The active form of vitamin D, calcitriol, induces a complex dual upregulation of endothelin and nitric oxide in cultured endothelial cells

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Vitamin D (calcitriol or 1,25-dihydroxyvitamin D₃) has been linked to the regulation of body levels of calcium, phosphorus, and bone mineralization. However, there is an increasing amount of data suggesting that vitamin D exerts its effects beyond kidney, intestine, and bone (35). Different epidemiological studies support that vitamin D has cardiovascular protective effects (7, 37), and its deficiency has been linked to an increased risk of hypertension, diabetes, congestive heart failure, peripheral arterial disease, myocardial infarction, stroke, and mortality (36). However, the mechanisms responsible for this protective effect are not fully understood.

In animals, the most well-documented cardiovascular consequence of vitamin D deficiency is hypertension. In fact, mice deficient in vitamin D receptor (VDR) develop hypertension, an effect that has been linked to the activation of the renin-angiotensin-aldosterone system (18), as vitamin D is a negative regulator of renin synthesis (12). Thus, in normal mice, the inhibition of calcitriol synthesis leads to an increased renin expression (24), and diet-induced vitamin D deficiency increases blood pressure (BP), an effect that can be reversed by returning to vitamin D-sufficient chow diet for 6 wk (36). However, the relation between vitamin D and blood pressure is not so clear in pathological conditions, particularly in humans. Inconsistent results have been obtained for hypertension, as positive and negative associations between serum calcitriol and BP have been observed in both hypertensive humans and rats (5, 11, 19, 31, 38, 40). Furthermore, several studies have shown no association between vitamin D treatment and BP, whereas others have reported decreases in BP induced by vitamin D (10, 11, 20, 40).

Vitamin D may also regulate vascular function through a direct effect on the vessel wall. The presence of VDR has been found in smooth muscle and endothelial cells (20), which are also able to locally produce calcitriol from calcidiol (41). The effects of calcitriol on vascular smooth muscle cells have been analyzed previously (3, 23), but the direct effect of calcitriol on endothelial cells has not been fully clarified. Dong et al. (4) observed that the attenuated nitric oxide (NO) production in ovarectomized rat aortic endothelial cells was restored following a 12-h treatment with calcitriol, whereas Molinari et al. (22) showed that calcitriol induced a very rapid increase in endothelial NO production via interaction with VDR. However, the endothelium synthesizes not only vasodilator autacoids such as NO but also vasoconstrictors such as endothelin-1 (ET-1), with the unbalance between these bioactive mediators being one of the main mechanisms responsible for endothelial dysfunction.

Herein, the present work was aimed to analyze the ability of calcitriol to modify the in vitro synthesis of endothelial vaso-
active factors, particularly ET-1 and NO, and the intracellular mechanisms responsible for this modulation. Moreover, the endothelial changes induced by calcitriol were also tested in vivo. Both ET-1 and NO synthesis increased after calcitriol treatment, but through different intracellular mechanisms. In vivo, calcitriol slightly but significantly raised BP in rats, an effect that was prevented by the inhibition of the endothelin-converting enzyme-1 (ECE-1).

