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Interaction between the effects of the selective estrogen modulator femarelle and a vitamin D analog in human umbilical artery vascular smooth muscle cells

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ABSTRACT

To further investigate the interaction between vitamin D system and estrogen-mimetic compounds in the human vasculature we studied the effect of the "less- calcemic" analog of $1,25(OH)_2D_3$ (1,25D); JK $1624F_2$-2 (JKF) in the presence of selective estrogen modulator femarelle (F), the phytoestrogen daidzein (D) and estradiol-17b (E_2) on ^{3}[H] thymidine incorporation (DNA synthesis) and creatine kinase specific activity (CK) in human umbilical artery vascular smooth muscle cells (VSMC). F, D and E_2, stimulated DNA synthesis at low concentrations, and inhibited it at high concentrations. All estrogen-related compounds increased CK dose- dependently. Daily treatment with JKF (1 nM for 3 days) resulted in decreased DNA synthesis, increased CK and up- regulation of the stimulation of DNA synthesis by low estrogen-related hormones whereas D- and E_2- mediated inhibition of cell proliferation was abolished by JKF. In contrast, inhibition of cell proliferation by F could not be blocked by JKF. JKF also up-regulated the stimulatory effects on CK by F, E_2 and D. VSMC expressed Estrogen Receptor (ER) a and ERb mRNA at a relative ratio of 2.7:1.0, respectively. JKF pretreatment increased ERa (~50%) and decreased ERb (~25%) expression. E_2 did not affect ERs whereas both D and F up-regulated ERb (~100%) and ERa (~50%). Additionally, JKF increased the intracellular competitive binding of F (from ~70 to ~310%), of D (from ~60 to ~250%) and of E_2 from (from ~ 70 to ~320%). F reciprocally modulated the vitamin D system by up-regulating VDR- and 25 hydroxyy vitamin D 1-a hydroxylase (1OHase) mRNA expression (~120%). F also stimulated 1OHase activity as indicated by an increase in the production of 1, 25D (~250%). A similar increase was elicited by D (~90%) but not by E_2. In conclusion, F has unique effects on human VSMC in that it can sustain inhibition of cell growth even in the presence of the vitamin D analog JKF. That JKF increases ER expression and F increased the endogenous production of 1, 25D and VDR expression offer new opportunities to modulate VSMC growth. Whether or not these mutual effects of F and JKF can be exploited to promote vascular health, particularly in estrogen-deficient states (e.g., menopause) is under investigation.

1. Introduction

Phytoestrogens vary considerably in terms of structure, estrogenic potency and availability in common food sources [1,2]. Human dietary sources contain two major chemical classes of phytoestrogens, isoflavones and lignans. The most-studied is perhaps the phytoestrogen genistein [2], whereas information on the biological effects of other isoflavones such as daidzein or its metabolite equol [2,3] are scarce. Based on the effects of these compounds on lipid oxidation [4] and vascular reactivity [5] it was suggested that dietary phytoestrogen consumption might cause cardiovascular protection [2].

The effect of estrogenic compounds on proliferation of vascular cells may be important in the vasculo-protective effects of estrogens, exemplified by the finding that estradiol-17b (E_2) inhibits the myointimal proliferative response to arterial injury in animal models [6,7] *in vivo* as well as inhibition of vascular smooth muscle cell (VSMC) proliferation *in vivo* and *in vitro* [3,8,9], but stimulates endothelial cell growth [10,11]. Recently it was reported that several phytoestrogens inhibit human aortic vascular smooth muscle cell growth [12].

We have previously reported that estrogens affect human VSMC growth in a bimodal way, at low concentration of E_2 stimulate, but at high concentration of E_2 inhibit DNA synthesis [13,14]. Genistein and daidzein (D) as well as femarelle (F) have E_2 −like activity in vascular cells in animal models *in vivo* and in vascular cells *in vitro* [15,16]. Vascular cells respond also to less-calcemic analogs of vitamin D such as the JKF by modulation the response of these cells to E_2 and to other

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estrogens [17,18]. CK stimulation in VSMC was up-regulated, whereas the inhibition of cell proliferation by high doses of E_2 was abolished by pre-treatment with JKF [17,18]. This was probably because of the differential modulation of ERs in these cells [18].

