificity; Creatine kinase

1. Introduction

The secosteroid 1,25-dihydroxyvitamin D_3 (1,25) generates a spectrum of biological responses via genomic [1] and nongenomic mechanisms [2]. For optimal bone growth and prevention of osteoporosis in post-menopausal women, adequate concentrations of both 17β-estradiol (E_2) [3,4] and Vitamin D_3 are required [3]. Low Vitamin D status is a common phenomenon in the elderly; the levels of Vitamin D as well as sex hormones are lower in older subjects [3,2] making post-menopausal women even more vulnerable to osteoporosis.

We have studied the interaction of Vitamin D analogs and estrogens in a rat model [6,7] using the increase in the specific activity of creatine kinase (CK) as a response marker. The brain type (BB) isoenzyme of creatine kinase, part of the "energy buffer" system, which regulates the cellular concentration of ATP and ADP, is the major component of the "E_2-induced protein" of rat uterus [8] and other tissues containing estrogen (E_2) receptors [9]. CK stimulation is an efficient response marker to detect activity of E_2 in bone cells [10], particularly in osteoblasts [11] which contain low concentrations of E_2 receptors [12,13]. Notably, the

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stimulation of CK in cultured bone cells, correlated with increased DNA synthesis in bone, requires the higher end of the physiological range of estrogen concentrations [10].

We demonstrated that pretreatment with 1,25 upregulated responsiveness and sensitivity to 17β-estradiol and to several selective estrogen receptor modulators (SERMS) in osteoblast-like cell lines (ROS 17/2.8 and SaoS 2) and rat bone, as measured by both the stimulation of the specific activity of CK and the increase in [^{3}H] thymidine incorporation into DNA [7,14–18]. This mutual interaction between E_2 and Vitamin D was also manifested by an increase in estrogen receptors (ER) after treatment with 1,25 [17,19].

However, the therapeutic use of Vitamin D is restricted as a consequence of its toxicity in humans, causing hypercalcemia [20,21]. Therefore, to avoid toxic effects, we tested structurally modified "non-hypercalcemic" analogues for their potency in upregulating estrogen activity. We reported that multiple treatments with "non-hypercalcemic" analogs of Vitamin D, such as MC 1288, CB 1093, EB 1089 and the demonstrably non-calcemic JKF 1624 F2-2 [22] stimulated the specific activity of CK in ROS 17/2.8 osteoblast-like cells [18,23]. Moreover, pretreatment of skeletal-derived cells with these analogs upregulated both responsiveness and sensitivity to E_2 [23] and SERMS [6].

In the most relevant system, human-derived primary osteoblasts, we found that E_2 and DHT increased the basal activity of CK in a gender specific manner [24]. Osteoblasts derived from males responded to DHT while female-derived cells responded only to 17β-estradiol [24]. In the present study, we show that an upregulation by Vitamin D analogs occurs in normal human primary osteoblasts, accompanied by increases in ERα and ERβ in female-derived cells.

2. Materials and methods

2.1. Reagents

All the reagents used were analytical grade. Chemicals, 17β-estradiol, dihydrotestosterone (DHT), and the creatine kinase assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO).

Raloxifene (RAL) was the gift of Dr. B. Fournier (Ciba-Geigy, Basel, Switzerland). 1,25$(OH)_2$ D_3, CB 1093, EB 1089 and MC 1288 were gifts of Leo Pharmaceutical Products, Ballerup, Denmark. JKF was synthesized as previously described [22].

Monoclonal anti-ERα antibodies (F-10: sc-8002) polyclonal anti-ERβ antibodies (H-150: sc-8974), and the secondary antibodies, rabbit anti mouse peroxidase, goat anti mouse peroxidase, and goat anti rabbit peroxidase were purchased from Santa Cruz Biotechnology Inc. Enhanced chemiluminescence reagents were purchased from Amersham (Amersham, Buckinghamshire, UK).

