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Calcitriol counteracts endothelial cell pro-inflammatory processes in a chronic kidney disease-like environment

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ABSTRACT

In advanced chronic kidney disease (CKD), hypocalcemia, high levels of advanced glycation end products (AGEs), and parathyroid hormone (PTH) coexist and are considered to play a role in the development of chronic vasculopathies. The aim of the present study was to evaluate the impact of a CKD-like environment on cultured endothelial cell (EC) functions and to assess the impact of calcitriol on the expression of parameters such as endothelial nitric oxide synthase (eNOS), receptor of AGEs (RAGE), interleukin 6 (IL-6) and nuclear factor kappa B (NFκB). Human umbilical vein cord endothelial cells (HUVEC) were grown in medium containing low Ca^{2+} concentration stimulated with AGE-HSA and PTH and treated with calcitriol for additional incubation. mRNA expression was established by reverse transcriptase-PCR, protein expression by Western blot analysis, IL-6 secretion by ELISA, NOS activity by conversion of $[^{14}C]$arginine to $[^{14}C]$citrulline and DNA-binding activity of NFκB-p65 assayed colorimetrically in nuclear extracts. The CKD-like environment characterized by the association of low Ca^{2+} and high levels of AGEs and PTH, depressed eNOS system activity and enhanced RAGE and IL-6 expression/secretion. DNA-binding activity of nuclear NFκB-p65 was increased and the expression of IκBα decreased. Addition of calcitriol normalized the expression, secretion and activity of eNOS, RAGE and IL-6. The enhanced NFκB activity was also counteracted probably due to the increased IκBα expression. The effect of CKD-like environment on EC may partly explain the increased vasculopathies in CKD patients, in contrast to calcitriol, which suggests a vascular protective action.

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1. Introduction

Cardiovascular complications are considered the main cause of morbidity and mortality in patients with chronic kidney disease (CKD) [1]. The abnormal biochemical environment that characterizes CKD and dialysis patients may contribute to the occurrence of cardiovascular disease (CVD), in addition to traditional risk factors [2–5]. Increased production and tissue accumulation of advanced glycation end products (AGEs) are involved in the development of CVD [3,6–8]. High blood levels of parathyroid hormone (PTH), reduced synthesis of calcitriol (1-25 dihydroxycholecalciferol, the active metabolite of vitamin D), and low ionized calcium concentrations in sera, may also participate in accelerating CKD-related atherosclerosis and increasing morbidity and mortality [2,9]. Clinical reports suggesting such interactions have been the basis of recent *in vitro* studies which demonstrated that low Ca^{2+} and high AGEs and PTH may each stimulate the endothelial expression of pro-inflammatory and pro-atherosclerotic parameters [10–14]. In addition, it was demonstrated that calcitriol might counteract some of these deleterious effects [14,15]. These results fit well with the protective effect of vitamin D on cardiovascular disease manifestations frequently observed in hemodyalized patients [16,17], as well as with its positive action on blood pressure and PTH levels in the elderly women [18].

The endothelium is involved in the development of vasculopathies, as well as in control of blood pressure homeostasis [19]. To evaluate the effects of CKD-like environmental modifications on endothelial cell functions, we used an *in vitro* model of endothelial cells in culture. The effect of extracellular stimulation with low Ca^{2+}, and high AGEs and PTH levels was studied both in the presence and in the absence of calcitriol. Endothelial expression, production and/or activity of endothelial nitric oxide synthase (eNOS), receptor of AGEs (RAGE), interleukin 6 (IL-6), and nuclear factor kappa

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B (NFκB) pathway were measured systematically and compared to those observed in standard conditions. Changes in the expressions of these parameters are recognized as markers of endothelial cell activation [19–22].

2. Materials and methods

2.1. Preparation of AGE-HSA

AGE-HSA was prepared as previously described [10]. Briefly, HSA (fraction V, 1.5 mmol/L) was dissolved with 1 mol/L of glucose in 100 mmol/L sodium phosphate buffer (pH 7.4) containing 200 U/mL of penicillin, 200 μg/mL streptomycin, 80 μg/mL of gentamycin, and 1.5 mmol/L of phenylmethylsulfonylfluoride (PMSF), under sterile conditions. Sterilization was initially performed with 0.22-μm pore size filters, after which the solution was incubated for 60 days in the dark at 37 °C. After incubation, the solution was dialyzed overnight against PBS. The HSA control was subjected to the same procedures (except for the presence of glucose in the incubating solution) as that used for AGEs. Endotoxin levels in all samples were measured by *Limulus* amoebocyte lysate assay (E-Toxate, Sigma, St. Louis, MO, USA), and were found to be less than 0.2 ng/mL (corresponding to 0.96 EU/mL). The concentrations of AGE-HSA and HSA were determined with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

