Vascular Medicine

Advanced Glycation End Products Activate a Chymase-Dependent Angiotensin II–Generating Pathway in Diabetic Complications

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Background—Angiotensin II is a key mediator of diabetes-related vascular disease. It is now recognized that in addition to angiotensin-converting enzyme, chymase is an important alternative angiotensin II–generating enzyme in hypertension and diabetes. However, the mechanism of induction of chymase in diabetes remains unknown.

Methods and Results—Here, we report that chymase is upregulated in coronary and renal arteries in patients with diabetes by immunohistochemistry. Upregulation of vascular chymase is associated with deposition of advanced glycation end products (AGEs), an increase in expression of the receptor for AGEs (RAGE), and activation of ERK1/2 MAP kinase. In vitro, AGEs can induce chymase expression and chymase-dependent angiotensin II generation in human vascular smooth muscle cells via the RAGE-ERK1/2 MAP kinase–dependent mechanism. This is confirmed by blockade of AGE-induced vascular chymase expression with a neutralizing RAGE antibody and an inhibitor to ERK1/2 and by overexpression of the dominant negative ERK1/2. Compared with angiotensin-converting enzyme, chymase contributes to the majority of angiotensin II production ($>70\%$, $P<0.01$) in response to AGEs. Furthermore, AGE-induced angiotensin II production is blocked by the anti-RAGE antibody and by inhibition of ERK1/2 MAP kinase activities.

Conclusions—AGEs, a hallmark of diabetes, induce chymase via the RAGE-ERK1/2 MAP kinase pathway. Chymase initiates an important alternative angiotensin II–generating pathway in diabetes and may play a critical role in diabetic vascular disease. (***Circulation***. 2006;113:1353-1360.)

Key Words: angiotensin ■ arteriosclerosis ■ coronary disease ■ diabetes mellitus ■ kidney

Vascular disease is the main cause of mortality, with coronary artery disease accounting for nearly 30% of all deaths in the Unites States, according to the Centers for Disease Control. Diabetes confers a 2- to 4-fold risk of cardiovascular disease.[1] Nearly 70% of diabetic patients have coexistent hypertension, which has an additional impact on diabetic vascular complications.[2]

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Angiotensin (Ang) II is a key mediator of diabetic vascular disease with biological effects on cardiovascular and kidney disease well beyond hypertension.[3–5] Although the prevailing view has been that angiotensin-converting enzyme (ACE) is the main Ang II–generating enzyme, there is much evidence to suggest the importance of alternative Ang II–generating pathways.[6–8] Chymase has emerged as the most important alternative Ang II–generating pathway, being responsible for up to 80% of local Ang II generation in the heart and coronary arteries.[9,10]

Chymase, a serine protease that is well characterized in mast cells, also is expressed by vascular smooth muscle cells (VSMCs) and glomerular mesangial and epithelial cells.[11,12] Mammalian chymases are α-chymases that are capable of cleaving Ang I to Ang II.[13]

Targeted overexpression of chymase in transgenic mice causes hypertension.[14] Blockade of chymase with chymase inhibitors has beneficial effects in animal models of myocardial infarction and vascular injury.[15] Moreover, valsartan (Ang II receptor 1 blocker) is able to produce an inhibitory effect on restenosis after percutaneous coronary interventions, whereas ACE inhibitors do not.[16] Furthermore, a number of clinical trials demonstrate that additional benefit in terms of slowing renal disease progression is obtained with dual blockade of Ang II using Ang receptor blockers with ACE inhibitors compared with ACE inhibitors alone in diabetic nephropathy.[17,18]

An important mediator of diabetes-related vascular injury is the production and deposition of advanced glycation end

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products (AGEs) as a result of prolonged hyperglycemia. Exogenous administration of AGEs in vivo promotes atherosclerosis,[19] whereas chemical degradation of AGEs or inhibition of AGE formation decreases microvascular and macrovascular diabetic complications in animal models.[20] We have previously demonstrated that vascular chymase is upregulated in diabetic nephropathy and is associated with the development of diabetic arteriopathy,[11,21] although the mechanisms that regulate vascular chymase expression in diabetes remain unknown. Thus, the present study tested the hypothesis that AGEs may induce vascular chymase expression and consequently chymase-dependent Ang II generation to mediate diabetic vascular complications. Furthermore, the signaling mechanism by which AGEs induce chymase expression and chymase-dependent Ang II generation was investigated.

