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A non-calcemic Vitamin D analog modulates both nuclear and putative membranal estrogen receptors in cultured human vascular smooth muscle cells☆

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Abstract

In cultured human vascular smooth muscle cells (VSMC), estradiol-17β (E_2) induced a biphasic effect on DNA synthesis, i.e., stimulation at low concentrations and inhibition at high concentrations. Additionally, E_2 increased the specific activity of creatine kinase (CK) in these cells. Observations that novel protein-bound membrane impermeant estrogenic complexes could elicit inhibition of DNA synthesis, suggested interaction via membranal binding sites. Nevertheless other effects, such as increasing CK activity were only seen with native E_2 but not with E_2–BSA, thus indicating that the classical nuclear receptor pathway was involved. In the present report, we confirm that human VSMC express both ERα and ERβ. Further, pretreatment of cultured VSMC with the Vitamin D non-calcemic analog JK 1624 F2-2 (JKF) increased ERα mRNA (100–200%) but decreased ERβ mRNA (30–40%) expression as measured by real time PCR. ERα protein expression assessed by Western blot analysis increased (25–50%) in parallel, whereas ERβ protein expression declines (25–55%). Using ovalbumin bound to E_2 (Ov–E_2) linked to Eu (Eu–Ov–E_2), to assess specific membrane binding sites, we observed that membranal binding was down regulated by JKF by 70–80%. In contrast, total cell binding of ^{3}[H] E_2, that nearly entirely represents intracellular E_2 binding, was increased by 60–100% by the same Vitamin D analog. The results provide evidence that the effects of JKF on ERα/ERβ as well as on membranal versus nuclear binding of estrogen are divergent and show differential modulation.

Keywords: Estrogen receptors α and β; Membranal binding; Non-hypercalcemic Vitamin D analogs; Estrogen; Phytoestrogens

1. Introduction

We have previously reported that estradiol-17β (E_2) and some phytoesrogens exert a biphasic effect on human vascular smooth muscle cells (VSMC) growth, such that low concentrations (in the range observed in men and post-menopausal women) increase, whereas higher concentrations (characteristic of reproductive females) decrease DNA synthesis in cultured VSMC [1–3]. That the inhibitory effect of E_2 on VSMC proliferation possibly depends on membrane-associated signaling was suggested by the finding that it requires the activation of MAP-kinase activity [4], and could be elicited by membrane impermeant estrogen or phytoestrogens conjugates. In a previous report, we also found that Vitamin D non-calcemic analogs modulated the effects of E_2 on VSMC [2].

The present study was undertaken to determine whether the vitamin D non-calcemic analog JK 1624 F2-2 (JKF) [5], modulates E_2- or phytoestrogens-dependent DNA synthesis in human vascular cells, via effects on the expression of estrogen receptors.

2. Materials and methods

2.1. Cell culture

Vascular smooth muscle cells were prepared from human umbilical artery as previously described with minor modifications [1,6].

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2.2. Association of the ^{3}[H] estradiol with vascular cells

Cells were incubated with ^{3}[H] E_2 [7] 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] E_2 was determined using a scintillating counter as described before [7].

2.3. Association of the membrane impermeant, estradiol–Ov conjugate labeled with europium with vascular cells

Cells were incubated with the steroid protein conjugates estradiol–ovalbumin (E_2–Ov) [8] labeled with Eu (Eu–Ov–E_2) at 4 °C for 90 min. Specific binding was defined as binding displaced by unlabeled E_2–BSA. Binding was determined by time-resolved fluorescence determination using an Arcus time resolved fluorometer (Wallac, Turku, Finland) as described before [9].

2.4. Estrogen receptor expression in vascular cells

Expression of ERα and ERβ in VSMC extracts was assessed by Western blotting using Anti-ERα antibodies (clone 13H2), kindly provided by Dr. H. Thole, Germany and polyclonal anti-ERβ antibodies from Dr. N. Ben-Jonathan from the University of Cincinnati. Additionally, ERα/ERβ mRNA was quantified using real time PCR (Applied Biosystems 7700).

2.5. Statistical analysis

Differences between mean experimental values and the control groups were evaluated by analysis of variance (ANOVA). A *P*-value less than 0.05 was considered significant.

