Calcitriol blunts the deleterious impact of advanced glycation end products on endothelial cells

Yeela Talmor,1,2 Eliezer Golan,1,2 Sydney Benchetrit,1,2 Jacques Bernheim,1,2 Osnat Klein,1,2 Janice Green,1 and Gloria Rashid1

1Renal Physiology Laboratory, Department of Nephrology and Hypertension, Meir Medical Center, Kfar-Saba and 2Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Submitted 29 January 2008; accepted in final form 17 March 2008

Calcitriol blunts the deleterious impact of advanced glycation end products on endothelial cells. Am J Physiol Renal Physiol 294: F1059–F1064, 2008. First published March 19, 2008; doi:10.1152/ajprenal.00051.2008.—Advanced glycation end products (AGEs), which are elevated in diabetic and uremic patients, may induce vascular dysfunctions, and calcitriol may improve the cardiovascular complications. Therefore, we examined whether calcitriol may modify the endothelial response to AGEs stimulation. Knowing the importance of nuclear factor-κB (NF-κB) in endothelial inflammatory responses, the effect of AGEs and calcitriol on this pathway was also studied. Calcitriol was added to endothelial cells previously incubated with AGE-human serum albumin (HSA). AGE-HSA induced a decrease in endothelial nitric oxide synthase (eNOS) mRNA expression and enzyme activity. Addition of calcitriol to AGE-HSA-treated endothelial cells improved the decreased action of AGEs on the eNOS system. AGE-HSA increased the AGEs receptor mRNA and protein, which were both blunted by calcitriol. The parallel elevation of interleukin-6 mRNA in the presence of AGE-HSA was also blunted by calcitriol. The NF-κB-p65 DNA binding activity was enhanced and associated with a decrease in inhibitor κBα (IkBα) and an increase in phosphorylated (p)-IkBα levels. Addition of calcitriol blunted the AGEs-induced elevation of NF-κB-p65 DNA binding activity, a phenomenon related to an increased expression of IkBα. This increase was correlated to declined p-IkBα levels. The present results support the concept that calcitriol may act as a vascular protective agent counteracting the probable deleterious actions of AGEs on endothelial cell activities.

Address for reprint requests and other correspondence: G. Rashid, Renal Physiology Laboratory, Dept. of Nephrology and Hypertension, Meir Medical Center, Kfar-Saba and Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel (e-mail: Gloriar@clalit.org.il).

MATERIALS AND METHODS

Preparation of AGE-HSA. AGE-HSA was prepared as previously described (23). Briefly, HSA (fraction V, 1.5 mmol/l; Sigma) was dissolved with 1 mol/l of glucose (Riedel-de Haen, Seelze, Germany) 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 phenylmethylsulfonyl fluoride 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 procedure (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) and were found to be <0.2 ng/ml (corresponding to 0.96 EU/ml). The concentrations of AGE-HSA and HSA were determined by the method of the BCA Protein Assay Kit (Pierce Biotechnology).

Endothelial cell culture and incubation. Endothelial cell cultures were obtained from human umbilical vein cord endothelial cells (HUVEC) as previously described (23). The ethical committee of Meir Medical Center approved the research program, and each participant signed an informed consent form. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
turer gave written consent permitting the use of her umbilical cord. 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 3–4 and were treated for 6–72 h with 200 µg/ml AGE-HSA. The cells were then treated with two physiological (10⁻¹¹ and 10⁻¹⁰ mol/l) and a supraphysiological (10⁻⁹ mol/l) concentration of calcitriol (Abbott Laboratories). Calcitriol (10⁻¹¹ and 10⁻¹⁰ mol/l) correspond to low and high physiological levels measured in human blood, and 10⁻⁹ mol/l, a supraphysiological concentration, is used in in vitro experiments (16). The control groups were treated with 200 µg/ml HSA.

RT-PCR. Total RNA was extracted from endothelial cells using the PUREscript RNA isolation kit (Gentra Systems), according to the manufacturer’s instructions. RNA (1 µg) was then reverse transcribed into single-strand DNA with 200 units of SUPERSCRIPT II RNase Reverse Transcriptase (Invitrogen) and oligo(dT)₁₅ primer (Promega, Madison, WI) at 37°C for 45 min, 42°C for 15 min, and 90°C for 5 min.