Methods

Reagents

Fetal bovine serum, penicillin/streptomycin/amphoterecin B, Dulbecco's modified Eagle's medium (DMEM), F-12K medium, insulin, transferrin, and selenium were obtained from Invitrogen (Carlsbad, Calif). Chymostatin, captopril, Ang I, endothelial cell growth supplement, HEPES, TES, AGE-BSA, and anti-RAGE antibodies were obtained from Sigma (St Louis, Mo). Antibodies to chymase and GAPDH were from Chemicon (Temecula, Calif) and Abcam (Cambridge, Mass). Antibodies to ERK1/2 p38, phosphorylated ERK1/2, and phosphorylated p38 were obtained from Santa Cruz Biotech Inc (Santa Cruz, Calif). The ERK1/2 kinase inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem (La Jolla, Calif).

Patients and Immunohistochemistry

Both normal and diabetic heart and kidney autopsy tissues (n=12 diabetic kidneys, n=12 diabetic hearts, n=12 normal heart and kidney tissues) were obtained from the Department of Pathology, Methodist Hospital following the approved protocol by the Institute Review Board of Baylor College of Medicine. All normal heart and kidney tissues were obtained from autopsy specimens without any known diabetes, hypertension, or coronary artery and kidney diseases. Of the 12 diabetic patients, 6 were male and 6 were female, with a mean age of 58 years (range, 38 to 72). Six of patients were diagnosed as having coexistent hypertension with a mean blood pressure of 143/80 mm Hg (range, 170 to 120/92 to 65 mm Hg). Sections (4 μm) of the formalin-fixed, paraffin-embedded human kidney and heart tissues were stained with antibodies to human AGEs, RAGE, phosphorylated ERK1/2, and chymase using the microwave-based antigen retrieval technique and a modified peroxidase anti-peroxidase method as described previously.[11,22]

Quantitative analyses of AGE accumulation, RAGE expression, pERK1/2 activation, and chymase expression were carried out using a quantitative image analysis system (Metamorph, Sunnyvale, Calif). Because the patterns of accumulation of AGEs and expression of RAGE and chymase are diffuse in nature, the percentage of vessel wall areas positive for AGEs, RAGE, and chymase were quantified under a ×20-power-field microscope. Briefly, up to 10 random areas of coronary and renal arteries were chosen from each tissue section and examined. The examined area was outlined, the positive staining patterns were identified, and the percent positive area in the examined area was then measured. Data were expressed as the percentage of mean±SEM. For analysis of pERK1/2 activation, nucleated positive cells within the arterial walls were identified under a ×40-power-field microscope, and the nucleated positive cells for pERK1/2 were counted in 10 random areas of coronary and renal arteries using a 0.02-mm^2 graticule fitted in the eyepiece of a microscope as described previously.[23] Data were expressed as cells per 1 mm^2. The arterial lumen and periarterial areas were excluded

from study. All examinations were performed blindly on coded slides.

Cell Culture

Human aortic VSMCs were obtained from ATCC (Mannasas, Va) and maintained in F-12K medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenite at 37°C in a 5% CO_2 incubator. For all experiments, VSMCs were grown to 80% confluence on 6- or 12-well plates (Falcon, Franklin Lakes, NJ) and made quiescent by incubation in serum-free DMEM for 24 hours before stimulation with AGEs. All reagents used were certified to be endotoxin free. Cells were stimulated with AGE-BSA at 50 μg/mL for 0, 6, 12, and 24 hours and at doses of 0, 25, 50, and 100 μg/mL for 24 hours to examine the time and dose response of chymase expression. BSA was used as a negative control.

The AGEs-RAGE interaction was inhibited by a rabbit neutralizing anti-RAGE antibody (10 μg/mL). An isotype rabbit IgG1 (10 μg/mL) was used as a negative control. To inhibit AGE-induced ERK1/2 MAP kinase or p38 MAP kinase activities, inhibitors to ERK1/2 (PD98059, 10 μmol/L) or p38 (SB203580, 10 μmol/L) MAP kinases and Adv-DN-ERK or Adv-DN-p38 adenovirus, respectively, were used. Recombinant adenovirus construct containing bacterial β-galactosidase gene (Adv-β-gal) was used as negative control. The characterization and transfection of these dominant negative vectors and negative control have been described elsewhere.[24,25] Briefly, VSMCs were incubated with the adenovirus at multiplicity of infection (MOI) of 50 in DMEM for 1 hour and then made quiescent for 24 hours before stimulation with AGEs. Each experiment was repeated at least 3 times throughout the study.

Reverse Transcription and Real-Time PCR

Total RNA was isolated with the Rnasy Mini kit (Qiagen, Valencia, Calif). Template cDNA was prepared using reverse transcriptase. Real-time PCR was performed with Sybrgreen (BioRad, Hercules, Calif) and the Opticon real-time PCR machine (MJ Research Inc, Waltham, Mass). The specificity of real-time PCR was confirmed by routine agarose gel electrophoresis and melting-curve analysis. Housekeeping gene GAPDH was used as an internal standard. The human chymase primers used in this study are as follows: forward, 5′-AACTTTGTCCCACCTGG-3′, and reverse, 5′-CGTCCATAG-GATACGATGC-3′; GAPDH: forward, 5′-CAATGACCCCTTC-ATTGACC-3′, and reverse, 5′ GTTCACACCCATGACGAACATG.