3. Results

Human vascular smooth muscle cells (VSMC) treated with E_2 showed a concentration-dependent increase in creatine kinase (CK) activity [2–4]. In VSMC pretreated for 3 days with JKF (1 nM), the CK specific activity response to E_2 (30 nM) increased by 230 + 28%. Pretreatment with JKF also modulated the effect of E_2 on DNA synthesis: the E_2-induced stimulation seen at low concentrations of E_2 (0.3 nM) was further increased by 75 ± 13%. In contrast, the inhibitory effect of E_2 exerted at a high concentration of E_2 (30 nM) was attenuated by 58 ± 13%.

Western immunobloting of cell extracts detected the presence of both ERα (67k) and ERβ (53k). In cells pretreated with JKF, the expression of ERα protein increased by 50% whereas ERβ protein expression decreased by 55% [7].

Table 1
Modulation by JKF of cytoplasmic/nuclear and membranal estrogen binding in human vascular cells

Percentage change in membranal binding after JKF treatment (Eu–Ov–E_2)		Percentage change in nuclear/cytoplasmic binding after JKF treatment (^{3}[H] E_2)	
+C	0 ± 8	+C	0 ± 5
+E_2–BSA	−70 ± 8	+E_2	102 ± 20
+CD–Ov	−79 ± 13	+D	60 ± 20
+Ral	6 ± 13	+Ral	50 ± 18
+ICI	−8 ± 13	+ICI	60 ± 15
+T–BSA	4 ± 12	+DHT	0 ± 10

Cells were incubated with JKF (1 nM for 3 days) or vehicle and then binding assays were performed. For membrane binding, cells were incubated with Eu–Ov–E_2 in the absence or presence of 500-fold excess of one of the following agents: vehicle (C), estradiol–BSA (E_2–BSA), carboxy daidzein–ovalbumin (CD–Ov), raloxifene (Ral), ICI 16480 (ICI) or testosterone–BSA (T–BSA). Results are expressed as percentage of Eu–Ov–E_2 binding in the presence or absence of any competitor. JKF by itself decreased total membranal binding by 30–40%. For intracellular (cytoplasmic/nuclear) binding competition was performed by 500-fold excess of estradiol 17β (E_2), daidzein (D), raloxifene (Ral), ICI 16480 (ICI) as well as dihydrotestosterone (DHT). Results are expressed as percentage of ^{3}[H] E_2 binding in the presence or absence of any competitor. JKF by itself increased total nuclear/cytoplasmic binding by 30–45%. Results are mean ± S.E.M. of three experiments each performed in triplicates. $^*P < 0.05$, $^{**}P < 0.01$ for the comparison with total binding C (no competitors). Data were assessed by ANOVA.

JKF also increased ERα mRNA, as assessed by real time PCR by 100–200%. In parallel to the effects of JKF on ERβ protein expression, VSMC treated with JKF also had decreased ERβ mRNA expression (−30 to 45%) compared with untreated cells.

Intact VSMC display specific binding sites for Eu–Ov–E_2: Eu–Ov–E_2 binding can be displaced by unlabeled E_2–BSA, the phytoestrogenic derivative–macroprotein complexes carboxy genistein–Ov (CG–Ov) or carboxy daidzein–Ov (CD–Ov), or the estrogen antagonist ICI 16480 (ICI), but not by the SERM raloxifene (Ral) or by testosterone–BSA (T–BSA). JKF (1 nM for 3 days) by itself, decreased total binding of Eu–Ov–E_2 in VSMC by 30–40%, but moreover it decreased specific binding of Eu–Ov–E_2 in VSMC by 70–80% (Table 1).

Free ^{3}[H] E_2 binding to VSMC could be displaced by excess unlabeled estradiol-17β, the phytoestrogen daidzein (D), ICI 16480 (ICI) or raloxifene (Ral), but not by dihydrotestosterone (DHT). JKF (1 nM for 3 days) by itself, increased specific ^{3}[H] E_2 binding in VSMC by 80–105% (Table 1).