To quantify the amounts of eNOS, RAGE, and IL-6 mRNA expressions in endothelial cells, real-time PCR was performed with a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) in glass capillary tubes. The Light Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics) and primers were added to cDNA dilutions: for eNOS mRNA amplification forward primer 5'-CCCCGACATGAGTCTG-3', and reverse primer 5'-CTGGCATGATGCTG-3'. α-Actin primers were as follows: forward primer 5'-GACCACACTTCTCTCTG-3', and reverse primer 5'-GGATTCTGTATGCTG-3'. The thermal profile for SYBR Green PCRs was 95°C for 10 min, followed by 35 cycles of 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 by 95°C for 5 s and 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.

Western blot analysis. To evaluate the protein expression of RAGE, inhibitor kBα (IkBα), phosphorylated (p)-IkBα, 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 and incubated with anti-RAGE monoclonal antibody (1:500; Chemicon, Temecula, CA), anti-IkBα monoclonal antibody (1:500; Santa Cruz, Santa Cruz, CA), anti-phosphorylated IkBα (p-IkBα) monoclonal antibody (1:400; Santa Cruz), or with α-tubulin monoclonal antibody (1:13,000; Sigma). The second antibody was sheep anti-mouse Ig conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs) and developed with the chemiluminescent reporter system. The nitrocellulose membranes were stripped and blocked before being reprobed with a different antibody. The expression of RAGE protein was detected as a single band at 48 kDa, the expression of IkBα or p-IkBα protein was detected as a single band at 37 kDa, and α-tubulin as loading control was detected as a single band at 50 kDa. The quantification of RAGE, IkBα, and p-IkBα expressions was normalized against the quantification of tubulin expression.

eNOS activity assay. To measure eNOS activity in cell lysates (150 µg total protein) the conversion of [¹⁴C]arginine to [¹⁴C]citrulline was used as previously described by Shah et al. (29).

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

**Statistical analysis.** The results are expressed as means ± SE. Student’s paired t-test or paired Wilcoxon test (for protein expression) were used for data analysis. P values of 0.05 or less were considered significant.

**RESULTS**

Calcitriol counteracts the AGE-HSA-dependent depression of the eNOS system. Incubation of HUVEC with AGE-HSA (200 µg/ml) for 24 h decreased the mRNA expression of eNOS compared with the control group, which was treated with HSA (200 µg/ml) (57 ± 10.8%, P = 0.015 vs. control; Fig. 1A). Addition of calcitriol (10⁻¹¹, 10⁻¹⁰, and 10⁻⁹ mol/l) upregulated the mRNA expression of eNOS up to the basal levels observed with HSA (10⁻¹¹, 80.3 ± 16.8%, P = 0.04; 10⁻¹⁰, 84.4 ± 13.2%, P = 0.032; 10⁻⁹, 98.1 ± 12.5%, P = 0.04 vs. AGE-HSA: 57 ± 10.8%; Fig. 1A). The values of eNOS mRNA expression in the control group (HSA) and in the groups treated with 200 µg/ml HSA.

![Fig. 1. Effect of calcitriol on the endothelial nitric oxide synthase (eNOS) system in human umbilical vein cord endothelial cells (HUVEC) stimulated with advanced glycation end products (AGE)-human serum albumin (HSA).](http://ajprenal.physiology.org/)

**A:** HUVEC were incubated for 24 h with 200 µg/ml HSA (control) or 200 µg/ml AGE-HSA, and calcitriol (10⁻¹¹, 10⁻¹⁰, and 10⁻⁹ mol/l) was added to AGE-HSA-stimulated HUVEC 1 h after stimulation for an additional 23 h. 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 were expressed as a degree of control. Data are expressed as means ± SE of 5 independent experiments. *P ≤ 0.015 vs. control (HSA); #P ≤ 0.05 vs. AGE-HSA.