2.2. Cell culture and treatments

Endothelial cell cultures were obtained from umbilical cords according to the method of Jaffe et al. [23]. The Ethical Committee of Meir Medical Center approved the research program and each parturient gave written consent permitting use of the umbilical cord. Only umbilical cords from normal pregnancies and deliveries were used. The cells were identified as endothelial cells by their typical cobblestone morphology and by immunostaining for von Willebrand factor. Confluent cultures of HUVEC were used for experiments at passages 4–5. HUVEC were grown in a low extracellular Ca^{2+} concentration (3.5 mg/dL) and stimulated with 200 μg/mL AGE-HSA and 10^{-10} mol/L PTH (CKD-like environment). In order to evaluate the effect of calcitriol on the CKD-like environment, calcitriol was added an hour later for additional incubation. The control group was grown in a standard of cell culture medium (containing 5.8 mg/dL concentration of Ca^{2+}) and treated with 200 μg/mL HSA. The effects on the expression, secretion, and/or activity of eNOS, RAGE and IL-6 as well as on the activity and/or expressions of the NFκB pathway were examined.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expressions of the eNOS, RAGE and IL-6 genes were performed by semi-quantitative multiplex RT-PCR and real-time PCR techniques. Total RNA was extracted from endothelial cells using the PUREscript RNA isolation kit (Gentra systems Inc., Minneapolis, MN, USA), according to manufacturer's instructions. RNA (1 μg) was then reverse transcribed into single-stranded DNA with 200 units of SUPERSCRIPT™II RNase Reverse Transcriptase and oligo $(dT)_{15}$ primer at 37 °C for 45 min, 42 °C for 15 min, and 99 °C for 5 min.

2.4. Real-time RT-PCR

To quantify the amounts of eNOS, RAGE and IL-6 mRNA expressions in endothelial cells, real-time RT-PCR was performed with a Light Cycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) in glass capillary tubes. The Light Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics GmbH) and primers for human eNOS, IL-6 and β-actin (the same as conventional PCR) and RAGE primers were: forward primer: 5′-TGGAACCGTAACCCTGACCT-3′, reverse primer: 5′-CGATGATGCTGATGCTGACA-3′ were added to cDNA dilutions. The thermal profile for SYBER Green PCRs was 95 °C for 10 min, followed by 35 cycles at 95 °C for 10 s, 58 °C for 7 s, 72 °C for 18 s, and 90 °C for 5 s. To prove the specificity of the PCR product, a melting curve analysis was performed at 95 °C for 5 s and at 70 °C for 20 s. A dilution series of a standard sample was run with the unknown samples. Gene expression was determined by normalization against β-actin expression.

2.5. Western blot analysis

To evaluate the protein expression of eNOS, RAGE, IκBα and α-Tubulin, total protein (50 μg) was electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk, incubated with anti-eNOS monoclonal antibody (1:2000), anti-RAGE monoclonal antibody (1:500), anti-IκBα monoclonal antibody (1:500) or α-Tubulin monoclonal antibody (1:13,000). The second antibody was sheep anti-mouse Ig conjugated with horseradish peroxidase and developed with chemiluminescent reporter system (ECL).

The nitrocellulose membranes were stripped and blocked before being reprobed with a different antibody. eNOS protein expression was detected at 136 kDa, RAGE protein at 48 kDa, IκBα at 37 kDa, and α-Tubulin as loading control was detected at 50 kDa.

2.6. eNOS activity assay

To measure eNOS activity in cell lysates (150 μg total protein), the conversion of [^{14}C]-arginine to [^{14}C]-citrulline was used as previously described by Shah et al. [24].

2.7. IL-6 immunoassay

IL-6 was measured in HUVEC supernatants using a DuoSet enzyme-linked immunosorbent assay (ELISA) development kit (R&D Systems, Minneapolis, MN, USA). IL-6 levels are given as pg/10^5 cells after correction of cell number.

