Advanced Glycation End Products Induce Calcification of Vascular Smooth Muscle Cells through RAGE/p38 MAPK

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Key Words

Advanced glycation end products · Vascular calcification · Receptor for advanced glycation end products · p38 mitogen-activated protein kinase

Abstract

Background: Mönckeberg's calcification in diabetes, known as medial artery calcification, is an independent predictor of cardiovascular mortality. However, the mechanism underlying this phenomenon remains to be elucidated. We demonstrate that advanced glycation end products (AGEs) induce calcification of vascular smooth muscle cells through the receptor for AGE (RAGE)/p38 mitogen-activated protein kinase (MAPK) signaling pathway. **Methods:** We detected vascular calcification by von Kossa staining. Alkaline phosphatase (ALP) activity was determined by measuring *p*-nitrophenol. Osteocalcin concentrations were measured using ELISA. Western blotting for protein phosphorylation and real-time RT-PCR for expression of mRNA were used. **Results:** AGEs induced calcification of vascular smooth muscle cells. AGEs also induced the expression of Runx2 mRNA. In addition, AGEs increased ALP activity and osteocalcin secretion. Furthermore, AGEs induced phosphorylation of p38 MAPK, and this phosphorylation was inhibited by the anti-RAGE block-

ing antibody. Increased ALP activity was inhibited by the p38 MAPK inhibitor or anti-RAGE blocking antibody. Furthermore, the p38 MAPK inhibitor and anti-RAGE blocking antibody both inhibited AGE-induced calcification of vascular smooth muscle cells. Diabetic serum induced calcification of smooth muscle cells and the calcification was inhibited by RAGE blocking. **Conclusion:** Our findings indicate that AGEs induce calcification of vascular smooth muscle cells by osteoblast-like differentiation of smooth muscle cells through RAGE/p38 MAPK.

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Introduction

Vascular calcification is associated with an increased risk of myocardial infarction, limb amputation and other adverse cardiovascular outcomes [1, 2]. Vascular calcification is a pathological process that occurs in many diseases, including atherosclerosis, diabetes and uremia [3]. Calcification of the tunica medial layer of arteries, also known as Mönckeberg's calcification, is thought to be significantly related to long-term diabetes mellitus [4, 5]. In type 2 diabetes mellitus, Mönckeberg's calcification is a significant independent predictor of cardiovascular

mortality, future cardiovascular events, cerebral infarctions and amputations [6].

Although the precise mechanism of vascular calcification has been and remains poorly understood, some new insights have recently been reported. Shanahan et al. [7] reported that diabetes-related Mönckeberg's calcification occurred in direct apposition to medial vascular smooth muscle cells and that smooth muscle cells expressed alkaline phosphatase (ALP), bone sialoprotein and bone Gla protein, which are all involved in osteogenesis; furthermore, phosphate [8], cAMP [9], 1,25-dihydroxyvitamin D₃ [10] and tumor necrosis factor- α [11] can all induce smooth muscle calcification through differentiation into osteoblast-like cells in vitro. The mechanism of vascular calcification in diabetes is not clear. Although severe vascular calcification is highly prevalent in patients with end-stage renal disease [12], vascular calcification also occurs in patients with type 2 diabetes mellitus who are free of diabetic nephropathy [13]. Considered together, these findings suggest that another mechanism exists to account for vascular calcification in diabetes.

Advanced glycation end products (AGEs), final products of the Maillard reaction, are formed at an accelerated rate in diabetic patients [14]. They are thought to contribute to the development of diabetic complications, including atherosclerosis and microvascular disease [15]. The best characterized AGE receptor is the receptor for AGE (RAGE) [16], although other AGE binding sites have also been reported [17]. Although Yamagishi et al. [18] reported that AGEs increased mineral deposition by pericytes, the mechanism behind this is unclear. We report herein that AGEs accelerate the calcification of smooth muscle cells by promoting osteoblast-like differentiation, which is mediated through the RAGE/p38 mitogen-activated protein kinase (MAPK) signaling pathway.

