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PH.D. THESIS

Vitamin D inhibits Tissue Factor and CAMs expression in Oxidized Low-Density Lipoproteins-treated human endothelial cells by modulating NF-κB pathway

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1. INTRODUCTION

Athero-thrombosis plays a pivotal role in the pathophysiology of cardiovascular disease and of its complications (1). Early stage of the atherosclerotic lesion formation involves oxidized low-density lipoproteins (oxLDL) (2,3). One of the major pathogenetic mechanisms of atherosclerosis is the recruitment of circulating leucocytes onto the vessel wall and their subsequent migration into the subendothelial spaces (4,5). This phenomenon appears to be mediated by cellular adhesion molecules (CAMs), usually expressed by endothelial cells only in response to several atherogenic stimuli (5-7). Among the cellular adhesion molecules, the Intercellular Adhesion Molecule-1 (ICAM-1) and the Vascular Cellular Adhesion Molecule-1 (VCAM-1), seem to play a pivotal role in mediating the transmigration of leucocytes, i.e. the final step of the leucocyte-endothelial cell cascade, involved in the progression of atherosclerosis (8). Atherosclerotic

plaque rupture causes TF exposition to other circulating coagulation factors leading to acute intracoronary thrombosis, responsible of acute coronary events (3,9). In physiological conditions, cells normally exposed to the blood flow, such as endothelial cells, do not express TF. However, several specific stimuli, such as inflammatory cytokines, oxidized lipids, oxygen free radicals and oxLDL have been demonstrated to induce TF expression in this cell population (10-13). Several epidemiologic studies have clearly demonstrated the correlation existing between vitamin D deficiency and increased risk of developing cardiovascular events (14,15). Moreover, recent studies have demonstrated that severe vitamin D deficiency has been associated with incident stroke (16), as well as with cardiovascular risk factors such as enhanced adiposity, and metabolic syndrome (17). Interestingly, many studies have pointed out the possible linkage of vitamin D deficiency and the development of cardiovascular disease (18-20). Thus, many

efforts have been done to study how Vitamin D might be involved in pathophysiology of cardiovascular events (21) and some studies have investigated its protective effects on some physiological functions of vessel wall cells such as endothelial and smooth muscle cell (14, 22-24). However, the effects of Vitamin D on these cells in preventing athero-thrombosis have not been completely investigated yet. Thus, in the present study, we have investigated whether vitamin D, besides regulating bone mineral balance, might exert cardiovascular protective effects by preventing the oxLDL-mediated effects on CAMs and TF expression in human endothelial cells in vitro.

2. MATERIALS AND METHODS

2.1 Cell cultures

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Basel, Switzerland) were grown in EGM 2 medium with 10 % FBS (Sigma Chemical Co., St Louis, USA), and used at passages 2 to 5. To evaluate TF expression and activity, cells were enzymatically harvested and counted in a haemocytometer and sub cultured in 24-well plates at an initial density of about 5 x 10^4 cell/well, while at confluence, cell density was of about 8.5 x 10^4 cell/well. For other set of experiments, cells were grown in 100 mm cell plates and, at confluence, cell density was of about 2 x 10^6 cells per plate. At confluence, cells were starved in serum-free medium for 24 hours and then used in the different set of experiments. Given the concern surrounding the potential contamination of Vitamin D or LDL/oxLDL with endotoxin, we analyzed our substances and found endotoxin level to be <0.125

EU/mL (<12.5 pg/mL) by Limulus assay (Bio Whittaker, Walkersville, USA). All media, reagents and water were also tested and endotoxin level found to be <0.125 EU/mL.

2.2 *Isolation of LDL and preparation of oxLDL*

LDL and oxLDL were isolated and prepared as previously described [1, 2] Briefly, LDL (density = 1.019–1.063 g/ml) were isolated from human plasma by sequential ultracentrifugation. For oxidation, LDL were diluted to 0.1 mg protein/ml with EDTA-free PBS and incubated with CuSO4 (10 µmol/l) for 18 hrs at 37°C. At the end of incubation, 0.1 mmol/l EDTA was added to prevent further oxidation and the ox-LDL were concentrated to 1 mg/ml. This procedure resulted in extensive LDL oxidation and the resulting preparations are referred to in the text as oxLDL.

2.3 ***Experimental protocol***

Confluent cells were starved in serum-free medium for 24 hours enriched with Vitamin-D (10^{-8} M) (Sigma Chemical Co., St Louis, USA) (14) and finally stimulated with native LDL (nLDL) or oxLDL (50 μg/mL). Tissue Factor gene and protein levels, surface expression and activity were evaluated at pre-specified time points. Non Vitamin D treated cells served as control.

