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1α,25-Dihydroxycholecalciferol Induces Nitric Oxide Production in Cultured Endothelial Cells

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Key Words
Vitamin D • Endothelium nitric oxide • Vitamin D receptor • eNOS

Abstract
Background: Recently, 1α,25-dihydroxycholecalciferol (vitD) has received increasing interest for its effects on many tissues and organs other than bone. A number of experimental studies have shown that vitD may have an important role in modifying risk for cardiovascular disease. Aims: This study was planned to test the effects of vitD on endothelial nitric oxide (NO) production and to study the intracellular pathways leading to NO release. Methods: In human umbilical vein endothelial cells (HUVEC) cultures the effects of vitD on NO production and p38, Akt, ERK and eNOS phosphorylations were examined in absence or in presence of the NO synthase inhibitor L-NAME and protein kinases specific inhibitors SB203580, wortmannin and UO126. Results: VitD caused a concentration-dependent increase in NO production. The maximum effect was observed at a concentration of 1 nM and the optimal time of stimulation was 1 min. Effects induced by vitD were abolished by L-NAME and by pre-treatment with protein kinases inhibitors. To verify the effective involvement of vitD receptor (VDR) in the action mechanism of vitD, experiments were repeated in presence of the specific VDR ligands ZK159222 and ZK191784. Conclusions: The results of this study demonstrate that vitD can induce a significant increase in endothelial NO production. VitD interaction with VDR caused the phosphorylation of p38, AKT and ERK leading to eNOS activation.

Introduction

It is well-known that 1α,25-dihydroxycholecalciferol, the most active form of vitamin D (vitD) plays an essential role in the regulation of calcium and phosphate homeostasis and in bone development and maintenance [1]. Classically, vitD is known to exert its actions on target organs, such as intestine, kidney, parathyroid glands, and bone. In recent years, however, there has been increasing evidence that vitD has an important function in

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the activity of a number of different tissues including skeletal muscle, vascular smooth muscle, myocardium and endothelium [2-4], and exerts a beneficial effect on cardiovascular function [4, 5]. In humans, the relationship among low levels of vitD, hypercalcaemia, osteoporosis, vascular calcification and cardiovascular diseases has been extensively studied. For example, in a recent study performed on Framingham Offspring Heart Study participants [6], vitD deficiency was associated with increased cardiovascular risk, above and beyond established cardiovascular risk factors. In another study, performed on elderly subjects, an association between low serum vitD levels and high arterial blood pressure was found [7].

As far as endothelial effects are concerned, it has been demonstrated that vitD modulates vascular tone by reducing calcium influx into the endothelial cells and hence decreasing the production of endothelium-derived contracting factors [8]. Endothelial cells are capable of synthesizing vitD as results from the expression of mRNA and protein for the enzyme 25(OH)D_3-1α-hydroxylase [9]. Moreover, studies elsewhere have demonstrated the presence of intracellular vitD receptors within endothelial cells (VDR) [10]. The coexistence of these two crucial elements of vitD metabolism suggests the hypothesis of a possible autocrine/intracrine mechanism exerted by vitD as a modulator of endothelial functions [9]. Other studies performed on myelomonocytic cells [11] have shown that synthesis of vitD requires coordinated interaction with nitric oxide (NO). Endothelial NO produced by endothelial isoform of NO synthase (eNOS) plays a key role not only in cardiovascular physiology but in the pathophysiology of vascular disorders as well. For example, is known that NO have a beneficial role in atherogenesis in part by limiting vascular calcification [12].

Because of the presence of vitD and VDR in the endothelial cells and the pivotal role of NO and eNOS in the endothelial activity [13], it is conceivable an interaction between vitD and NO capable of influencing vascular function.

Although the relationship among vitD, endothelium and cardiovascular disease is well established, little is known about the effect of vitD on endothelial NO production.

Therefore, this research was carried on in HUVEC cultures in order to observe the effect of vitD administration on NO production and to study the intracellular pathways activated by vitD leading to eNOS activation. In this study, the role of VDR in endothelial NO production has been investigated as well.