METHODS

Materials. Calcitriol (1α-25 dihydroxy-vitamin D3) was obtained from Sigma-Aldrich-Fluka Chemical (St. Louis, MO) and in its intravenous form (Calcijex) from Abbott Laboratories (Madrid, Spain). Culture media, culture plates, Blue Star-prestained protein marker, BCA protein assay reagent, nitrocellulose membranes, secondary horseradish peroxidase-conjugated goat anti-mouse IgG, CLXposure films, and SuperSignal West Pico detection system were from Cultek (Pierce, Rockford, IL). The ET-1 ELISA system was from Biomedica Gruppe (Vitro; R & D Systems, Madrid, Spain). Mouse monoclonal anti-VDR, rabbit polyclonal anti-c-Fos and anti-c-Jun antibodies, and rabbit polyclonal anti-ECE-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-eNOS and anti-P-eNOS Ser1177 were from BD Bioscience (Franklin Lakes, NJ). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). Protease inhibitor cocktail tablets and FastStart LongRead (Leiti, Alcalá de Henares, Madrid, Spain). Electrophoresis equipment was from Bio-Rad Laboratories (Richmond, CA). Protease inhibitor cocktail tablets and FastStart universal probe master were from Roche Diagnostics (Madrid, Spain). High-capacity cDNA reverse transcription kit and TaqMan gene expression assays for human ECE-1, eNOS, prepro-ET-1, and β-actin were purchased from Applied Biosystems (Life Technologies, Madrid, Spain). Canfass transfection reagent was from MobiBioLab (Laboratorios Leti, Dominion MLB, Madrid, Spain). Dual luciferase reporter Aaas system, pGL3 vector, and pRL-SV40 vector were from Promega (Walkersville, MD). VDR siRNA, Hyperfect transfection reagent, and AllStars negative control used as a nonsilencing control were from ZAP (Qiagen, Barcelona, Spain). LightShift Chemiluminescent EMSA Kit was from Thermo Scientific (Pierce). Mouse monoclonal anti-VDR, rabbit polyclonal anti-c-Fos and anti-c-Jun antibodies, and rabbit polyclonal anti-ECE-1 were from Sigma-Aldrich-Fluka Chemical.

Cell culture. EA.hy926 (EA), a human endothelial cell line, was obtained from American Type Culture Collection (Manassas, VA). EA were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μl/g streptomycin in a 5% CO2 atmosphere. Supernatants were collected to measure ET-1, and NO production reagents were obtain from Sigma-Aldrich-Fluka Chemical.

Animal studies. Animal studies were performed in two-mo-old male Wistar rats with free access to water, maintained at 24°C, and kept on a 12:12-h light-dark cycle. Arterial BP was measured in conscious animals by a tail cuff sphygmomanometer (LE 5001 Pressure Meter; Leica Scientific Instruments, Hospitalia, Spain). Animal studies were performed on 3 days before the measurement was started to prevent stress and were warmed at 30°C with a heater (LE5660/6; Letica Scientific Instruments). Basal BP was recorded for 2 consecutive days, with 20 determinations/day. Then, BP was registered 24 h after a single intraperitoneal (ip) dose of either calcitriol (400 ng/kg body wt) or vehicle (0.2 ml phosphate-buffered saline). BP was also registered 30 min after a single ip dose of FR-901533 (1 mg/kg body wt) (17, 33), an ECE-1 blocker provided by Dr. Yuriyo Yamamoto (Fujisawa Pharmaceutical, Osaka, Japan). After that, animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and a blood sample was collected through right ventricular puncture. Plasma was also collected separated (3,500 rpm, 10 min) and stored until ET-1 determination. Aorta and lungs were removed through a thoracic-abdominal incision and stored until analysis. Aorta and lung portions were collected in 4% paraformaldehyde for histological studies. The study design and the experimental protocols were performed in agreement with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union regulations. The study was revised and approved in accordance with the Ethics Committee of Alcalá University, Alcalá de Henares, Madrid, Spain. All experiments done in this work complied with the current laws of the country (Spain) in which they were performed.

Slices of aorta and lung underwent immunostaining (16) with anti-ECE-1 and anti-eNOS antibodies. Antibody-protein complexes were detected with anti-horseradish peroxidase secondary antibodies using diaminobenzidine reagent, following the manufacturer’s instructions (Dako Cytomixetrix, Fort Collins, CO). At least four sections per animal were analyzed for each immunostaining.

Measurement of ET-1 levels and ECE-1 activity. Supernatants of the confluent monolayer of EA cells treated with 100 nM calcitriol for 16 h were collected, lyophilized, and stored at −70°C until assay. ET-1 synthesis was measured by enzyme-linked immunosorbent assay (ELISA) according to the kit instructions (Biomedica Gruppe) using a 96-well microtiter plate reader.