2.4 Effects of Vitamin D on oxLDL-induced TF-mRNA levels in endothelial cells

Tissue Factor gene levels have been evaluated in stimulated HUVECs as previously described (26). At 60 and 120 minutes after stimulation, cells were washed with phosphate-buffered saline (PBS) and total mRNA was extracted using TRIzol reagent (GIBCO, Carlsbad, USA), according to the manufacturer's

instructions. Reverse transcription was performed using SensiFAST cDNA (BIOLINE) and 100 ng of the RNA samples from each culture condition. Samples were run in triplicate in 20 μL reactions by using an Rotor-Gene Q sequence detector system (QIAGEN). Samples were incubated at 95ºC for 2 min and then underwent 40 cycles at 95ºC for 5 s and 60 ºC for 1 min. SYBR green chemistry was used to detect fluorescence and an internal standard (SensiFAST SYBR, BIOLINE). Specific oligonucleotides for human GAPDH and human TF were designed on the basis of published sequences using PRIMER EXPRESS Software (Applied Biosystems) and validated for their specificity (11). The results were analysed using a comparative method, and the values were normalised to the GAPDH expression and converted into percentage change. Three different experiments were performed for each experimental condition.

2.5 Effects of Vitamin D on TF protein expression in oxLDL-treated endothelial cells

Tissue Factor protein expression was measured in HUVECs incubated with nLDL or with oxLDL for 6 hours. TF expression was evaluated in cell lysates by Western blotting. The samples (30 µg) were treated with SDS-PAGE sample buffer, followed by heating and then subjected to 10% gel. The protein was transferred onto membranes with a iBLOT2 Dry Blotting System (invitrogen), according to the manufacturer's instructions.TF was detected with a specific antibody (1:1000, American Diagnostica Inc, Greenwich, CT, USA). After detecting TF, the membranes were stripped and then treated to detect tubulin expression as housekeeping protein. Band intensities were quantified using Image 1 J software (densitometric units x 1000) and graphically expressed as mean ± SD. Six different experiments were performed for each experimental condition.

2.6 Effects of Vitamin D on TF surface expression and activity in oxLDL-treated endothelial cells

Evaluation of TF levels on cell surface was investigated by FACS analysis. After 6 hours incubation, endothelial cells were detached with 10 mmol/L EDTA in PBS (without trypsin) and stained with FITC-labelled monoclonal antibody (Pharmingen, Franklin Lakes, USA) against TF, or with the appropriate isotype IgG (phycoerythrin or FITC) as control. Fluorescence intensity of 9000 cells for each sample was quantified by a FACS Calibur analyzer (Becton-Dickinson, Franklin Lakes, USA). Finally, To evaluate whether oxLDL-induced TF was functionally active, TF pro-coagulant activity was determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation FXa, as previously described (27).

Briefly, after stimulation with oxLDL, cells were washed and incubated with 1 nM of recombinant human FVIIa (Novo

Nordisk A/S Gentofte, Denmark), followed by 100 nM of purified human factor X (Calbiochem Novobiochem, La Jolla, CA, USA) and 5mM CaCl2 for 15 min at 37 °C. A chromogenic substrate, specific for factor X (Cromozym X, Roche Diagnostics, Mannheim, Germany, 0.5 mmol/l) was then added and incubated for 30 min at 37 °C. The reaction was stopped by adding 200 µl/ml of sample of a 30% solution of acetic acid and the change in optical density at 405 nm was quantified with a spectrophotometer. Purified factor Xa of known concentration (Sigma Chemical Co., St Louis, USA) allowed generation of calibration curves. Six different experiments were performed for each experimental condition.

2.7 Effects of Vitamin D on adhesion molecule expression.

Endothelial cells were incubated as above described with oxLDL in presence or not of vitamin D for 12 hours and ICAM-1 and

VCAM-1 were evaluated by specific ELISA assay (BMS201 and KHT0601, respectively, purchased from Thermofisher). Both assays have been run according manufacturer's instructions. Six different experiments were performed for each experimental condition.

2.8 Effects of Vitamin-D and oxLDL-induced TF: the role of NF-κB nuclear translocation.

Since it has been already reported that upregulation of TF expression in different cell types occurs via NF-κB pathway [3, 4] we have investigated whether vitamin-D was able to inhibit TF expression by modulating the activation of this transcription factor. Thus, in an additional set of experiments, endothelial cells were treated as above described for 4 hours and NF-κB translocation was measured by using a non-radioactive, sensitive method for detecting the specific transcription factor DNA

binding activity, following manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). In parallel, IκB protein levels were assessed by western blot analysis and using anti-IκB antibody 1:1000 dilution (Thermofisher). Three different experiments were performed for each experimental condition.

2.9 Statistical Analysis

Data are presented as mean ± SD. Differences between groups were determined by a one way ANOVA followed by a Student's t test with Bonferroni's correction. A p value < 0.05 was considered statistically significant. In all statistical analysis SPSS 22.0 Statistical Package Program for Windows (SPSS Inc., Chicago, IL, USA) was used.