Western Blot Analysis

Cultured VSMCs were lysed, protein extracted, denatured at 99°C for 5 minutes and then transferred to a polyvinylidene difluoride membrane. Nonspecific binding to the membrane was blocked for 1 hour at room temperature with 5% BSA in Tris-buffered saline buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20). The membranes then were incubated overnight at 4°C with primary antibodies against chymase phosphorylated ERK1/2, phosphorylated P38, total ERK, total P38, and anti-GAPDH. After being washed extensively, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature in 1% BSA/Tris-buffered saline buffer. The signals were visualized by an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Chymase-Dependent Ang II–Generating Activity

This was measured by ELISA, adapting the method of Lundequist et al.[26] Briefly, VSMCs were distributed onto 24-well plates at a concentration of 0.5×0^6 cells per well and grown to confluence. After being rendered quiescent as described above, they were stimulated with AGEs (50 μg/mL) or control BSA (50 μg/mL) for 24 hours. Subsequently, 400 ng/mL Ang I was added to the culture in the presence or absence of captopril (100 μmol/L) and/or chymostatin (20 μmol/L), and 100 μL of medium was taken after 1 hour for measurement of Ang II levels using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Mich). To investigate the

signaling mechanisms by which AGEs regulate chymase-dependent Ang II generation, VSMCs were treated with a neutralizing anti-RAGE antibody (10 μg/mL) or control rabbit antibody (10 μg/mL), SB203580 (10 μmol/L), or PD 98059 (10 μmol/L), along with AGEs (50 μg/mL), or infected with Adv-DN-ERK, Adv-DN-p38, or Adv-β-gal (MOI of 50) before AGE stimulation as described above. Supernatants obtained from each treatment were measured for Ang II generation in the presence of Ang I as described above. Results were expressed as mean±SEM.

Statistical Analysis

All data are expressed as mean±SEM. Statistical significance was determined with 1-way ANOVA. We used t tests for multiple comparisons. Differences were considered statistically significant at $P<0.05$. Statistical analysis was conducted with STATA version 6 (College Station, Tex) and Microsoft Excel.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written

Results

Upregulation of Vascular Chymase Is Associated With the Deposition of AGEs

We first examined the hypothesis that upregulation of vascular chymase is associated with the deposition of AGEs in diabetic vascular disease. Serial sections of coronary and renal arteries obtained from autopsies of patients with diabetes were examined for both chymase expression and AGE deposition through the use of immunohistochemistry. As shown in Figures 1A through 1D and 2A through 2D, compared with normal human coronary and renal arteries in which chymase was weakly expressed by VSMCs (Figures 1A and 2A), vascular chymase was markedly upregulated in coronary and renal arteries exhibiting severe arteriopathy characterized by massive collagen matrix deposition and intimal thickening in patients with diabetes and hypertension (Figures 1B and 2B). Upregulation of vascular chymase in diabetic coronary and renal arteriopathy was associated with a strong deposition of AGEs in serial sections (Figures 1C, 1D, 2C, and 2D). These observations were further demonstrated by quantitative analysis (Figures 1K and 2K).

AGEs Are Able to Induce Vascular Chymase Expression and Chymase-Dependent Ang II Generation

