

Nanomomedical studies of the restoration of nitric oxide/peroxynitrite balance in dysfunctional endothelium by 1,25-dihydroxy vitamin D₃ – clinical implications for cardiovascular diseases

Alamzeb Khan
Hazem Dawoud
Tadeusz Malinski

Department of Chemistry &
Biochemistry, Nanomedical Research
Laboratories, Ohio University, Athens,
OH, USA

Background: Clinical studies indicate that vitamin D₃ improves circulation and may have beneficial effects in hypertension. This study uses nanomedical systems to investigate the role of 1,25-dihydroxy vitamin D₃ in the preservation/restoration of endothelial function in an angiotensin II (Ang II) cellular model of hypertension.

Methods: 1,25-dihydroxy vitamin D₃-stimulated nitric oxide (NO) and peroxynitrite (ONOO⁻) concentrations were measured in situ with nanosensors (200–300 nm diameter with a detection limit of 1 nM) in human umbilical vein endothelial cells of African American (AA) and Caucasian American (CA) donors exposed to Ang II. The balance/imbalance between NO and ONOO⁻ concentrations ([NO]/[ONOO⁻]) was simultaneously monitored and used as an indicator of endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction.

Results: [NO]/[ONOO⁻] imbalance in Ang II-stimulated dysfunctional endothelium was 0.20±0.16 for CAs and 0.11±0.09 for AAs. Uncoupled eNOS and overexpression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contributed to high production of ONOO⁻. Vitamin D₃ treatment reversed [NO]/[ONOO⁻] to 3.0±0.1 in CAs and 2.1±0.1 in AAs – exceeding that observed in normal endothelium. Vitamin D₃ restored uncoupled eNOS and endothelial function by increasing cytoprotective NO and decreasing the cytotoxic ONOO⁻. The beneficial effect of vitamin D₃ is associated with a favorable rate of NO and ONOO⁻ release, restoration of the [NO]/[ONOO⁻] and the overall decrease in the overexpression of eNOS, inducible nitric oxide synthase and NADPH oxidase. This effect of vitamin D₃ may prove to be beneficial in the treatment of hypertension and other cardiovascular diseases, including heart failure, myocardial infarction, vasculopathy, stroke and diabetes.

Keywords: vitamin D₃, hypertension, nitric oxide, peroxynitrite, endothelial dysfunction, nanomedicine, restoration of endothelium

Introduction

There is a large body of observational data that links vitamin D₃ active metabolite 1,25-dihydroxy vitamin D₃ to the function of the cardiovascular system.^{1,2} This non-classical effect of vitamin D₃ is additional to the more classical role of vitamin D₃ on the mineral-calcium metabolism in bone.^{3,4}

Clinical studies suggest (somewhat inconsistently) that elevated vitamin D₃ levels can lower arterial blood pressure.⁵ However, there is a strong correlation between insufficient serum levels of vitamin D₃ that are observed in heart failure, myocardial infarction and elevation of arterial blood pressure.⁶ In cross-sectional studies (National

Correspondence: Tadeusz Malinski
Nanomomedical Research Laboratory,
Ohio University, 350 West State
Street, Athens, OH 45701, USA
Tel/fax +1 740 597 1247
Email malinski@ohio.edu

Health and Nutrition Examination Survey III), a deficiency in vitamin D₃ metabolites correlated with hypertension, diabetes mellitus, hyperglyceridemia and obesity.⁷

Studies carried out on vitamin D₃ receptor (VDR) knock-out and 1- α hydroxylase-deficient mouse models showed elevated arterial blood pressure.^{8,9} Other studies carried out on spontaneous hypertensive rats have indicated that vitamin D₃ administration suppresses endothelium-dependent contraction of aorta in these models.^{10,11} Vitamin D₃ has antihypertrophic, anti-inflammatory and antiproliferative properties and may reduce cardiac hypertrophy in spontaneous hypertensive rats.¹² Furthermore, vitamin D₃ has a direct effect on endothelial and smooth muscle cells and may decrease coagulation and increase re-endothelialization and fibrinolysis.¹²

The dysfunction of endothelium in the cardiovascular system is a common denominator of several diseases such as hypertension, diabetes, obesity and heart failure – all of which are diseases where an insufficient level of vitamin D₃ is observed.^{13–15} The dysfunction of the endothelium is characterized by low production of bioavailable nitric oxide¹⁶ (NO) – a cytoprotective vasorelaxant – and a high concentration of cytotoxic vasoconstrictor, peroxynitrite (ONOO⁻).¹⁷ ONOO⁻ is one of the most powerful oxidants in the biological milieu, and at high concentrations ONOO⁻ can considerably shift the redox balance in endothelium and negatively affect vascular function and hemostasis.^{18,19}

Endothelial peroxynitrite is produced in the rapid diffusion-controlled reaction of superoxide (O₂⁻) with NO.²⁰ There are two major sources of O₂⁻ in dysfunctional endothelium: NADPH oxidase and uncoupled endothelial nitric oxide synthase (eNOS). Partially uncoupled eNOS can concomitantly produce NO and O₂⁻, in close proximity, potentially resulting in high levels of ONOO⁻. Therefore, uncoupled eNOS can be a very effective generator of ONOO⁻, which can trigger a cascade of redox events leading to endothelial dysfunction.

The study presented here uses nanomedical methods of measurement and analysis to elucidate the role of 1,25-dihydroxy vitamin D₃ (abbreviated as vitamin D₃ throughout the text) in the stimulation of NO and ONOO⁻. Normal and angiotensin II (Ang II)-induced dysfunctional human umbilical vein endothelial cells (HUVECs) of African Americans (AAs) and Caucasian Americans (CAs) were used in this study. Nanosensors were used for the in situ monitoring of the concentrations of NO and ONOO⁻ after the treatment of dysfunctional endothelial cells with vitamin D₃. We developed a unique nanosystem that can be used for the simultaneous direct measurement in situ of bioavailable NO and ONOO⁻ in single endothelial cells.^{21,22} No other

currently available methods of NO and ONOO⁻ measurements (chemoluminescence, UV spectroscopy, fluorescence) are suitable for these kinds of measurements. The balance between NO concentration (NO) and ONOO⁻ concentration (ONOO⁻) was used to evaluate the level of eNOS uncoupling and endothelial function/dysfunction. Vitamin D₃ treatment can effectively restore [NO]/[ONOO⁻] to a level similar to normal endothelium for both CAs and AAs. We believe that the vitamin D₃-stimulated improvement of endothelial function may directly benefit the treatment of the dysfunction of the cardiovascular system.

Methods

Reagents

Vitamin D₃ (1,25-dihydroxy vitamin D₃, calcitriol) was purchased from Cayman Chemicals. 7-(1,3-Benzoxazol-2-ylsulfanyl)-3-benzyl-3H-[1,2,3]triazolo[4,5-d]pyrimidine (VAS2870), an inhibitor of NADPH oxidase, and Ang II (human) were from Sigma Aldrich. Material for the preparation of NO and ONOO⁻ sensors: Mn (III) meso-tetra (*N*-methyl-4-pyridyl) porphyrin pentachloride, TMHPPMn (CAS # 125565–45-9) and nickel (II) tetrakis (3-methoxy-4-hydroxy-phenyl) porphyrin, TNMPPNi (Cat # T40113) (Frontier Scientific). MCDB-131 complete medium (VEC Technologies). Bovine serum albumin, primary antibodies: rabbit polyclonal immunoglobulin G (IgG) anti eNOS (SC-654), rabbit polyclonal IgG anti-inducible nitric oxide synthase (iNOS) (SC-650), rabbit polyclonal IgG anti-Nox4 (SC-30141) and goat anti-rabbit IgG-horseradish peroxidase conjugate (SC-2004, secondary antibody) (Santa Cruz biotechnologies). BCA kit (Thermo Fisher Scientific).