2.8. NFκB DNA-binding activity assay

Nuclear protein extracts were prepared using the NucBuster Protein Extraction Kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions. DNA-binding activity of NFκB was assayed colorimetrically, using NoShift NFκB (p65) reagents and the NoShift Transcription Factor Assay Kit (Novagen, Madison, WI, USA). Negative controls consisted of reactions performed in the absence of nuclear extract. As secondary antibody, we used HRP-conjugated goat antimouse immunoglobulin G, which targets anti-NFκB-p65 mouse monoclonal antibody. All assays were performed in duplicate. Binding activity was measured via colorimetric absorbance at 450 nm on a spectrophotometer (Sunrise, NEOTEC, Grodig, Austria) using 3,3′,5,5′-tetramethylbenzidine as substrate.

2.9. Statistical analysis

The results are expressed as mean ± standard error (SE). Student's *t*-test or Wilcoxon test were used for data analysis. A *p*-value of 0.05 or less was considered significant.

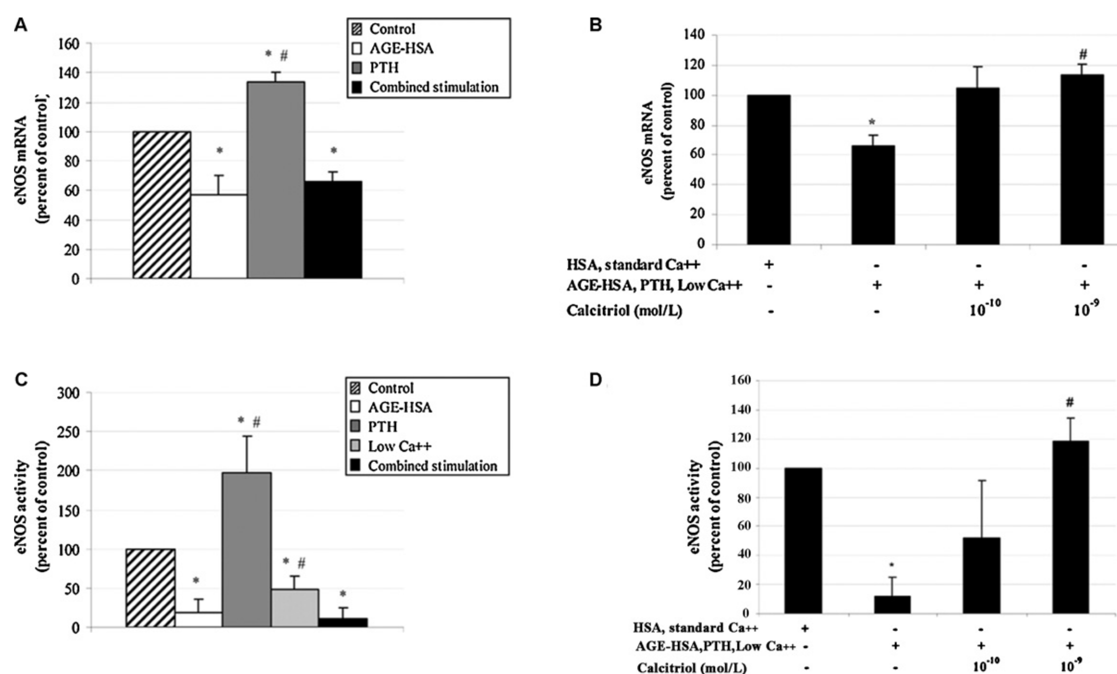

Fig. 1. Effect of calcitriol on eNOS system in HUVEC stimulated with CKD-like environment. The control group was grown in standard calcium (5.8 mg/dL extracellular ionized calcium), without AGE-HSA or PTH. AGE-HSA group was treated by AGE-HSA (200 μg/mL) in standard calcium without PTH. PTH group was treated by PTH (10^{-10} mol/L) in standard calcium without AGE-HSA. Low calcium group was grown in low calcium (3.5 mg/dL extracellular ionized calcium). Combined stimulation group (CKD-like environment) was grown in low calcium and treated by AGE-HSA and PTH as described in Section 2. (A) 24 h or (C) 72 h incubation time. (B and D) Calcitriol (10^{-10} or 10^{-9} mol/L) was added to the stimulated HUVEC an hour after stimulation for an additional 23 h (B) or 71 h (D). (A and B) Total RNA was extracted and the levels of eNOS and β-actin mRNA expression were assessed by real-time PCR. eNOS mRNA levels normalized to the levels of β-actin mRNA expression and relative mRNA content was expressed as fold of control. Data are expressed as mean ± SE of 3–4 independent experiments. $^{*}p \leq 0.01$ vs control (HSA, standard Ca^{2+}); $^{\#}p \leq 0.05$ vs CKD-like environment (AGE-HSA, low Ca^{2+}, PTH). (C and D) eNOS activity in total cell lysates was measured by conversion of [^{14}C]-arginine to [^{14}C]-citrulline. Data are expressed as mean ± SE of 4 independent experiments. $^{*}p \leq 0.005$ vs control; $^{\#}p \leq 0.05$ vs CKD-like environment.