Here we compare the modulations by a vitamin D analog JKF on the effects of F, D and E_2 on cell proliferation of human VSMC cells *in vitro*.

VSMC cells express high-affinity receptors for 1,25$(OH)_2$ vitamin D_3 (VDR) [18,19] and vitamin D metabolites exert numerous effects in VSMC [17] which involve vital aspects of VSMC function and pathology, including contractility, growth and migration and the evolution of vascular calcifications. Evidence also suggests that 1, 25 $(OH)_2D_3$ inhibits VSMC replication and diminishes the mitogenic response to growth enhancers [17].

Given the high circulating levels of the precursor of 1,25 $(OH)_2D_3$, we reasoned that a locally expressed 1OHase system enabled VSMC to regulate the concentrations of 1,25D in an autocrine and/or paracrine manner such that it can be governed by local factors and provide intracellular ligands for the VSMC vitamin D receptor. In the present study we show that a functional 1OHase is regulated in cultured human VSMC by F as well as by D and E_2 leading to changes in 1OHase expression and activity.

2. Materials and methods

2.1. Materials

Estradiol-17b (E_2), Daidzein (D) and creatine kinase assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). Femarelle (F) was purchased from Se- Cure Pharmaceuticals (Teradion, Israel). JKF was provided by Dr. G.H. Posner. All other materials were of analytical grade.

2.2. Umbilical vascular smooth muscle cells (VSMC)

Umbilical VSMCs were prepared as previously described with minor modifications [13,14]. In brief, umbilical cords were collected shortly after delivery, and arteries were dissected, cleaned of blood and adventitia, and cut into tiny slices (1 to 3 mm). The segments were kept in culture in Medium 199 containing 20% fetal calf serum, glutamine, and antibiotics. Cell migration was detected within 5 to 7 days. Cells were fed twice a week and, on confluence, trypsinized and transferred to 24-well dishes. Cells were used only at passages 1 to 3 when expression of smooth muscle actin was clearly demonstrable.

2.3. Assessment of DNA synthesis

Cells were grown until sub-confluence and then treated with various hormones or agents as indicated for 24 h either without or with pretreatment for three days of 1 nM JK-1264 F_2-2 (JKF) [18]. ^{3}[H] thymidine was added for the last 2 h and its incorporation into DNA was assayed [13,14], using Beckman scintillator beta counter.

2.4. Creatine kinase extraction and assay

Cells were treated for 24 h with the various hormones or agents either without or with pretreatment for three days of 1 nM JKF [16], protein extraction and assessment of CK activity were as previously described [13,14].

2.5. Association of ^{3}[H] estradiol −17b with VSMC

Cells were incubated with ^{3}[H] E_2 [18] at 37 ∘C; in the presence or absence of 500-fold excess of unlabeled hormones for 60 min. Cells were subsequently washed three times with PBS containing 1% BSA; and cell-associated ^{3}[H] E2 was determined using a Scintillating counter as described before [18].

2.6. Determination of mRNA by real time PCR

RNA was extracted from cells, and was subjected to reverse transcription as previously described [18,19]. ERa, ERb, VDR and 1OHase cDNA were used as standard controls.

2.7. Assessment of 1OHase in VSMC

1OHase activity was assessed by the measurement of 1,25D generated within 60 min after the addition of 25$(OH)D_3$ (200 ng/ml) to culture, using the 1, 25 D I ^{125} RIA kit from DiaSorin, Mn, USA [19].

2.8. Statistical analysis

Comparison between the control and various treatments were made by Anova. A P-value less than 0.05 was considered significant.