Next, we examined the effect of AGEs on vascular chymase expression in vitro. Quiescent cultured human VSMCs were stimulated with AGE-BSA or control BSA for 6, 12, 24, and 72 hours at various doses (0, 25, 50, 100 μg/mL), and cells were collected for chymase mRNA and protein expression. As shown in Figures 3 and 4, real-time PCR and Western blot analyses demonstrated that addition of AGEs induced a marked expression of vascular chymase mRNA and protein in a time and a dose-dependent manner, being significant at 12 hours and peaking at 24 hours. Although maximal upregulation of chymase was achieved by addition of AGEs at a dose of 50 μg/mL, expression of chymase at both mRNA and protein levels was decreased when the AGE dose was increased to 100 μg/mL, presumably because of the toxicity of AGEs (Figure 4).
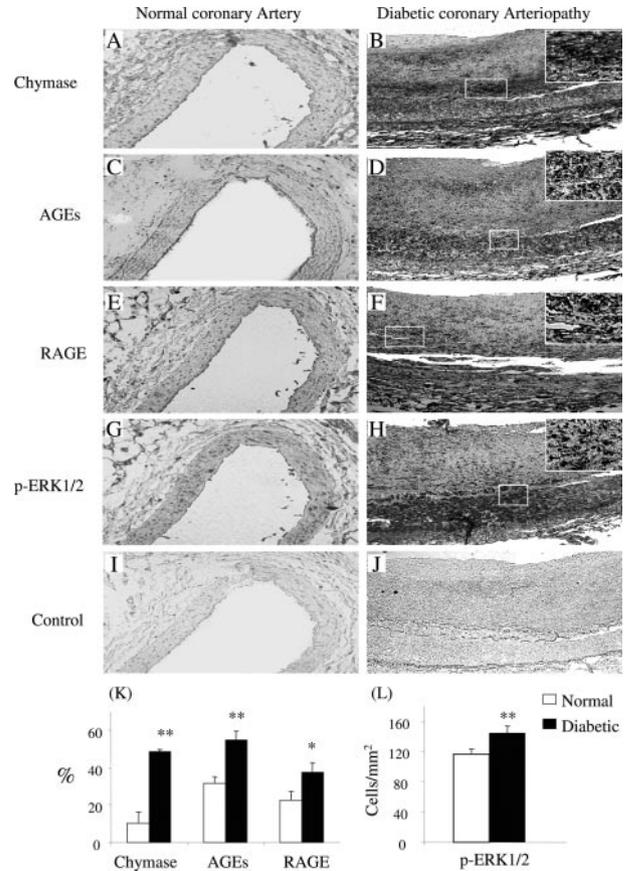

Figure 1. Immunohistochemistry demonstrates that vascular chymase expression in diabetic coronary arteriopathy is associated with a strong deposition of AGEs, a marked upregulation of RAGE, and activation of ERK1/2 MAP kinase. Serial sections of normal human and diabetic coronary arteries are stained for antibodies to human chymase, AGEs, RAGE, and p-ERK 1/2. In contrast to the normal coronary artery (A, C, E, G), chymase is markedly increased in a coronary artery with severe diabetic arteriopathy (B). This is associated with a strong deposition of AGEs (D), along with upregulation of RAGE (F) and phosphorylation of ERK1/2, as indicated by nuclear staining pattern (H). The specificity of staining is confirmed by a negative staining with nonimmune serum in both normal and diabetic arteries (I, J). More detailed information is illustrated in the insets. K, L, Quantitative analysis. Data are expressed as mean±SEM for groups of 12 patients. *$P<0.05$, **$P<0.01$ vs normal control. Magnification ×100.

The functional activity of AGEs-induced chymase was examined by the ability of chymase to convert Ang I to Ang II. As shown in Figure 5A , addition of AGEs resulted in a marked increase in Ang II generation (a 50-fold increase) by VSMCs. To examine the relative proportions of chymase- and ACE-dependent Ang II generation (because VSMCs express ACE), we conducted the experiment in the presence of ACE inhibitor (captopril, 100 μmol/L), chymase inhibitor (chymostatin, 20 μmol/L), and their combination. Both captopril and chymostatin treatment resulted in a significant decrease in Ang II generation in response to AGEs (Figure 5A). Importantly, compared with ACE inhibitor, which inhibited 40% of Ang II generation, blockade of chymase with chymostatin reduced <70% of Ang II production (Figure 5B). Addition of Ang I to the medium alone did not result in an increase in Ang II levels.
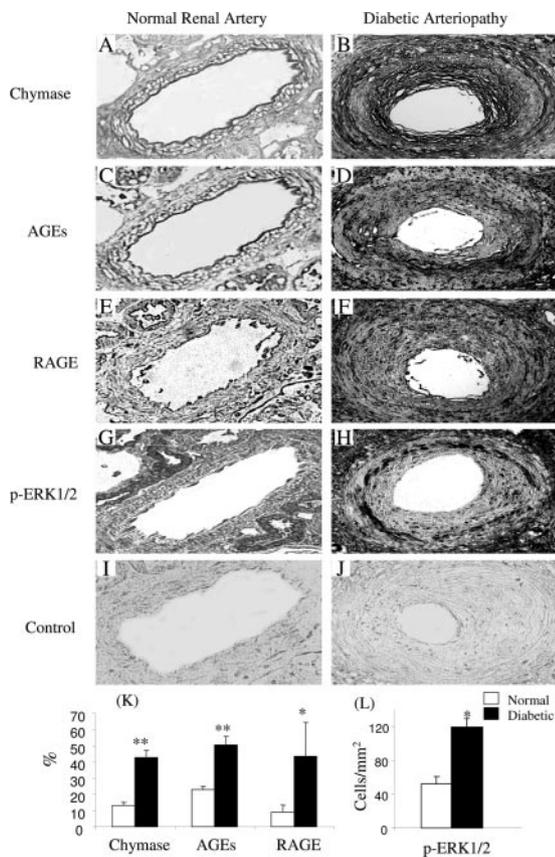
Figure 2. Immunohistochemistry demonstrates that vascular chymase expression in diabetic renal arteriolopathy is associated with a strong deposition of AGEs, a marked upregulation of RAGE, and activation of ERK1/2 MAP kinase. Serial sections of normal human and diabetic renal arteries are stained for antibodies to chymase, AGEs, RAGE, and p-ERK 1/2. In contrast to the normal renal artery (A, C, E, G), chymase is markedly increased in a renal artery with severe diabetic arteriopathy (B). This is associated with a strong deposition of AGEs (D), along with upregulation of RAGE (F) and phosphorylation of ERK1/2 as indicated by nuclear staining pattern (H). The specificity of staining is confirmed by a negative staining with nonimmune serum in both normal and diabetic arteries (I, J). K, L, Quantitative analysis. Data are expressed as mean±SEM for groups of 12 patients. *$P<0.05$, **$P<0.01$ vs normal control. Magnification ×100.
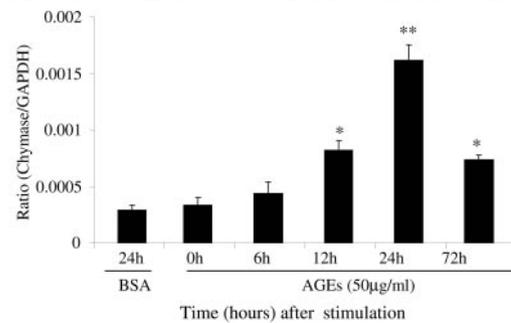
AGEs Induce Vascular Chymase Expression and Ang II Generation via the RAGE-ERK1/2 MAP Kinase