3. Results

3.1. CKD-like environment and the eNOS system – effect of calcitriol

PTH treatment increased the mRNA expression and activity of eNOS [12]. Extracellular ionized calcium decreased the activity of eNOS, however did not affect its mRNA expression [13]. AGEs on the other hand decreased both mRNA expression and activity [10,14]. Calcitriol and low calcium did not affect the eNOS system [15].

In a CKD-like environment eNOS mRNA (66 ± 6.57% vs control: 100%; $p = 0.01$) (Fig. 1A) and activity (11.5 ± 13% vs control: 100%; $p = 0.005$) (Fig. 1C) were markedly depressed. The combined stimulation with CKD-like environment, completely overshadowed the stimulatory action of PTH alone on mRNA expression (134 ± 13%), vs CKD-like environment (66 ± 6.5%); $p = 0.03$ (Fig. 1A) and on the activity of eNOS (198 ± 44%) vs CKD-like environment (11.5 ± 13.3%); $p = 0.01$ (Fig. 1C). In addition, the inhibitory effect of the CKD-like environment on eNOS activity was stronger than the effect of low Ca^{2+} alone (48 ± 17%) vs CKD-like environment (11.5 ± 13.3%); $p = 0.05$ (Fig. 1C).

Addition of calcitriol to EC in a CKD-like environment normalized eNOS mRNA expression (113 ± 7.4%), vs CKD-like environment (66 ± 6.57%); $p = 0.03$ (Fig. 1B) and the activity of eNOS (118.3 ± 16%) vs CKD-like environment (11.5 ± 13%); $p = 0.02$ (Fig. 1D).

3.2. CKD-like environment and endothelial RAGE mRNA expression – effect of calcitriol

The mRNA expression of RAGE was enhanced in the presence of AGE-HSA, low Ca^{2+}, or PTH [11,13,14] and decreased in the presence of calcitriol [15]. As expected, the combined stimulation with AGE-HSA, low Ca^{2+}, and PTH (CKD-like environment) had a similar effect on RAGE mRNA expression (209 ± 46% vs control: 100%; $p \leq 0.05$) (Fig. 2A). The stimulatory effect of CKD-like environment (209 ± 46%) was significantly stronger than the effect of PTH alone (142 ± 16%); $p \leq 0.05$ (Fig. 2A), however not stronger than low Ca^{2+} or AGEs separately.

Addition of calcitriol decreased the over-expression of RAGE mRNA (10^{-10} mol/L calcitriol: 168.7 ± 56%, $p = 0.04$; 10^{-9} mol/L calcitriol: 138 ± 16.4%, $p = 0.05$ vs CKD-like environment: 209 ± 46%) (Fig. 2B).

3.3. CKD-like environment and endothelial IL-6 expression and secretion – effect of calcitriol

Incubation of EC with low Ca^{2+}, AGEs, or PTH enhanced the endothelial expression of IL-6 mRNA, as previously reported [11,13,14]. Calcitriol did not effect IL-6 expression [15]. As expected, the combined incubation (CKD-like environment) had a significant stimulating impact on IL-6 mRNA expression (271.7 ± 80% vs control: 100%; $p \leq 0.05$) (Fig. 3A). The stimulating effect of CKD-like environment was stronger than the effects of low Ca^{2+} or PTH separately (low Ca^{2+}: 177 ± 34.5%, $p \leq 0.05$; PTH: 166 ± 10.9%, $p \leq 0.05$, vs CKD-like environment: 271 ± 79.8%) (Fig. 3A).

IL-6 secretion was also augmented in the presence of low extracellular Ca^{2+} and high AGEs and PTH (751.63 ± 187 pg/10^5 cells vs control: 452.93 ± 93 pg/10^5 cells; $p = 0.03$) (Fig. 3C).