Endothelial cell culture

HUVECs of AA (pooled, n=6, age 26±3 years) and CA (pooled, n=7, age 27±2 years) donors were purchased from Lonza (Walkersville, MD, USA). Cells were grown in T-75 flasks (Greiner bio-one, Cat # 658175) and incubated in MCDB-131 complete medium (VEC technologies) at 37°C under 5% CO₂ and 95% air and passaged every 3–4 days. After reaching confluence, the monolayer cells were trypsinized and fully detached. MCDB-131 (10 mL) complete medium was then added to trypsinized cell suspension in order to inactivate trypsin. Cells were then centrifuged at 1,500 rpm for 5 minutes. The cell pellet was then resuspended in 3 mL of fresh MCDB-131 complete media. To maintain the cell culture passage, 1 mL of the cell suspension was transferred to each of the three T-75 flasks followed by addition of 11 mL of fresh MCDB-131 complete media. For measurement, cells were resuspended in MCDB-131

and 1×10^5 cells were seeded in each well of 24-well cell culture plates.

Preparation of electrochemical nanosensors

NO and ONOO⁻ nanosensors (diameter 200–300 nm) were prepared according to the published procedure.^{21,22} Briefly, a carbon fiber (6 μm in diameter) was inserted into a glass capillary (80–100 μm in diameter). The tip (8–15 μm) of the capillary holding protruded carbon fiber was sealed with mixture of bee wax and rosin, and shortened/reduced to the diameter of 200–300 nm by heating in propane microburners. The active tip of the carbon fiber was then coated by electrochemical polymerization of monomeric TMHPPNi to form a conductive polymeric film. The polymeric TMHPPNi film was then coated with Nafion (1% in ethanol). Similarly, the ONOO⁻ nanosensor was prepared by deposition of a conductive TNMPPMn polymeric film on the tip of the carbon fiber by cyclic voltammetry. The surface of the polymeric TNMPPMn sensor was then coated with poly(4-vinylpyridine). The sensors were stored at room temperature in a phosphate buffer (pH 7.4). The NO and ONOO⁻ nanosensors have a detection limit of 1 and 3 nmol/L, respectively, and can be used separately or in tandem for the simultaneous measurements of NO and ONOO⁻ concentrations. Each of the nanosensors sample a volume of about 3–10 picoliters.

The sensor's selectivity is based on the potential of NO or ONOO⁻ oxidation/reduction, very rapid electron transfer (high current) generated in this reaction, as well as the preventative barriers for negatively charged species (NO₂⁻, NO₃⁻, dopamine, etc.) for the NO sensor and a barrier for positively charged species for the ONOO⁻ sensor. Additionally, the response selectivity for NO was confirmed in separate experiments in the presence of inhibitor(s) of eNOS (N(G)-nitro-L-arginine methyl ester [L-NAME]) or scavengers of ONOO⁻ (Mn(III) tetrakis (4-benzoic acid) porphyrin chloride). The detection limit of sensors is 1×10^{-9} mol/L for NO and 3×10^{-9} mol/L for ONOO⁻. The response time (limited by sensor response and analytical data collection system) is better than 5 μs and was estimated based on the response time of the sensor and processing/storage time of analytical (electrical current) signal.

Amperometric measurement of NO and ONOO⁻

Three-electrode system consisting of a platinum wire (counter electrode), Ag/AgCl (reference electrode) and a tandem of NO and ONOO⁻ nanosensors (working electrodes) was used. Amperometric curves of current (proportional to concentration) versus time were measured using a GAMRY dual

potentiostat, at a potential of 0.56 V (versus Ag/AgCl) for NO and -0.32 V (versus Ag/AgCl) for ONOO⁻. HUVECs cultured in a well were incubated under 5% CO₂ and 95% O₂ and allowed to grow for 1–2 days. Nanosensors were positioned at a well-defined and reproducible distance from the membrane of a single endothelial cell. X, Y and Z positions of the nanosensors were monitored according to the following procedure: first, the sensor was placed about 50 μm above endothelial cells, with the help of a stereotactic remote-controlled micro-manipulator (Sexsapex, Finland) and gradually lowered closer to the membrane of the “sacrificial” single endothelial cell. When the sensor touched the membrane, a small signal (piezoelectric current) and a mechanically stimulated NO signal were recorded. The position of the sensor was assumed as zero distance from the cell membrane (axis Z=0). From Z=0, the sensor was raised about 5 μm and transferred along the X and Y axes (keeping the Z axis constant) and positioned above another endothelial cell, separated about 50–70 μm from the “sacrificial” cell. The “sacrificial” cell could not be used for further measurements because it was mechanically distorted by the sensor. The positioning of the nanosensor at the well-defined distance (5 ± 2 μm) from the cell membrane is necessary to obtain reproducible results. NO concentration decreases with the increase of distance away from the membrane surface. A distance higher than 100 μm, NO can no longer be detected by the nanosensor.²⁶

Vitamin D₃ (concentration, 1 μmol/L in 0.1 mol/L phosphate buffer) was injected with a microinjector in a well and NO and/or ONOO⁻ release was measured with a time response better than 5 μs. A stock solution of vitamin D₃ was prepared as follows: vitamin D₃ was initially dissolved in several microliters of dimethyl sulfoxide (DMSO) and subsequently diluted in water and an aqueous solution of 0.1 mol/L of phosphate buffer. The trace amounts of DMSO present in aqueous stock solution of vitamin D₃ did not have any significant effect on NO or ONOO⁻ release.

NO and ONOO⁻ concentration was calculated by the standard addition method and/or by a standard calibration curve for each sensor before and after measurement. The nitric oxide nanosensor was calibrated by using a standard solution (range of about 20–600 nmol/L) prepared from stock solution of NO (saturated solution of NO 1.8 mM) in phosphate buffer (pH 7.4). A linear calibration curve was constructed from these measurements for each sensor. Also, a standard addition method was used to monitor the response of the sensor to subsequently added standard solution of NO. The concentration of NO standard was confirmed with UV-visible spectrophotometry (hemoglobin method) and/or coulometry.

The peroxynitrite nanosensor was also calibrated in amperometric mode by both, the calibration curve and the standard addition method. The absorbance of ONOO^- was measured in standard solution using a UV-visible spectroscopy at a wavelength of 303 nm. The molar absorptivity coefficient for peroxynitrite is $1,670 \text{ M}^{-1} \text{ cm}^{-1}$. The electrochemical NO or ONOO^- nanosensor measured the net concentration of diffusible NO and ONOO^- .

In a separate set of experiments, cells were incubated with Ang II ($1 \mu\text{mol/L}$, 1 hour) to make them dysfunctional and vitamin D_3 -stimulated NO and ONOO^- were measured. Additionally, dysfunctional (Ang II treated) endothelial cells were incubated with different concentrations of vitamin D_3 before measuring NO and ONOO^- in the presence or absence of L-arginine (eNOS substrate), VAS 2780 (NADPH oxidase inhibitor) or PEG-SOD (membrane-permeable dismutase of O_2^-).