3. Results

3.1. The effects of JKF on the modulation of DNA synthesis and CK by femarelle in VSMC

JKF (1 nM daily for 3 days), which, by itself, decreased ^{3}[H] thymidine incorporation in VSMC [9], increased the stimulation of DNA synthesis resulting from low dose of F (2 ng/ml) (about 2–3fold) and maintained the stimulation of DNA synthesis in the presence of D (30 nM) or E_2 (0.3 nM) (Fig. 1B.). High concentrations of D (3000 nM), as well as a high concentration of E_2 (30 nM) inhibit DNA synthesis; but did not inhibit this after pre-treatment with JKF were no more inhibitory (Fig. 1B.). In contrary, the effect on cell proliferation of F at a high concentration (200 ng/ml) remained inhibitory even after pretreatment with JKF (Fig. 1C).

JKF (1 nM daily for 3 days), which increased the specific activity of CK in VSMC, further increased the stimulation of CK induced by the high doses of all the hormones tested: 200 ng/ml F as well as 3000 nM D or 30 nM E_2 increased CK by about 2–3 fold (Fig. 1A.). Similar results were also obtained at low F concentrations (data not shown).

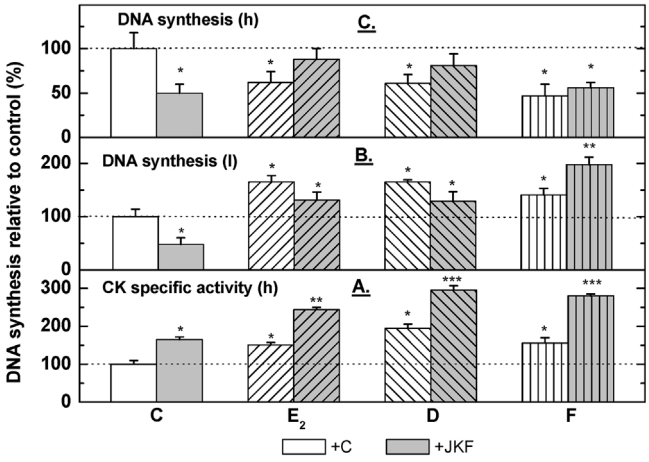

Fig. 1. Modulation by daily treatment with JKF on DNA synthesis and CK specific activity by high and low doses of F and D compared to E_2, in VSMC cells. JKF was added daily for 3 days (at 1 nM) followed by 24 h treatment with F (2 (low) or 200 (high) ng/ml), D (30 or 3000 nM) or E_2 (0.3 (i) or 30 (h) nM). Results are means ± SEM of 8–16 incubates from 2 to 4 experiments and are expressed as% change from basal ^{3}[H] thymidine incorporation in hormone-treated and of% change in basal CK specific activity in hormone-treated compared to untreated cells. *p < 0.05; **p < 0.01; ***p < 0.001 from vehicle-treated cells. The basal level of ^{3}[H] thymidine incorporation into DNA in VSMC was 8200 ± 1080 dpm/well and the basal level of CK in VSMC was 0.055 ± 0.010 mmol/min/mg.

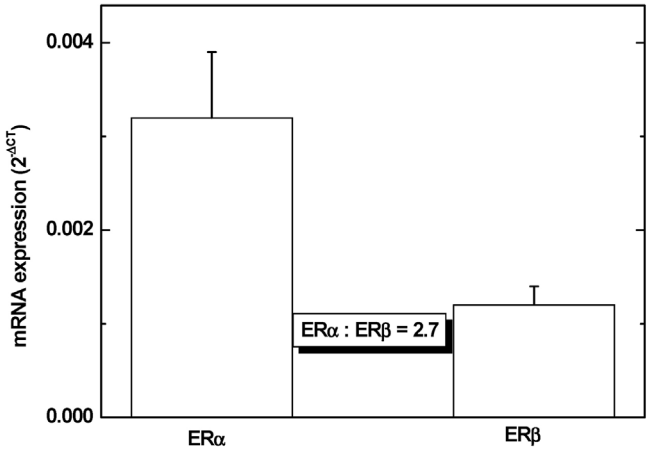

Fig. 2. Real time PCR assay of mRNA levels of ERa and ERb in VSMC cells. Conditions are explained in Materials and Methods. Results are means of 5 experiments and are expressed as number of cycles needed to get these mRNA levels (2^{-DCT}).