Calcitriol treatment decreased the elevated IL-6 mRNA expression in EC in a CKD-like environment (10^{-10} mol/L calcitriol: 146 ± 21%, $p = 0.05$; 10^{-9} mol/L calcitriol: 167 ± 23.4%, $p = 0.05$ vs CKD-like environment: 209 ± 46%) (Fig. 3B). The elevated IL-6 secretion in the CKD-like milieu was significantly decreased when

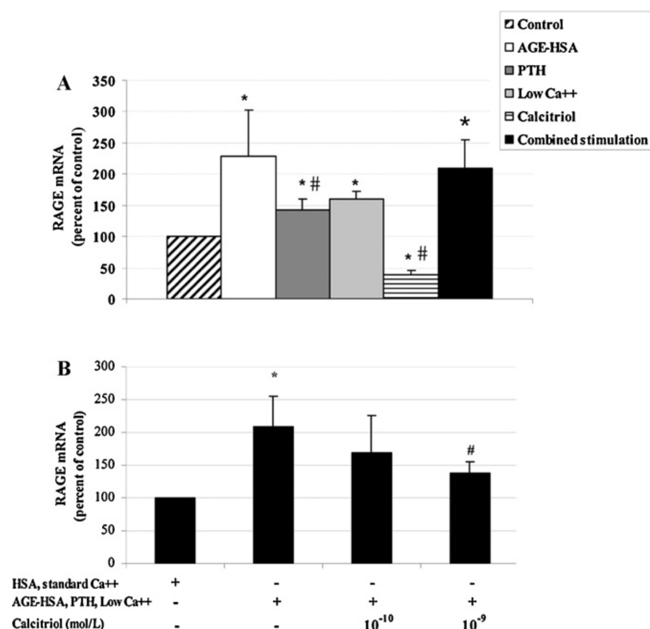

Fig. 2. Effect of calcitriol on RAGE mRNA expression in HUVEC stimulated with CKD-like environment. The control group was grown in standard calcium (5.8 mg/dL extracellular ionized calcium), without AGE-HSA or PTH. AGE-HSA group was treated by AGE-HSA (200 μg/mL) in standard calcium without PTH. PTH group was treated by PTH (10^{-10} mol/L) in standard calcium without AGE-HSA. Low calcium group was grown in low calcium (3.5 mg/dL extracellular ionized calcium). Calcitriol group was treated with calcitriol (10^{-9} mol/L) in standard calcium without other stimulations. Combined stimulation group (CKD-like environment) was grown in low calcium and treated by AGE-HSA and PTH as described in Section 2. (A) 24 h incubation time. (B) Calcitriol (10^{-10} or 10^{-9} mol/L) was added to the stimulated HUVEC an hour after stimulation for an additional 23 h. (A and B) Total RNA was extracted and the levels of RAGE and β-actin mRNA expression were assessed by real-time PCR. RAGE mRNA levels normalized to the levels of β-actin mRNA expression and relative mRNA content was expressed as fold of control. Data are expressed as mean ± SE of 3–4 independent experiments. $^{*}p \leq 0.01$ vs control (HSA, standard Ca^{2+}); $^{\#}p \leq 0.05$ vs CKD-like environment (AGE-HSA, low Ca^{2+}, PTH).

calcitriol was added (10^{-10} mol/L calcitriol: 591.5 ± 149 pg/10^5 cells, $p \leq 0.05$; 10^{-9} mol/L calcitriol: 612 ± 161 pg/10^5 cells, $p \leq 0.05$ vs CKD-like environment: 751.63 ± 187 pg/10^5 cells) (Fig. 3D).

3.4. CKD-like environment and endothelial NFκB pathway – effect of calcitriol

Incubation of EC with either low extracellular Ca^{2+} or AGE-HSA alone induced an elevation in NFκB-p65 DNA-binding activity [13,14]. Calcitriol on the other hand decreased the activity of NFκB-p65 [15]. PTH alone did not affect this pathway (data not shown). The combined stimulation of AGE-HSA, low extracellular Ca^{2+} and PTH increased the NFκB-p65 DNA-binding activity (155.7 ± 5.36% vs control: 100%; $p = 0.01$) (Fig. 4A), probably in relation to the combined impact of low extracellular Ca^{2+} and AGE-HSA. An additive effect was detected, the increased activity was stronger compared to that induced by either low Ca^{2+} or AGEs alone (low Ca^{2+}: 114 ± 3.8%, $p = 0.005$; AGEs: 133 ± 7%, $p = 0.05$ vs CKD-like environment: 155.7 ± 5.36%) (Fig. 4A). In addition, a decrease in IκBα levels was found after incubation in the CKD-like environment (Fig. 4C). α-Tubulin was used as the loading control (Fig. 4C).

Calcitriol treatment was associated with a normalization of p65 DNA-binding activity (115 ± 5.5% vs CKD-like environment: 155.7 ± 5.36%; $p = 0.05$) (Fig. 4B) and with an increase in IκBα expression (Fig. 4D). α-Tubulin was used as the loading control (Fig. 4D).