Immunoassay

For detection of our targeted proteins (eNOS, iNOS, NADPH oxidase), the indirect enzyme-linked immunosorbent assay

was performed as per the instructions of Abcam's protocol and plates were read in a microplate reader (BioTek Synergy HT). Protein samples were analyzed in triplicate and the results were recorded from absorbance at 450 nm.

Statistical analysis and calculations

All data presented here are mean \pm standard error, $n=3-10$. One-way analysis of variance with Student–Newman–Keuls multiple comparisons post hoc analysis was used to statistically analyze the mean difference between multiple comparisons. A P -value of <0.05 was considered statistically significant. Origin (v 6.1 for windows, originLab, Northampton, MA, USA) and GraphPad Prism were used to analyze and plot data.

Results

Typical amperograms showing changes of NO and ONOO^- concentrations with time after stimulation of normal HUVECs with vitamin D_3 ($1 \mu\text{mol/L}$) are shown in Figure 1A and B. Vitamin D_3 stimulated NO release from endothelium. The maximal NO concentration of $370 \pm 15 \text{ nmol/L}$ was reached

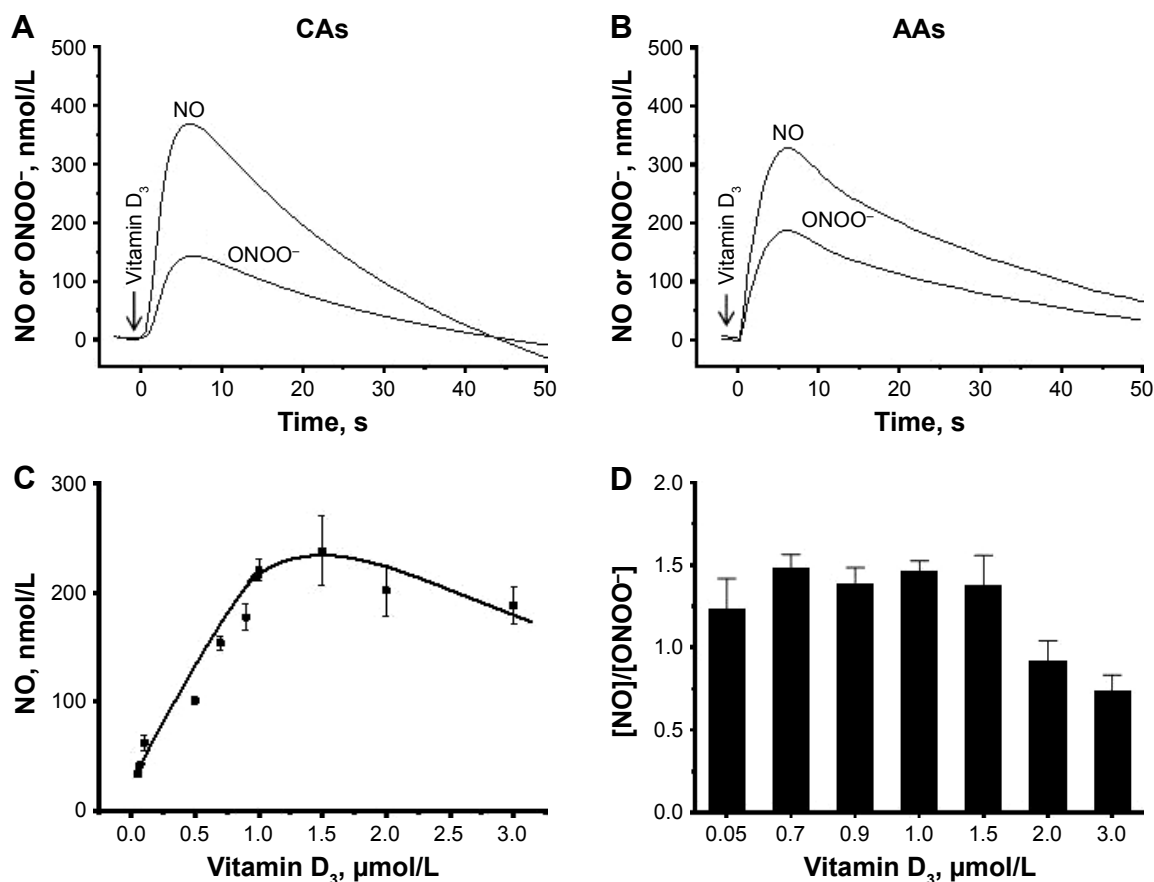


Figure 1 Typical amperograms showing the change of nitric oxide (NO) and peroxynitrite (ONOO^-) concentration with time.

Notes: The release of NO and ONOO^- from human umbilical vein endothelial cells (HUVECs) of Caucasian Americans (CAs) (A) and African Americans (AAs) (B) was stimulated with vitamin D_3 ($1 \mu\text{mol/L}$). Maximal NO concentration (C) and the ratio of maximal $[\text{NO}]/[\text{ONOO}^-]$ concentrations (D) recorded after stimulation of HUVECs of AAs with different concentrations of vitamin D_3 .

after about 7 s for CAs. For AAs, the peak of 328 ± 21 nmol/L was reached after about 6 s. NO release was accompanied by relatively low production of ONOO^- , with maximal concentrations of 150 ± 12 nmol/L for CAs and 190 ± 17 nmol/L for AAs. A relationship between the maximal NO concentration and the concentration of vitamin D₃ is shown in Figure 1C. A linear increase in NO was observed up to about $1 \mu\text{mol/L}$ concentration of vitamin D₃. At concentrations of D₃ higher than $1.5 \mu\text{mol/L}$, the NO concentrations linearly decreased. A plot of the ratio of NO concentrations (NO) and ONOO^- concentrations (ONOO^-) versus the concentration of vitamin D₃ is shown in Figure 1D. The $[\text{NO}]/[\text{ONOO}^-]$ ratio, which reflects the balance between these two molecules, increased slightly, but not significantly, with the increase of vitamin D₃ levels. However, with concentrations of vitamin D₃ higher than $1.5 \mu\text{mol/L}$, the ratio of $[\text{NO}]/[\text{ONOO}^-]$ unfavorably shifted to low levels.

Figure 2A and B shows concentration changes of NO and ONOO^- after stimulation by vitamin D₃ in HUVECs treated

with Ang II ($1 \mu\text{mol/L}$, 4 hrs) – cellular model of hypertension. For Ang II-treated CA endothelial cells, both maximal (NO) and maximal (ONOO^-) changed significantly. NO decreased to 62 ± 11 nmol/L, while ONOO^- increased to 334 ± 13 nmol/L. Maximal (NO) and (ONOO^-) in AAs also changed considerably: (NO) decreased to 50 ± 5 nmol/L and (ONOO^-) increased to 430 ± 19 nmol/L after treatment with Ang II and indicated significant eNOS uncoupling. The treatment of cells with vitamin D₃, in this cellular model of hypertension, significantly improved the function of eNOS and endothelium (Figure 2C and D). Vitamin D₃ restored (NO) and diminished (ONOO^-) to levels similar to those observed for fully functional endothelial cells, in both AAs and CAs.