Measurement of NO production. Nitrite synthesis was evaluated by fluorimetric assay (21). The medium used in these experiments was a glucose buffer that did not contain phenol red (in mM): 130 NaCl, 5 KCl, 16 sodium acetate, 5 glucose, and 20 Tris-HCl, pH 7.5. Aorta and lungs from control rats were extracted and incubated at 37°C in the presence or absence of 100 nM calcitriol using the glucose buffer. At the end of the incubations, cell or tissue supernatants were mixed with freshly prepared, light-protected 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) and incubated for 10 min at room temperature. The reaction was stopped with 2.8 M NaOH, and the fluorescent signal was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. The fluorescent signal was compared with the values given by nitrite standards (purity 98%) freshly prepared and dissolved in glucose buffer. Nitrite production in aorta or lung was corrected per milligram of tissue.

Western blot assays. Proteins were obtained from EA and tissues from rats by using the lysis buffer (in mM) containing a protease inhibitor cocktail: 20 Tris-HCl, pH 7.5, 150 NaCl, 1 EGTA, 1 EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, and 10 sodium pyrophosphate. Protein concentration was determined with the BCA protein assay kit. Proteins were separated on 6% SDS-PAGE (30 μg protein/lane) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk in Tris-buffered saline and Tween 20 (TBST; in mM): 20 Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween 20 at room temperature. Detection of endothelial nitric oxide synthase (eNOS) and ECE-1 proteins was done by immunoblotting, as described previously (17), using monoclonal anti-eNOS (1:3,000 dilution) and anti-ECE-1 (10 μg/ml) antibodies (mAb AEC32-236 for EA and AEC27-236 for rats, provided...
by Dr. Kohei Shimada, Biological Research Laboratories, Sankyo, Tokyo, Japan) diluted in TBST, respectively. Detection of active phosphorylated eNOS was done using a monoclonal antibody P-eNOS Ser1177 (1:2,500 in 3% BSA TBST, 1 h at room temperature). Detection of VDR was done using a monoclonal antibody anti-VDR (1:250 overnight at 4°C). Similar experiments were performed in cytosolic and nuclear fractions from control and calcitriol-treated cells. Proteins were separated on 8% SDS-PAGE and detected by immunoblot with rabbit anti-c-Fos and anti-c-Jun antibodies. Blots were incubated overnight at 4°C with each antibody (1:750 in 3% BSA TBST). After washing in TBST, blots were incubated with 1/10,000 horseradish peroxidase-conjugated goat anti-rabbit IgG or with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The immunoreactive bands were visualized with the SuperSignal West Pico detection system after 30 s of exposure to CL-Xposure film. Blots were then rebotted with a rabbit anti-actin antibody to normalize ECE-1, eNOS, p-eNOS Ser1177, c-Fos, c-Jun, and VDR levels.

Quantitative RT-PCR. Total RNA extracted from EA was reverse transcribed with the High Capacity cDNA reverse transcription kit. ECE-1, prepro-endothelin-1, eNOS, and β-actin complementary DNA were amplified by quantitative PCR using the TaqMan gene expression assay on 96-well plates using the 7500 Fast Real-Time PCR System and analyzed with 7500 Fast sequence detection software version 1.3.1 (Applied Biosystems).

Transient transfection experiments. To determine whether the effect of calcitriol on ECE-1 and eNOS gene expression was mediated by the 5′-flanking region of each gene, endothelial cells were transfected using plasmids containing complete human ECE-1 or eNOS promoter. The pGL3-ECE-1 plasmid containing either complete human ECE-1 promoter or serial deletion fragments (A to G) linked to luciferase reporter gene was used as described (13, 17). Complete human eNOS promoter/luciferase reporter gene plasmid, supplied by Dr. C. J. Lowenstein from the University of Rochester School of Medicine and Dentistry (Rochester, NY) (8, 25), and serial deletions (A to D) were used based on the sequence of the eNOS gene (15).

EA were grown at 60–80% confluence in 12-well plates and transfected with each luciferase construct by mixing plasmid DNA (0.1 μg/μl of luciferase reporter, 1 ng/μl of plasmid control from Renilla luciferase, pRL-SV40 vector, and 10 μg/ml of Canfast into complete DMEM). Twenty-four hours after transfection, cells were incubated with complete DMEM for 24 h, and then calcitriol was added in some wells at different times using serum-free DMEM. Luciferase activity was assessed using a dual-luciferase reporter assay system and expressed as relative light units of each plasmid DNA per Renilla per milligram protein of each well.