**B:** HUVEC were incubated for 24 h with 200 µg/ml HSA (control) or 200 µg/ml AGE-HSA, and calcitriol (10⁻¹¹, 10⁻¹⁰, and 10⁻⁹ mol/l) was added to AGE-HSA-stimulated HUVEC 1 h after stimulation for an additional 23 h. 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 were expressed as a degree of control. Data are expressed as means ± SE of 5 independent experiments. *P ≤ 0.015 vs. control (HSA); #P ≤ 0.05 vs. AGE-HSA.
with both AGEs and calcitriol were not significantly different. The eNOS activity was markedly depressed after 72 h of incubation with AGE-HSA (18.4 ± 16.8%, P = 0.04 vs. control; Fig. 1B). Addition of calcitriol (10⁻⁹ mol/l) clearly improved this depressed activity of eNOS up to values equivalent to that found in the control group (10⁻⁹: 101.3 ± 26%, P = 0.038 vs. AGE-HSA: 18.4 ± 16.8%; Fig. 1B).

Calcitriol induces a decrease in the AGE- and HSA-related elevation of endothelial RAGE expression. AGE-HSA upregulates mRNA (200 ± 33.3%, P = 0.043 vs. control; Fig. 2A) and protein (197 ± 43%, P = 0.035 vs. control; Fig. 2B) expressions of RAGE compared with the control group (HSA). Calcitriol blunted this increase in RAGE mRNA (10⁻¹¹: 72 ± 9.4%, P = 0.034; 10⁻¹⁰: 57.9 ± 15.4%, P = 0.017; 10⁻⁹: 55 ± 21.8%, P = 0.015 vs. AGE-HSA: 200 ± 33.3%; Fig. 2A) and protein (10⁻⁹: 96 ± 59%, P = 0.05 vs. AGE-HSA: 197 ± 43%; Fig. 2, B and C) expressions. The depressed RAGE expression after calcitriol addition was even lower than the basal mRNA (10⁻⁹: 55 ± 21.8%, P = 0.03 vs. control; Fig. 2A) expression.

Calcitriol decreases the AGE-HSA-stimulated endothelial IL-6 expression. IL-6 mRNA expression was significantly enhanced after stimulation with AGE-HSA (185 ± 48%, P = 0.04 vs. control; Fig. 3); in presence of 10⁻⁹ mol/l calcitriol, this elevation was completely blunted, and the values were equivalent to that found with HSA (10⁻⁹: 98 ± 39%, P = 0.043 vs. AGE-HSA: 185 ± 48%; Fig. 3).

Calcitriol prevents the stimulating actions of AGE-HSA on NF-κB activity. AGE-HSA induced a significant increase in nuclear endothelial p65 DNA binding activity after 6 (133 ± 7%, P = 0.03 vs. control; Fig. 4A) and 24 (143 ± 7%, P = 0.026 vs. control; Fig. 4A) h. IκBα levels were decreased (47.5 ± 9%, P = 0.035 vs. control; Fig. 4, B and C) and p-IκBα levels increased (197 ± 23.5%, P = 0.035 vs. control; Fig. 4, D and E) 24 h after AGE-HSA stimulation. Addition of calcitriol was associated with a decrease in p65 DNA binding activity (6 h: 113 ± 2.6%, P = 0.033 vs. AGE-HSA: 133 ± 7%; Fig. 4A; 24 h: 113 ± 4.9%, P = 0.026 vs. AGE-HSA: 143 ± 7%; Fig. 4A). This was in parallel with higher levels of IκBα (155.7 ± 56%, P = 0.05 vs. AGE-HSA: 47.5 ± 9%; Fig. 4, B and C) and a decline in IκBα phosphorylation as demonstrated by the presence of lower levels of p-IκBα (92.6 ± 63%, P = 0.05 vs. AGE-HSA: 197 ± 23.5%; Fig. 4, D and E).

**DISCUSSION**

Atherosclerotic vasculopathy is particularly severe in patients with CRF, DM, and ageing (1). In such conditions, the formation and accumulation of AGEs is accelerated (1). Overproduction of AGEs is accompanied by changes in matrix components as well as an increase in platelet aggregation, inflammatory cell chemotaxis, and oxidant stress, leading to reduced vascular relaxation and rapidly progressive atherosclerotic processes (2, 15, 36). Recent reports have shown a beneficial effect of calcitriol on cardiovascular functions and structures, especially in clinical conditions associated with accelerated AGE accumulation (diabetes and diabetic patients) (17, 19, 20, 30). On the basis of these data, we decided to study the impact of AGE-HSA on cultured endothelial functions and to evaluate the possible effect of calcitriol treatment on these eventual AGE-HSA-related changes. We have chosen to use both physiological concentrations of calcitriol (10⁻¹¹ and 10⁻¹⁰ mol/l) to mimic in vivo conditions and a supraphysiological concentration of calcitriol (10⁻⁹ mol/l) as used by in vitro protocols (16). This supraphysiological concentration of calcitriol (10⁻⁹ mol/l) has been considered to be relevant and is used in experimental designs because HUVEC express 1α-hydroxylase and therefore can explain higher local concentrations and production of calcitriol (39). The most significant effects of calcitriol were obtained at 10⁻⁹ supraphysiological concentration, a relevant concentration in in vitro studies.

