Vitamin D Analogs Modulate the Action of Gonadal Steroids in Human Vascular Cells In Vitro

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We have previously reported that estradiol (E2) and dihydrotestosterone (DHT) regulate cell growth in human umbilical arterial smooth muscle cells (SMC) and in an endothelial cell line (E304). In SMC both gonadal steroids stimulated DNA synthesis at low concentrations and suppressed 3[H] thymidine incorporation at high concentrations, whereas in E304 cells E2 and DHT dose dependently enhanced DNA synthesis. In both cell types gonadal steroids also induced the specific activity of creatine kinase BB (CK). Previous evidence suggests that the in vitro and in vivo CK responses to gonadal steroids in bone cells are upregulated by pretreatment with vitamin D analogs due to increased level of cellular estrogen receptors (ER). Here we analyzed the interaction of the vitamin D analogs hexafluorovitamin D (FL), JK-1624 F2-2 (JKF), and CB 1093 (CB) with gonadal steroids in regulating DNA synthesis and CK activity in human vascular cells in vitro. In E304 cells, daily treatment with FL, JKF, or CB (1 nmol/L for 3 days) increased DNA synthesis by 110±66%, 65±16%, and 88±23% respectively. In contrast, the same analogs inhibited 3[H] thymidine incorporation by 52±21%, 46±19%, and 50±10%, respectively, in SMC. In both cell types all three analogs increased CK by 25% to 75% and amplified the CK response to E2 and to DHT by twofold to threefold. In E304 cells the vitamin D analogs also increased DNA response to gonadal steroids from 50% to 60% to 200% to 280%. In SMC these analogs did not modify the DNA synthetic response to a low E2 concentration, but prevented the suppression of DNA synthesis exerted by high concentrations of E2 and DHT. Vitamin D inhibitors known to block cellular calcium mobilization, had no effect on the proliferative activity induced by vitamin D analogs. However, the inhibitor of the nuclear effects of vitamin D, ZK 159222, blocked the stimulatory effects of CB on DNA synthesis in E304 cells. Finally, both 1,25(OH)2 D3 and JKF decreased the expression of ERβ proteins in SMC and increased the ERA isoform in E304 cells by 40% to 75%. The results indicate that vascular cells are targets for both vitamin D and gonadal steroid action and suggest a possible interaction between these hormones in the regulation of cell proliferation via modulation of vascular ER or interaction with proteins associated with ER.


KEY WORDS: Vitamin D, estradiol, androgens, vascular smooth muscle cells, endothelial cells.

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We have previously reported that 17β estradiol (E₂) and dihydrotestosterone (DHT) regulate cell growth in human umbilical arterial smooth muscle cells (SMC) and in a human endothelial cell line (E304).¹ In SMC both gonadal steroids had a biphasic effect, such that DNA synthesis was stimulated at low concentrations and inhibited at high concentrations.¹ In E304 cells E₂ and DHT dose dependently enhanced DNA synthesis.¹ Additionally, in both cell types E₂ and DHT stimulated, in a dose-related manner, the activity of the brain isozyme of creatine kinase (CK), an enzyme linked to cell metabolism and steroid hormone action in many cell types.²³ Earlier reports indicated that the response of bone and cartilage cells to gonadal steroids is upregulated by pretreatment with vitamin D metabolites and their nonhypercalcemic analogs,⁴⁻⁷ presumably secondary to increased expression of estrogen receptors (ER).⁷⁻⁸ In contrast, in the uterus cellular CK responses to E₂ were downregulated by vitamin D in association with a slight decrease in ERα. These observations lend support to the concept that vitamin D may be a modulator of gonadal steroid action in several sex-steroid–responsive tissues.

Relative or frank sex hormone deficiency is a hallmark of human aging.¹⁰ Likewise, a low vitamin D status is quite common in older subjects.¹¹ Indeed, vitamin D supplementation in the form of multivitamins, vitamin-D–enriched dairy products, or prescribed medications is widespread in Western countries. Thus, combined sex hormone and vitamin D replacement therapy is a common practice in older patients. The cardiovascular implications of this combination in human subjects have not been explored. In the present study we examined the effects of several nonhypercalcemic vitamin D analogs on [³H] thymidine incorporation and CK activity in human SMC and E304 cells in the presence and absence of gonadal steroids. The results indicate that vitamin D analogs are, by themselves, modulators of DNA synthesis in human vascular cells and suggest that they modify the effects of gonadal steroids on [³H] thymidine incorporation in both SMC and endothelial cells.

**MATERIALS AND METHODS**

**Experimental Procedures**

**Reagents** All reagents used were of analytical grade. Chemicals, steroids, and the CK assay kit were purchased from Sigma Chemical Co. (St. Louis, MO).