4. Discussion

Cardiovascular diseases, especially atherosclerosis, have been reported to be the main causes of dialysis-related morbidity and mortality [1]. In the development of these vasculopathies, not only are traditional risk factors involved [4,5], but also CKD-related biochemical changes such as hypocalcemia, vitamin D deficiency, hyperparathyroidism, and elevated AGEs, which are considered promoting factors [2,3]. Previous *in vitro* studies have demonstrated that endothelial cells are stimulated and respond to extracellular changes by increasing the expression and production of pro-inflammatory and atherosclerotic parameters [13]. Similar results were obtained after stimulation with high concentrations of AGEs and PTH [10–12,14]. These studies evaluated the stimulating effect of calcium, AGEs and PTH separately, however, in advanced CKD and hemodialysis patients, an abnormal cellular environment of low Ca^{2+} and high AGEs and PTH blood levels coexist [9,25]. Therefore, in the present study the cellular environment was modified to mimic conditions considered to be similar to advanced CKD by stimulating the cells with low Ca^{2+} and high levels of AGE-HSA and PTH together.

The impact of the different combinations of stimuli on the eNOS system was not uniform. Low extracellular Ca^{2+} decreased the activity of eNOS, even though the mRNA expression did not significantly change [13]. High AGE-HSA depressed both mRNA expression and activity of eNOS [10,14], while PTH was found to enhance them [12]. The combined effect manifested by a strong blunting action on the eNOS system even in the presence of PTH, characterize the dominance of the inhibitory effect of low extracellular levels of Ca^{2+} and AGE-HSA. The combined effect was stronger than the effect of low Ca^{2+} alone, but it was not significantly stronger than the effect of AGE-HSA alone. This response suggests that AGEs accumulation may be of primary importance in the pathophysiology of CVD seen in conditions associated with high AGEs production such as diabetes mellitus (DM) ageing and/or CKD [3,6,7]. One of the main features in EC dysfunction is reduced bioavailability of nitric oxide (NO) which has important anti-atherogenic properties [19,26,27]. The decreased eNOS expression and activity suggest that these CKD-like environmental conditions could be considered a “non-traditional” cardiovascular risk factor.

Addition of calcitriol improved and even normalized the parameters associated with the eNOS system. Such phenomenon may be particularly relevant to maintaining appropriate endothelial function, knowing that nitric oxide may affect vascular cell function, protect vascular structures, and modulate coagulation processes [19,26,27].

Unlike the variable effect on the eNOS system, each of the stimuli used, separately, increased RAGE and IL-6 expression [11,13,14]. As expected, their combined effects were similar. The increased expression of RAGE in a CKD-like milieu was significantly stronger than that observed with PTH alone. In addition, the expression on IL-6 expression was stronger than that found in an extracellular milieu characterized by either low Ca^{2+} or high PTH.

The stimulating effects of both RAGE and IL-6 were blunted by calcitriol. The present findings corroborate previous reports showing that calcitriol affects non-stimulated and stimulated EC functions [14,15].

In the present model, the impact of AGEs on EC functions seems dominant when we consider that the effects of the CKD-like environment on the eNOS, RAGE and IL-6 expressions were not significantly stronger than the effects obtained with AGEs alone. Since the deleterious action of AGEs is mainly mediated through RAGE [28,29], the impact of calcitriol on RAGE expression may be essential in preventing or delaying the development of vasculopathies related to increased production of AGEs.