Next, we tested the hypothesis that AGEs may act by activating the ERK1/2 MAP kinase pathway to induce vascular chymase expression after binding to RAGE.

Immunohistochemically, vascular chymase expression was tightly associated with the upregulation of RAGE and an increase in phosphorylated ERK1/2 MAP kinase in serial sections of diabetic coronary and renal arteriopathy (Figures 1E through 1H and 2E through 2H). This was confirmed by quantitative analysis (Figures 1K, 1L, 2K, and 2L).

In vitro, we first demonstrated that AGEs are capable of inducing phosphorylation of ERK1/2 and p38 MAP kinases at 30 to 60 minutes (Figure 6), which was blocked by adding anti-RAGE antibody and inhibitors to ERK1/2 kinase (PD98059, 10 μmol/L) or p38 MAP kinase (SB203580, 10 μmol/L) and by infecting cells with adenovirus-mediated
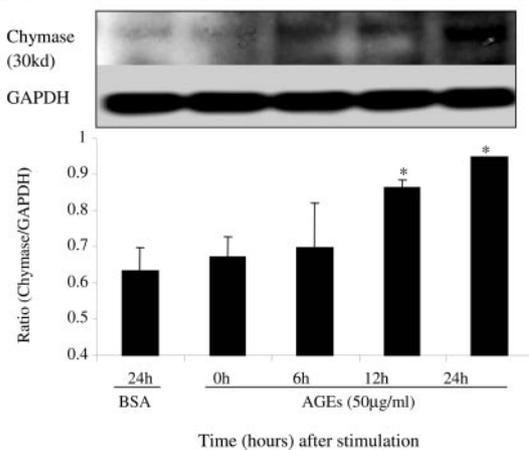

Figure 3. Real-time PCR and Western blot analyses show that AGEs induce chymase expression by VSMCs in a time-dependent manner. Real-time PCR (A) and Western blot analyses (B) show that AGE-BSA (50 μg/mL), but not BSA (50 μg/mL), induces chymase mRNA and protein expression in a time-dependent manner, which is significant at 12 hours and peaks at 24 hours. Each bar represents the mean±SEM for 5 independent experiments. *$P<0.05$, **$P<0.01$ vs time 0 and/or BSA control at 24 hours.

dominant negative ERK 1/2(Adv-DN-ERK) or Adv-DN-p38 (Figure 6). Then, we investigated the signaling mechanism whereby AGEs induce vascular chymase by blocking the AGE-RAGE interaction and either ERK1/2 or p38 MAP kinase signaling pathway with the same strategies. As shown in Figure 7, real-time PCR and Western blot analyses showed that blockade of RAGE with a neutralizing anti-RAGE antibody resulted in complete inhibition of AGE-induced chymase mRNA and protein expression. Similarly, blockade of ERK 1/2 kinase with PD98059 (10 μmol/L) and Adv-DN-ERK resulted in diminished upregulation of chymase mRNA and protein expression (Figure 7). In contrast, addition of p38 MAP kinase inhibitor (SB203580, 10 μmol/L) and overexpression of Adv-DN-p38 did not have any detectable inhibitory effect on AGE–induced chymase mRNA or protein expression. The specificity of the anti-RAGE antibody and Adv-DN-ERK or Adv-DN-p38 MAP kinases was confirmed by the inability of the isotype control antibody or control Adv-β-gal plasmid to inhibit AGE-induced chymase mRNA and protein expression (Figure 7).