AGE-HSA induced a decrease in eNOS activity and mRNA expression and an increase in the expressions of the proatherosclerotic/proinflammatory parameters RAGE and IL-6. Addition of calcitriol to the AGE- and HSA-stimulated endothelial
cells led to a normalization of eNOS mRNA expression and enzymatic activity as well as decreased RAGE and IL-6 expressions. In addition, calcitriol did counteract the stimulating impact of AGE-HSA on NF-κB pathway activities.

We had previously shown that different types of prepared AGEs (AGE-HSA, \(N\)-ε-carboxymethyllysine, and AGE-B2-microglobulin) were able to inhibit mRNA and protein expressions of eNOS in HUVEC (23). In the present study, we confirmed the decrease in eNOS mRNA expression by using a real-time PCR technology. The physiological impact of this inhibiting action was strengthened by showing a significant suppressive effect of AGE-HSA on eNOS activity. In correlation to these findings are other reports that have shown an AGE- and BSA-dependent depression of the eNOS system in bovine endothelial cells (3, 24). Nitric oxide (NO) acts as a vascular immunomodulator; therefore, it may affect the development of atherosclerotic vasculopathy when its production is depressed; low NO production was found to play a role in the pathogenesis of atherosclerosis by disturbing the endothelial functions (8, 12, 13). The lower eNOS system activity induced by AGEs may partly explain why impaired vasodilation and endothelial dysfunctions may be observed in clinical conditions associated with high AGE formation (1, 18). The decrease in eNOS expression and activity was significantly counterbalanced by adding calcitriol, even at physiological concentrations. These findings support the results of recent studies that have suggested that vitamin D plays other roles besides calcium homeostasis (19, 30, 38).

RAGE, which is expressed in membranes of different cell types, including endothelial cells, mediates important cellular functions by binding to AGEs with high affinity. AGEs can activate RAGE, leading to the induction of various inflammatory mediators and the development of atherosclerotic vasculopathy (19, 30, 38). In the present study, we examined the effect of calcitriol on the expression of RAGE in HUVEC stimulated with AGE-HSA. Calcitriol significantly decreased the expression of RAGE in these cells, indicating that calcitriol may also play a role in inhibiting the development of atherosclerosis by downregulating RAGE expression.

**Fig. 3.** Effect of calcitriol on interleukin (IL-6) expression by HUVEC stimulated with AGE-HSA. HUVEC were incubated for 24 h with 200 µg/ml HSA (control) or 200 µg/ml AGE-HSA, and calcitriol (10^{-11}, 10^{-10}, and 10^{-9} mol/l) was added to AGE-HSA-stimulated HUVEC 1 h after stimulation for an additional 23 h. 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 a degree of control. Data are expressed as means ± SE of 4 independent experiments. *P = 0.05 vs. control (HSA); #P ≤ 0.012 vs. AGE-HSA.