Materials and Methods

Materials

Media and fetal calf serum (FCS) were purchased from Gibco (Grand Island, N.Y., USA). Bovine serum albumin (BSA; fraction V, endotoxin free), silver nitrate and β -glycerophosphate (β -GP) were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Anti-RAGE blocking antibody was purchased from R&D Systems Inc. (Minneapolis, Minn., USA). Anti-p38 MAPK and anti-phospho-p38 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, Mass., USA). Multiple inhibitors of intracytoplasmic signaling including PD98059 (ERK inhibitor), SB202190 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) were purchased from Cell Signaling Technology.

Cell Culture and in vitro Calcification of Human Aortic Smooth Muscle Cells

Human aortic smooth muscle cells (HASMCs) were purchased from Kurabo (Osaka, Japan). They were cultured in Dulbecco's modified Eagle's medium (DMEM) (low glucose: 5.5 mM) containing 10% heat-inactivated FCS and supplemented with sodium pyruvate (1 mmol/l), penicillin (100 U/ml) and streptomycin (100 U/ml). At confluence, the cells were inoculated in DMEM with 10% FCS in the presence of 10 mM β -GP. This medium was replaced with fresh medium every 3 days.

Preparation of AGEs

BSA was incubated with 0.5 M glucose in phosphate-buffered saline (PBS) for 6 weeks at 37°C in the presence of 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM ethylenediaminetetraacetic acid, penicillin (100 U/ml) and streptomycin (100 U/ml) under sterile conditions as described previously [19]. The unincorporated sugars were removed utilizing dialysis against PBS. Control nonglycated BSA was incubated under the same conditions, except for the absence of glucose. Endotoxin levels were checked using an endotoxin testing kit (Limulus J Single Test, Wako Pure Chemical Industries, Osaka, Japan). AGE-BSA solutions at the concentrations used in this study were confirmed to be endotoxin free (<2.5 U/ml of endotoxin).

Von Kossa Staining for Calcification

Cell monolayers were fixed in 0.1% glutaraldehyde in PBS for 15 min at room temperature. Cells were then washed twice with double-distilled water (ddH₂O) and incubated with 5% silver nitrate for 30 min at room temperature in darkness. The silver nitrate was removed and the cells were again rinsed twice with ddH₂O. Cultures were then air-dried and exposed to sunlight until color development was complete. Cells were rinsed with ddH₂O and prepared for phase microscopy.

Quantification of Calcium Deposition

Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically utilizing the O-Cresolphthalein Complexone method (Calcium C-test, Wako Pure Chemical Industries) [20]. After decalcification, cells were washed 3 times with PBS and solubilized with 0.1 N NaOH-0.1% SDS. Protein content was measured utilizing Bradford's method [21]. Calcium content of the cell layer was normalized with regard to protein content.

Quantitative Real-Time RT-PCR

HASMCs were grown in triplicate 60-mm dishes for the indicated time with or without AGEs. Total RNA samples were isolated utilizing the Isogen Kit (Nippon Gene Co., Tokyo, Japan) following the manufacturer's recommended protocol. Fluorescent real-time PCR was performed utilizing a one-step RT-PCR Kit (Takara Shuzou, Shiga, Japan) with SYBR Green I Nucleic Acid Gel Stains (Cambrex Bio Science, Rockland, Me., USA) on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The reaction mixture (20 μ l in volume) contained 2 μ l of total RNA (5 ng/ μ l), 12.5 μ l of 2 $\times$ OneStep RT-PCR buffer as specified in the instructions provided by the manufacturer, 0.5 μ l of RNase inhibitor (40 U/ μ l), 0.5 μ l of Takara Ex Taq HS (5 U/ μ l; Taq DNA polymerase), 0.25 μ l of M-MLV reverse transcriptase (200 U/ μ l), 0.5 μ l of forward and reverse primers (10