Materials and Methods

Experimental chemicals

1α,25-dihydroxycholecalciferol, L-NAME, detanonoate, wortmannin, DMSO, ethanol, acetylcholine chloride, sodium orthovanadate, protease inhibitor cocktail, Hepes, NaCl, SDS, Triton-X100, sodium deoxycholate, glycerol, $MgCl_2$, EGTA, NaF, gelatin, penicillin-streptomycin, L-glutamine, Dulbecco Modified Eagle's Medium (DMEM), β-actin, horseradish peroxidase-coupled goat anti-rabbit Ig and anti-mouse Ig were obtained from Sigma-Aldrich (Milan, Italy). Endothelial growth media 2 (EGM-2), hEGF, hydrocortisone, gentamicin-amphotericin B, 2% fetal bovine serum (FBS), VEGF, hFGF, recombinant analogue insulin-like growth factor 1 human, ascorbic acid, heparin were purchased from Lonza Inc. (Basel, Switzerland). Griess reagent system, SB203580, UO126 were obtained from Promega Corporation (Madison WI, USA). DAF-FM was purchased from Molecular Probes (Eugene, OR, USA). BCA was obtained from Pierce Rockford, USA. SDS-PAGE and molecular weights were obtained from Bio-Rad Laboratories (Hercules, CA, USA). PVDF membrane was purchased from GE-Heathcare (Buckinghamshire, UK). Anti p-Akt^{Ser473}, anti-Akt, anti-pERK$^{Thr202/Tyr204}$, anti-ERK1/2, anti-p-p38$^{Thr180/Tyr180}$, anti-p38, anti-p-eNOS^{Ser1177} and anti-eNOS were obtained from Cell Signaling Technologies (Beverly, MA, U.S.A.). Western Lightning Chemiluminescence was obtained from Perkin Elmer (Boston, MA, U.S.A.). VDR ligands ZK159222 and ZK191784 were a gift from Bayer Schering Pharma AG (Berlin, Germany).

Cell cultures

HUVEC were isolated from voluntary umbilical cord donors who gave informed consent in accordance with the procedures approved by the local institutional ethics committee and according to the Declaration of Helsinki. Cells were cultured as previously described [14]. HUVEC were plated into 0.1% gelatin-coated flask in a specific culture medium (EGM-2, endothelial growth media 2) with the addition of hEGF, hydrocortisone, gentamicin-amphotericin B, 2% fetal bovine serum (FBS), VEGF, hFGF, recombinant analogue insulin-like growth factor 1 human, ascorbic acid, heparin, 2mM glutamine and 1% penicillin-streptomycin and maintained at 37°C with 5% CO_2. The cells used for the experiments were obtained from passage 3 to passage 6. HUVEC were used for NO production measurement (first set of experiments) and protein activation (second set of experiments).

NO production detection

$1x10^5$ cells were plated in gelatin-coated 24-well plates in EGM-2 complete medium in incubator since adhesion and then incubated for 4-6 h in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 2mM glutamine and 1% penicillin-streptomycin without fetal bovine serum (FBS) and red phenol (starvation medium). NO production was measured in culture supernatants in which an equal volume of Griess reagents was added following the manufacturer's instructions. After 10 min of incubation the absorbance of supernatants was measured by means of a spectrometer (BS1000 Spectra Count) at 570nm.

