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Anti-endothelial properties of 1,25-dihydroxy-3-epi-vitamin D_3, a natural metabolite of calcitriol☆

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Abstract

Background: Calcitriol [1,25-$(OH)_2D_3$] is a strong anti-proliferative agent both in vitro and in vivo. Earlier studies have established that calcitriol inhibits the growth factor-stimulated proliferation of endothelial cells (EC) and angiogenesis. However, the lethal calcemic side effects of calcitriol prohibit its use as a therapeutic agent. Several analogs of vitamin D have been developed to minimize these calcemic side effects. 1,25-dihydroxy–3–epi–vitamin D_3 (3-epiD_3), a naturally formed vitamin D metabolite is one such analog.

Objective: To demonstrate that 3-epiD_3, a calcitriol analog, inhibits endothelial cell proliferation and induces apoptosis.

Results: Treatment of EC with 3-epiD_3 showed 60% inhibition ($P < 0.006$) of proliferation. Cell viability assays corroborated these results. Pro-apoptotic caspase-3 activity was increased fourfold ($P < 0.01$) in 3-epiD_3-treated cells over controls. 3-epiD_3 induced apoptosis in EC as shown by genomic DNA fragmentation. Cell cycle analysis of 3-epiD_3-treated EC revealed a G0/G1 arrest.

Conclusions: 3-epiD_3, a low-calcemic, natural analog of calcitriol, inhibits EC proliferation by causing a G0/G1 arrest and induces apoptosis more effectively than 1,25-$(OH)_2D_3$. These results suggest that 3-epiD_3 is a potent inhibitor of EC growth.

Keywords: Calcitriol; 1,25-Dihydroxy–3–epi–vitamin D_3 (3-epiD_3); Endothelial cells; Proliferation inhibition; Apoptosis; Caspase-3; G0/G1 arrest

1. Introduction

The active form of vitamin D, calcitriol [1,25-$(OH)_2D_3$] is a key regulator of calcium homeostasis [1] and has anti-proliferative activity in a wide variety of normal and cancer cells [2]. Recent studies have shown that calcitriol inhibits proliferation of endothelial cells and angiogenesis [3,4]. Angiogenesis is known to be required for growth of tumors larger than a few millimeters [5], and agents that selectively inhibit endothelial cells have been shown to limit tumor growth [6,7]. Furthermore, endothelial cells are attractive therapeutic targets because of their greater genomic stability and are less likely to rapidly develop resistance [8]. Expression of the vitamin D receptor (VDR) has been observed in endothelial cells [9], and calcitriol and its analogs have been demonstrated to inhibit embryonic angiogenesis in chick chorioallantoic membranes [10].

Since the use of calcitriol as a therapeutic agent is limited by its hypercalcemic effects [11], low calcemic analogs of calcitriol have been used to develop calcitriol-based therapeutic strategies [12]. 1,25-Dihydroxy–3–epi–vitamin D_3 (3-epiD_3) is a naturally formed analog of calcitriol that demonstrates low calcemic side effects [13,14]. 3-epiD_3 differs from calcitriol in the orientation of 3-OH group (Fig. 1). In the present study, we investigated the ability of 3-epiD_3 to inhibit proliferation and induce apoptosis, and its effect on cell cycle progression in human umbilical vein endothelial cells (HUVEC).

2. Materials and methods

2.1. Chemicals and reagents

Calcitriol was from Calbiochem and 3-epiD_3 was provided by Dr. G.S. Reddy (Brown University, Providence, RI). Calcitriol and 3-epiD_3 were reconstituted in ethanol at 100 μM concentration and were diluted directly into the culture media. MTS cell viability assay kit was from Promega Corporation, Madison, WI, BrdU assay kit was from Roche Diagnostics, Indianapolis, IN, and RNase A was from Sigma Chemical Company (St. Louis, MO).

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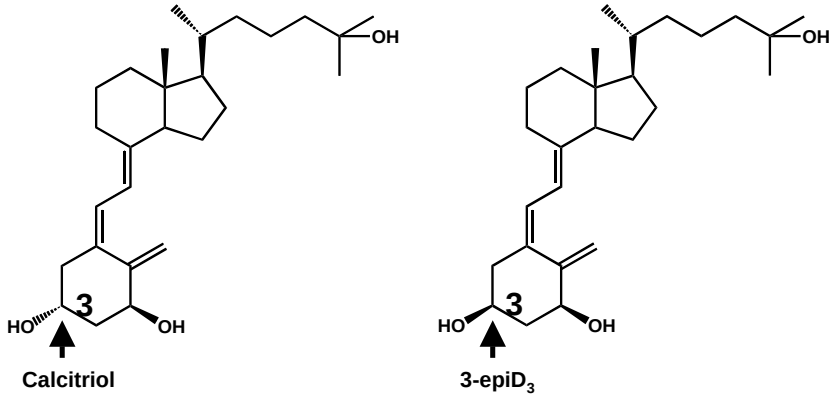

Fig. 1. Structure of calcitriol and 3-epiD_3. Calcitriol and 3-epiD_3 differ in the orientation of the hydroxyl group at 3 position (arrow mark).