μM), 2.5 μl of SYBR Green I and RNase-free ddH₂O. PCR was initiated at 42°C for 15 min and 95°C for 2 min, followed by 45 cycles of denaturing at 95°C for 5 s, annealing and extending at 60°C for 31 s. After PCR, a dissociation curve was constructed at 65–95°C. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate (Applied Biosystems). PCR was performed in triplicate. All data were analyzed with ABI Prism SDS 2.0 software. Reaction product was quantified using a simultaneously amplified series of dilutions of a sequence of known concentration (in this case, Runx2 gene in human osteoblasts) to generate a standard curve. Human osteoblasts were purified from metaphyseal trabecular bone in the proximal femur of patients presenting with osteoarthritis during hip arthroplasty. RT-PCR results were normalized to the amounts of GAPDH mRNA. The following Runx2 primers were used: forward 5'-TCATGGCGG-GTAACGATGAA-3' and reverse 5'-TTGTGAAGACGGTTA-TGGTCAAGG-3'. The following GAPDH primers were used: forward 5'-CCACCCATGGCAAATTCATGGCA-3' and reverse 5'-TGAGACGGCAGGTCAGGTCCACC-3'.

Assay of ALP Activity

After the cells were washed 3 times with PBS, cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and then centrifuged. The supernatants were next assayed for ALP activity as described previously [22]. One unit was defined as the activity producing 1 nm of *p*-nitrophenol over 30 min. Protein concentrations were measured using Bradford's method [21].

Secretion of Osteocalcin

Cells were treated with BSA or AGEs at various concentrations, in the absence or presence of 10 mM β -GP, for various periods of time. Cell supernatants were collected and osteocalcin concentrations were measured utilizing the Osteocalcin ELISA Kit (Bender Medsystems, Vienna, Austria) according to the manufacturer's recommended instructions.

Western Blotting

Cells (1×10^6) were cultured in DMEM containing 10% FCS on 6-well plates. The medium was changed when cell growth reached 80% confluence. Cells were cultured in DMEM containing 1% FCS for 12 h, then BSA or AGE-BSA was added at varying concentrations. Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with a lysis buffer of the following composition: 20 mM Tris, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin. They were then cleared by centrifugation at 12,000 g at 4°C for 15 min. Total protein concentrations were measured utilizing a Bio-Rad Protein Assay Kit (Bio-Rad, USA). After the samples were heat-denatured, they were analyzed by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Invitrogen, San Diego, Calif., USA). Nonspecific binding sites were blocked by immersing the membranes in 5% skim milk in PBS for 1 h at room temperature, and then the membranes were washed 5 times with PBS containing 0.1% Tween-20. The membranes were incubated with 1 $\mu\text{g}/\text{ml}$ primary antibody in PBS containing 0.1% Tween-20 overnight at 4°C. After the membranes were washed 5 times, a secondary antibody was added. Chemiluminescence was produced using ECL reagent (Amersham Biosciences, Arlington Heights, Ill., USA) and detected with Hyperfilm-ECL (Amersham Biosciences).

Calcification of HASMCs Induced by Diabetic Serum

Serum samples collected every month from diabetic patients who visited our hospital were pooled together. Patients with renal insufficiency were excluded from analysis. Serum samples from age-matched normal healthy individuals served as controls. The pooled serum was frozen at -20°C in aliquots for use in tissue culture. After cells reached confluence, 10% of sera from the controls and diabetics were each added to the media for use with cell cultures. The control group consisted of 5 healthy individuals (age 65 ± 8.5 years; males/females 2/3; fasting plasma glucose 93.3 ± 8.9 mg/dl; insulin 7.0 ± 4.6 $\mu\text{U}/\text{ml}$; HbA1c $4.8 \pm 0.3\%$). The diabetic group comprised 16 patients (age 62.5 ± 9.5 years; males/females 5/11; fasting plasma glucose 134.6 ± 25.5 mg/dl; insulin 7.6 ± 3.4 U/ml; HbA1c $6.9 \pm 0.8\%$).

Statistical Analysis

All data were obtained from at least 5 independent experiments performed in triplicate. Results were expressed as means $\pm$ SD. Analyses of differences were carried out by ANOVA following by a post hoc Student-Newman-Keuls test. A *p* value of <0.05 was considered statistically significant.