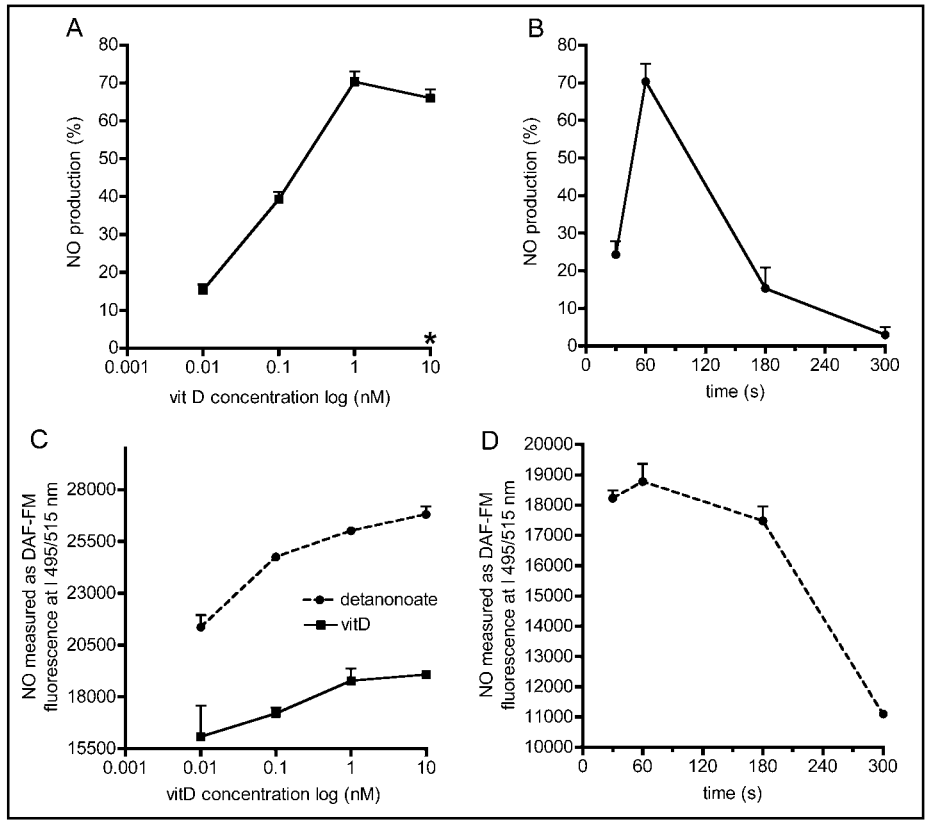

Fig. 1. Time-course and dose-response study of vitD. In A, the increase of NO production measured through Griess assay in 1 min of stimulation induced by vitD range 0.01nM-10nM. In B, the time-course of the maximum effect induced by vitD 1nM determined by Griess. In C and D, the same experiments performed with DAF-FM. In C, results obtained after vitD administration are compared to a standard curve generated by the NO donor detanonoate. Reported data are means ± SD (%) of 5 independent experiments. *, administration of the vehicle alone to HUVEC, induced a very limited response (3%).

To quantify NO production a standard nitrate curve was generated, in the same medium in which the experiments were performed. The results were expressed as a percentage.

The results obtained were also validated by diamino-fluorescein fluorophore system (DAF-FM). The cells prepared in same condition used for Griess assay, after the stimulation, were washed with PBS 1X sterile and incubated with DAF-FM 0.8μM for 25 min at the dark in the incubator. The cell bathing medium was taken to measure the fluorescence using a fluorescence spectrometer at λex/em 495/515 nm. The results obtained in the samples were compared to a standard curve generated by the NO donor detanonoate (100nM-0.01nM).

Western blot analysis

In the second series of experiments the same cells were used to evaluate the protein expression. HUVEC were plated on 0.1% gelatin-coated flask with EGM-2 complete medium. The cells at confluence were washed with PBS1x and then incubated overnight in starvation medium in incubator. After this time, the cells were stimulated with the same agents used for NO detection and then washed with iced-PBS1x supplemented with 2mM sodium orthovanadate and lysed in a iced-Ripa buffer (50mM Hepes, 150mM NaCl, 0.1% SDS, 1% Triton-X100, 1% sodium deoxycholate, 10% glycerol, 1.5mM MgCl2, 1mM EGTA, 1mM NaF) supplemented with 2mM sodium orthovanadate and 1:100 protease inhibitor cocktail. The protein extract was quantified by using a bicinchoninic acid protein assay (BCA) and 30 μg of each sample was dissolved in Laemmli buffer 5x, heated at 95°C for 5 min, resolved on 8% or 15% SDS PAGE and transferred to a PVDF membrane. The membranes were incubated overnight at 4°C in agitation with a specific primary antibody (anti-phospho-ERK$^{Thr202/Tyr204}$, anti-ERK1/2, anti-phospho-p38$^{Thr180/Tyr180}$ MAPK, anti-p38 MAPK, anti-phospho-Akt^{Ser473}, anti-Akt, anti-p-eNOS^{Ser1177}, anti-eNOS, anti-β-actin). The membranes were washed and incubated with horseradish peroxidase-coupled goat anti-rabbit Ig and anti-mouse Ig and were developed by use of a non-radioactive method using Western Lightning chemiluminescence. Densitometric analysis was performed using Quantity One image analysis software (BioRad). The quantification of protein expression was normalised to specific total protein, which was loaded on each respective blot, and to β-actin detection and was expressed as a percentage. Net intensity values were calculated by subtracting the background within the area measured for each band from the total intensity within this same measured area to account for any variation in background intensity across the band.