3.2. The effects of JKF and femarelle on ERa and ERb mRNA expression in VSMC

VSMC express mRNA for both ERa and ERb as measured by real time PCR with a higher abundance of ERa (ERa: ERb of 2.7:1) (Fig. 2). When cells were pretreated with JKF (1 nM daily for 3 days) there was a ~ 50% increase in ERa (Fig. 3A.) but a ~75% decrease in ERb (Fig. 3B.). On the other hand pre-treatment with E_2 (30 nM daily for 3 days) did not change their expression of either of the ERs (Fig. 3A. and B.). Still, pretreatment with F (20 ng/ml daily for 3 days) up regulated mRNA for both ERa [by ~200%] (Fig. 3A.) and ERb [by ~150%] (Fig. 3B.), D was similar to F in modulation of ERs.

3.3. The effects of JKF on the competitive ^{3}[H]E_2 binding by the estrogens in VSMC

VSMC cells bind specifically ^{3}[H] E_2 to intracellular sites, which are both nuclear and cytoplasmic. F as well as D or E_2 itself but not JKF competes for these specific binding sites. Pre-treatment of the cells with JKF (1 nM daily for 3 days) increased significantly the specific binding of ^{3}[H] E_2 in the presence of F (from ~70 to ~310%), D (from ~60 to ~50%) or E_2 itself (from ~70 to ~320%) (Fig. 4),

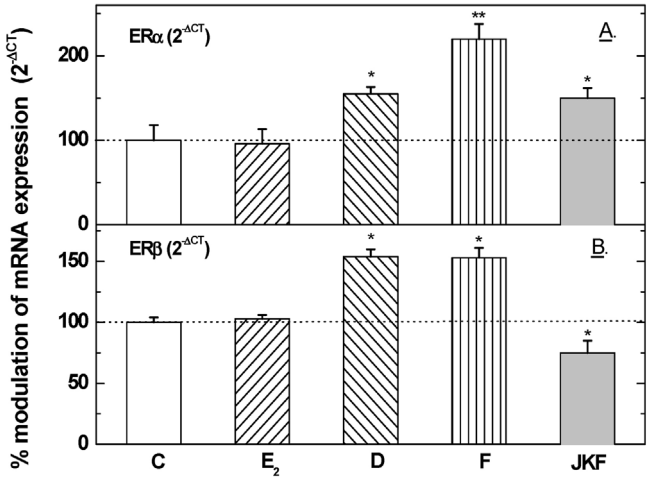

Fig. 3. The effect of daily treatment with JKF compared to E_2, D and F on the mRNA levels of ERa and ERb in VSMC cells as measured by real time PCR. JKF was added daily for 3 days (at 1 nM) E_2 (at 3 nM), D (30 nM) and F (at 20 ng/ml). Results are means of 4 experiments and are expressed as% change in mRNA levels compared to untreated cells; *p < 0.05; **p < 0.01.

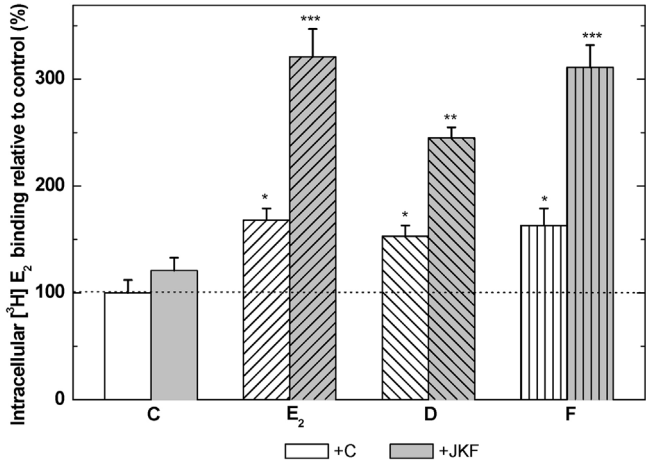

Fig. 4. The effect of daily treatment with JKF on intracellular binding of F, D or E_2 in VSMC cells, as measured by competitive binding of ^{3}[H] E_2. Results are means of 4 experiments and are expressed as% modulation of the binding in JKF treated cells compared to untreated cells; **p < 0.01.The basal level of E_2 binding was 2500 ± 250dpm/well.