**Fig. 4.** Effect of calcitriol on nuclear factor-κB (NF-κB) DNA binding activity in HUVEC stimulated with AGE-HSA. A: HUVEC were incubated for 6 and 24 h with 200 µg/ml HSA (control) or 200 µg/ml AGE-HSA; in addition, 10^{-9} mol/l calcitriol were given to AGE- and HSA-stimulated HUVEC 1 h after the stimulation for an additional 5 or 23 h. Nuclear protein extracts were prepared. DNA binding activity of NF-κB was measured by enzyme-linked immunosorbent assay. Results are means ± SE of 3 independent experiments. P < 0.05 vs. control (●) and vs. AGE-HSA (●●). B–E: HUVEC were incubated for 24 h with 200 µg/ml HSA (control) or 200 µg/ml AGE-HSA; in addition, 10^{-9} mol/l calcitriol was given to AGE- and HSA-stimulated HUVEC 1 h after the stimulation for an additional 24 h, and protein lysates were prepared. Inhibitor IκB (IκB) and phosphorylated (p)-IκB protein expressions were assessed by Western blot analysis. B and D: level of α-tubulin is shown as a loading control. C and E: protein expressions were determined by normalization against tubulin, and the relative protein content was expressed as a percentage of control. Data are expressed as means ± SE of 3–4 experiments. *P ≤ 0.05 vs. control and (●) vs. AGE-HSA (●●).
effects of AGEs (31). In vasculopathies, the endothelial expression of RAGE are markedly elevated (25, 26). In our present study, AGE-HSA was found to stimulate both mRNA and protein expressions of RAGE in cultured endothelial cells, a phenomenon that may amplify the effects of AGEs, particularly when its production is elevated. Our results confirm previous studies that showed that AGEs increase the endothelial RAGE expressions (40).

We have previously shown that calcitriol may blunt the mRNA expression of RAGE in nonstimulated HUVEC (33). In the present study, we have demonstrated that the addition of calcitriol was able to blunt the expressions of RAGE in AGE- and HSA-stimulated cells, suggesting that the inhibiting action of calcitriol on the AGE- and HSA-related effects may be caused by the downregulation of RAGE expression. Further investigations will have to be done to confirm this possibility.

Interaction between AGEs and RAGE may stimulate the expression of IL-6 (37). The increased mRNA expression of IL-6, found after incubation with AGEs, may confirm these results. Calcitriol induced a decrease in IL-6 mRNA expression, suggesting that vitamin D may eventually diminish or even prevent endothelial inflammation. These findings support the concept that vitamin D may have anti-inflammatory and vascular protective properties (8, 30). These data confirm and strengthen our previous study (33). They also are compatible with the concept that AGEs may be involved in the development of atherosclerotic processes encountered in CRF, DM, ageing, and hypertension (1, 32). The blunting effect of calcitriol on the AGE-related abnormalities may explain some of the positive actions of vitamin D compounds observed in patients with cardiovascular complications (30).

The cellular mechanism underlying the impact of calcitriol on the AGE-related cell dysfunctions was evaluated by studying its impact on NF-κB-p65 pathway activities, known to be involved in endothelial inflammatory responses (34). A previous report showed that ligation of endothelial RAGE activates the transcription factor NF-κB (27), a phenomenon confirmed and strengthened here by an increased nuclear NF-κB-p65 DNA binding activity in AGE-treated endothelial cells. This increased DNA binding activity was associated with lowered IkBα levels, which have probably facilitated the transition of NF-κB-p65 to the nucleus (10). The depressed IkBα expression may be explained by an increase in its phosphorylation, as suggested by the elevated p-IkBα levels presently detected. This increased NF-κB activity was normalized by addition of calcitriol, which blunted the AGE-dependent stimulation of nuclear DNA binding activity. This was characterized by increased IkBα levels, whereas p-IkBα levels were diminished after treatment with calcitriol. This higher IkBα is explained by the lowered phosphorylation that targets IkBα for ubiquitination and degradation (10). A similar impact of calcitriol on NF-κB activity was demonstrated in peritoneal macrophages (5) and melanoma cells (9).

In summary, our present results have shown that calcitriol counteracts the AGE-dependent dysfunctions found in cultured endothelial cells. The blunting effect of calcitriol on the impact of AGEs on NF-κB pathway activities may explain the normalization of the endothelial cell dysfunction. The positive impact of vitamin D on endothelial cell function may partially explain the beneficial effect of vitamin D metabolites on the cardiovascular morbidity and mortality reported in patients with CRF (30).

ACKNOWLEDGMENTS

This study is part of the requirements of the Doctorate of Philosophy of Y. Talmor from Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel.

GRANTS

This study was supported by Hendrich and Irene Gotwirth (O. Klein) and Carol and Leonora Fingerhut (S. Benchetrit) grants from Sackler Faculty of Medicine, Tel-Aviv University and the Israeli Diabetes Association (G. Rashid) and the Dr. Y. Chechekel and Pearl Klyaman, Cathedra of Urology of whom J. Bernheim is incumbent.

REFERENCES


F1064 CALCITRIOL AFFECTS AGE-STIMULATED HUVEC