3. RESULTS

3.1 Effects of Vitamin D on TF gene and protein levels in oxLDL treated cells

As already reported (10,12,26), TF mRNA, evaluated by RT-PCR was undetectable in unstimulated endothelial cells. Incubation with native LDL did not cause any increase of TF-mRNA levels. On the contrary, oxLDL stimulation resulted in a significant induction of TF gene as compared to unstimulated cells (Figure 1). These oxLDL mediated effects on TF-mRNA levels were significantly reduced in HUVECs incubated with Vitamin-D (Figure 1). Similarly, the levels of TF protein evaluated by Western Blot, were almost undetectable in unstimulated HUVECs and in native LDL-treated cells. Stimulation with oxLDL caused a significant increase of TF protein expression which was decreased in cells preincubated with Vitamin D (Figure 2).

3.2 *Effects of Vitamin D on oxLDL-induced TF surface expression and activity in endothelial cells*

Tissue Factor expression on cell surface was measured by FACS analysis. TF was almost undetectable on unstimulated HUVECs, at baseline as well as in cells stimulated with native LDL. Incubation with ox-LDL caused significant increase of TF expression on HUVEC surface (Figure 3). Similarly, TF procoagulant activity, determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation FXa, was almost undetectable at baseline, and on cells stimulated with native LDL. The procoagulant activity increased after stimulation with ox-LDL (Figure 4). Vitamin D prevented the effects exerted by ox-LDL on TF expression (Figure 4) and activity (Figure 4).

3.3 *Effects of Vitamin D on adhesion molecule expression.*

Expression of CAMs on HUVECs were measured by specific ELISA kits. Unstimulated cells as well as cells stimulated with native LDL did not express VCAM-1 and ICAM-1. The significant increase of CAMs expression was observed in cells incubated with ox-LDL. Vitamin D preincubation significantly prevented ICAM-1 and VCAM-1 expression at similar extent (Figure 5).

3.4 *Vitamin D and oxLDL-stimulated cells: the role of NF-κB nuclear translocation.*

Nuclear translocation of NF-κB was assessed by a non-radioactive, sensitive method for detecting the specific transcription factor DNA binding activity. NF-κB translocation was not observed in unstimulated cells and in cells stimulated with native LDL. Incubation with oxLDL resulted in a significant NF-κB translocation from the cytosol to the nucleus.

Preincubation with Vitamin D prevented these oxLDL effects (Figure 6). As expected and in line with NF-κB translocation, IκB cytoplasmatic levels were reduced in HUVECs stimulated with oxLDL and normalized in those cells preincubated with Vitamin D.

4. DISCUSSION

The main results of the present study are: 1. oxLDLs promote expression of gene and of functionally active Tissue Factor in endothelial cells, shifting them to a prothrombotic phenotype; 2. oxLDLs induce expression of the adhesion molecules ICAM-1 and VCAM-1, both implicated in early stage of the atherosclerotic process; 3. Vitamin D seems able to prevent these phenomena by modulating the activity of the transcription factor NF-κB.

Athero-thrombosis plays a pivotal role in the pathophysiology of cardiovascular disease and of its complications. In this phenomenon, oxLDL are actively involved since they participate in the atherosclerotic process by various mechanisms, including endothelial cell activation and dysfunction, macrophage foam cell formation, and vascular smooth muscle cell migration and proliferation (2). One of the major pathogenetic mechanisms of atherosclerosis is the recruitment of circulating leucocytes onto the vessel wall and their subsequent migration into the subendothelial spaces (4). This phenomenon appears to be mediated by cellular adhesion molecules (CAMs), such as

ICAM-1 and VCAM-1, usually expressed by endothelial cells only in response to several atherogenic stimuli and able to mediate the transmigration of leucocytes, i.e. the final step of the leucocyte-endothelial cell cascade, involved in the progression of atherosclerosis (4,6-8). Acute coronary events occur when a coronary atherosclerotic plaque cracks and causes TF exposition to other circulating coagulation factors leading to acute intracoronary thrombosis (3,9). Moreover, several evidences have clearly demonstrated that vascular thrombosis might be due also to TF exposition on endothelial cells, in response to specific stimuli including oxLDL (10-13). Thus, oxLDL might be actively involved in the progression of athero-thrombosis, from its early steps to its acute thrombotic complication. In line with these observations we show that oxLDL are able to induce CAMs expression of endothelial cells. Furthermore, we demonstrate that oxLDL promote TF gene as protein expression in this cell population that in the vessel wall is at the interface with other coagulation factors vehiculated in the blood flow. Importantly, the TF expressed was functionally active and with procoagulant activity. Vitamin D is a fat soluble vitamin with two dominant forms, vitamins D2 (ergocalciferol) and D3