3.4. The effects of femarelle on VDR, 1OHase expression and 1,25D production in VSMC

VSMC express VDR and 1OHase mRNA as demonstrated by Real Time PCR. These cells also produce 1,25D. In cellstreated daily for 3 days with 20 ng/ml F, 30 nM D or 3 nM E_2, VDR mRNA was increased by 120, 100 and 50% respectively (Fig. 5A), 1OHase mRNA is increased by 130%, 100% and 50% respectively (Fig. 5B). Basal production of 1, 25D in VSMC was 1.25 + 0.18 pmoles/mg protein. E_2 (30 nM for 24 h) did not affect the production of 1, 25D whereas F (200 ng/ml for 24 h) as well as D (3000 nM for 24 h) increased the production of 1,25 D by 250% and 90% respectively (Fig. 5C)

4. Discussion

We have previously found that the exposure of the human vascular cells to phytoestrogens results in different effects on cell growth [15,16] such as clear inhibition of VSMC growth, but in contrast stimulation of endothelial cell replication [15,16]. It appears that these compounds accelerate cell growth in some cell types but inhibit cell proliferation in others.

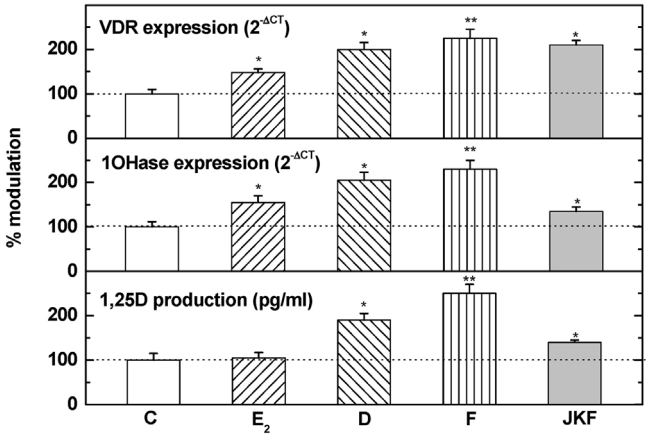

Fig. 5. The effect of daily treatment with F (20 ng/ml), D (30 nM) or E_2 (3 nM) for 3 days on the mRNA levels of VDR, 1OHase mRNA in VSMC cells as measured by real time PCR and on the enzymatic conversion of 25(OH)D_3 into 1,25D after 24 h treatment with F (200 ng/ml), D (3000 nM) or E_2 (30 nM). Results are means of 4 experiments and are expressed as% change compared to untreated cells; *p < 0.05; **p < 0.01. The basal level of 1,25D is 18.06 + 0.29 pg/ml.

The heterogeneity of the effect of the different phytoestrogens on human VSMC growth, probably depend on the interaction of each of the phytoestrogen with the variety of the estrogen receptors isoforms. ERb is probably predominantly expressed in human VSMC [6]. But there is also evidence that estrogen-induced inhibition of VSMC growth may take place even in the absence of either estrogen receptors, as was shown in either ERa or ERb knockout mice, in which the myointimal proliferative response to intimal injury can still be reduced by E_2 [7]. On the other hand, some phytoestrogens could affect DNA synthesis by estrogen receptor-independent mechanisms such as via the inhibition of tyrosine kinases [24] or protein kinase C [20], or via antioxidant activity [21] which leads to altered NO/O_2^- ratio. The inhibition of VSMC replication may retard atherogenesis while enhancement of DNA synthesis may promote myointimal proliferative response to injury. This requires caution in indiscriminate extrapolation of estrogenic properties to phytoestrogens with respect to cardiovascular disease.