Results

To investigate the mechanisms involved in vascular calcification in diabetes, we examined the mineralization of vascular smooth muscle cells cultured in vitro. In vitro models of smooth muscle cell calcification have already been developed by others [8–11]. We used a similar model in which smooth muscle cells were cultured with β -GP. We found that AGEs, but not BSA, induced vascular calcification (fig. 1a) and increased the calcium content of the cell layer in a dose-dependent manner (fig. 1b). These results suggest that AGEs could be a potent inducer of vascular calcification in diabetes. AGEs did not induce calcification of HASMCs in the absence of β -GP (data not shown).

Previous studies reported that calcification of smooth muscle cells was due to differentiation to osteoblast-like cells [7–11]. Runx2 is a bone-determining transcription factor [23] and is thought to be one of the master genes accounting for osteoblast differentiation. We examined the expression of Runx2 mRNA in smooth muscle cells. AGEs upregulated Runx2 mRNA expression in a dose-dependent manner (fig. 2a). These results suggest that AGEs can potentially transform smooth muscle cells into osteoblast-like cells.

ALP is well recognized as an early differentiation marker of osteoblasts and is thought to be essential for bone mineralization [24]. ALP activity was increased in both BSA- and AGE-treated cells in the presence of β -GP; however, AGEs increased ALP activity in a dose-depen-

Fig. 1. AGEs induce calcification of HASMCs. After cells reached confluence, they were cultured in the presence of 10 mM β -GP with the indicated concentrations of BSA or AGE-BSA for 14 days. **a** Morphology of calcified HASMCs. Mineral deposition was assessed at the light microscopic level utilizing von Kossa staining. Magnification $\times 200$. **b** Calcium contents were measured utilizing the O-Cresolphthalein Complexone method and normalized according to cellular content. HASMCs were incubated with the indicated concentrations of BSA or AGE-BSA for 7 days or 14 days. Data are expressed as means $\pm$ SD of 5 independent experiments, each performed in triplicate. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. ** $p < 0.01$ compared with the untreated control.

dent manner even in the absence of β -GP (fig. 2b). AGE-treated samples secreted significantly higher levels of osteocalcin than controls. Osteocalcin is a late differentiation marker of osteoblasts and is secreted extracellularly. However, the secretion at the highest AGE concentration was decreased compared with the secretion at other AGE concentrations, and the addition of β -GP did not significantly change AGE-induced osteocalcin secretion (fig. 2c). In osteoblasts, it is reported that osteocalcin secretion increases in the early mineralization phase, while the secretion decreases in the late mineralization phase [25, 26]. We suggest that since at high levels of AGEs, smooth muscle cells rapidly reach the late mineralization phase, osteocalcin secretion might rather be decreased. Considered together, these findings suggest that AGEs

accelerate the vascular calcification process by inducing osteoblast-like differentiation of smooth muscle cells.

AGEs phosphorylated p38 MAPK in HASMCs in a time-dependent manner (fig. 3a). AGEs bind to several cell surface sites including RAGE [27], galectin-3/p60/p90 [28] and macrophage scavenger receptors [29]. RAGE appears to modulate various cellular functions and is reported to be expressed on smooth muscle cells, endothelial cells, monocytes/macrophages, mesangial cells and neurons [30]. In our experiments, anti-RAGE blocking antibody inhibited AGE-induced p38 MAPK phosphorylation (fig. 3b), suggesting that AGEs induce p38 MAPK phosphorylation through RAGE.