VitD receptor agents

To investigate the role of VDR in the endothelial effects induced by vitD, ZK159222 and ZK191784 compounds were used. The former is described as an antagonist with residual agonistic activity [15], whereas the latter is the most representative of a novel class of vitD analogs, initially studied for its immunomodulatory effects on T cells [16]. As regards effects of ZK159222, according to the analysis in *in vitro* systems, this VDR ligand appears to be a functional antagonist in all cellular systems, whereas the agonistic action is very low [17].

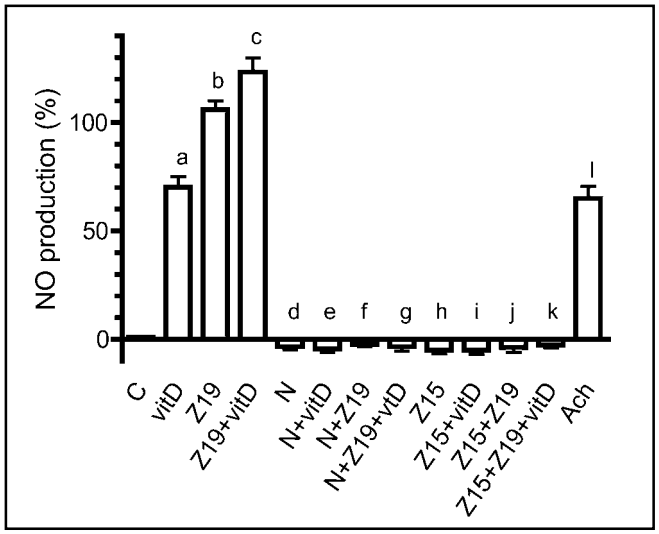

Fig. 2. Effects on NO production, determined by means of Griess method, induced in HUVEC cultures by stimulation with vitD (1nM) alone or in co-stimulation with various agents. C = control; Z19 = ZK191784 (1nM); Z19+vitD = co-stimulation; N = L-NAME (10mM); N+vitD = co-stimulation; N+Z19 = co-stimulation; N+Z19+vitD = co-stimulation; Z15 = ZK159222 (1nM); Z15+vitD = co-stimulation; Z15+Z19 = co-stimulation; Z15+Z19+vitD = co-stimulation; Ach = acetylcholine (10μM). a, b, d, h, l, P<0.05 versus control; c, P<0.05 versus a,b; e, I, P<0.05 versus a; f, j, P<0.05 versus b; g, k, P<0.05 versus c. Reported data are the means ±SD of 5 independent experiments.