Anti-ERα antibodies (clone 13H2) were kindly provided by Dr. H. Thole, Germany. Polyclonal anti-ERβ antibodies were the kind gift of Dr. N. Ben-Jonathan from the University of Cincinnati. 1Chloro β25 (OH) vitamin D₃ (HL) was a gift of GBF, Braunschweig Germany, and ZK 159222 was kindly provided by the Schering Co., Berlin, Germany. The secondary antibodies were rabbit antimouse IgG1 peroxidase (Zymed, San Francisco, CA), goat antimouse peroxidase, and goat antirabbit peroxidase (Sigma, Rehovot, Israel). The enhanced chemiluminescence reagents were from Amersham (Amersham, Buckinghamshire, UK).

**Cell Cultures**

**Umbilical Artery Smooth Muscle Cells (SMC)** Umbilical SMC were prepared as previously described, with minor modifications.¹¹ In brief, umbilical cords were collected shortly after delivery, cleaned of blood and adventitia, and cut into tiny slices (1–3 mm). The segments were kept in culture in medium 199 containing 20% fetal calf serum (FCS), glutamine, and antibiotics. Cell migration was detected within 5 to 7 days.

Cells were fed twice a week and, upon confluence, trypsinized and transferred to 24-well dishes. Cells were used only at passages 1 to 3, when expression of smooth muscle action was clearly demonstrable.¹¹

**Endothelial Cells (E304)** E304 cells, an endothelial cell line derived from a human umbilical vein, were purchased from ATCC and grown in medium 199 containing 20% FCS, glutamine, and antibiotics.¹

**Pretreatment With Vitamin D Analogs and Inhibitors** Cells were seeded as described and treated daily for 3 days with 1 nmol/L of 1,25 (OH)₂ D₃ hexafluoro vitamin D (FL)⁶⁻⁷ JK-1264 F₂-2 (JKF)¹³ or CB 1093 (CB).⁴ In other experiments, either the inhibitor of the membranous effects of vitamin D, HL, or the inhibitor of its nuclear action, ZK 159222 (ZK), were used. On day 4 the cells were treated with E₂, DHT, or 3000 nmol/L raloxifene (RAL) for 24 h for the assessment of DNA synthesis or 4 h for the measurement of CK activity.⁷

**Assessment of DNA Synthesis** Cells were grown until subconfluence and then treated with various hormones or agents as indicated. Twenty-two hours later, [³H] thymidine was added for 2 h. Cells were then treated with 10% ice-cold trichloro acetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 mL of 0.3 mmol/L NaOH, samples were aspirated, and [³H] thymidine incorporation into DNA was determined.¹

**Creatine Kinase Extraction and Assay** Cells were treated for 4 h with the various hormones and agents, as specified, and were then scraped off the culture dishes and homogenized by freezing and thawing three times in an extraction buffer, as previously described.¹ Supernatant extracts were obtained by centrifugation of homogenates at 14,000 × g for 5 min at 4°C in an Eppendorf microcentrifuge. CK was determined by a coupled spectrophotometric assay described previ-
ously. Protein was determined by Coomasie blue dye binding using bovine serum albumin as the standard.

Statistical Analysis Comparisons between the control and various treatments were made by analysis of variance.

Western Blot Analysis of ER Western blot analysis of ER was performed using specific antibodies against the two forms of the receptors. Cells from treated and control incubates were homogenized in a homogenization buffer consisting of 50 mmol/L β-glycerophosphate (pH 7.3), 1.5 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L dithiotreitol, 0.1 mmol/L sodium orthovanadate, 1 mmol/L benzamidine, aprotinin (10 μg/mL), and leupeptin (10 μg/mL), and the protein content of the homogenate was determined. For Western blot analysis equal amounts (30 μg) of homogenates and 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 5% milk or 2% bovine serum albumin (BSA) membranes were incubated overnight with the antibodies diluted in TBS buffer (20 mM Tris and 0.14 M NaCl pH 7.6) containing 1% milk or 1% BSA, anti-ERα (0.5 μg/mL), and anti-ERβ (at 1:3000 dilution). Membranes were washed and processed for enhanced chemiluminescence using the ECL reagents. Signals were then quantified by densitometry.

RESULTS
Effects of Vitamin D Analogs on [3H] Thymidine Incorporation and CK Activity Daily treatment with FL, JKF, or CB (1 nmol/L) for 3 days increased DNA synthesis by 110%, 65%, and 88%, respectively, in E304 cells, whereas in SMC cells, cell proliferation was inhibited by 45% to 55% by these agents (Figure 1). In both cell types the specific activity of CK increased by 75% to 120% after daily treatment with FL, JKF, or CB (Figure 2).