Vitamin D₃ improves NO production in HUVECs of CAs and AAs

Vitamin D₃ stimulated NO release, and the rate of NO release was moderate in normal HUVECs (Figure 3). The rate of NO release was faster in normal CA cells (120 ± 12 nmol/L s)

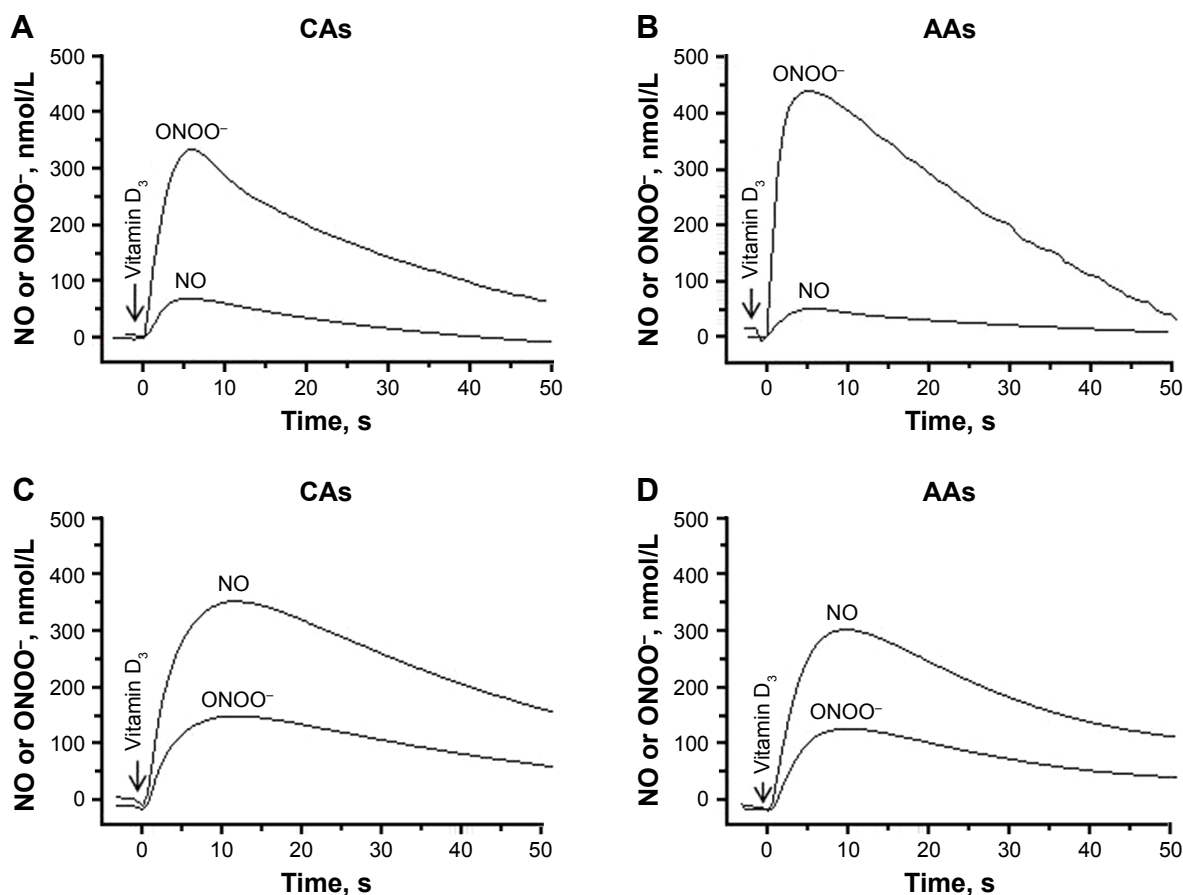


Figure 2 Typical amperograms showing the vitamin D₃ ($1 \mu\text{mol/L}$)-stimulated changes of nitric oxide (NO) and peroxynitrite (ONOO^-) concentrations, with time, in a cellular model of hypertension.

Notes: Human umbilical vein endothelial cells of Caucasian Americans (CAs) and African Americans (AAs) were incubated with angiotensin II (Ang II) ($1 \mu\text{mol/L}$ for 4 hours) in the absence (A and B) and presence (C and D) of vitamin D₃ (100 nmol/L). After incubation with Ang II, cells were washed with buffer and after 30 minutes were then stimulated with vitamin D₃ ($1 \mu\text{mol/L}$) to release NO and ONOO^- .

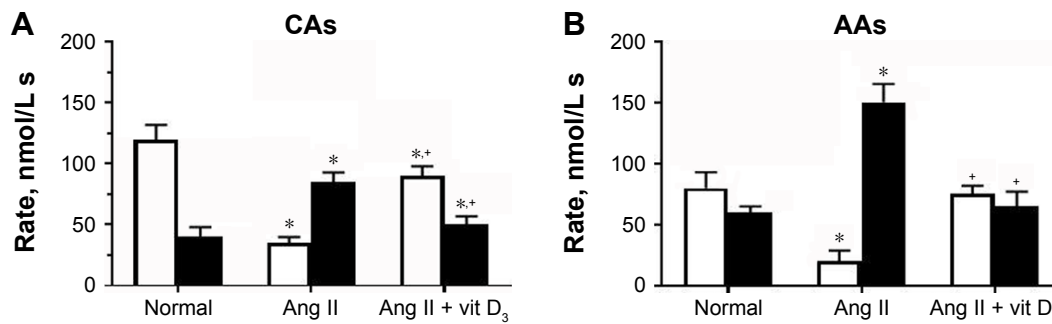


Figure 3 The maximal rate of nitric oxide (NO) release (open bars) and peroxynitrite (ONOO⁻) release (solid bars) measured from normal and angiotensin II (Ang II)-treated Caucasian American (CA) (A) and African American (AA) (B) human umbilical vein endothelial cells (HUVECs) after stimulation with vitamin (vit) D₃ (1 μmol/L). **Notes:** The maximal rate of NO and ONOO⁻ release was measured in normal HUVECs and HUVECs treated with Ang II (1 μmol/L) for 4 hours in the presence and absence of vitamin D₃ (100 nmol/L). All data represented here are mean ± SE (N=5–15). One-way analysis of variance followed by Student–Newman–Keuls multiple comparison test were used to compare groups. **P*<0.05 versus normal ***P*<0.05 versus Ang II incubated.

than in normal AA cells (80±13 nmol/L s). In contrast, the rate of ONOO⁻ production was much faster in AA than in CA cells—about 55 and 30 nmol/L s, respectively. Ang II treatment slowed the rate of NO release by about 70%–75% for both CAs and AAs. As expected, the rate of ONOO⁻ generation increased by about 80%. Vitamin D₃ restored the rate of NO

release and significantly decreased the rate of ONOO⁻ production to a level similar to that observed in normal CA and AA cells. Vitamin D₃ treatment significantly increased maximal NO concentration as compared to Ang II-treated HUVECs for both ethnic groups (Figure 4). [NO] increased linearly with the time of incubation with vitamin D₃. The increase in