4. Discussion

The present report confirms and extends our previous observations that JKF modulates both the CK and the growth responses to E_2 in human VSMC [2]. Further, these effects can now be examined in the context of the effects of JKF

on ERs expression. First, JKF increased ERα and decreased ERβ protein expression. This was associated with parallel effects on ER mRNA as quantified by real time PCR; i.e., JKF markedly increased ERα mRNA, but slightly decreased ERβ mRNA in these cells. Since JKF increased the CK response and the growth enhancing response to E_2, it is possible that these effects in VSMC, which are apparently modulated via classical nuclear receptor pathways, are exerted via ERα, the expression of which is also upregulated by JKF. In contrast, JKF blocks the inhibitory effect of high concentrations of E_2 on ^{3}[H] thymidine incorporation in VSMC in association with down regulation of ERβ expression. Hence, it appears plausible that E_2-induced inhibition of VSMC is mediated via ERβ.

Pretreatment with JKF modified not only ERα/ERβ expression, but also the binding of Eu–Ov–E_2 to VSMC. Eu–Ov–E_2 binding, apparently representing putative membranal receptors was down regulated, whereas free E_2 binding, presumably reflecting cytoplasmic/nuclear E_2 binding was upregulated in JKF-treated VSMC. Although the data presented herein do not provide definitive characterization of the ER subtype(s) responsible for either the membranal or the nuclear binding, the parallel JKF-induced increases in ERα and nuclear receptor binding activity, suggest that classical nuclear ERα receptors are affected. Further, since JKF inhibited both ERβ expression and membranal binding activity, it is possible that ERβ operate at the cell membrane as well.

Overall, the results provide evidence for the presence of membrane estrogen binding in addition to nuclear estrogen receptors, and suggest that these divergent binding sites are differentially modulated by JKF.

References

[1] D. Somjen, F. Kohen, A. Jaffe, Y. Amir-Zaltsman, E. Knoll, N. Stern, Effects of gonadal steroids and their antagonists on DNA synthesis in human vascular cells, Hypertension 32 (1998) 39–45.

[2] D. Somjen, F. Kohen, Y. Amir-Zaltsman, E. Knoll, N. Stern, Vitamin D analogs modulate the action of gonadal steroids in human vascular cells in vitro, Am. J. Hypertens. 13 (2000) 396–404.

[3] D. Somjen, E. Knoll, F. Kohen, N. Stern, Effects of phytoestrogens on DNA synthesis and creatine kinase activity in vascular cells, Am. J. Hypertens. 14 (2001) 1256–1266.

[4] D. Somjen, C.J. Paller, F. Kohen, B. Gayer, E. Knoll, N. Stern, Chronic exposure to high glucose blocks estradiol-induced proliferation and ERK1/ERK2/ERK7 activation in human vascular cells: a comparison with raloxifene, in: Proceedings of the 83rd Annual Meeting of The Endocrine Society, Denver, Colorado, 2001, p. 209.

[5] G.H. Posner, J.K. Lee, Q. Wang, S. Peleg, M. Burke, H. Brom, P. Dolan, T.W. Kensler, Noncalcemic, antiproliferative, transcriptionally active, 24-fluorinated hybrid analogues of the hormone 1α,25-dihydroxyvitamin D_3. Synthesis and preliminary biological evaluation, J. Med. Chem. 41 (1998) 3008–3014.

[6] R. Limor, G. Weisinger, S. Gilad, E. Knoll, O. Sharon, A. Jaffe, F. Kohen, E. Berger, B. Lifshizt, N. Stern, A novel form of platelet type 12-lipoxygenase mRNA in human vascular smooth muscle cells, Hypertension 38 (2001) 864–871.

[7] D. Somjen, F. Kohen, B. Gayer, O. Sharon, R. Limor, T. Kulik, E. Knoll, N. Stern, Role of putative membrane receptors in the effects of estradiol on human vascular cells growth, J. Steroid Biochem. Mol. Biol., submitted for publication.

[8] F. Kohen, S. Bauminger, H. Lindner, Preparation of antigenic steroid–protein conjugates, in: E.D.H. Cameron, S.G. Hillier, K. Griffiths (Eds.), Steroid Immunoassay, Alpha Omega publishing, Cardiff, 1975, pp. 11–31.

[9] O. Mazor, M. Hillairet de Boisferon, A. Lombet, A. Gruaz-Guyon, B. Gayer, D. Skrzydelsky, F. Kohen, P. Forgez, A. Scherz, W. Rostene, Y. Salomon, Europium-labeled epidermal growth factor and enurotensin: novel probes for receptor-binding studies, Anal. Biochem. 301 (2002) 75–81.