Interaction Between Vitamin D Analogs and Gonadal Steroids in E304 Cells Hexafluorovitamin D, JKF, and CB increased basal [3H] thymidine incorporation and further augmented the response to E2 (30 nmol/L) and DHT (300 nmol/L) by approximately twofold to sixfold, (from 50% to 60% to 200% to 400% in the absence and presence of vitamin D analogs, respectively (Figure 3). A parallel upregulation of the response of E304 cells to gonadal steroids by vitamin D analogs was observed with respect to CK activity (Figure 4); the E2- or DHT-induced increase in CK, on the order of 60% to 80%, was further enhanced to 300% to 320% in the presence of FL, JKF, or CB (Figure 4).

Interaction Between Vitamin D Analogs and Gonadal Steroids in SMC Pretreatment with vitamin D analogs affected the response of the SMC to gonadal steroids in a complex manner. FL, JKF, and CB reduced basal [3H] thymidine incorporation but did not alter the increase induced by a low concentration of E2 (30 nmol/L) or DHT (300 nmol/L) decreased DNA synthesis by ~50%. However, in the presence of vitamin D analogs these inhibitory effect of E2 and DHT were no longer seen (Figure 3). FL, JKF, and CB also amplified the CK response to gonadal steroids (Figure 4) from ~30% to 170% to 390% (with and without pretreatment with vitamin D analogs, respectively).

Effect of the Vitamin D Analog CB on RAL-Mediated DNA Synthesis Because our previous studies showed that estrogen receptor antagonists mimic the effect of E2 on DNA synthesis in SMC and E304 cells,
we examined the effect of the vitamin D analog CB on DNA synthesis in the presence of RAL. As depicted in Figure 5, in SMC a low concentration of RAL (30 nmol/L) increased $[^3H]$ thymidine incorporation, whereas a high concentration (3000 nmol/L) decreased DNA synthesis. CB augmented the proliferative response to a low concentration of RAL and prevented the suppression of $[^3H]$ thymidine incorporation induced by a high concentration of RAL. In E304 cells, CB amplified the stimulatory effect of RAL (3000 nmol/L) on DNA synthesis (Figure 5).

**Effect of Vitamin D Analogs on Receptor Proteins for E2 (ER) in Vascular Cells** Western immunoblotting of SMC extracts detected the presence of two isoforms (32 k and 63 k) of ER$\alpha$ protein and a single 53-k band of ER$\beta$ protein. After 72 h of incubation with 1,25 (OH)$_2$ vitamin D$_3$ (added daily at 1 nmol/L), the expression of the 32-k and 63-k ER$\alpha$ peptides was unchanged, whereas ER$\beta$ levels declined by 70% to 90%; (Figure 6). Similarly, incubation with the vitamin D analog JKF (daily addition, 1 nmol/L) resulted in a 25% to 50% increase in the 63-k ER$\alpha$ associated with a 25% to 60% reduction in ER$\beta$ protein. In E304 cells, only high concentrations of E$_2$ (30 nmol/L) or DHT (300 nmol/L) were used (as in these cells gonadal steroids stimulated DNA synthesis dose dependently). DNA synthesis was assessed by $[^3H]$ thymidine incorporation. The results are means ± SEM of eight to 12 incubates from at least two different cultures and are expressed as % change from basal $[^3H]$ thymidine uptake compared with untreated cells. **P < .05, ***P < .001.

**Effect of Vitamin D Inhibitors on Vitamin D Analog Action** We used two chemically and biologically distinct inhibitors of vitamin D action, 1 chloro $\beta$25 (OH)$_2$ D$_3$ (HL), a blocker of transmembranous vitamin-D-
dependent calcium influx, and ZK 159222, an inhibitor of the nuclear action of vitamin D. Neither had an independent effect on DNA synthesis in either SMC or E304 cells and, similarly, these two inhibitors did not modify the effect of estradiol on DNA synthesis. In E304 cells, only the nuclear blocker of vitamin D effects, ZK 159222, (but not the membranous inhibitor HL; data not shown) prevented the amplification of E2-dependent DNA synthesis induced by the vitamin D analog CB (Figure 7). In SMC, after pretreatment with CB, high E2 concentrations no longer prevented DNA synthesis in SMC (Figure 7). When the vitamin D nuclear antagonist ZK 159222 was also added—so that cells were pretreated with both the vitamin D analog and its nuclear antagonist, followed by high concentration of E2—DNA synthesis was actually stimulated, an effect seen with low E2 concentrations alone. In contrast, the membranous antagonist of vitamin D action HL did not modify the interaction between the vitamin D analog and high concentrations of E2 (data not shown).