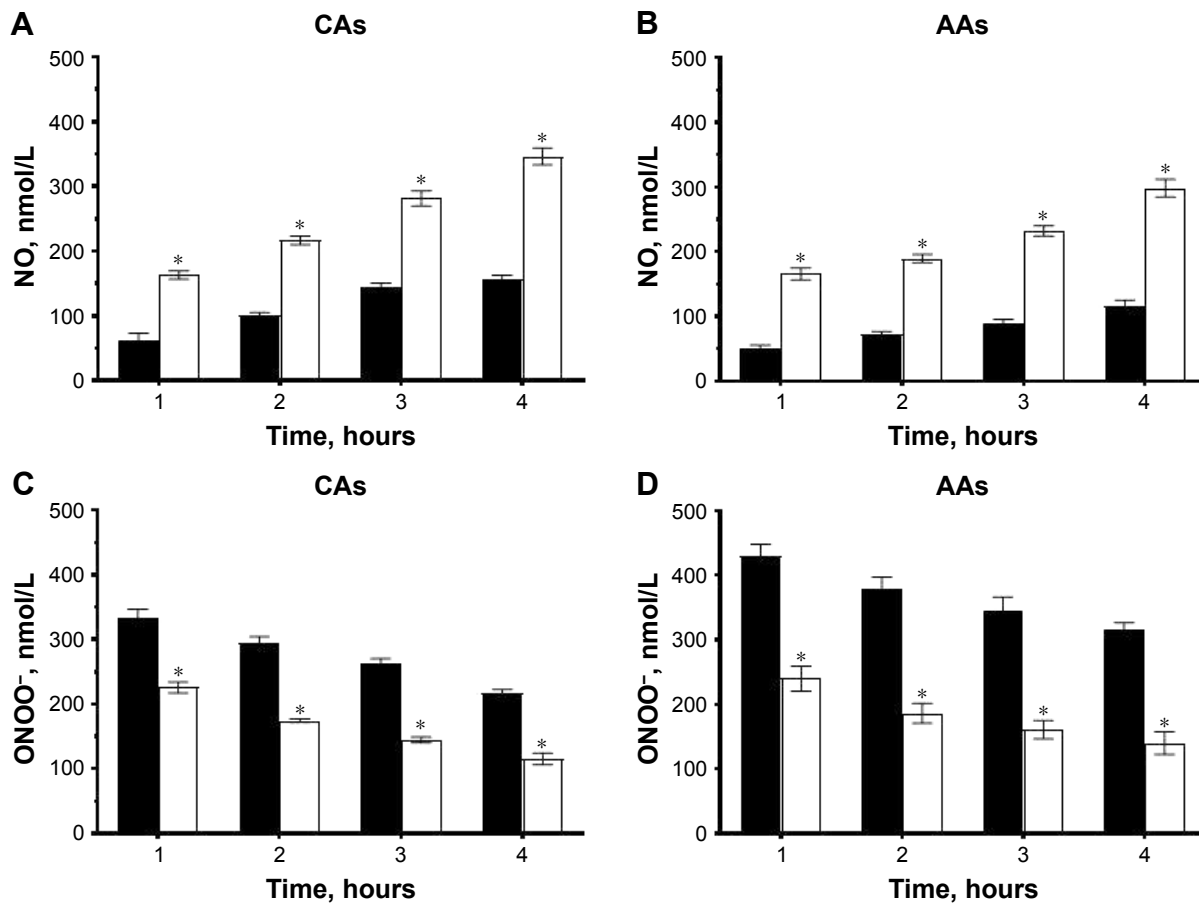


Figure 4 Maximal nitric oxide (NO) concentration (A and B) and maximal peroxynitrite (ONOO⁻) concentration (C and D) stimulated by vitamin D₃ (1 μmol/L) from the human umbilical vein endothelial cells (HUVECs) of Caucasian Americans (CAs) and African Americans (AAs).

Notes: HUVECs were incubated at different time intervals with angiotensin II (Ang II) (1 μmol/L) (solid bars) or with Ang II (1.0 μmol/L) + vitamin D₃ (100 nmol/L) (open bars). All data represented here are mean ± SE (N=5–15). One-way analysis of variance followed by Student–Newman–Keuls multiple comparison test was used to compare groups. **P*<0.05 versus Ang II incubated for both ethnic groups.

the level of NO with vitamin D₃ treatment was accompanied with a linear decrease of ONOO⁻. After 4 hours of incubation with vitamin D₃, the concentration of NO increased by about 5 times, while there was an inverse fivefold decrease observed with ONOO⁻ after the same incubation period. The beneficial effects of vitamin D₃ was observed for both CA and AA HUVECs that were treated with Ang II (1 μmol/L).

Vitamin D₃ improves the [NO]/[ONOO⁻] in HUVECs of CAs and AAs

To evaluate the status of endothelial function, we used the ratio of NO to ONOO⁻ concentration [NO]/[ONOO⁻]. The ratio of [NO]/[ONOO⁻] reflects on the relative balance between cytoprotective NO and cytotoxic ONOO⁻.

In Ang II cellular model of hypertension, [NO]/[ONOO⁻] was 0.20±0.16 for CA and 0.11±0.09 for AA

cells (Figure 5A and B). After 1 hour of treatment with vitamin D₃, there was an increase in the [NO]/[ONOO⁻] ratio to 0.70±0.05 for both CAs and AAs. The increase in [NO]/[ONOO⁻] ratio was linear with vitamin D₃ incubation time and reached a maximum of 3.0±0.1 for CAs and 2.10±0.11 for AAs after 4 hours, which exceeded the ratio observed in normal HUVECs. In normal HUVECs studied, the [NO]/[ONOO⁻] ratio was 2.5±0.2 and 1.7±0.2 for CA and AA cells, respectively (Figure 5C and D).

Effect of modulators of eNOS and NADPH pathway in Ang II cellular model of hypertension

We used the ratio of [NO]/[ONOO⁻] to elucidate the function of eNOS and NADPH in the Ang II cellular model of hypertension (Figure 5C and D). L-NAME, a nonselective