VDR was silenced in endothelial cells by transfecting a specific small interfering RNA (siRNA) against VDR (Qiagen). An unspecific scrambled was used as transfection control. Scrambled and VDR siRNA were transfected into the Hyperfect transfection reagent for 72 h. Cells were incubated with complete DMEM for 24 h, and then calcitriol was added in some wells for 6 h using serum-free DMEM. Cells were processed for immunoblotting using a specific mouse monoclonal anti-VDR and anti-eNOS, as described above (Western blot assays).

Cellular localization of c-Fos, c-Jun, and VDR by immunofluorescence. Cells were grown on coverslips for 24 h. Calcitriol was added at different times. After being washed twice, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then 0.5% Triton X-100 was added for 10 min at room temperature. After this, cells

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**Fig. 1.** Effect of calcitriol on endothelin-1 (ET-1) and nitric oxide (NO); analysis of synthesis, enzymatic activities, and mRNA content. A: EA were incubated with 100 nM calcitriol for 16 h, and then synthesis of ET-1 (black bars) was measured as ET-1-immunoreactive concentration in supernatants, and membrane-linked endothelin-converting enzyme-1 (ECE-1) activity (open bars) was assayed from cells by measuring the amount of newly produced 35 fmol/ml ET-1. B: EA cells were incubated with 100 nM calcitriol (CT) for 6 and 24 h, and then nitrite production (black bars) was measured in supernatants by the nitric assay, and total endothelial NO synthase (eNOS; striped bars) and Ser1177-phosphorylated eNOS (open bars) were measured by Western blot. A representative experiment is shown at the top, whereas at the bottom the densitometric analysis of the results of 5 independent experiments is given (means ± SE). Values are expressed as %basal conditions. Control cells produced 0.32 mM nitrite. C: EA cells were incubated with 100 nM calcitriol for 6 h. ECE-1 (black bars), preproET-1 (striped bars), and eNOS (open bars) mRNA expression was analyzed by quantitative PCR. Values are expressed as %basal conditions, and they are the mean ± SE of 4 independent experiments. *P < 0.05.
were blocked with 5% BSA for 1 h at room temperature and then incubated overnight with antibodies anti-c-Fos and anti-c-Jun (1:200) or with antibody anti-VDR (1:100). After washing in phosphate-buffered saline (in mM: 139 NaCl, 8.66 Na₂HPO₄, 1.4 NaH₂PO₄, pH 7), cells were incubated with 200-fold-diluted goat anti-rabbit IgG labeled with Alexa Fluor 488 for detecting c-Fos and c-Jun or with 500-fold-diluted goat anti-mouse IgG labeled with Alexa Fluor 647 (Invitrogen, Molecular Probes) for detecting VDR. Coverslips were mounted on prolonged gold antifade reagent with DAPI to stain nuclei. Preparations were visualized in confocal microscope (TCS-SP5; Leica Scientific Instruments).

Electrophoretic mobility shift assays. Nuclear extracts from EA treated with calcitriol at different times and electrophoretic mobility shift assays (EMSA) were displayed to check the activation of AP-1 and VDR. To detect DNA-protein interactions, we used the LightShift Chemiluminescent EMSA Kit, which uses a nonisotopic method. Oligonucleotide sequences were based on the putative AP-1-binding element in the ECE-1 promoter (from nucleotides –640 to 669: 5'-CCC TGC ACT TCC TCT CAT GGC TCC-3') (34) and VDR-binding element in eNOS promoter (from nucleotides –1,360 to 1,390: 5'-CGA GGT GGT GGG GTG GGG TGG GGC CTC AGG-3'). Biotin end-labeled DNA containing the binding site of interest (AP-1 from ECE-1 and VDR from eNOS) was briefly incubated with 1 μg/μl nuclear extracts. Protein-DNA complexes were subjected to gel electrophoresis on a native polyacrylamide gel in 0.5x Tris buffer EDTA and then transferred to a positively charged nylon membrane. The biotin end-labeled DNA was detected using the streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate as described in the kit. For competition experiments, 200-fold molar excess of competitor DNA (AP-1, VDR, or NF-κB oligonucleotides) was coincubated with biotin end-labeled DNA (biotin-AP-1 or biotin-VDR). Sequence of the oligonucleotide for NF-κB in the ECE-1 promoter (from nucleotides –591 to 617) was 5'-GGC TGG AGG GAT TTT TCC TCC TTT CA-3'. To analyze the specificity of the DNA-protein complex, 200-fold molar excess of rabbit polyclonal IgG antibodies for anti-c-Jun or anti-c-Fos was coincubated with the biotin-AP-1.