To test whether blockade of RAGE and ERK1/2 kinase inhibited chymase-dependent Ang II generation, a neutraliz-
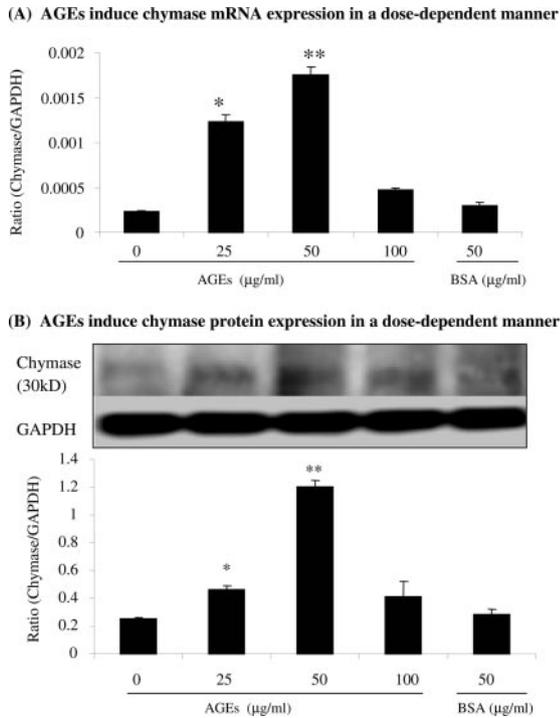
(A) AGEs induce chymase mRNA expression in a dose-dependent manner

(B) AGEs induce chymase protein expression in a dose-dependent manner

Figure 4. Real-time PCR and Western blot analyses show that AGEs induce chymase expression by VSMCs in a dose-dependent manner. Real-time PCR (A) and Western blot (B) analyses show that AGE-BSA, but not BSA, induces chymase mRNA and protein expression at 24 hours in a dose-dependent manner, which is significant at 25 μg/mL and peaks at 50 μg/mL. Note that an increase in AGE dose to 100 μg/mL results in a reduction in chymase mRNA expression, presumably because of the cytotoxic effect of high concentrations of AGEs on VSMCs. Each bar represents mean±SEM for 5 independent experiments. *$P<0.05$, **$P<0.01$ vs dose 0 and/or BSA control (50 μg/mL) at 24 hours.

(A) AGEs induce chymase-dependent Ang II Generation

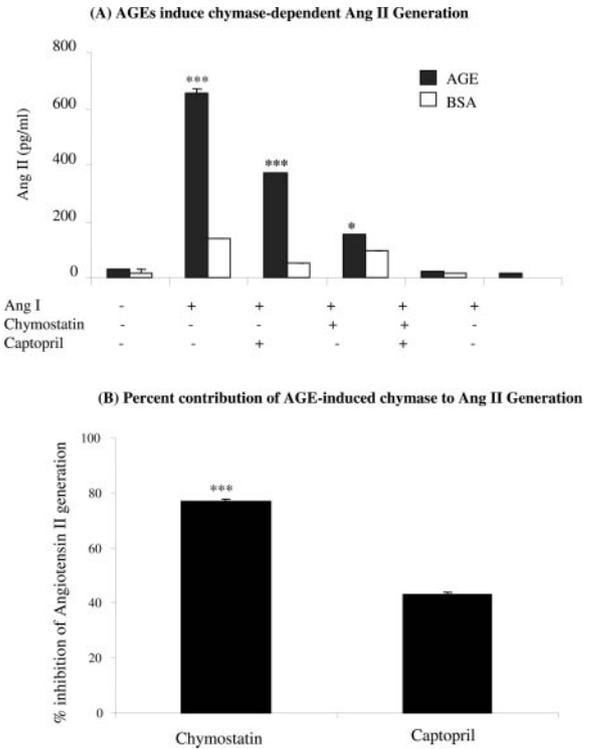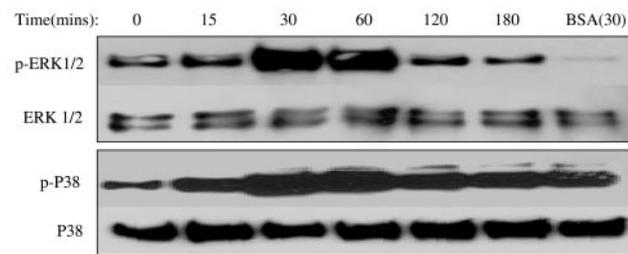**(B) Percent contribution of AGE-induced chymase to Ang II Generation**