2.2. Cell cultures

Human bones were obtained from biopsies of patients undergoing corrective surgery following accidental injury, hip or knee replacement. All patients (women and men) were healthy, non-osteoporotic and not receiving hormonal replacement treatment. Four groups were defined: pre-menopausal, ranging between 37 and 50 years old, normally menstruating women ($n = 5$ per analog treatment group, $n = 10$ for vehicle controls). Post-menopausal women, ranging between 59 and 84 years old ($n = 5$ per analog treatment group, $n = 10$ for vehicle controls). Mature men, ranging between 32 and 53 years old ($n = 7$ per analog treatment group, $n = 10$ for vehicle controls). Older men, ranging between 57 and 76 years old ($n = 5$ per analog treatment group, $n = 10$ for vehicle controls).

The non-enzymic method for isolation and culture of human bone cells and their characterization as osteoblasts was described previously [25]. In brief, samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm^3 pieces and extensively and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants, with no enzymatic digestion, were seeded in 100 mm diameter tissue culture dishes and incubated in DMEM medium without Ca^{2+}, to avoid fibroblastic growth [25,26], containing 10% fetal calf serum (FCS) and antibiotics. Cell outgrowth from the bone explants was apparent after 6–10 days. The cultures consisted of osteoblast-like cells (with negligible fibroblasts) showing the characteristic high basal alkaline phosphatase activity and levels of osteocalcin, and their stimulated production by 1,25 as well as increased cAMP formation in response to PTH [25]. First passage cells were then seeded at a density of 3×10^5 cells/35 mm tissue culture dish in phenol red free DMEM with 10% charcoal stripped FCS and incubated at 37 °C in 5% CO_2.

2.3. Hormonal treatment

Cells were pre-treated daily with vehicle (0.01% ethanol in medium) or Vitamin D analogs, for 3 days, starting on day 1 after seeding, at 1 nM final concentration [7]. On day 4 after seeding, 30 nM E_2, 300 nM DHT or 3 μM RAL, or RAL together with E_2 were added to the cultures for 4 h, followed by harvesting for CK assay.

2.4. Creatine kinese activity assay

Cells were scraped from culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction buffer [10]. Supernatant extracts were obtained by centrifugation at 14,000 × g for 5 mm at 4 °C in an Eppendorf micro centrifuge. CK activity was measured in a Kontron Model 922 Uvicon Spectrophotometer at 340 nm using a Sigma coupled assay kit (procedure 47-UV). Protein was

assayed by Coomassie brilliant blue dye binding [27], using bovine serum albumin (BSA) as the standard.

2.5. Western blot analysis of ER

Western blot analysis of ER was performed using specific antibodies against ERα and ERβ [28]. Cells from treated and control incubations were homogenized in buffer containing 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM benzamidine, aprotinin (10 μg/ml), and leupeptin (10 μg/ml), and the protein content of the homogenate was determined. Equal amounts (30 μg) of homogenate protein, along with molecular-weight markers were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes and stained with Ponceau red to verify equal protein loading and transfer. After blocking with full milk or 2% bovine serum albumin, membranes were incubated overnight with the antibodies diluted in TBS buffer (20 mM Tris, 0.14 M NaCl pH, 7.6) containing 1% milk or 1% BSA, anti-ERα (0.5 μg/ml), or anti-ERβ (at 1:3000 dilution). Membranes were washed and processed for enhanced chemiluminescence. Signals were then quantified by densitometry.

2.6. Statistical significance

The significance of differences between experimental and control values was evaluated using a non-paired, two-tailed Student's *t*-test in which *n* is the number of donors.

3. Results

3.1. The effect of Vitamin D analogs on CK specific activity in human-derived osteoblasts

Treatment with Vitamin D analogs modified the response of osteoblast cells in diverse ways, depending on the specific analog and the sex and age of the cell donor.

In cells derived from both pre- and post-menopausal women, three daily-treatments with the non-hypercalcemic analog JKF [22] or the less hypercalcemic CB, at a concentration of 1 nM, led to an insignificant increase in CK specific activity (Fig. 1). EB significantly reduced CK specific activity in cells of both age groups of cells (Fig. 1). MC increased CK activity in cells derived from pre-menopausal women by 42%, while in cells derived from post-menopausal women, CK activity was significantly decreased (Fig. 1).