(cholecalciferol) (28,29). Vitamin D2 is synthesized by invertebrates and plants after exposure to ultraviolet radiation while Vitamin D3 is naturally present in a small range of foods (such as oily fish, egg and fortified dairy products) and is also made endogenously in the skin (28,29). Dietary Vitamin D typically comprises only 10.0% to 20.0% of circulating levels of Vitamin D. Recently, it has been seen that vitamin D3 is significantly more effective than D2 in increasing serum 25(OH) vitamin D (25(OH)D) concentrations (29). Vitamin D receptor is ubiquitously expressed in almost all body cells, including vascular or myocardial cells (30), suggesting that this vitamin might exert its effects in several other systems apart from musculoskeletal tissues being involved in infectious or autoimmune diseases, cancer or cardiovascular diseases (31). Interestingly, many studies have pointed out the possible linkage of Vitamin D deficiency and the development of cardiovascular disease (18-20). Specifically, it has been demonstrated that Vitamin D deficiency is associated with higher blood pressure levels (32). and some observational studies have indicated the linkage between Vitamin D deficiency, lower high density lipoprotein and higher triglycerides, as well as higher

apolipoprotein E levels (33,34). Moreover, some contrasting studies have suggested that Vitamin D deficiency might be associated with the risk of developing diabetes in the future and obesity (35). Finally, several epidemiological studies have clearly indicated that Vitamin D deficiency is associated to an increased risk of cardiovascular disease (15,21,36,37) such as stroke (16), myocardial infarction (38,39) [5, 6] and heart failure (40-42). At the same time, many experimental studies have been addressed to elucidate the role of Vitamin D in pathophysiology of cardiovascular disease. Thus, it has been demonstrated that this vitamin modulates numerous genes involved in fundamental processes of potential relevance to cardiovascular disease, such as cell proliferation and differentiation, apoptosis, oxidative stress, membrane transport, matrix homeostasis, and cell adhesion (21). In this context, the effects of Vitamin D on vessel wall cells have been extensively investigated. Specifically, in endothelial cells Vitamin D reduces inflammation, improves flow–mediated dilation finally modulating the progression of atherosclerosis. Moreover, in the same cells, this vitamin reduces endothelial mediated contraction and, consequently, the risk of hypertension (22). However, the effects observed in this report

that Vitamin D inhibits CAMs expression in endothelial cells, have not been investigated before. Similarly, the Vitamin D "antithrombotic" effects as inhibitor of TF expression have been investigated in smooth muscle cells only (14) and not in endothelial cells as we did. This is a very important pathophysiological issue, considering that endothelial cells, being at interface with other coagulation factors vehiculated in blood, do not express constitutively TF but only in response to specific stimuli including oxLDL (10-13). Taken together, these data permit to draw a hypothetical pathophysiological scenario for patients with Vitamin D deficiency, which have increased risk of developing cardiovascular disease: lower levels of this vitamin might be associated with dyslipidemia, and with an "unprotected" endothelium that might "easily" shift to an atherothrombotic phenotype. Another interesting finding of our study was that Vitamin D was able to inhibit the activation of the transcription factor NF-kB. This transcription factor is present in an inactive form in the cytoplasm of many cells such as lymphocytes, monocytes, endothelial and smooth muscle cells. NF-kB has been demonstrated to be activated in the unstable plaques of patients with acute coronary syndromes by several

stimuli including oxidative stress and ox-LDL (26,43-45). Moreover, and, interestingly, binding sites for this transcription factor are contained in the promoter for TF (46). In line with these previous observations, data of the present study permit to speculate that oxLDL, activate NF-kB pathway that, in turn, finally leads to expression of functionally active TF on endothelial cells. Interestingly, Vitamin D interferes with this molecular mechanism by inhibiting oxLDL-mediated NF-κB activation and TF expression.

An important limitation of this study is that it is an in vitro study and that several in vivo studies, addressing the effect of vitamin D supplementation for cardiovascular prevention have generated controversial results (47-49). The recently published VITAL study has clearly indicated that supplementation with vitamin D did not result in a lower incidence of cardiovascular events or invasive cancer than placebo (50). However, in the VITAL study most of the individuals supplemented were not Vitamin D deficient (only 12.7% had levels below 20 ng/dL), while the epidemiological observations clearly state that the increased cardiovascular risk was observed in those patients with Vitamin

D value below 20 ng/mL (36). Another potential limitation is that we have studied Vitamin D in the context of atherosclerosis by evaluating only VCAM/ICAM expression. that are only a piece in the complex pathophysiological puzzle of this disease. However, these CAMs are considered a marker of endothelial dysfunction, a phenomenon that represents one of the first steps of atherosclerosis. Thus, keeping in mind these potential limitations, the results of the present study, should be considered by a pathophysiological point of view, since they shed a brighter light to understand the mechanisms by which Vitamin D might exert protective cardiovascular effects. Specifically, we have observed that this vitamin reduces TF and CAMs expression in endothelial cells stimulated with a pathophysiological stimulus such as oxLDL. Further studies will be necessary to translate these findings to a clinical scenario to better define the potential therapeutical role of Vitamin D supplementation in the management of cardiovascular disease in patients with Vitamin D deficiency.

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6. FIGURE LEGENDS

FIGURE 1

Effects of Vitamin D on ox-LDL-induced TF transcription in human endothelial cells assessed by Real Time quantitative PCR. TF mRNA was undetectable at in unstimulated control cells (CTRL) human endothelial cells. Incubation with ox-LDL caused significant increase in TF mRNA levels, as compared to unstimulated cells. Preincubation with Vitamin D inhibited the ox-LDL effect on TF-mRNA. Data are expressed as % change versus control gene represented by GAPDH. Each bar represents the mean ± SD of 3 different experiments. (*=$p<0.001$ vs Control; (**=$p<0.001$ vs oxLDL; one-way ANOVA with Tukey's post hoc test).