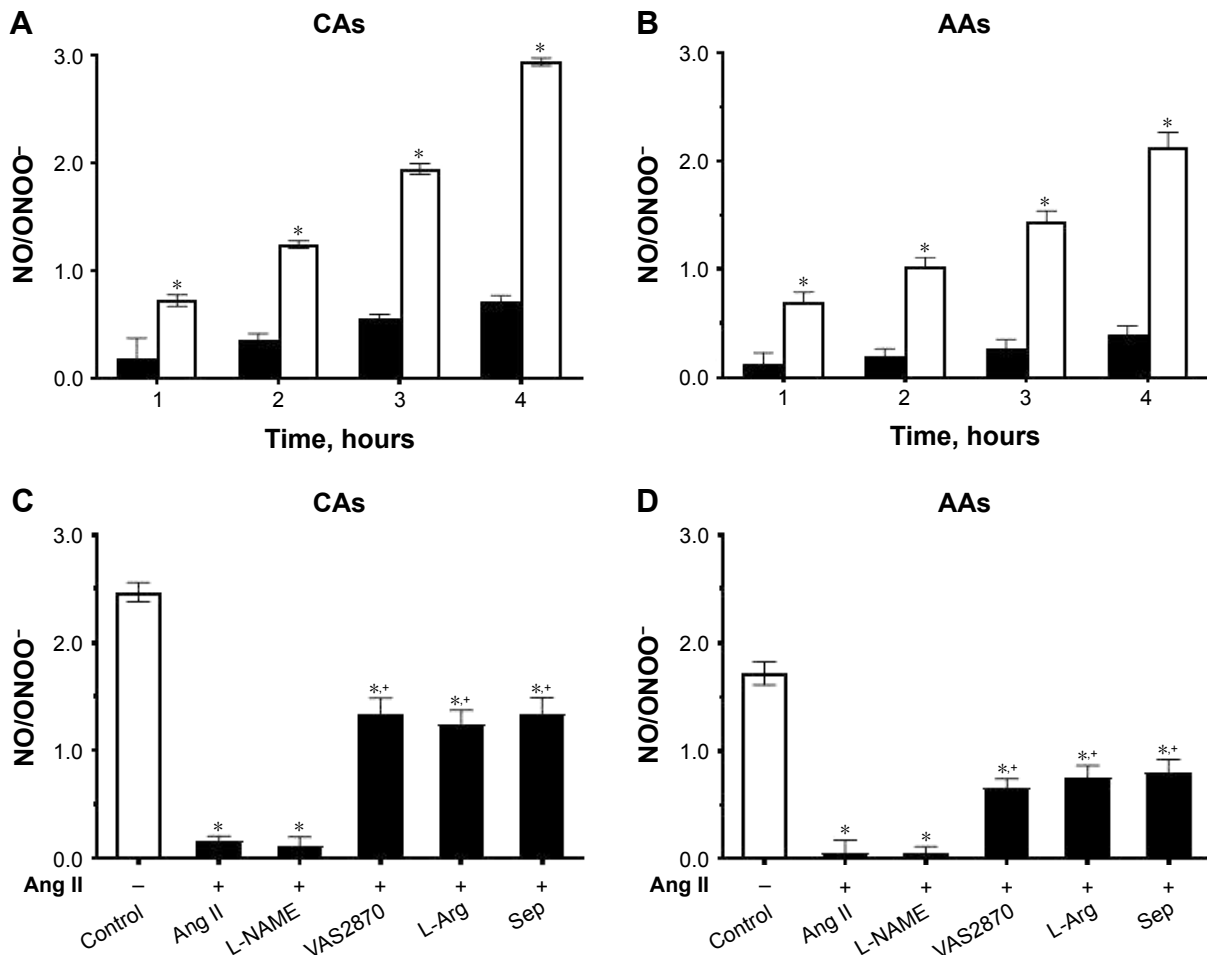


Figure 5 The change of [NO]/[ONOO⁻] balance in HUVECs of Caucasian Americans (CAs) (A) and African Americans (AAs) (B) as a function of incubation time. Vitamin D₃ (100 nmol/L)-stimulated maximal (NO) and (ONOO⁻) were measured after 4 hours of incubation of HUVECs with angiotensin II (Ang II) (1 μmol/L) (solid bars) and with Ang II (1 μmol/L) + vitamin D₃ (100 nmol/L) (open bars). Vitamin D₃ (1 μmol/L)-stimulated maximal (NO) and (ONOO⁻) measured in HUVECs of CAs (C) and AAs (D) incubated with Ang II (1 μmol/L for 4 hours) in the presence of L-NAME (0.3 μmol/L), VAS2870 (10 μmol/L), L-Arg (3 mmol/L) or Sep (0.1 μmol/L).

Notes: All data represented here are mean ± SE (N=5–15). One-way analysis of variance followed by Student–Newman–Keuls multiple comparison test was used to compare groups. *P<0.05 versus control, †P<0.05 versus dysfunctional (Ang II-incubated) for both ethnic groups.

Abbreviations: vitamin D₃, 1, 25-dihydroxy vitamin D₃; Ang II, human angiotensin II; HUVECs, human umbilical vein endothelial cells; VAS2870, 7-(1,3-Benzoxazol-2-ylsulfanyl)-3-benzyl-3H-[1,2,3]triazolo[4,5-d]pyrimidine; Sep, sepiapterin; L-Arg, L-arginine; L-NAME, N(G)nitro-L-arginine methyl ester.

eNOS inhibitor, does not have a significant effect on [NO]/[ONOO⁻]. However, at elevated concentrations of L-arginine (eNOS substrate) a substantial increase of the [NO]/[ONOO⁻] was observed. A similar increase in [NO]/[ONOO⁻] was also observed in the presence of sepiapterin, which is a precursor of tetrahydrobiopterin – an important cofactor in stabilizing eNOS dimer. An inhibitor of NADPH (VAS 2870) also significantly increased [NO]/[ONOO⁻] to the level observed in the presence of L-arginine or sepiapterin. The changes of [NO]/[ONOO⁻] due to cofactors, inhibitors and substrates were slightly more pronounced in CA cells than in AA cells.

Vitamin D₃ downregulates expression of NADPH oxidase, eNOS and iNOS in cultured HUVECs of CAs and AAs

We hypothesized that the overexpression of NADPH oxidase, eNOS and iNOS may play a role in an unfavorable shift in the [NO]/[ONOO⁻] balance and endothelial function.

After 4 hours of incubation of HUVECs with Ang II, the eNOS expression was significantly upregulated in CAs and slightly in AAs (Figure 6A). Ang II further upregulated eNOS in HUVECs of both ethnic groups, and this effect was very significant after 12 hours of incubation. In contrast, vitamin D₃ treatment downregulated eNOS expression in HUVECs of both ethnic groups. This effect was highly significant after 12 hours of incubation with vitamin D₃.

iNOS expression can be upregulated due to oxidative and nitroxidative stresses and/or inflammation. Therefore, we estimated iNOS expression in both Ang II- and vitamin D₃-treated HUVECs. Ang II significantly upregulated expression of iNOS in HUVECs of both ethnic groups versus control groups after 4 and 12 hours of incubation (Figure 6B). Treatment with vitamin D₃ downregulated the expression of iNOS in HUVECs of both ethnic groups.

Ang II had minimal effect on the NADPH oxidase 4 expression after 4 hours of incubation; however, NADPH oxidase expression was upregulated after 12 hours (Figure 6C), indicating the effect of Ang II on the expression of enzymes