In men, only JKF increased CK specific activity, while CB or EB decreased CK specific activity and MC had no effect (Fig. 2). The stimulatory effect of JKF was greater in cells derived from older males (Fig. 2). A significant decrease in CK specific activity after CB and EB treatment was found in cells derived from older males (Fig. 2).

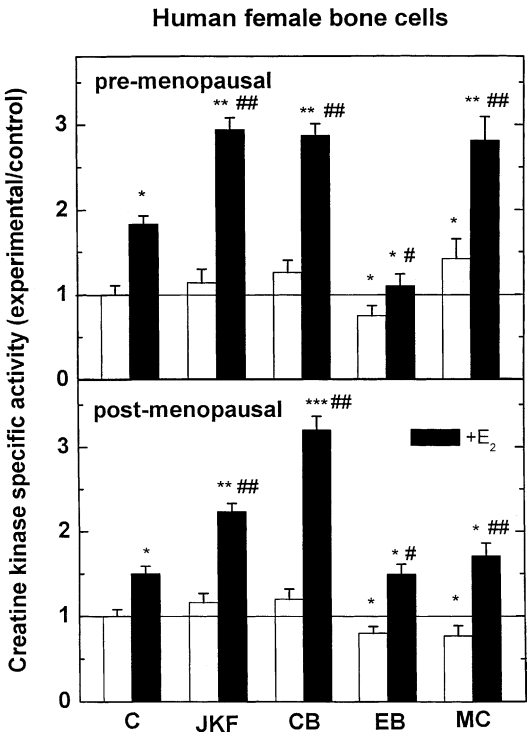

Fig. 1. Stimulation of CK activity by non-hypercalcemic Vitamin D analogs, E_2, or their combination, in primary bone-derived cells from pre- and post-menopausal women. Bone cells were cultured, treated and assayed for CK activity as described in Section 2. Cells were treated daily for 3 days with vehicle or Vitamin D analogs: JKF 1624 F2-2 (JKF), CB 1093 (CB), EB 1089 (EB), or MC 1288 (MC) at a final concentration of 1 nM. On day 4, the cells were treated for 4 h with vehicle or 30 nM 17β-estradiol (E_2). Results are mean ± S.E.M. for triplicate cultures from five women/group for analog treated cultures and 10 women for vehicle control (C) cultures. Control means were 22.6 ± 3.2 and 20.8 ± 3.7 nmol/mol/mg protein, for pre- and post-menopausal women, respectively. Experimental means compared to control means: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Means of cells treated with Vitamin D analog + E_2 vs. cells treated with analog alone: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

3.2. Up regulation of CK specific activity in response to E_2 by pretreatment with Vitamin D analogs in osteoblasts derived from bones of human females

Bone cells derived from women treated with 30 nM E_2 for 4 h, showed a significant increase in CK activity (Fig. 1). The response to E_2 was significantly greater in cells derived from pre-menopausal women (84%) than in cells derived from post-menopausal women (50%), Fig. 1. In all cell cultures derived from pre-menopausal women, 3 days pretreatment with JKF, CB, or MC produced significant upregulation of CK specific activity in response to 30 nM E_2 (Fig. 1). Although EB reduced basal CK activity by 25%, the addition of E_2 elevated the response beyond the control level (Fig. 1).