FIGURE 2

Effects of Vitamin D on ox-LDL-induced TF protein evaluated by Western Blot analysis of cell lysates. Ox-LDL caused a significant increase of TF protein levels. These effects were prevented by treatment with Vitamin D. Tubulin served as loading control. Each bar represents the mean ± SD of 6 different experiments (*=$p<0.001$ vs Control; **=$p<0.001$ vs oxLDL; one-way ANOVA with Tukey's post hoc test). The insert shows results of a representative experiment.

FIGURE 3

Effects of Vitamin D on TF surface expression in oxLDL-stimulated HUVEC.

Panel A. FACS analysis showed that oxLDL-induced TF expression on cell surface. Control, Vitamin D alone and LDL stimulated cells did not significantly express TF on their membrane. Stimulation with oxLDLs, caused TF^+ in 69.5±2.1%

of cells. In oxLDL treated cells preincubated with Vitamin D, TF^+ cells were reduced to 47.7±3.3%. Each column represents the mean ± SD of 6 experiments (*=p<0.001 vs Control; **=p<0.001 vs oxLDL).

Panel B. Representative histograms of a typical FACS analysis experiment.

FIGURE 4

Effects of Vitamin D on ox-LDL-induced TF activity evaluated by a two-step colorimetric assay based on the ability of TF/FVIIa to promote generation of coagulation FXa. OxLDL induced-TF activity reflects results observed for TF expression, confirming that TF was functionally active. Vitamin D preincubation significantly reduces TF activity. Each column represents the mean ± SD of 6 experiments in triplicate (*=p<0.001 vs Control; **=p<0.001 vs oxLDL, one-way ANOVA with Tukey's post hoc test).

FIGURE 5

Effects of Vitamin D on adhesion molecules in oxLDL-treated ECs evaluated by ELISA. In basal conditions, HUVEC expressed very low basal levels of ICAM-1 and VCAM-1. Stimulation with oxLDL, induced the expression of these adhesion molecules in treated cell at significant extent for both, VCAM-1 (***Panel A*** 22.12±0.35ng/mL, *=p<0.001 vs Control; one-way ANOVA with Tukey's post hoc test) and ICAM-1 (***Panel B*** 110.55±3.78ng/mL, (*=p<0.001 vs Control; one-way ANOVA with Tukey's post hoc test). Vitamin D preincubation significantly prevented VCAM-1 (***Panel A*** 6.23±0.32ng/mL, **=p<0.001 vs oxLDLs-treated ECs; one-way ANOVA with Tukey's post hoc test) and ICAM-1 expression (***Panel B*** 37.46±3.59ng/mL, **=p<0.001 vs oxLDLs-treated ECs; one-way ANOVA with Tukey's post hoc test). Each column represents the mean ±SD of 6 experiments in triplicate.

FIGURE 6

Effects of Vitamin D on NF-κB translocation in oxLDL-treated HUVEC.

Panel A. In HUVEC incubated with ox-LDL, a significant increase of NF-kB levels was observed. Pretreatment of endothelial cells with Vitamin D prevented the ox-LDL effects on NF-kB.

Each bar represents the mean ± SD of 3 different experiments. (*=p<0.001 vs Control; **=p<0.001 vs oxLDL)

Panel B: Western blot analyses. OxLDLs/stimulation results in significant reduced levels of IκB. Conversely, preincubation with Vitamin D restores IκB levels.

7. FIGURE

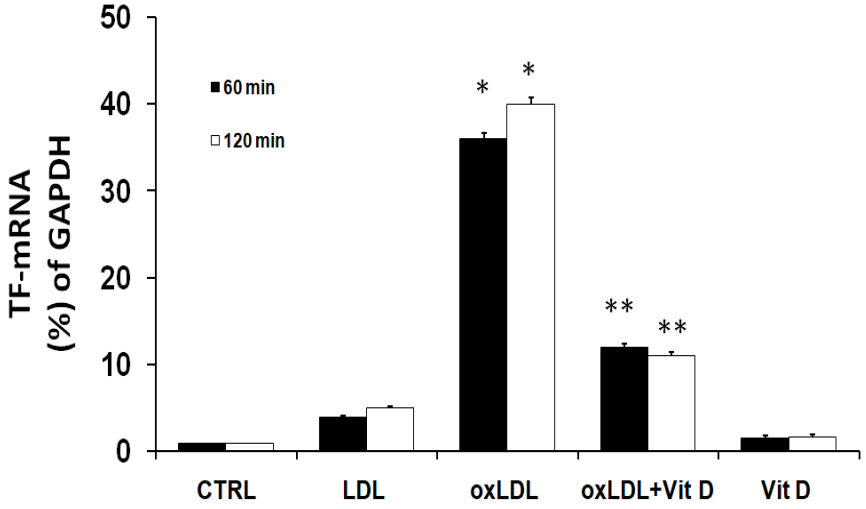


Fig. 1 Effects of Vitamin D on ox-LDL-induced TF transcription in human endothelial cells assessed by Real Time quantitative PCR.