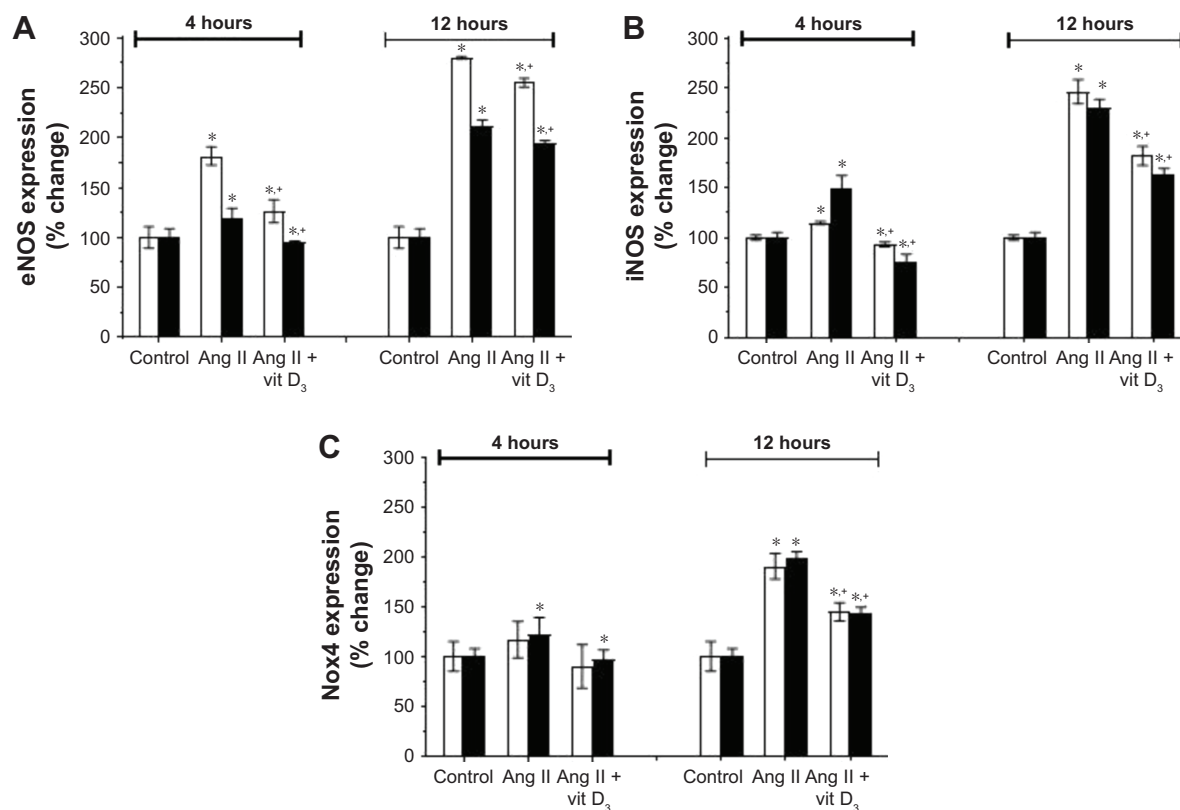


Figure 6 The effect of Ang II and vitamin D₃ on the expression of eNOS (A), iNOS (B) and NADPH oxidase (Nox 4) (C) in HUVECs of CAs (open bars) and AAs (solid bars).

Notes: HUVECs were incubated with Ang II (1 μmol/L) in the presence and absence of vitamin (vit) D₃ (100 nmol/L). Total protein samples, collected from HUVECs of CAs (open bar) and AAs (solid bar), were screened, and expressions of eNOS (A), iNOS (B) and NADPH oxidase 4 (C) were recorded from the absorbance at 450 nm and presented here as % change versus control (mean ± SE [N=3–5]). One-way analysis of variance followed by Student–Newman–Keuls multiple comparison test was used to compare treated groups versus control. **P*<0.05 versus control and ***P*<0.05 versus Ang II.

Abbreviations: AAs, African Americans; CAs, Caucasian Americans; vitamin D₃, 1, 25-dihydroxy vitamin D₃; eNOS, endothelial nitric oxide synthase; Ang II, human angiotensin II; HUVECs, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate.

responsible for O₂⁻-induced oxidative stress. Vitamin D₃ treatment significantly downregulated the expression of NADPH oxidase in HUVECs of both ethnic groups. The effect of vitamin D₃ was more pronounced in HUVECs of AAs than in those of CAs. These results indicate that vitamin D₃ may particularly protect endothelial cells from NADPH-generated O₂⁻, and oxidative stress may reduce ONOO⁻ formation and improve endothelial function.

Discussion

The nanomedical system utilized in these studies offers a unique opportunity to monitor in situ, the near real-time molecular changes of two signaling molecules, NO and ONOO⁻, produced by endothelial cells. The production of ONOO⁻ increases with the increased rate of NO release. Therefore, any rapid stimulation of NO could be considered unfavorable for endothelial function due to the potential uncoupling of eNOS and enhanced ONOO⁻ generation. Therefore, a modest rate of vitamin D₃-stimulated NO release is of crucial importance to the proper function of endothelium and the cardiovascular system. This is one of the important findings of this study. Here, we used Ang II to produce dysfunctional endothelium, similar to that observed in hypertension. Treatment with vitamin D₃, in this cellular model of hypertension, significantly restored bioavailable NO with the concomitant decrease in nitroxidative stress that is associated with high ONOO⁻. The moderate NO production by vitamin D₃ does not cause eNOS uncoupling. As a net result, vitamin D₃ maintains a favorably high ratio of [NO]/[ONOO⁻] in the endothelium with a relatively high concentration of cytoprotective vasorelaxant NO and a relatively low level of cytotoxic vasoconstrictor ONOO⁻.²⁴ This favorable kinetics of NO generation and the subsequent low production of ONOO⁻ after stimulation with vitamin D₃ are crucial factors in the process of restoring dysfunctional endothelium.

In long-term (hours) treatment, vitamin D₃ effectively reversed the imbalance between [NO] and [ONOO⁻] in Ang II cellular model of hypertension. Importantly, vitamin D₃ restored eNOS coupling in dysfunctional HUVECs in both CAs and AAs as evidenced by increased NO bioavailability and reduced nitroxidative stress. The uncoupling of eNOS was significantly higher in AAs than in CAs, as indicated by [NO]/[ONOO⁻]. However, treatment with vitamin D₃ produces proportional results for both ethnic groups. There was no significant difference in the % of [NO] and [ONOO⁻] changes between AAs and CAs versus control group. A decrease in the expression of eNOS, as well as

NADPH oxidase, that occurs after vitamin D₃ treatment of the Ang II model of hypertension is an important factor in the overall restoration of endothelial function. Even a small decrease in eNOS expression improved the availability of substrates (especially L-arginine) and other cofactors, and more effectively stabilized (coupled) eNOS dimer, preventing the generation of significant O₂⁻ by this enzyme.

The dominating product of coupled eNOS dimer is NO, while in the uncoupled dimer of eNOS, a rapid direct one-electron transfer to oxygen produces O₂⁻. NO is the most effective scavenger of O₂⁻, which produces ONOO⁻ in a diffusion-controlled reaction.^{23,24} Therefore, vitamin D₃ treatment actively reduced endothelial production of O₂⁻ from two major sources: NADPH oxidase and uncoupled eNOS. The efficiency of O₂⁻ production by uncoupled eNOS in dysfunctional endothelium can be comparable to that generated by NADPH. Vitamin D₃ simultaneously decreases the level of O₂⁻ generated by these two sources, by reducing the level of NADPH on one side and then by limiting the generation of O₂⁻ from coupled eNOS dimer on the other side.

This effect accounts for the reduction of oxidative/nitroxidative stress and the overall increase in bioavailable NO – both a highly beneficial effect of vitamin D₃ treatment on endothelium and the cardiovascular system. The process of restoring bioavailable NO production by vitamin D₃ is more efficient and comprehensive than other possible pathways of the restoration of dysfunctional endothelium, like elevated levels of L-arginine, sepiapterin treatment, scavenging or dismutase of O₂⁻. As shown here, L-arginine, sepiapterin or NADPH inhibition can only partially restore (20%–30%) endothelial function under physiologically acceptable concentrations.

In this study, we used the [NO]/[ONOO⁻] ratio as a precise indicator of eNOS coupling/uncoupling and endothelial function/dysfunction.²⁵ In normal, functional endothelium, the [NO]/[ONOO⁻] ratio varies from 2 to 5.²⁶ At an [NO]/[ONOO⁻] level of <2, the eNOS is considered partially uncoupled. When the [NO]/[ONOO⁻] ratio falls below 1, uncoupled eNOS become the dominant factor in the production of O₂⁻ and the highly efficient generator of ONOO⁻. Therefore, when the [NO]/[ONOO⁻] ratio is below 1, ONOO⁻ is the main determinant of the oxidative/nitroxidative stress in endothelium. The nanomedical approach used in this study allowed us to directly measure in situ, with a time resolution of better than 5 ms, the concentration of NO and ONOO⁻ in normal/fully functional and dysfunctional endothelium. In the HUVECs studied here, the initial [NO]/[ONOO⁻] was 2.5±0.2 and 1.7±0.2 for CAs and AAs, respectively. As we

have shown in a previous study, the eNOS uncoupling is more advanced in AAs than in CAs.¹⁷ Treatment with Ang II further increased eNOS uncoupling, which was reflected by a decrease in [NO]/[ONOO⁻] ratio to 0.20 ± 0.16 for CAs and 0.11 ± 0.09 for AAs. This suggests that potential damage to endothelial function imposed by Ang II in hypertension is more severe in AAs than in CAs. It is interesting to note that the treatment of endothelial cells of AAs (Ang II cellular model of hypertension) with vitamin D₃ significantly improved the [NO]/[ONOO⁻] ratio above the original levels observed in normal functioning HUVECs of CAs and AAs. The [NO]/[ONOO⁻] gradually increased in both groups after 1–4 hours of vitamin D₃ treatment.