In cells derived from bones of post-menopausal women, CB was most potent; JKF and MC were less effective. The combination of JKF and E_2, caused a 127% increase in the response, compared to treatment with E_2 alone (Fig. 1). The comparable response to the combined treatment of CB

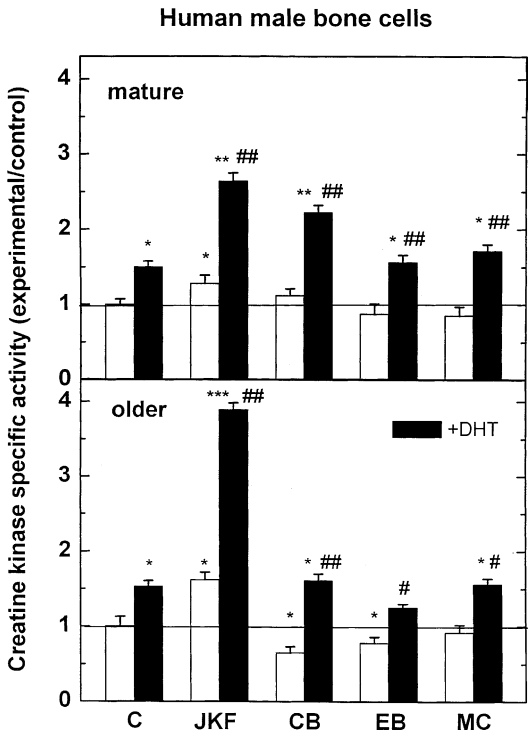

Fig. 2. Stimulation of CK activity by non-hypercalcemic Vitamin D analogs, dihydrotestosterone (DHT), or their combination, in human primary bone-derived cells from mature and older men. Bone cells were cultured, treated and assayed for CK activity after pretreatment with Vitamin D analogs as described in Fig. 1. On day 4, the cells were treated for 4 h with vehicle or 300 nM DHT. Results are mean ± S.E.M. for triplicate cultures from seven mature or five older men/group for analog treated cultures and 10 men for vehicle control (C) cultures. Control means were 28.0 ± 6.5 and 24.6 ± 4.0 nmol/mol/mg protein, for mature and older men, respectively. Experimental means compared to control means: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Means of cells treated with Vitamin D analog + DHT vs. cells treated with the analog alone: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

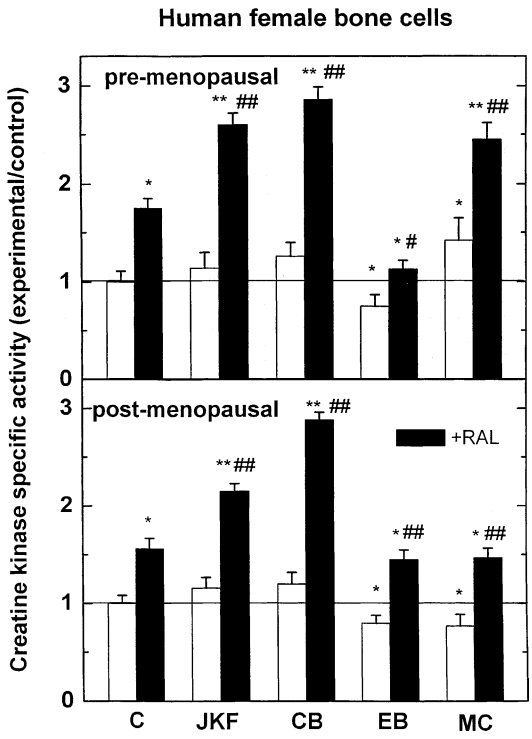

Fig. 3. Stimulation of CK activity by non-hypercalcemic Vitamin D analogs, raloxifene (RAL), or their combination, in human primary bone-derived cells from pre- and post-menopausal women. Bone cells were cultured, treated and assayed for CK activity after pretreatment with Vitamin D analogs as described in Fig. 1. On day 4, the cells were treated for 4 h with vehicle or 3 μM RAL. Results are mean ± S.E.M. for triplicate cultures from 5 women/group for analog treated cultures and 10 women for vehicle control (C) cultures. Control means were presented in the legend to Fig. 1. Experimental means compared to control means: $^{*}P < 0.05$; $^{**}P < 0.01$. Means of cells treated with Vitamin D analog + RAL vs. cells treated with analog alone: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

and E_2 was even greater (223%). Although EB and MC reduced the specific activity of CK, the addition of E_2 restored the response to that after treatment with E_2 alone (Fig. 1).