Statistical analysis. Unless otherwise specified, data are expressed as means ± SE and as the percentage of the control values. All experiments were performed at least five times in duplicate (no. of experiments is given in each figure legend). Comparisons were made by nonparametric statistics, particularly the Wilcoxon (2 groups) or
Friedman (more than 2 groups) tests, with the Bonferroni correction. BP data of animal studies were analyzed by two-way ANOVA, followed by multiple-comparison tests. The level of statistical significance was defined as $P < 0.05$.

**RESULTS**

Effect of calcitriol in human endothelial cells. The effect of calcitriol on the regulation of endothelial vasoactive factors was assessed in human endothelial cells. Calcitriol induced a rise in ET-1 (Fig. 1A, black bars) and nitrite content (Fig. 1B, black bars) in cell supernatants after 16 h. ECE-1 activity (Fig. 1A, open bars) also increased 16 h after treatment of calcitriol. Total eNOS (Fig. 1B, striped bars) and active eNOS content (Fig. 1B, open bars), measured as eNOS phosphorylated in Ser1177, also increased after cell incubation with calcitriol. ECE-1 (Fig. 1C, black bars) and eNOS (Fig. 1C, open bars) mRNA increased when human endothelial cells were treated with calcitriol, whereas prepro-ET-1 mRNA expression did not change with this treatment (Fig. 1C, striped bars). In addition, ECE-1 and eNOS protein content were upregulated in human endothelial cells incubated with calcitriol at different doses and for different times (Fig. 2).

Mechanism involved in calcitriol-induced ECE-1 and eNOS expression. To analyze the mechanisms involved in the ECE-1 and eNOS regulation by calcitriol, experiments combining the analysis of promoter activities and the study of transcription factor regulation were performed. ECE-1 and eNOS promoter activities were measured by luciferase activity assay in cells transfected with constructions containing both complete promoters linked to luciferase. Calcitriol increased the promoter activity of ECE-1 (Fig. 3A) and eNOS (Fig. 3B). For the analysis of the transcription factors that could be involved in this promoter activity upregulation, experiments similar to the previous ones were performed, although constructions with several deletions of each promoter were used. The results are shown in Fig. 3. The calcitriol-dependent stimulation of the ECE-1 promoter activity disappeared when cells were transfected with the first deletion (A, Fig. 3A), whereas changes in...
eNOS promoter activity were not observed in cells transfected with the second deletion (B, Fig. 3B).

The best-recognized response element in the promoter region deleted in the first deletion of ECE-1 is an AP-1 binding site. Thus, we tested AP-1 activation by two means. First, the intracellular localization of c-Fos and c-Jun after cell incubation with calcitriol was studied by immunofluorescence (Fig. 4A) and by Western blot of cytosolic and nuclear extracts (Fig. 4B).
A rapid and transient nuclear accumulation of both proteins was observed after treatment with calcitriol. Second, the binding of the transcription factor AP-1 to the nuclei of cells stimulated with calcitriol was tested by EMSA. A rapid increase in the biotin-labeled AP-1 binding was observed (Fig. 4C). This effect was specific for AP-1 because the signal disappeared with 200-fold excess of unlabeled AP-1 oligonucleotide (Fig. 4C, bottom, lane 3), remained almost unchanged with 200-fold excess of unlabeled NF-κB oligonucleotide (Fig. 4C, bottom, lane 4), and was supershifted with antibodies c-Jun and c-Fos (Fig. 4C, bottom, lane 5).