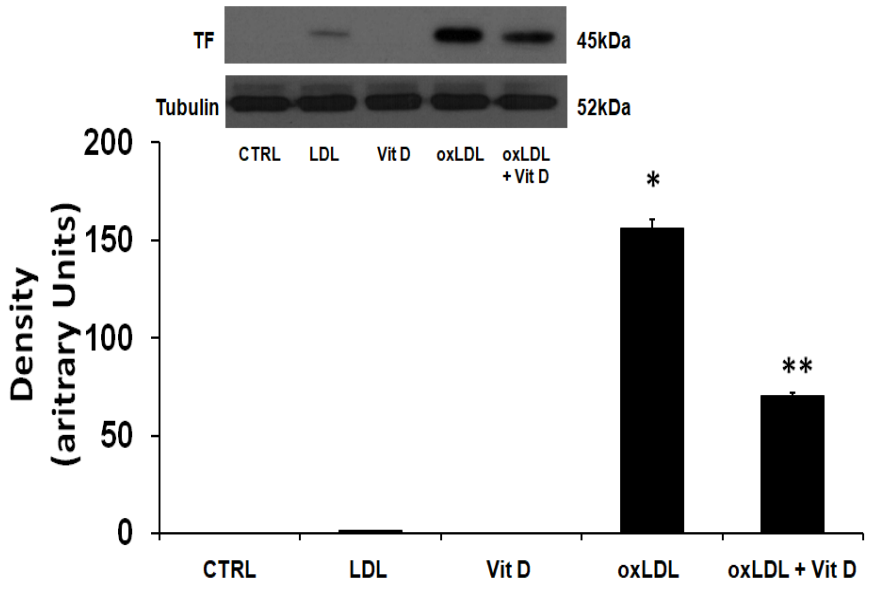
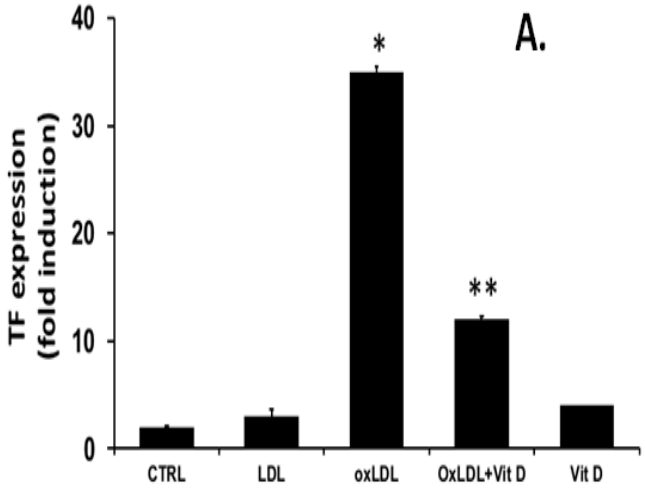


Fig. 2 Effects of Vitamin D on ox-LDL-induced TF protein evaluated by Western Blot analysis of cell lysates.



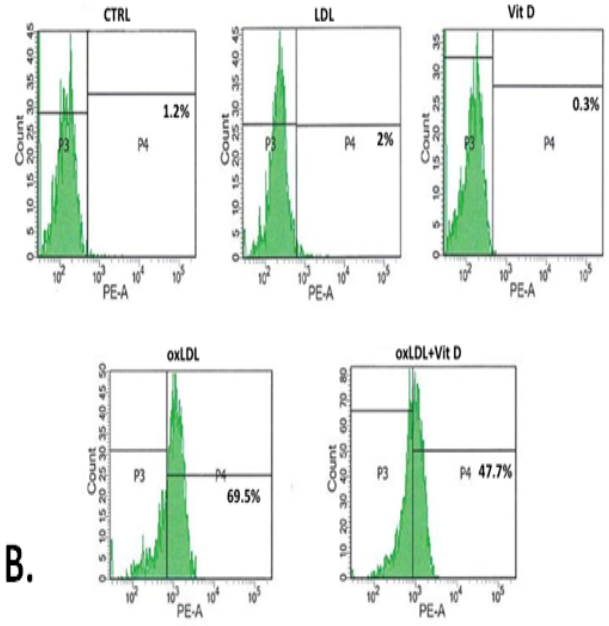


Fig. 3 Effects of Vitamin D on TF surface expression in oxLDL-stimulated HUVEC.