2.2. Cell culture and treatment

Human umbilical vein endothelial cells were obtained from Cascade Biologics, Inc. (Portland, OR) and cultured as recommended by the manufacturer. All experiments utilized HUVEC at passage 6 or less. Cells were plated in 96-well plates or T-25 flasks and allowed to attach for 24 h, serum deprived for 24 h, and treated with the test compounds or vehicle (control) in complete medium for 48 or 72 h.

2.3. Cell viability assay

Cell viability was measured by using MTS cell viability assay kit. Briefly, cells were incubated with 0.25, 0.5, 2.5, 5, 25, 50, and 250 nM 3-epiD_3 or calcitriol in complete medium for 48 h, after which MTS reagent was added to the cultures and the optical absorbance at 490 nm was measured after 3 h using a microplate reader. Cell viability was represented as the mean percentage of absorbance before and after treatment.

2.4. BrdU incorporation assay

HUVEC were treated with 10 and 100 nM 3-epiD_3 or calcitriol as above. The effect of 3-epiD_3 on proliferation of HUVEC cells was assessed by measuring BrdU incorporation during DNA synthesis [15]. The assay was performed according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). The results are expressed as percentage BrdU incorporation.

2.5. DNA fragmentation analysis

The cells (3×10^6) were treated with 10 nM 3-epiD_3 for 72 h and lysed in 0.5 ml of lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 200 mM NaCl, 0.4% Triton X-100, and 0.1 mg/ml Proteinase K) for 20 m; extracted with phenol chloroform; DNA was precipitated in cold ethanol and analyzed on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

2.6. Cell cycle analysis

Cell cycle analysis and quantification of apoptosis were carried out by flow cytometry as described earlier [16]. Briefly, the cells were treated with 10 nM 3-epiD_3 for 72 h. The floating and adherent cells were collected, washed with PBS, fixed and permeabilized with ice-cold 70% ethanol, and stained with propidium iodide. Data acquisition and analysis were performed on a FACScan flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

2.7. Caspase activity

Apotarget Caspase-3/CPP32 kit (BioSource International Inc., Camarillo, CA) was used to determine the caspase-3 activity following the manufacturer-suggested protocol. Caspase-3 activity was expressed as absorbance at 405 nm.

2.8. Data analysis

Statistical analysis of the data was performed with linear regression analysis and one-way ANOVA followed by Fisher's protected least significant difference tests.

3. Results

3.1. 3-epiD_3 inhibits the proliferation of HUVEC

We used a tetrazolium (MTS) reduction assay and a BrdU incorporation assay to test the anti-proliferative effects of 3-epiD_3 on HUVEC. We observed a dose-dependent inhibition of HUVEC proliferation (Fig. 2). There was 78% ($P < 0.01$) inhibition of HUVEC proliferation by 3-epiD_3 at 50 nM dose while calcitriol showed only 37% ($P < 0.01$) inhibition. The IC_{50} value (concentration needed to reach 50% inhibition of proliferation) for 3-epiD_3 was 3.7 nM. Similarly, there was 75% ($P < 0.01$) inhibition of BrdU incorporation by 3-epiD_3, while calcitriol showed only 52% inhibition (Fig. 3). These proliferation studies clearly show

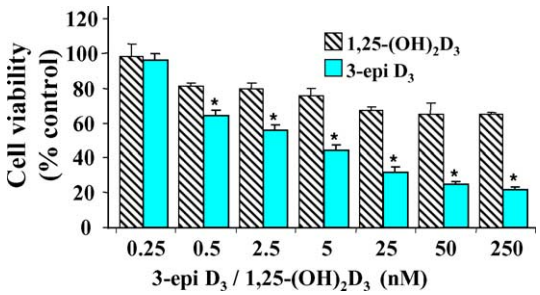

Fig. 2. 3-epiD_3 inhibits the proliferation of HUVEC. HUVEC were treated with increasing concentrations of calcitriol or 3-epiD_3 for 48 h. The cell viability was determined by using tetrazolium reduction assay as described in Section 2 ($^{*}P < 0.01$ vs. calcitriol).

that 3-epiD_3 inhibits HUVEC proliferation more efficiently compared to calcitriol.

3.2. 3-epiD_3 induces apoptosis in HUVEC

We treated HUVEC cells with 10 nM 3-epiD_3 for 72 h. Genomic DNA was isolated and analyzed for apoptotic DNA fragmentation by agarose gel electrophoresis. DNA fragments characteristic of apoptosis were observed in the case of 3-epiD_3-treated cells (Fig. 4). However, control cells did not show any DNA laddering, indicating that 3-epiD_3 induces cell death by apoptosis in HUVEC.