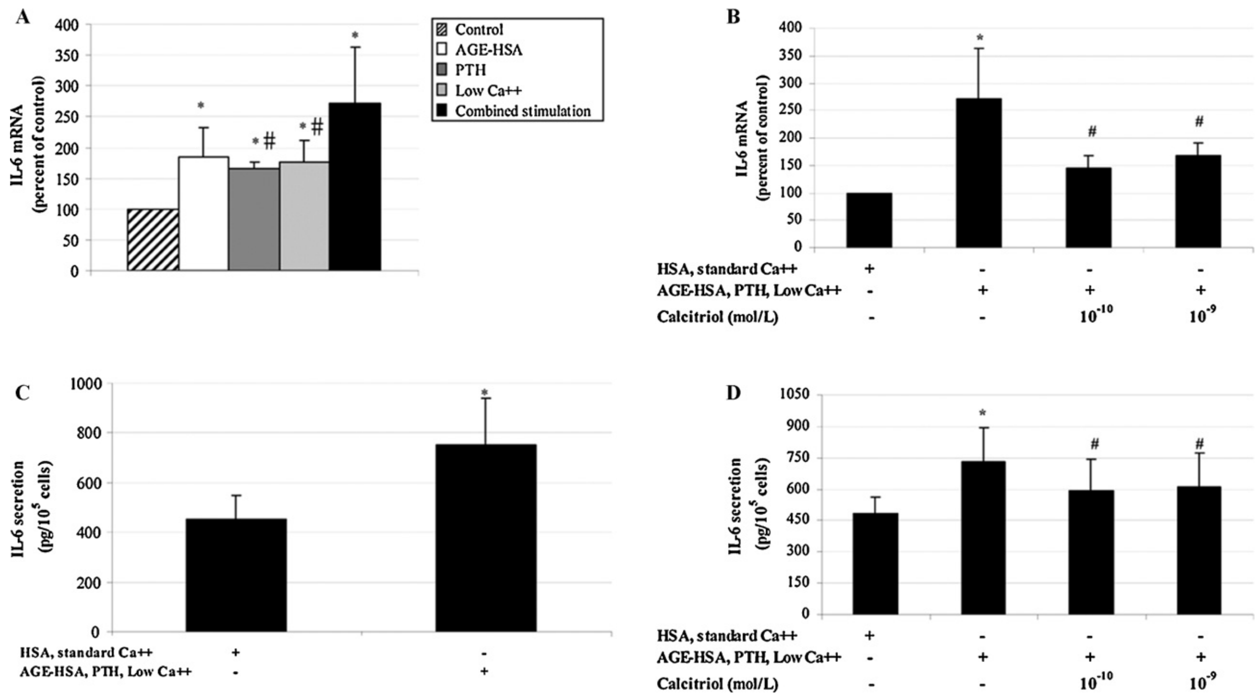

Fig. 3. Effect of calcitriol on IL-6 mRNA and protein secretion in HUVEC stimulated with CKD-like environment. For details see Fig. 2. (B and D) Calcitriol (10^{-10} or 10^{-9} mol/L) was added to the HUVEC an hour after stimulation for an additional 23 h. (A and B) Total RNA was extracted and the levels of IL-6 and β-actin mRNA expression were assessed by real-time PCR. IL-6 mRNA levels normalized to the levels of β-actin mRNA expression and relative mRNA content was expressed as fold of control. Data are expressed as mean ± SE of 3 independent experiments. $^*p \leq 0.05$ vs control (HSA, standard Ca^{2+}); $^{\#}p \leq 0.05$ vs CKD-like environment (AGE-HSA, low Ca^{2+}, PTH). (C and D) IL-6 in supernatants was measured by ELISA. Results are mean ± SE of 7–8 independent experiments. $^*p \leq 0.05$ vs control; $^{\#}p \leq 0.05$ vs CKD-like environment.

As previously shown, a low concentration of extracellular Ca^{2+} or AGEs may stimulate NFκB-p65 DNA-binding activity [13,14], unlike PTH which did not stimulate this pathway (data not shown). The effect observed in the CKD-like environment demonstrates the existence of an activated NFκB pathway, which was significantly stronger than that detected with either low Ca^{2+} or high AGEs. The lower endothelial IκBα levels found in these EC, may explain why p65-NFκB is activated.

Calcitriol once again was found to counteract the activity of stimulated NFκB. The elevated IκBα levels in the EC incubated with calcitriol in the CKD-like environment may explain why the NFκB pathway activation was blunted by calcitriol. The activation