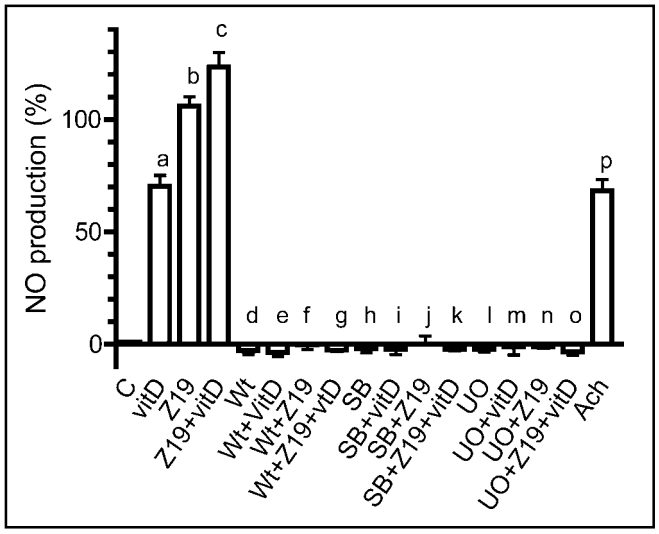

Fig. 3. NO production induced by vitD (1nM) and ZK191784 (1nM) in presence of inhibitors. C = control; Z19 = ZK191784 (1nM); Z19+vitD = co-stimulation; Wt = wortmannin (100nM); Wt+vitD = co-stimulation; Wt+Z19 = co-stimulation; Wt+z19+vitD = co-stimulation SB = SB203580 (1μM); SB+vitD = co-stimulation; SB+Z19 = co-stimulation; SB+z19+vitD = co-stimulation; UO = UO126 (10μM); UO+vitD = co-stimulation; UO+Z19 = co-stimulation; UO+Z19+vitD = co-stimulation; Ach = acetylcholine (10μM). a, b, d, h, l, p, j, P<0.05 versus control; e, i, m, j, P<0.05 versus a; f, j, n, j, P<0.05 versus b;g, k, o j P<0.05 versus c. Reported data are means ± SD (%) of 5 independent experiments.

Statistical analysis

Student's t test and ANOVA test were used to examine changes in NO production and protein expression. The non-parametric Mann Whitney U test was used to compare percentage of responses. Data are the means of at least five independent experiments both for NO production and signaling experiments and were expressed as means±SD and correspond to the NO (μM) released by samples. P<0.05 was considered statistically significant.

Results

NO production, dose and time-course study

The NO release induced by stimulation of HUVEC with vitD (from 0.01 to 10nM, dissolved in ethanol), was measured in a dose-response and time-course study by Griess assay and verified through DAF-FM. The maximum effect between 30 and 300 sec was tested as well. As illustrated in Fig. 1A and C, the effect of vitD on NO production was concentration-dependent and the maximum effect was observed at 1nM concentration after 1min of stimulation (70.33±4.73%, P<0.05), measured through Griess assay and DAF-FM. This value corresponds to a NO production of 1.17 μg/s. This concentration was used for all successive experiments. The optimal time of stimulation was at 1min because after this time the effects of vitD on NO production, measured through Griess method, progressively decreased (Fig. 1B). The results obtained by Griess assay were similar to the one obtained by DAF-FM (Fig.1D).

Role of VDR and eNOS in NO production

To verify the effective involvement of VDR in the action mechanism of vitD, HUVEC were treated with VDR ligands ZK159222 1nM (antagonist), ZK191784 1nM (agonist). alone or in co-stimulation with vitD. Moreover, the effects of 1nM vitD on NO release were examined in presence or absence of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester 10mM (L-NAME). As described in Fig. 2, 1nM vitD induced NO release of about 70.33±4.73% (P<0.05). The stimulation of HUVEC with the VDR agonist ZK191784 (1nM) caused an increase in NO production amounting to 106±4% (P<0.05) and in presence of 1nM vitD the effect was amplified compared to