The balance/imbalance of [NO]/[ONOO⁻] is influenced by NO, O₂⁻ and ONOO⁻; coupled/uncoupled eNOS, iNOS and NADPH oxidase. All of these molecules and their sources may contribute to shift in oxidative/nitroxidative stress and bioavailability of NO. Upregulated expression of these three enzymes may lead to excessive oxidative stress in cellular environment, which effectively diminished the level of bioavailable NO. Furthermore, upregulation in the expression of iNOS, which is usually associated with inflammation, can contribute to this unfavorable effect. By design, iNOS is a secondary source of NO synthesis when there is a shortage of the NO generated by eNOS. High NO production by iNOS depletes L-arginine levels and accelerates the further uncoupling of eNOS and makes the recovery of eNOS dimer even more difficult. Additionally, the NO produced from iNOS contributes minimally to the overall level of bioavailable NO, but has a significant effect on the overall increase of ONOO⁻ due to the scavenging of O₂⁻. This further exposes the cellular environment to severe oxidative and nitroxidative stress and endothelial dysfunction. Also, excessive O₂⁻ and ONOO⁻ can oxidize tetrahydrobiopterin, BH₄, an important cofactor of eNOS and a stabilizer of eNOS dimer.^{27,28} In the absence of BH₄, eNOS produces mostly O₂⁻. Supplementation of L-arginine and/or sepiapterin (precursor of BH₄) improved endothelial function by diminishing O₂⁻ and ONOO⁻ concentrations.

Our study provides direct molecular insight to previously published observations that have suggested that vitamin D₃ deficiency-induced hypertension is associated with vascular oxidative stress.²⁹ It is well documented that oxidative stress is involved in vascular complications and development of hypertension.³⁰ Oxidative and nitroxidative stress impairs endothelium-dependent relaxation of the blood vessels and is involved in the facilitation of smooth muscles contractions.^{31–33} It has been suggested that oxidative

stress is the main source of endothelial damage, and that it is generated by NADPH oxidase – one of the key enzymes of facilitating oxidative stress. We found here that the contributions of NADPH to the total oxidative stress is dominant in the initial stage of endothelial dysfunction. However, in severely dysfunctional endothelium, the contribution of NADPH to the total O₂⁻ was less than that generated from uncoupled eNOS (40% and 60%, respectively). Results of the studies presented here are coherent with and complementary to the results of previous observations. Scavengers and inhibitors of oxidative and nitroxidative stress partially prevent the dysfunction of endothelium.³⁴ It has been suggested that protective effect of AT1R blockers, the major class of antihypertensive drugs in patients with hypertension, is associated with their antioxidative action.^{35,36}

In SHR, it has been found that vitamin D₃ reduces the expression of NADPH oxidase in the vasculature.³⁷ Vitamin D₃ treatment of renal arteries of hypertensive patients showed improved endothelial function and reduction in oxidative stress.³⁷ It was suggested that the downregulation of NADPH oxidase and the upregulation of SOD1 and SOD2 may be responsible for this effect.³⁷ Our studies support and confirm this hypothesis.

Based on clinical studies, it has been suggested that vitamin D₃ may improve NO, modulate vascular tone and lower blood pressure in patients with hypertension.²⁹ However, none of these studies provided any direct proof of how vitamin D₃ improves NO production and decreases nitroxidative stress. Our studies are not only coherent with previous observations, but provide new direct evidence, on the molecular level, why vitamin D₃ can be highly beneficial for the treatment of a dysfunctional cardiovascular system. We proved here by direct NO measurements that vitamin D₃ stimulated NO release within the first second of its exposure to endothelial cells. This rapid response of endothelium to vitamin D₃ suggests that this process is most likely controlled by VDRs that are present on endothelial cell membrane,³⁸ and is followed by the signal transduction through the calcium–calmodulin pathway. We suggest that the beneficial action of vitamin D₃ on the cardiovascular system can be explained solely based on the enhanced effect on endothelial NO levels and its depreciative effect on ONOO⁻ levels.

While the beneficial effects of vitamin D₃ on dysfunctional eNOS and endothelium is well supported by this research, the effect of a high level of vitamin D₃ on normal cells will require more studies to establish the potential for an unfavorable shift of [NO]/[ONOO⁻] to a level higher than 5. The ideal ratio of NO to ONOO⁻ for fully functional

endothelium varies from 3 to 5 and depends on the location of the endothelial cells in the vasculature. Generally, at [NO]/[ONOO⁻] higher than 7, the NO may become a dominating factor, which can potentially lead to excessive production of cyclic guanosine monophosphate, excessive smooth and cardiac muscle relaxation and a dramatic decrease in blood pressure. Therefore, one can expect that excessive vitamin D₃ treatment of fully functional endothelial cells may produce risk for the cardiovascular system.

The studies presented here strongly indicate that vitamin D₃ restores endothelial function by balancing [NO]/[ONOO⁻], increasing bioavailable NO and reducing oxidative and nitroxidative stress in HUVECs, thus confirming the potential role of vitamin D₃ in the prevention and/or treatment of vascular complications. The improvement in the [NO]/[ONOO⁻] by vitamin D₃ is mainly due to the restoration of eNOS coupling and decrease in eNOS, iNOS and NADPH expressions. Vitamin D₃ does not significantly scavenge for superoxide or peroxynitrite but attenuates oxidative/nitroxidative stress and increases NO bioavailability by transcriptional regulation of the enzymes responsible for generating them. The net result is a decrease of the expression of iNOS and eNOS in the cellular environment. At low eNOS and iNOS expression, the available concentrations of major substrate, L-arginine and cofactor (BH₄) become more available for eNOS coupling.

Therefore, these studies are the first to identify the molecular mechanism of vitamin D₃-triggered restoration of the function of eNOS and the function of endothelial cells in the cardiovascular system. While these studies were performed using a cellular model of hypertension, the implications of the influence of vitamin D₃ on dysfunctional endothelium is much broader. The dysfunction of endothelium is a common denominator of several cardiovascular diseases, particularly those associated with ischemic events. Therefore, we suggest that vitamin D₃ treatment may be of clinical importance in the restoration of dysfunctional cardiac endothelium after heart ischemia, capillary endothelium after brain ischemia, hypovolemia, vasculopathy, diabetes and atherosclerosis. Our suggestion is strongly supported by several clinical studies indicating that vitamin D₃, at doses significantly higher than those currently used for the treatment of bone diseases, can be highly beneficial for the treatment of the dysfunctional cardiovascular system.

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Disclosure

The authors report no conflicts of interest in this work.

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