To confirm the relevance of AP-1 activation in the calcitriol-dependent ECE-1 activation, some experiments with pharmacological inhibitors were performed. PD-98059, which inhibits phosphorylation of ERK1/2 by MEK and subsequently c-Fos, and SP-600125, which inhibits JNK and prevents c-Jun activation, were selected. Calcitriol was unable to induce the overexpression of ECE-1 protein (Fig. 5A) and ECE-1 promoter activity (Fig. 5B) in the presence of such inhibitors.

The analysis of the results of the serial deletion experiments performed with the eNOS promoter suggests that a response element deleted in the second deletion was responsible for the calcitriol-induced eNOS changes. Because it has been shown that a VDR-responsive site is present in this region, the activity of VDR as a transcription factor was studied. The intracellular localization of VDR after cell incubation with calcitriol was evaluated by immunofluorescence (Fig. 6A) and Western blot in cytosolic and nuclear extracts (Fig. 6B). Data demonstrated that calcitriol induced a displacement of VDR from cytosol to nucleus. Also, the binding of the VDR transcription factor to nuclei of cells stimulated with calcitriol was tested by EMSA. A rapid increase in the binding of biotin-labeled VDR to nuclei of cells stimulated with calcitriol was tested by EMSA. An increase in the binding of biotin-labeled VDR to nuclear extracts was observed after treatment (Fig. 6C), an effect that disappeared when 200-fold excess of unlabeled VDR oligonucleotide (Fig. 6C, bottom, lane 2) was added and remained unchanged with 200-fold excess of unlabeled NF-κB oligonucleotide (Fig. 6C, bottom, lane 3). To confirm the relevance of VDR activation in the calcitriol-dependent eNOS activation, VDR expression was silenced by transfecting siRNA against VDR. Calcitriol was unable to upregulate eNOS protein content (Fig. 7) when cells did not express VDR. In these VDR-depleted cells, calcitriol was also unable to increase ECE-1 protein content (Fig. 7).

In vivo effects of calcitriol in rats. To test the relevance of these in vitro findings, the effect of calcitriol was tested in normal Wistar rats treated with a single intraperitoneal injection of the drug. A significant rise in plasma ET-1 levels (Fig. 8A) and nitrite production in aorta and lung tissue (Fig. 8B) were observed. These changes were paralleled by similar increases observed in ECE-1 (Fig. 9, A and B) and eNOS (Fig. 9, C and D) protein content in lung and aorta tissues and measured by Western blot (Fig. 9, A–D, top) and immunohistochemistry (Fig. 9, A–D, bottom). As a result of both upregulations, a slight but significant rise in BP was observed in

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Fig. 5. Role of AP-1 in calcitriol-dependent ECE-1 upregulation: effect of different blockade strategies. A: human endothelial cells (EA) were incubated for 30 min with 50 μM PD-98059, an ERK1/2 inhibitor, or with 25 μM SP-600125, a JNK inhibitor, and then incubated for 8 h with 100 nM calcitriol. ECE-1 protein expression was analyzed by Western blot. The densitometric analysis of 5 independent experiments is given (means ± SE). Values are expressed as %control cells. B: EA cells were transfected with the human ECE-1 promoter linked to luciferase, preincubated for 30 min with 50 μM PD-98059 and 25 μM SP-600125, and then incubated for 6 h with 100 nM calcitriol; promoter activity was measured using a luciferase assay. The mean ± SE of 4 independent experiments in triplicate is expressed as %control cells. *P < 0.05 vs. other groups.