To investigate the mechanism of AGE-induced osteoblast-like differentiation, we used several MAPK inhibi-

Fig. 2. AGEs induce expression of Runx2 mRNA and osteoblast differentiation markers on HASMCs. After cells reached confluence, the experiments described below were performed in DMEM with 0.5% FCS. **a** Cells were treated for 12 h in the presence of the indicated concentrations of BSA or AGE-BSA without β -GP. The expression level of Runx2 mRNA was determined by real-time RT-PCR and normalized to GAPDH mRNA expression. The indicated numbers are fold increases compared to untreated control group cells. Data are expressed as means $\pm$ SD of 3 independent experiments. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. ** $p < 0.01$ compared with the control. Representative data from 3 independent assays are shown. **b** Cells were cultured for 72 h in the presence of the indicated concentrations of BSA or AGE-BSA with (+) or without (-) 10 mM β -GP. ALP activity was measured and normalized according to the cellular protein content. **c** Cells were cultured for 72 h in the presence of the indicated concentrations of BSA or AGE-BSA with (+) or without (-) 10 mM β -GP. Osteocalcin concentrations were measured in the culture medium by ELISA. Data are expressed as means $\pm$ SD of 5 independent experiments performed in triplicate. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. * $p < 0.05$ and ** $p < 0.01$ compared with the untreated control in each group with or without β -GP; ^a $p < 0.05$ and ^b $p < 0.01$ compared with the value at the same concentration but in the absence of β -GP.

Fig. 3. AGEs phosphorylate p38 MAPK through RAGE. **a** HASMCs were incubated for the indicated times with 10 μ g/ml AGEs. After cell lysis, expression of phosphorylated p38 MAPK and total p38 MAPK was detected using specific antibodies. These are representative results of 5 experiments performed with similar results. **b** HASMCs were incubated with various concentrations of anti-RAGE blocking antibody or control IgG, followed by 10 μ g/ml AGEs for 60 min. After cell lysis, phosphorylated p38 MAPK and total p38 MAPK were detected using specific antibodies. These are representative results of 5 experiments performed with similar results.

tors. Previous studies reported that ERK [31], p38 MAPK [32] and JNK [33] are involved in osteoblast differentiation. In our experiments, SB202190 (a p38 MAPK inhibitor), but not PD98059 (an ERK inhibitor) and SP600125 (a JNK inhibitor), suppressed an AGE-induced increase

in ALP activity (fig. 4). Furthermore, in this study, the anti-RAGE antibody inhibited AGE-induced ALP activity. These findings emphasize the importance of the AGE-RAGE interaction.

Fig. 4. Inhibition of p38 MAPK and RAGE suppresses ALP activity. HASMCs were incubated for 72 h with 10 μ g/ml AGEs and each of the various MAPK inhibitors at the indicated concentrations. ALP activity was measured and normalized according to the cellular protein content. Data are expressed as means $\pm$ SD of 5 experiments performed in triplicate. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. ** $p < 0.01$ compared with the group treated with 10 μ g/ml AGEs alone.

Furthermore, the anti-RAGE blocking antibody and p38 MAPK inhibitor inhibited AGE-induced calcification of HASMCs (fig. 5a) and decreased the calcium content of the cell layer (fig. 5b).

Sera from diabetic patients induced calcification of smooth muscle cells. The calcification was partially inhibited by the anti-RAGE blocking antibody (fig. 6). Therefore, the AGE/RAGE cascade is thought to be related to calcification of smooth muscle cells in diabetes.

Discussion

Although vascular calcification in diabetes is a strong predictor of cardiovascular events [6], the underlying mechanism remains unclear. Elucidation of this mechanism is important for the establishment of new therapies to prevent vascular calcification. In the present study, we demonstrated that AGEs accelerate vascular calcification by promoting osteoblast-like differentiation of HASMCs. Our results showed that AGEs induced expression of the bone-determining transcription factor Runx2 mRNA and of osteoblast differentiation markers such as ALP