3.3. 3-epiD_3 activates caspase-3

It has been well established that the activation of caspase-3 is essential for apoptosis [17]. We determined the activity of caspase-3 in calcitriol and 3-epiD_3-treated cells using specific substrates as described in Section 2. 3-epiD_3 induced increased caspase-3 activity by a factor of 4.1 over the control, whereas calcitriol showed a 1.75-fold increase (Fig. 5). These studies support that the induction of apoptosis in HUVEC by 3-epiD_3 is through the caspase-mediated pathways.

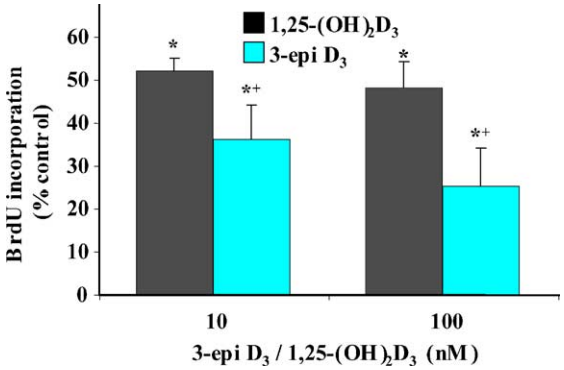

Fig. 3. 3-epiD_3 inhibits BrdU incorporation in HUVEC. HUVEC were treated with 10 or 100 nM calcitriol or 3-epiD_3 for 48 h. DNA synthesis was measured by using BrdU incorporation assay kit as described in Section 2 ($^{+}P < 0.01$ vs. calcitriol; $^{*}P < 0.01$ vs. control).

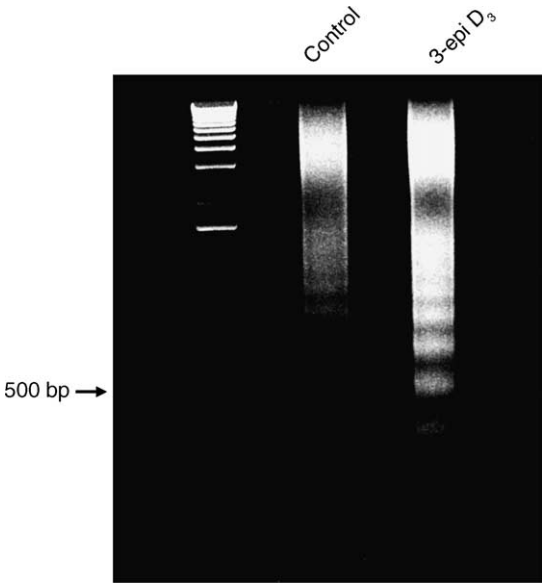

Fig. 4. 3-epiD_3 induces apoptosis in HUVEC. HUVEC were treated with 10 nM 3-epiD_3 for 72 h. Floating and attached cells were pooled and DNA was extracted as described in Section 2. The DNA was analyzed on 1.5% agarose gel along side of markers.

3.4. Cell cycle analysis of 3-epiD_3-treated HUVEC

To understand the mechanism of growth suppression of HUVEC by 3-epiD_3 and to determine the extent of apoptosis quantitatively, cell cycle analysis was performed. HUVEC were treated with vehicle control or 10 nM 3-epiD_3 for 72 h and processed for FACS analysis as described in Section 2. In vehicle-treated HUVEC, only a small fraction of apoptotic cells (5.1%) were detected (Fig. 6a). Treatment with 3-epiD_3 resulted in extensive apoptosis (25.5%) (Fig. 6b). Furthermore, 3-epiD_3 treatment increased the proportion of cells in the G0/G1 phase of the cell cycle from 58.3% (in vehicle-treated control) to 77.3% and decreased the proportion of cells in the S phase from 26.8% (in vehicle-treated control) to 10.7%, suggesting a G0/G1 block in cell cycle progression.

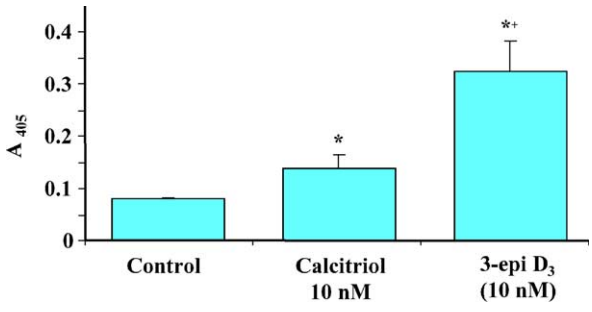

Fig. 5. 3-epiD_3 activates caspase-3. HUVEC were treated with 10 nM calcitriol or 3-epiD_3 for 72 h. Caspase-3 activity was determined using Apotarget Caspase-3/CPP32 kit after cell lysis. Caspase-3 activity is expressed as absorbance at 405 nm ($^{+}P < 0.01$ vs. calcitriol;$^{*}P < 0.01$ vs. control).