Figure 5. AGEs induce chymase or ACE-dependent Ang II generation determined by ELISA. Ang II generation assay was performed as described in Methods. A, Addition of AGE-BSA (50 μg/mL), but not BSA (50 μg/mL), results in a marked increase in Ang II generation as determined by the ability of converting Ang I (400 ng/mL) into Ang II measured by Ang II enzyme immunoassay as described in the Methods. Addition of chymostatin (20 μmol/L) or captopril (100 μmol/L) inhibits AGE-induced Ang II generation to different degrees, reflecting the relative contributions of chymase and ACE to Ang II generation. Note that dual blockade of chymase and ACE completely suppresses Ang II generation compared with BSA alone. The specificity of chymase or ACE-dependent Ang II generation in response to AGEs is confirmed by no detectable Ang II generation in cell-free medium in the presence of Ang I (last lane). B, Comparison of chymase and ACE-dependent Ang II generation. Blockade of chymase activities with chymostatin (20 μmol/L) significantly reduces Ang II generation (70% reduced), whereas inhibition of ACE with captopril (100 μmol/L) reduces Ang II by only 40%. Each value represents mean±SEM for 3 independent experiments. *$P<0.05$, ***$P<0.001$ vs BSA stimulation (A) or ACE inhibition (B).

ing anti-RAGE antibody, inhibitors to ERK1/2 or p38 MAP kinases, and Adv-DN-ERK or Adv-DN-p38 MAP kinases were added to AGE-stimulated VSMCs. Blockade of RAGE or ERK1/2 MAP kinase, but not p38, abolished Ang II generation in response to AGEs stimulation, demonstrating the functional importance of this pathway in diabetic vascular complications (Figure 8).

Discussion

Microvascular and macrovascular complications remain the major cause of morbidity and mortality in diabetics.[27] Major microvascular complications include nephropathy, retinopathy, and neuropathy with characteristic arterial sclerosis, whereas macrovascular complications manifest as accelerated atherosclerosis.[28] Arterial hypertension is present in up to 40% of patients with type 1 and up to 70% of those with type 2 diabetes.[3] Hypertension in diabetes may amplify or accelerate the development and progression of both the macrovascular and microvascular complications of diabetes by contributing to endothelial dysfunction and vascular remodeling, including increased vascular wall thickness, VSMC hyperplasia, and extracellular matrix accumulation in resistance arteries.[29] The renin-angiotensin-aldosterone system may act beyond their hemodynamic effects to cause vascular

complications in diabetes because changes in blood pressure cannot entirely explain the antiproteinuric effect of both ACE inhibitors and AT1 receptor antagonists in patients with diabetes.[30] However, mechanisms of local Ang II generation in diabetic vascular complications remain largely unclear.

Increasing evidence demonstrates that dual blockade of Ang II with ACE inhibitors and Ang II receptor blocker produces additional benefit in cardiovascular and kidney disease with diabetes and/or hypertension,[17,18,30] indicating that alternative Ang-generating pathways are existent in these disease conditions in addition to ACE. Among these alternative pathways, chymase is important. Recent studies suggest that the chymase-dependent Ang-generating pathway is active and contributes largely to Ang II generation in cardiovascular disease with hypertension and atherosclerosis.[13]

(A) AGEs induce ERK1/2 and p38 MAP kinase activation in a time-dependent manner


(B) AGEs activate ERK1/2 and p38 MAP kinases via RAGE
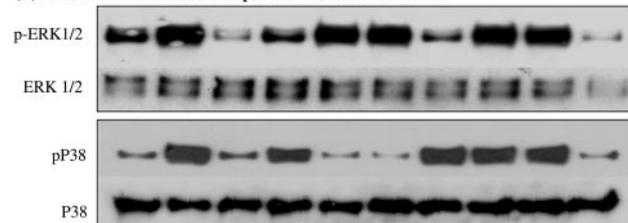
Figure 6. Western blot analysis demonstrates that AGEs signal through RAGE to activate the ERK1/2 signaling pathway and p38 signaling pathways. A, Representative Western blot shows that AGE-BSA (50 μg/mL) induces ERK1/2 and p38 phosphorylation in VSMCs in a time-dependent manner, being significant by 30 minutes. BSA (50 μg/mL) at 30 minutes does not induce such phosphorylation. B, AGE-induced phosphorylation of p38 and ERK1/2 is blocked by a neutralizing rabbit anti-RAGE antibody (10 μg/mL) but not by the isotype control rabbit IgG (10 μg/mL), confirming the involvement of RAGE in the signaling pathway. Furthermore, addition of inhibitors to ERK1/2 (PD98059, 10 μmol/L) or p38 (SB203580, 10 μmol/L) and infection with Adv-DN-ERK1/2 or Adv-DN-p38 (MOI of 50) also inhibit AGE-induced ERK1/2 and p38 phosphorylation respectively, whereas the control Adv-β-gal (MOI of 50) does not, confirming the efficacy of the dominant negative constructs. BSA (50 μg/mL) has no effect on either ERK1/2 or p38 phosphorylation.