3.3. Up regulation of CK specific activity in response to DHT by pretreatment with Vitamin D analogs in osteoblasts derived from bones of human males

In male-derived cells, treatment with 300 nM DHT for 4 h increased CK specific activity significantly to the same extant in cells derived from the two age groups (Fig. 2). In cells derived from bones of mature men, daily pretreatment for 3 days with JKF increased synergistically CK specific activity by 163% and the combination of CB followed by DHT resulted in a synergistic CK response of 122%. Although EB and MC reduced the basal CK activity, the combined treatment of EB or MC followed by DHT, restored the response to that of DHT alone (Fig. 2). In cells derived from older males a highly significant upregulation of the response to DHT by pretreatment with Vitamin D analogs was restricted to JKF (Fig. 2).

3.4. Up regulation of CK specific activity in response to raloxifene, by pretreatment with Vitamin D analogs, in cells derived from human female bones

Raloxifene (at 100 times the concentration of E_2) stimulated CK activity to the same extent as E_2 and this action was similarly augmented after pretreatment with Vitamin D analogs (Fig. 3, cf. Fig. 1). Treatment with 3 μM RAL for 4 h, increased CK specific activity to a significantly greater extent in cells derived from bones of pre-menopausal compared to post-menopausal women (Fig. 3). Daily treatment for 3 days with any of the Vitamin D analogs tested (at 1 nM), increased significantly the response of CK to RAL (compared to the increase of CK activity in response to the analog alone); this response was lower in cells derived from post-menopausal women (Fig. 3). In both age groups CB was most potent, JKF was effective, and EB was the least effective. MC that was as potent as JKF in

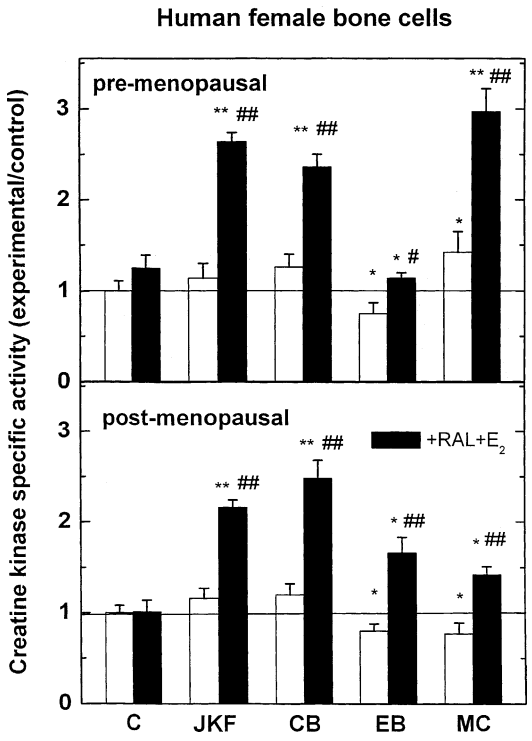

Fig. 4. Stimulation of CK activity by non-hypercalcemic Vitamin D analogs, E_2 combined with raloxifene (RAL), or their combination, in human primary bone-derived cells from pre-and post-menopausal women. Bone cells were cultured, prepared and assayed for CK activity, after pretreatment with Vitamin D analogs as described in Fig. 1 On day 4, the cells were treated for 4 h with vehicle or 30 nM 17β-estradiol (E_2) combined with 3 μM RAL. Results are mean ± S.E.M. for triplicate cultures from 5 women/group for analog treated cultures and 10 women for vehicle control (C) cultures. Control means were presented in the legend to Fig. 1. Experimental means compared to control means: $^{*}P < 0.05$; $^{*}P < 0.01$. Means of cells treated with Vitamin D analog followed by RAL + E_2 vs. cells treated with analog alone: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