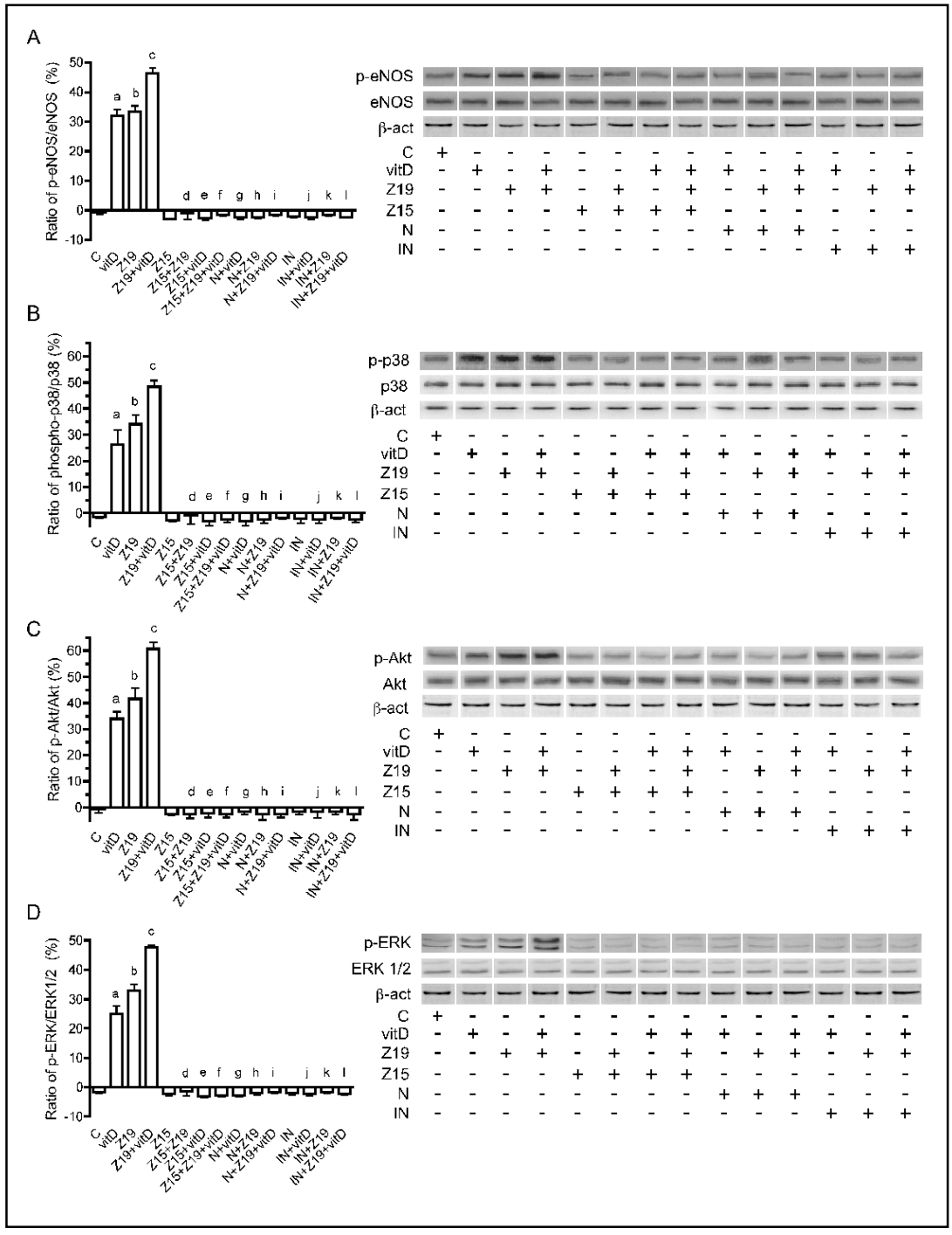

Fig. 4. Effects of vitD (1nM) and various agents on the level of phosphorylation (percent) of eNOS (A), p38 (B), Akt (C), and ERK 1/2 (D). In each panel both the densitometric analysis and immunoblots of phosphorylation relative to specific proteins are represented. IN = kinases inhibitors (SB+UO+Wt). Other abbreviations are the same as used above. Reported data are the means ±SD of 5 experiments. a, b, P<0.05 versus control; c P<0.05 versus control, a, b; e, g, j, P<0.05 versus a; d, h, k, P<0.05 versus b; f, i, l, P<0.05 versus c.

ZK191784 or vitD alone 123.3±6.5% (P<0.05). These effects were abolished in presence of antagonist of VDR ZK159222 (1nM). These results confirmed the involvement of VDR in the signaling leading to NO release in HUVEC and were also validated by the experiments performed in presence of 10mM L-NAME in which NO production was completely abolished.

Intracellular signaling activated by vitD leading to eNOS activation

To explain the action mechanism of vitD involved in the activation of NO production in HUVEC, the role of p38/MAPK, PI3K/Akt and MEK1/MAPK pathways was examined in experiments performed with their specific inhibitors. Cell cultures were separately pre-incu-

bated for 15 min with 1μM SB203580, 100nM wortmannin and 10μM UO126. The vehicles of inhibitors and vitD were tested in the basal medium without agents. Acetylcholine chloride 10μM was used as positive control. In these conditions the effects on NO production of vitD or ZK191784, alone or in co-stimulation, were abolished, thus demonstrating the involvement of the above kinases in the signaling leading to eNOS activation and NO production (Fig. 3).