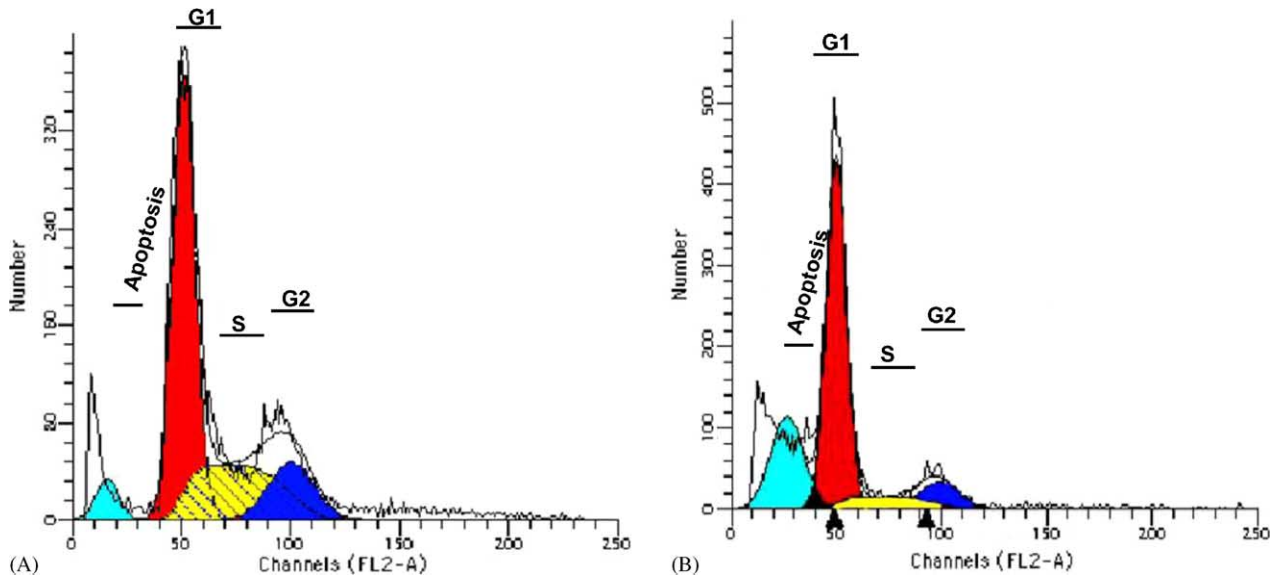

Fig. 6. Cell cycle analysis of 3-epiD_3-treated HUVEC. The cells were treated with 10 nM 3-epiD_3 or vehicle (control) for 72 h. The cells were stained with propidium iodide and subjected to cell cycle analysis as described in the Section 2. (A) Vehicle-treated control and (B) 3-epiD_3 treated.

4. Discussion

Angiogenesis is the process by which new blood vessels sprout from pre-existing vasculature. Endothelial cells play a crucial role in angiogenesis. Regulation of angiogenesis therefore carries tremendous potential in treating diseases such as cancer [18–20]. Previous studies have demonstrated that calcitriol exerts anti-proliferative activity on endothelial cells. There is also considerable data suggesting that calcitriol inhibits angiogenesis [3,4]. However, since a significant hypercalcemia induced by calcitriol is dose limiting [11], uses of analogs with reduced hypercalcemic activity have been investigated [21]. 3-epiD_3 is one such analog of calcitriol, which is formed naturally [13,14]. The calcemic side effect of 3-epiD_3 is considerably low compared to calcitriol making it a potential candidate in anti-angiogenesis-based therapeutic strategies.

Since EC demonstrate a greater genomic stability than many cancer cells, they are attractive targets for new anti-cancer agents. Additionally, the signaling pathways involved in EC apoptosis can also be targeted. Therefore, understanding the mechanism(s) by which calcitriol and its analogs inhibit angiogenesis should allow a more rational basis for designing therapies involving such agents [22]. In the current investigation, we evaluated the anti-proliferative activity of 3-epiD_3 in inhibiting EC. 3-epiD_3 was found to be a potent anti-endothelial agent; it inhibited the proliferation of HUVEC at low nanomolar concentrations and induced apoptosis. Apoptosis was preceded by a G0/G1 cell cycle block. The caspase-3 cascade pathway was involved in apoptosis induced by 3-epiD_3.

In summary, our studies show that 3-epiD_3 is a potent anti-endothelial factor. This coupled with low calcemic activity makes it a suitable molecule for developing further therapies that target angiogenesis.

Acknowledgements

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