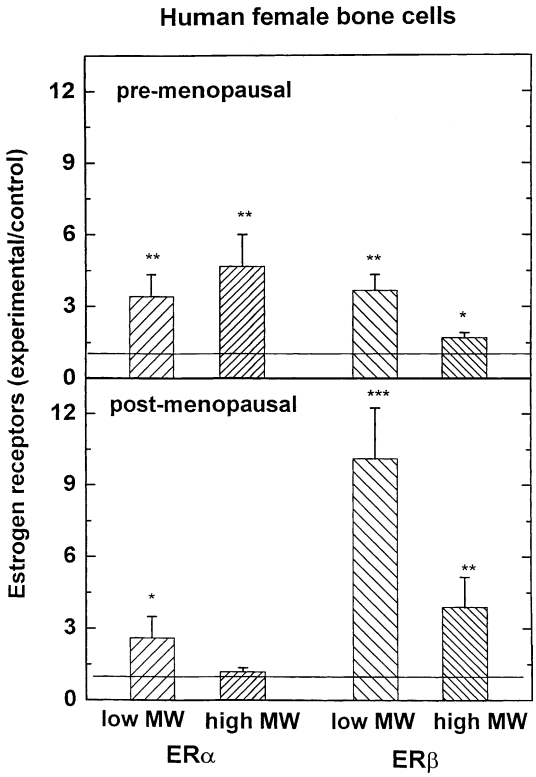

Fig. 5. Stimulation of the expression of estrogen receptors α and β by JKF in primary human female bone-derived cells. Bone cells were cultured treated and extracts prepared for Western immunobloting analysis as described in Section 2. Cells were treated daily for 3 days with vehicle (C), or JKF. Results are mean ± S.E.M. for triplicate cultures from 5 women/group for analog treated cultures and 10 women for vehicle control (C) cultures. Experimental means were compared to control (vehicle alone) means: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

cells from pre-menopausal women, had no effect on cells of post-menopausal women (Fig. 3).

Treatment with RAL in the presence of E_2 (Fig. 4), inhibited the stimulatory effect of estradiol alone (cf. Fig. 1) in cells derived from both pre- and post-menopausal women, nor conversely was the positive effect of RAL (cf. Fig. 3) observed in the presence of E_2. Pretreatment with Vitamin D analogs abolished this reciprocal inhibition (Fig. 4).

3.5. Stimulation by JKF of estrogen receptor expression in human bone derived cells

Western immunobloting of cell extracts detected the presence of two forms of both ERα (32 and 67 K) and ERβ (37 and 63 K, Fig. 5). Three daily additions of JKF modulated the expression of these two molecular weight forms of ERα and ERβ in a complex manner (Fig. 5). In cell cultures derived from bones of pre-menopausal women, pre-incubation with JKF resulted in a significant increase in expression of all ER forms detected.

In cells derived from bones of post-menopausal women, the increase of 32 K ERα was significant (161%, Fig. 5), while 67 K ERα levels were not elevated. Both 37 K and 63 K ERβ forms were highly significantly increased, reaching a 10-fold increase in the expression of 37 K ERβ (Fig. 5).

In cells derived from male bones, all four of the ER forms seen in female-derived bone cells were detected. After treatment with JKF, the only changes seen were a significant increase (1.7-fold) in the 67 K ERα form and a slight decrease in 32 K ERα form. No age difference was found in the expression levels of any of the ER forms (not shown).

4. Discussion

The increased responsiveness to estrogens after pretreatment with Vitamin D or Vitamin D analogs, previously demonstrated in skeletal-derived cells in vitro [7,17,18,29] and pre-pubertal female rat bone in vivo [7,16] has now been demonstrated using Vitamin D analogs in primary human bone cells. This is apparently an indirect effect of multiple Vitamin D or Vitamin D analog treatment due to an increase

in estrogen receptors [14,19] (Fig. 5). All forms of the estrogen receptors were increased significantly by JKF treatment except the 67 K ERα in post-menopausal women. While in pre-menopausal women the increased expression of ERα was greater than that of ERβ, in cells derived from bones of post-menopausal women, both 37 and 63 K forms of ERβ forms were more highly expressed. Preliminary study of real time PCR measurements of ERα and ERβ mRNA in primary human osteoblasts (unpublished data) show confirmatory changes in ER, in cells derived from both pre- and post-menopausal women. These observations raise the possibility that, with aging of osteoblasts, the relative importance of ERβ increases. An indication that the relative proportion of ER may affect the age related responses to estrogen is the observation that genistein, which shows preferential activity with ERβ, is more active in post-menopausal women [30].