To confirm that eNOS activation was mediated by the p38, Akt, ERK1/2 pathways, protein activation of the signaling leading to NO release was analysed by Western blot. HUVEC were stimulated by means of vitD, alone or in co-stimulation with the same agents used before and the levels of phosphorylation of p38, Akt, ERK and eNOS were analysed. As shown in Fig. 4 and 5, in the experiments performed with vitD or ZK191784 all levels of activation were greater than control and in co-stimulation they were amplified. The immunoblots and densitometric analysis showed that these effects in presence of L-NAME were abrogated and in presence of single blockers they were abolished as well. It is noteworthy that in presence of ZK159222 effects induced by vitD, alone or in co-stimulation with ZK191784, were abolished. These data indicate that vitD/VDR complex was was able to activate intracellular pathways (p38/MAPK, PI3K/Akt, ERK1/2) leading to eNOS activation.

Discussion

The results of this study demonstrate for the first time that vitD is able to stimulate NO production in HUVEC cultures through eNOS activation. This effect is dose-dependent and is accompanied by a significant increase in the level of phosphorylation of intracellular kinases. Data from this study clearly demonstrate that the administration of vitD, at a concentration that in the present study induced the highest production of NO, acutely increased the phosphorylation of eNOS, p38, AKT and ERK, which are known to be involved in the intracellular signaling leading to NO production [18]. The effects on eNOS and on the most important pathways stimulating NO production were prevented by the concomitant administration of L-NAME or specific protein kinases inhibitors like SB203580, wortmannin and UO126, using doses similar to the ones previously used by others to block intracellular pathways related to p38, AKT and ERK in endothelial cells [19].

The finding of an involvement of vitD in NO production by endothelial cells is quite relevant. NO is an important bioregulatory molecule and serves multiple functions including vasodilatation and many antiatherogenic properties. Indeed, dysregulation of eNOS activity is thought to contribute to the pathogenesis of certain vascular diseases such as atherosclerosis and hypertension [20].

Moreover, this study could be of great interest in the light of the reported association among low levels of serum vitD, arterial calcification and cardiovascular disease. Data available in literature show that treatment with vitD can lower blood pressure in patients with hypertension and modify the cytokine profile in patients with heart failure [21].

Although the correlation between endothelial NO production and ectopic vascular calcification has been clearly demonstrated, the connection between vitD and endothelial NO synthesis was still lacking. The results of the present study demonstrate that vitD can affect the intracellular pathways leading to activation of eNOS.

Another important finding of this study is the demonstration of the involvement of VDR in vitD-induced endothelial NO production. This fact is shown by the administration of VDR antagonist ZK159222 and by the increase in NO production induced by the VDR agonist ZK191784. It is noteworthy that the concomitant administration of ZK191784 and vitD induced effects which are greater than the vitD or VDR agonist alone. The reason why vitD and ZK191784 combined administration induces a more potent effect on HUVEC could be an interesting issue for successive research.

These data on the role of VDR ligands on endothelial NO production add new information on a possible therapeutic role of these substances. In fact, emerging evidence suggests that VDR plays an important role in modulating cardiovascular function and early interventional studies in humans demonstrated that VDR analogues therapy seems more effective than native vitD supplementation in modulating cardiovascular disease risk factors [22].

In the present study, vitD response occurs within seconds and, for this reason, it appears to be a non-genomic effect. This hypothesis is supported by studies performed on a variety of cell types in which it was demonstrated that the rapid response induced by the hormone reflects interaction with a separate, membrane

localised, signaling system [23]. Membrane receptor responsible for vitD rapid response seems to be the VDR, which is normally found in the nucleus, but can also be resident near or associated with caveolae present in the plasma membrane [24].

This work adds new information to the debate on the benefits of vitD supplementation. This issue is still controversial, as demonstrated by two very recent systematic reviews [25, 26]. The former shows that the association between vitD status and cardiometabolic outcomes is uncertain and that no clinically significant effect of vitD supplementation at the dosages given is found. The latter suggests that vitD supplements at moderate to high doses may reduce cardiovascular disease risk.

In conclusion, findings of this work can suggest that VitD alone or in combination with a synthetic VDR agonist, because of its capacity to increase endothelial NO production, it can exert a beneficial action on vascular function. This could be relevant in the light of the use of vitD supplementation as potentially useful tool to treat or prevent cardiovascular diseases.

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