Fig. 4. Role of activator protein-1 (AP-1) in the calcitriol-dependent ECE-1 upregulation. A: cultured human endothelial cells (EA) were grown on coverslips and incubated with 100 nM calcitriol for 15 and 30 min. c-Fos and c-Jun were detected by immunofluorescence (green) in cells in which nuclei were stained with DAPI (blue). B: EA cells were incubated with 100 nM calcitriol at different times as indicated. c-Fos and c-Jun protein expression was analyzed by Western blot in cytosolic (left) and nuclear (right) extracts. A representative Western blot is shown for 4 independent experiments. C: nuclear extracts were harvested from EA cells that were previously incubated with 100 nM calcitriol for different times and then incubated with biotin-labeled oligonucleotide containing the AP-1 consensus sequence from ECE-1 gene. A representative electrophoretic mobility shift assay (EMSA) is shown on the left with a competition assay using nuclear extracts from control cells: lane 1, negative control without nuclear extract; lane 2, biotin-labeled AP-1; lane 3, an excess of unlabeled AP-1; lane 4, an excess of unlabeled NF-κB; lane 5, supershift with c-Jun and c-Fos antibodies. The densitometric analysis of gel shift experiments (means ± SE) of 4 different experiments is given on the right and is expressed as %control cells (time 0). *P < 0.05 vs. time 0.
calcitriol-treated rats, an effect that was not observed when rats were pretreated with an antagonist of the ECE-1, FR-901533 (Fig. 8C).

**DISCUSSION AND CONCLUSIONS**

The cardiovascular pleiotropic effects of vitamin D have been subject to special attention during the last 10–12 years; thus, some studies have suggested that vitamin D may play a role in blood pressure (32) and vascular function regulation (22). However, there are few studies involving vitamin D and endothelial function regulation analysis and in particular concerning the synthesis of vasoactive endothelial factors. Our results show that calcitriol was able to increase the synthesis of both endothelial factors NO and ET-1 in cultured endothelial cells. The rise in ET-1 synthesis depended on an increment in the activity and protein content of ECE-1, as changes in prepro-ET-1 were not observed when cells were treated with calcitriol, whereas the rise in NO levels seemed to be due to an increase in eNOS in cultured cells.

There is no previous evidence on the ability of calcitriol to modulate ECE-1 or on the treatment with active vitamin D derivatives inducing ET-1 synthesis. Absi and Ward (1) have demonstrated recently that 1,25-dihydroxyvitamin D₃ enhances endothelin responsiveness in human aortic smooth
muscle cells through the ETₐ receptor, but they did not measure ET-1 levels after the addition of vitamin D. Seals et al. (30) showed several correlations between aging and vascular endothelial function in humans. In their study, they found that calcitriol and endothelin levels as well as eNOS protein expression and eNOS activity were higher in older adults than in younger ones; also, oxidative stress and expression of several inflammatory mediators increased with aging. As a consequence, older adults presented impaired endothelial function compared with young ones (31). On the other hand, it is known that calcitriol induces NO production through interaction with VDR (22) and can also regulate arterial blood pressure by either reducing it (10, 11, 19, 40) or increasing it (2, 26, 27, 31), although the mechanisms involved are completely unknown.

NO and ET-1 systems are closely related due to their reciprocal regulation (6, 9, 25, 28, 29), and it could be suggested that the observed changes could be modulated by this interaction. NO reduces ECE-1 cellular content by increasing the degradation of its mRNA (25), and ET-1 induces NO production through ETB receptor activation (28). Changes observed in the ET-1 system in our data cannot be associated with NO changes, as a reduced ECE-1 content would be expected in the presence of increased NO levels. However, it could be suggested that the rise in NO could be inducing an inhibitory effect on ECE-1, which would minimize the observed stimulatory effect of calcitriol on this protein. In contrast, we could suggest that the rise in ET-1 could be responsible for increasing NO production, but the activation of ETB receptor by ET-1 induces changes in the eNOS activity, and our results also demonstrate increased eNOS mRNA and protein content, suggesting that other mechanisms of stimulation must be involved. Here, we did not assess the effect of different antagonists of soluble guanylate cyclase or ET receptors to

Fig. 7. Role of VDR in the calcitriol-dependent ECE-1 and eNOS upregulation: effect of silent VDR. EA cells were transfected with a small-interfering RNA against VDR (siVDR) or control (scrambled) for 72 h, and then cells were treated with 100 nM calcitriol (CT) for 8 h. Protein levels of ECE-1, eNOS, and VDR were analyzed by Western blot. A representative Western blot is shown at the top, whereas below the densitometric analysis of 4 independent experiments is given at the bottom (means ± SE). Values are %basal (B) conditions transfected with scrambled (%C). *P < 0.05 vs. %C.