The modulation of ERs is a recent addition to the spectrum of actions of Vitamin D [31], which stimulates the differentiation of osteoblasts and osteoclasts and stimulates osteoblasts to produce osteocalcin and alkaline phosphatase [31]. The effect of 1,25 on bone mineralization is indirect, by stimulation of the supply of calcium and phosphate, mainly by absorption from the gut. In the elderly, the formation of Vitamin D is much less efficient, causing Vitamin D deficiency. This condition stimulates the parathyroid glands which leads to high bone turnover, resulting in bone loss, and osteoporosis, facilitating vertebral and hip fractures [5].Although, in our human osteoblast cell system, a single treatment with 1,25 had no effect on the specific activity of CK [24], multiple 3 day treatments with Vitamin D analogs EB or MC modified the specific activity of CK, depending on the analog and the sex and age of the cell donor (Figs. 1 and 2). However, in both mature and older males, JKF increased basal CK specific activity. Pretreatment with any of the analogs upregulated the responses to estrogen in our human osteoblast cell system as it had in ROS 17/2.8 cells [18,23] or in prepubertal rat diaphyseal bone [6,7]. The up regulation of androgen action (Fig. 2) raises the possibility of combination therapy with Vitamin D analogs against male osteoporosis.

In our human cell system, similar upregulation of CK specific activity was obtained after combined treatment with Vitamin D analogs, and the SERM raloxifene (Fig. 3). RAL at 3 μM, when provided alone, acted as a full estrogen agonist and stimulated CK to the same extent as 30 nM E_2 (cf. Fig. 1). When RAL was provided simultaneously with E_2, RAL acted as a complete antagonist (Fig. 4). This mutual annihilation phenomenon was demonstrated previously in ROS 17/2.8 osteoblast-like cells, human SaoS2 osteoblast-like cells, Ishikawa endometrial cancer cells, as well as in prepubertal rat diaphysis, epiphysis and uterus [6,32,33] and an explanation based on the formation of mixed heterodimers was proposed [32]. Simultaneous treatment of E_2 and RAL following 3 days treatment with all "non-hypercalcemic" Vitamin D analogs resulted in stimulation of CK specific activity similar to that when either E_2 or RAL were given alone, presumably due to an increase in ER's, which was able to prevent the mutual annihilation.

The increase in ERs caused by Vitamin D and its analogs in osteoblast-like cells, contrasts sharply with its action in cancer cells where it acts as a potent inhibitor of cell growth [34–36]. Swami et al. [37] showed decreases in ER expression, accompanied by significant decreases in steady state levels of ER mRNA, in MCF-7 breast cancer cells treated with 1,25 for 24 h and suggested that 1,25 exerts a direct negative effect on ER transcription in MCF-7 cells [37].

In the vascular system, Vitamin D analogs can either increase or decrease the content of ER in a complex manner. Both 1,25 and JKF decreased the expression of ER in umbilical smooth muscle cells and increased ERα in E304 endothelial cell lines [28].

The favorable cell specific effects of Vitamin D metabolites, i.e. the upregulation of E_2 action by non-calcemic Vitamin D analogs in normal human bone and in human vascular cells [28] and the parallel down regulation of E_2 action by 1,25 in MCF-7 breast cancer cells [37], raises the possibility that by use of "non-hypercalcemic" Vitamin D analogs, lower doses of E_2 could be administrated to post-menopausal women to achieve multiple positive effects. Our in vivo studies demonstrated promising results; pretreatment with JKF injections augmented the response of prepubertal female rat diaphysis and epiphysis to E_2 [7]. Therefore, such treatment could potentially reduce the threat of estrogen stimulated breast and endometrial cancer while preserving its protective effect on the skeletal system.

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