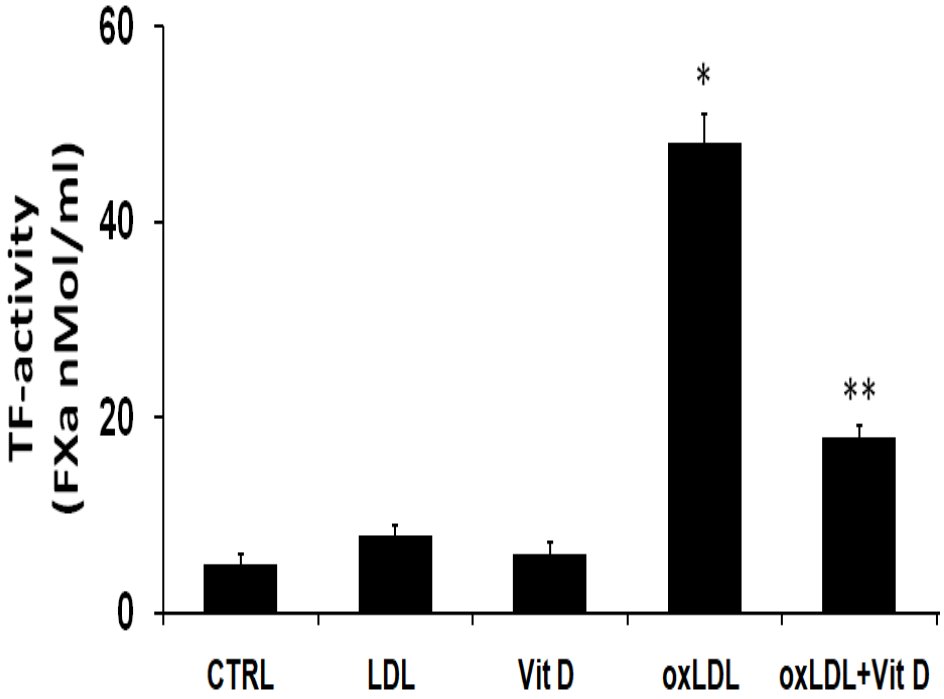


Fig. 4 Effects of Vitamin D on ox-LDL-induced TF activity evaluated by a two-step colorimetric assay based on the ability of TF/FVIIa to promote generation of coagulation FXa.