Fig. 8. Effect of calcitriol on vasoactive endothelial factors in rats. Wistar rats were treated with either calcitriol (Calciex; 400 ng/kg body wt ip) or vehicle (basal, 0.2 ml phosphate-buffered saline) for 24 h. A: plasma ET-1 levels were measured after 24 h. Control rats showed 3.5 fmol/ml ET-1. B: aorta and lung tissue were extracted from untreated rats and then incubated in the presence or not of 100 nM calcitriol for different times. Aorta tissue (black bars) was incubated for 6 h, and lung tissue (open bars) was incubated for 2 h in glucose buffer at 37°C, and then nitrite production was measured in their supernatants. Basal nitrite production expressed as nmol·mg⁻¹·h⁻¹ was 0.94 in lung tissue and 2.24 in aorta tissue. C: mean blood pressure was registered 24 h after vehicle or calcitriol injection (black bars). Rats were then treated with FR-901533 (FR; 1 mg/kg body wt ip), an ECE-1 antagonist, and blood pressure was registered for the next 30 min (open bars). Results are the mean ± SE of 8 independent animals/group. *P < 0.05 vs. basal; **P < 0.05 vs. without FR.
explain whether changes observed in both factors could be completely independent.

Present results were also devoted to evaluate the intracellular mechanisms responsible for the increase in ECE-1 and eNOS cellular content. Changes in both proteins were due to an increase in their mRNA and promoter activities. However, the mechanism involved in each one seemed to be different. Calcitriol-induced ECE-1 upregulation depended on the inter-
action of calcitriol with VDR, with the subsequent activation of AP-1 that was the main transcription factor involved in the genesis of this upregulation. On the other hand, calcitriol-induced eNOS stimulation depended directly on VDR activation, with the calcitriol-VDR complex being the transcription factor that mediated this effect. Both signaling pathways are normally used by vitamin D to perform its different functions in cells (39).

Considering these results, it is difficult to determine the biological effect of calcitriol at the vascular level because of this apparently dual effect. If we consider the data quantitatively, we could suggest that changes in ET-1 induced by calcitriol could be more relevant than those obtained for NO, as the increment in ECE-1 was higher than in eNOS, despite the increase in ET-1 and NO levels being similar. To evaluate the physiological/functional role of calcitriol in vivo, Wistar rats were treated for 24 h with a single injection of calcitriol. Arterial blood pressure was measured as a parameter that is regulated mainly by vasoactive endothelial factors to modulate vascular tone.

The acute effect of calcitriol in rats caused an increase in the levels of ET-1 in plasma and tissue NO production as well as an upregulation of ECE-1 and eNOS protein content in aorta and lung tissues. Results from the in vivo experiments were in accord with the in vitro ones. In addition, rats treated with calcitriol showed a slight rise in arterial blood pressure, suggesting again that changes in ET-1 predominated over NO changes. The use of FR-901533, an ECE-1 antagonist, confirmed this hypothesis as FR-901533 prevented the rise in blood pressure induced by calcitriol.

The effect of calcitriol on blood pressure is controversial. As stated in the introduction, several studies have shown no association between vitamin D treatment and blood pressure, whereas others have reported decreases in blood pressure induced by vitamin D (10, 11, 20, 40). Regulation of blood pressure is a very complex phenomenon, and vitamin D exerts numerous biological effects, which are the reasons why associations between vitamin D and blood pressure are difficult to interpret. Concerning our results, an unbalance between NO and ET-1 could induce endothelial dysfunction and contribute to an increase in blood pressure. In addition, this unbalance may decrease blood flow in peripheral tissues, thus contributing to organ damage.

In conclusion, we hypothesized that the changes in blood pressure elicited by vitamin D could be due to its ability to modify the synthesis of endothelial vasoactive factors. Our data suggest an independent upregulation of ECE-1 and eNOS by calcitriol not only in endothelial cells but also in normotensive rats, which as a result of that upregulation presented a slight hypertension. The effect of calcitriol on eNOS depended on VDR activation, whereas its effect on ECE-1 depended on AP-1 activation.

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