**DISCUSSION**

Smooth muscle cells express high-affinity receptors for 1,25(OH)2 vitamin D3, which suggests that the arterial wall may be a target for vitamin D action. Classic nuclear receptors of vitamin D could mediate several reported vascular effects of this hormone, such as increased expression of Ca-ATPase, induction of contractile protein synthesis, and hence, increased arterial resistance. That vitamin D may also be a modulator of vascular wall growth has been suggested by several reports that 1,25(OH)2 vitamin D3 either suppresses or stimulates [3H] thymi-
To our knowledge, this is the first study addressing the effect of vitamin D on growth in human vascular cells.

A key finding in this study is that vitamin D analogs exert opposite effects on human SMC and endothelial cells, ie, they inhibit growth in SMC while stimulating \[^{3}H\] thymidine incorporation in E304 cells (Figure 1). The observation that the analogs are inhibitors of cell growth in human SMC is in agreement with a previous report that 1,25(OH)\(_2\)D\(_3\) inhibits growth in human mesangial cells, presumably a uniquely differentiated form of SMC. Extrapolation of these dual endothelial-SMC effects to the in vivo situation would suggest that vitamin D analogs provide a substantially different research opportunity in vivo, because their calcemic effect is negligible.

The major finding in this study is that vitamin D analogs modulate the effects of gonadal steroids on vascular cell growth in a complex and cell-specific manner. In E304 cells, vitamin D analogs enhanced E\(_2\) and DHT-induced \[^{3}H\] thymidine incorporation. Because pretreatment with each of the analogs tested alone increased \[^{3}H\] thymidine incorporation by 60% to 110%, and the response to estrogens seen in analog-treated cells rose, in relative terms, by 100% to 180% compared with \[^{3}H\] thymidine incorporation into DNA with the various treatments, compared with that seen in untreated cells. *P < .01, **P < .05, ***P < .001.
ure 3 for DNA synthesis and in Figure 4 for CK activity. To the extent that enhanced endothelial cell replication favors vascular protection, the combination of vitamin D analogs and E₂ may offer an advantage over E₂ alone.

In SMC, FL, JKF, and CB did not affect the stimulatory influence seen with low concentrations of gonadal steroids, yet they prevented the suppression of DNA synthesis exerted by high doses of E₂ and DHT. This disinhibition of SMC growth was, in essence, duplicated when RAL (an estrogen agonist in this system) rather then E₂ was present, thus further confirming that vitamin D analogs block the inhibitory effects on SMC growth exerted by high concentrations of estrogentic agents (Figures 3, 5). Of note is the observation that a nuclear inhibitor of vitamin D action (but not an inhibitor of its membranous effects) prevented the analog-induced enhancement of the stimulatory effect of E₂ in E304 cells (Figure 7). This suggests that the analog-dependent augmentation of [³H]thymidine incorporation and CK activity induced by E₂ in endothelial cells was mediated by classic nuclear routes of vitamin D action. It is not clear why the disinhibition of the suppressive effect of E₂ on DNA synthesis exerted by vitamin D analogs in SMC was not reversed by the inhibitor, such that the combination of the vitamin D agonist and its nuclear antagonist resulted in stimulation of growth after high concentrations of E₂ (rather than the restoration of the inhibitory effect of high concentrations of E₂; Figure 7). In fact, this effect resembled the induction of growth seen in SMC with low concentrations of E₂.

One potential mechanism by which vitamin D analogs may modify the vascular actions of estrogens is via modulation of ER expression. Indeed, there are precedents for such interactions in bone-marrow–derived stromal cells, as well as in bone and cartilage cells. Results depicted in Figure 6 appear consistent with this possibility; 1,25(OH)₂ vitamin D₃, as well as its analog JKF, reduced the expression of ERβ protein in SMC, but not in E304 cells. Observations that the inhibitory effect of estrogen on SMC proliferation is preserved in ERα knockout mice suggest that the ERβ isoform is involved in E₂-mediated suppression of SMC growth. If the ERβ isoform is also involved in the inhibitory effect of E₂ on DNA synthesis in human SMC, its downregulation by vitamin D analogs (Figure 6) could negate this effect of E₂. How the observed increase in the ERα isoforms induced by 1,25(OH)₂ vitamin D₃ and its analog in E304 cells relates to the enhancement of E₂-dependent growth attained by these agents cannot be determined based on these preliminary observations.

In summary, vitamin D analogs interact with gonadal steroids in a dose- and cell-specific manner. Vitamin D analogs modulate the effects of E₂ on DNA synthesis, apparently via classic nuclear routes of vitamin D action. Downregulation of ERβ expression and upregulation of ERα protein by vitamin D analogs may contribute to the interaction between these agents and estrogens in human vascular cells.

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