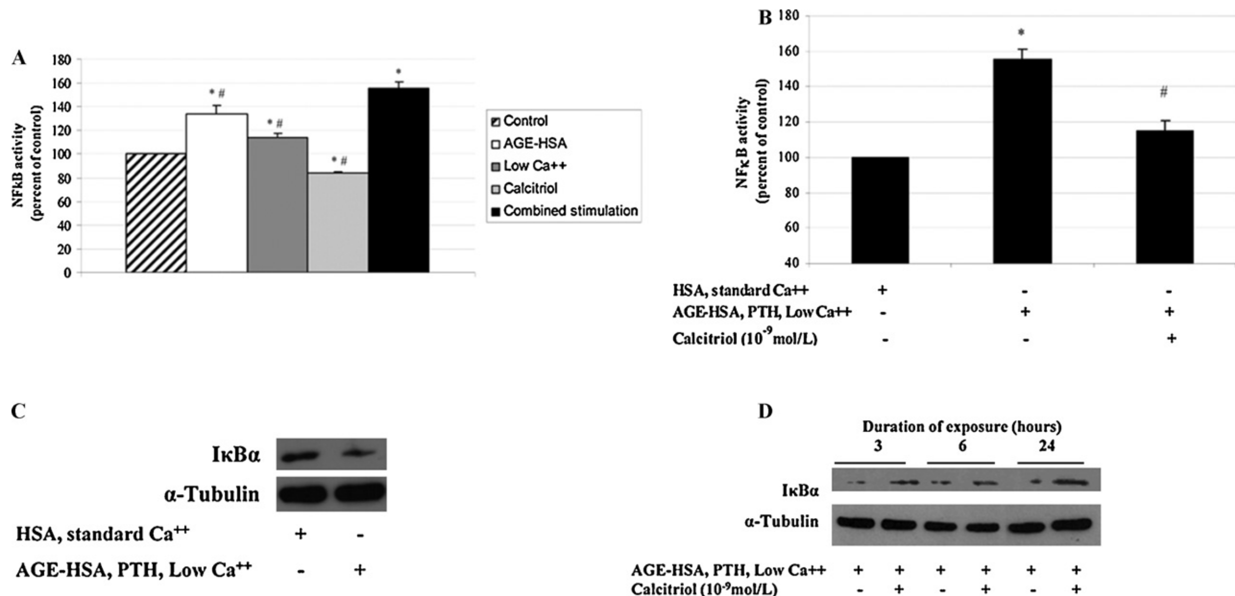

Fig. 4. Effect of calcitriol on NFκB DNA binding activity in HUVEC stimulated with CKD-like environment. (A and C) HUVEC were grown in 3.5 mg/dL extracellular ionized calcium and incubated for 24 h with 200 μg/mL AGE-HSA and 10^{-10} mol/L PTH (CKD-like environment). The control group was grown in 5.8 mg/dL extracellular ionized calcium (standard medium) and treated with 200 μg/mL HSA for 24 h. (B and D) Calcitriol (10^{-9} mol/L) was added to the HUVEC an hour after stimulation for an additional 23 h. (A and B) Nuclear protein extracts were prepared. DNA-binding activity of NFκB was measured by ELISA. Results are mean ± SE of 3 independent experiments. $^*p \leq 0.05$ vs control; $^{\#}p \leq 0.05$ vs CKD-like environment. (C and D) Protein lysates were prepared. IκBα protein expression was assessed by Western blot analysis. The level of α-Tubulin is shown as a loading control. Similar results were obtained in 3 independent experiments.

of the endothelial NFκB pathway may represent an activation of inflammatory processes in the cultured EC and may explain why the eNOS system was depressed and RAGE and IL-6 expressions were enhanced [21]. The impact of such environmental changes, which exists in CKD, may explain the endothelial dysfunction and long-term vasculopathies observed in CKD patients [1,2].

In conclusion, we observed that the combination of low extracellular Ca^{2+} and high concentrations of AGEs and PTH significantly affected the functions of cultured EC. This effect is characterized by diminished activity of the eNOS system and increased expression and secretion of the pro-inflammatory parameters RAGE and IL-6. These changes may be partially explained by activation of the intracellular NFκB pathway. Calcitriol, the active vitamin D metabolite, was found to blunt these effects. This moderating effect of calcitirol was also observed on the activity of the NFκB pathway.

The level of 10^{-10} mol/L concentration of calcitriol is in the normal range of calcitriol in the blood. Concentration of 10^{-9} mol/L calcitriol is supra-physiological but may be considered relevant and is used in experimental protocols since HUVEC express 1α-hydroxylase [30] and can explain higher local concentrations and production of calcitriol.

To the best of our knowledge, this is the first *in vitro* study that examines the effect of several stimuli on EC functions. Knowing that each of these stimuli may play a role in the development of CKD-related vasculopathies and eventually increase morbidity and mortality in CKD and dialysis patients, we wanted to evaluate their possible interactions with EC functions, looking for possible additive effects.

Calcitriol had a protective action on the EC in spite of the dysfunctions induced by the CKD-like environment. While it is always difficult to extrapolate results obtained from *in vitro* studies, the present data suggest that the association of high extracellular AGEs and PTH in the presence of hypocalcemia may accelerate the occurrence of CKD-related CVD and vasculopathies by affecting the endothelium. In addition, the effects observed with calcitriol *in vitro* may explain the clinical improvements in CVD observed in dialysis patients after treatment with vitamin D compounds [16,17].

Conflict of interest statement

None declared.

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