VSMC cells express high affinity receptors of 1, 25$(OH)_2D_3$ suggesting that the arterial wall is a target for vitamin D action [18,22]. Vitamin D analogs were found by several reports to either suppress or stimulate [27] cell proliferation in vascular cells. We found [18] that vitamin analogs including JKF inhibit DNA synthesis in VSMC [18]. Vitamin D analogs also interact differently with E_2 and some phytoestrogens [18]. This heterogeneity of interaction could result from differences in estogenic/phytoestrogenic effects on ERs expression, such as an increase in ERa and a decrease in ERb [25].

In the present study, the inhibitory effect of F on VSMC growth [26,27] was not affected by JKF, which differs from the inhibitory effects of E_2 and D, both of which were aborted by JKF. Whether or not this results from ligand specific interaction profiler with the various known estrogen receptors is presently unclear and requires further elucidation. Pretreatment with JKF slightly increased the nuclear binding of E_2 and D, but abolished the membranal binding of E_2 [18].

The effects of vitamin D and vitamin D analogs in human vascular cells responses, including their interaction with phytoestrogens, are probably modulated via classical vitamin D nuclear receptors [23]. In a previous report we showed that vitamin D analogs induce changes in both nuclear (ERa and ERb) and non-nuclear estrogen receptors/binding elements [18]. Other mechanisms may also play a role, including interactions with co activators or co repressors of the different receptors [29]. Hence, further studies will be needed to elucidate the mechanism of action before the new combinations of the compounds can be assumed to be vasculoprotective.

Further, in was reported that the receptor isoform that is mainly expressed in human VSMC is ERb although the quantities of both ERs are quite similar [26]. But, there is evidence that estrogen- induced inhibition of VSMC growth may take place even in the absence of either estrogen receptors, since in either ERa or ERb knockout mice, the proliferative response of the myointima to injury can still be reduced by E_2 [27].

Here we show that JKF and F independently affect DNA synthesis and CK activity in human VSMC cells. The effects of JKF on human vascular cells responses to F and other estrogens like D are probably modulated via classical vitamin D nuclear receptors [17] resulting in changes in both ERa and ERb, and also by modulating interactions with co activators and/or co repressors of the different receptors. Obviously further studies will be needed to elucidate the exact mechanism before the combinations of these compounds might be assumed to be vasculoprotective.

The synthesis of 1,25 $(OH)_2D_3$ (1,25D) from its precursor 25$(OH)D_3$, is catalyzed by 1OHase, an enzyme most abundantly expressed in cells of the nephron [19]. Renal 1OHase is subject to tight systemic metabolic control by calciotrophic hormones and by 1,25D itself. Nevertheless, extra renal production of 1,25D and/or discrete expression of 1OHase are now well documented in several other human cell types [28]. While the function of 1OHase, in many of these extra renal tissues is presently undefined, it appears that 1OHase is differentially regulated in some of these sites.

VSMC are likely sensitive to the effects of the circulating 1,25D. Additionally, it appears that a locally regulated generation of 1,25D might provide yet another route to directly affect VSMC independent of systemic vitamin D [19]. Thus, regulation of 1OHase expression and activity in VSMC by several estrogens, offers a potentially important mechanism to further modulate the intracellular production rate of 1,25D. Further, locally generated 1,25 D might inhibit VSMC cell growth but clearly increases metabolic turnover as assessed by the CK activity [18,19]. That F modulates 1OHase expression and activity in VSMC complements observations that estrogen increases circulating levels of 1,25D in post-menopausal women [29] although the mechanism underlying these *in vivo* changes remains unexplored. The present experiments show that the expression of an active 1OHase in human VSMC can be also up regulated by F, which complements our previous report that this system is subject to regulation by native estrogens. As active vitamin D, in turn, inhibits VSMC proliferation, the potential role of this system as an autocrine mechanism to modulate VSMC growth and differentiation merits further investigation.

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