and osteocalcin. In particular, AGEs markedly increased ALP activity even in the absence of β -GP. ALP is thought to promote mineralization by providing a source of orthophosphate for incorporation into CaPO_4 mineral [34]. There are some reports about vascular calcification related to the diabetic condition. Yamagishi et al. [18] reported previously that AGEs accelerated calcification of microvascular pericytes. Our results are in agreement with the findings of their study, which indicated that AGEs increased ALP activity and calcification of vascular cells. However, the mechanism of induction of osteoblast-like differentiation was not clear in their study. The concentrations of glucose vary in diabetic patients. In this regard, Chen et al. [35] described that high blood glucose levels induced calcification of vascular smooth muscle cells. In their study, the glucose concentration was 25 mM, which was considered too high. In fact, it is very rare to find such high glucose levels in diabetic patients. In comparison, our study was performed under low-glucose conditions, and our results showed that AGEs induced calcification of vascular smooth muscle cells irrespective of the glucose concentration. In addition, diabetic serum-induced calcification was inhibited by blockade of RAGE. Therefore, our study suggests that AGEs may be one of the factors responsible for vascular calcification in diabetes. Furthermore, the combination of high glucose levels and AGEs seems to influence mineralization by affecting osteoblast function, while AGEs alone have no such effect [36]. The response to AGEs may be different among different cell types.

In osseous tissues, insulin induces differentiation of osteoblasts and mineralization [37]. It is conceivable that certain factors, such as glucose and insulin, are involved in diabetes-related vascular calcification [38]. In our study, sera of diabetic patients induced calcification of smooth muscle cells and such calcification was partially inhibited by RAGE blockade. These results suggest the involvement of the AGE/RAGE cascade in the induction of vascular calcification in diabetes.

RAGE is a major receptor for AGEs, although other AGE binding sites have also been reported [17]. In this study, the anti-RAGE antibody inhibited AGE-induced ALP activity and calcification of smooth muscle cells. These findings emphasize the importance of the AGE-RAGE interaction. A key consequence of ligand engagement of RAGE is activation of multiple signaling pathways, including p21 Ras, ERK 1/2 MAPK, p38 MAPK, JNK MAPK, Rho GTPases, phosphoinositol-3-kinase and JAK/STAT, as well as downstream effector molecules such as NF- κ B and CREB [39]. In osteoblasts, MAPKs such as

Fig. 5. Inhibition of p38 MAPK and RAGE suppresses AGE-induced calcification of HASMCs. Cells were cultured in the presence of 10 mM β -GP with 10 μ g/ml AGEs alone or 10 μ g/ml AGEs plus 10 μ M SB202190, 10 μ g/ml anti-RAGE antibody or 10 μ g/ml anti-IgG control for 14 days. **a** Morphology of calcified HASMCs. Mineral deposition was assessed on the light microscopic level by von Kossa staining. Magnification $\times 200$. **b** Calcium content was measured by the O-Cresolphthalein Complexone method and normalized according to the cellular content. Data are expressed as means $\pm$ SD of 5 independent experiments, each performed in triplicate. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. ** $p < 0.01$ compared with the group treated with 10 μ g/ml AGEs alone.

Fig. 6. Diabetic serum induced calcification of HASMCs and blockage of RAGE inhibited the calcification. Cells were cultured in the presence of 10 mM β -GP with control serum, control serum plus 10 μ g/ml anti-RAGE antibody, diabetic serum, diabetic serum plus 10 μ g/ml anti-RAGE antibody, diabetic serum plus 10 μ g/ml anti-IgG antibody or diabetic serum plus SB202190 10 μ M for 14 days. Calcium content was measured by the O-Cresolphthalein Complexone method and normalized according to the cellular content. Data are expressed as means $\pm$ SD of 5 independent experiments, each performed in triplicate. Statistical analysis of the results was carried out using ANOVA followed by a post hoc Student-Newman-Keuls test. * $p < 0.05$ and ** $p < 0.01$ compared with the group treated with diabetic serum alone.

ERK 1/2, p38 and JNK are associated with osteoblast differentiation [31–33]. In our study, p38 MAPK was associated with increased ALP activity in smooth muscle cells.

In conclusion, our results demonstrated that AGEs induced calcification of vascular smooth muscle cells by promoting osteoblast-like differentiation. These findings suggest that AGEs can induce vascular calcification in diabetes. Our results also indicated that AGE-induced calcification of smooth muscle cells was mediated through the RAGE/p38 MAPK signaling pathway, suggesting that inhibition of the AGE/RAGE pathway could be potentially effective therapeutically in preventing vascular calcification in diabetic patients.

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