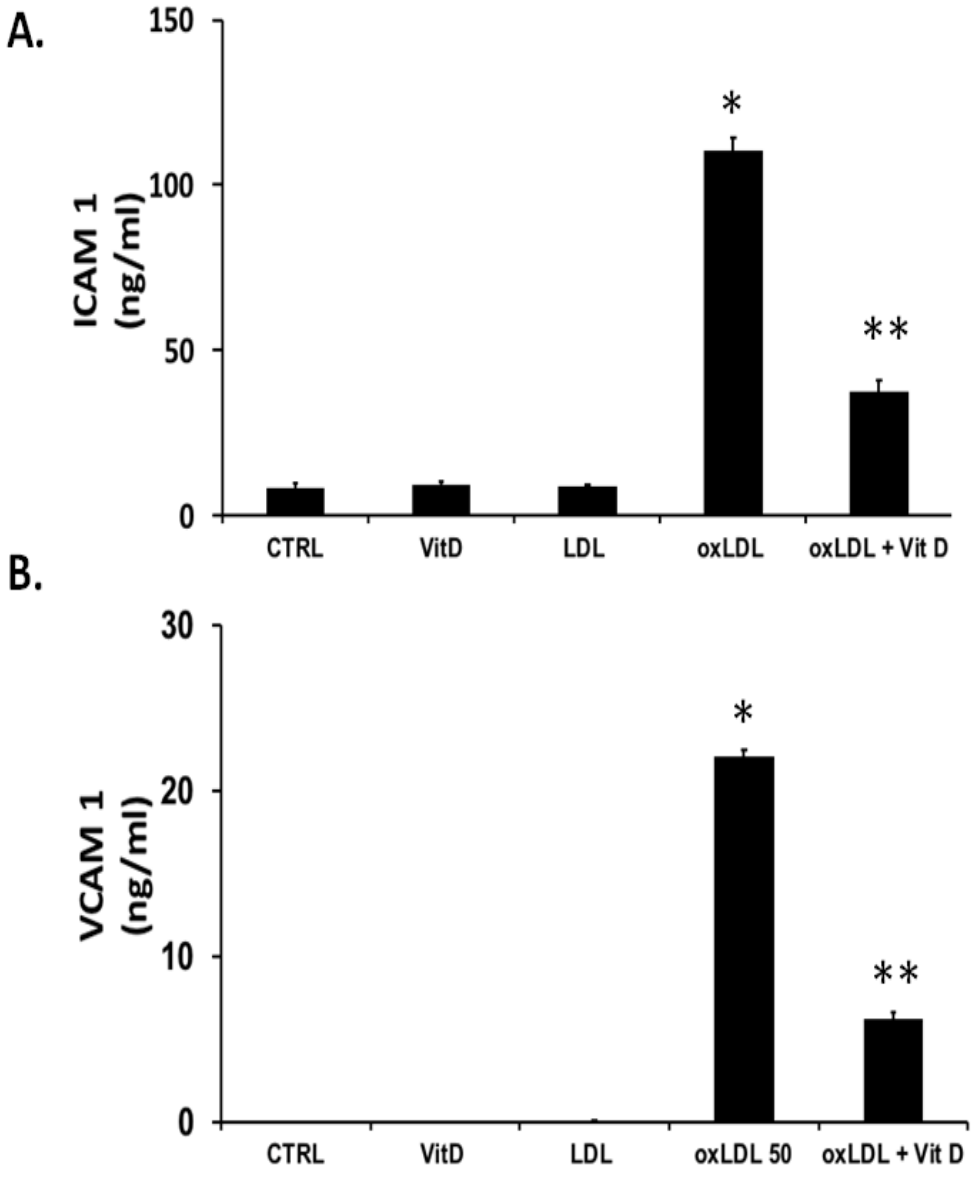


Fig. 5 Effects of Vitamin D on adhesion molecules in oxLDL-treated ECs evaluated by ELISA.

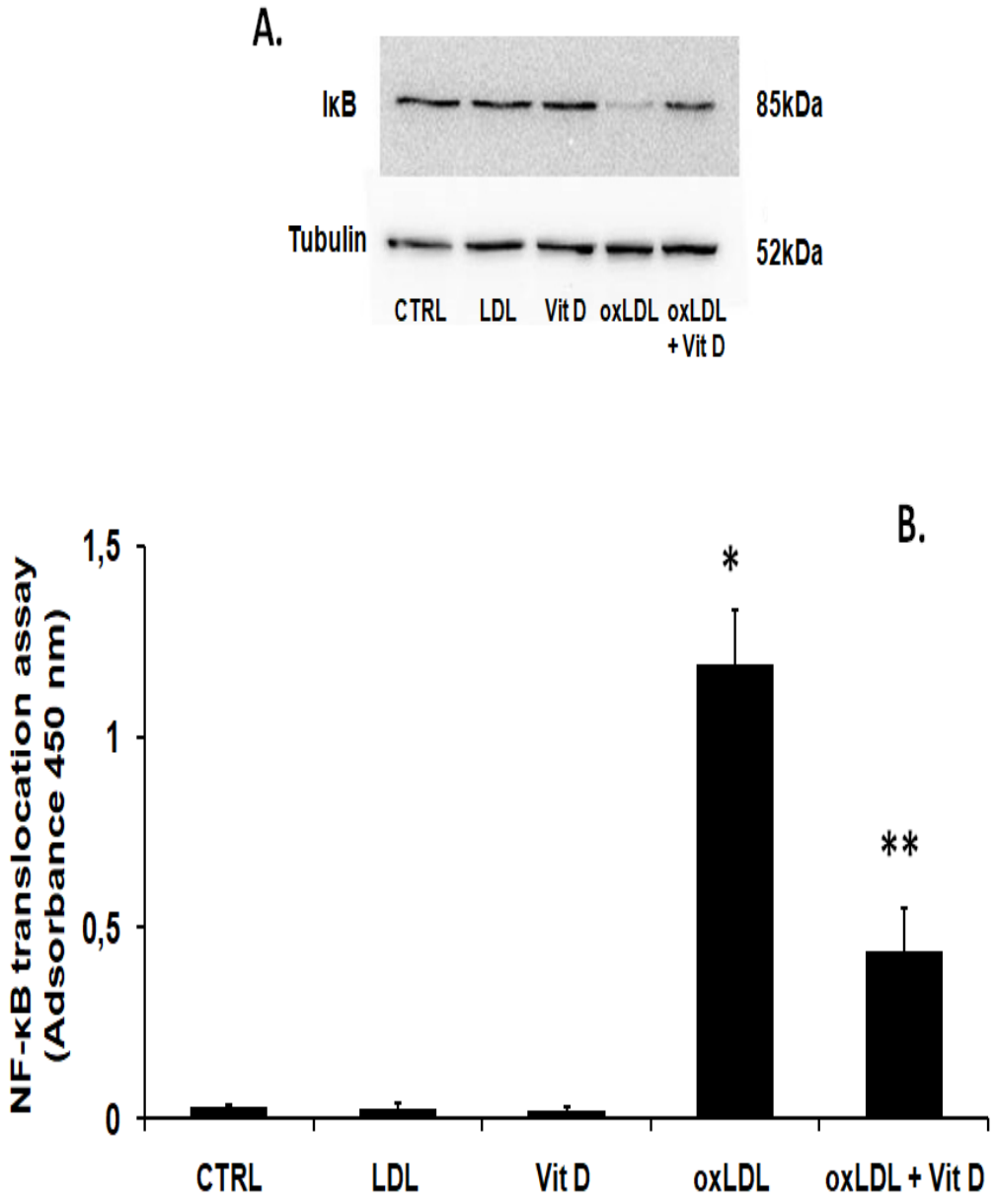


Fig. 6 Effects of Vitamin D on NF-κB translocation in oxLDL-treated HUVEC.