Chymase is a serine protease, which is synthesized as an inactive prochymase and stored in intracellular granules in which the pH is maintained at 5.5. Prochymase is then cleaved by dideptidyl peptidase I, which has an optimum pH of 6.0, into chymase. Chymase is then stored as a macromolecular complex bound to heparin proteoglycans, thereby rendering it resistant to endogenous protease inhibitors. Chymase itself is inactive at a pH of 5.5. On stimulation of mast cells by factors such as inflammation, the chymase heparin proteoglycan complex is released into the interstitium where the pH is between 7 and 8, and the chymase is rendered active. Although these mechanisms have been well studied in mast cells, activation of vascular chymase remains unclear. It is highly possible that similar mechanisms may involve the activation of chymase in vascular smooth cells because the vascular chymase may share the similar features of mast cell

(A) AGEs induce chymase mRNA via RAGE and ERK ½ MAP Kinase
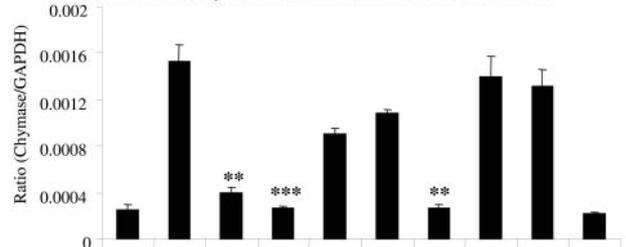
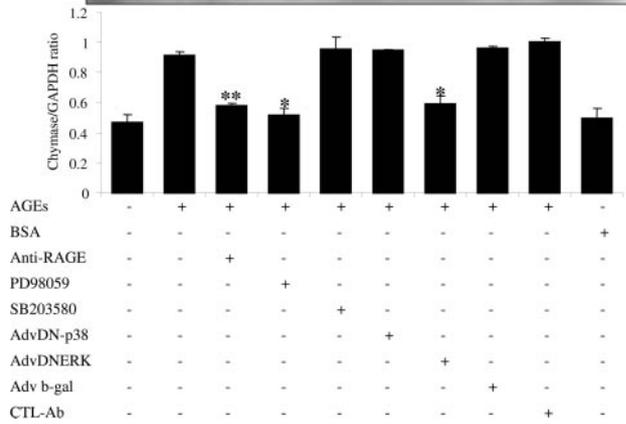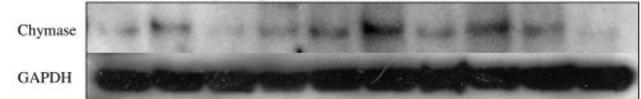**(B) AGEs induce chymase protein via RAGE and ERK ½ MAP Kinase**

Figure 7. Blockade of RAGE and ERK1/2 kinase inhibits AGE-induced chymase mRNA and protein upregulation at 24 hours. Real-time PCR (A) and Western Blot analyses (B) demonstrate that AGE (50 μg/mL)–induced chymase mRNA and protein expression at 24 hours is blocked by the addition of an anti-RAGE antibody (10 μg/mL) and ERK1/2 MAP kinase inhibitor (PD98059, 10 μmol/L) and by overexpression of Adv-DN–ERK MAP kinase, but not by blockade of p38 MAP kinase with the inhibitor (SB203580, 10 μmol/L) and Adv-DN-p38 MAP kinase. The specificity of the anti-RAGE antibody, Adv-DN-ERK, and Adv-DN-p38 MAP kinases is confirmed by the inability to block AGE-induced chymase mRNA and protein expression by the isotype control rabbit IgG (10 μg/mL) and control Adv-β-gal construct. Results are shown as mean$\pm$SEM for 6 independent experiments. $*P<0.05, **P<0.01, ***P<0.001$ vs AGE stimulation and/or Adv-β-gal or control antibody, respectively.

chymase because it is detected by the same primers and the antibody against mast cell chymase (Figures 1 through 4 and 7). Furthermore, the ability of chymostatin to inhibit Ang II generation (Figure 5) demonstrates the functional similarities between the mast cell and smooth muscle cell chymase.

There are several novel and significant findings in the present study. First, we demonstrated that upregulation of vascular chymase in coronary and renal arteriopathy was associated with the deposition of AGEs in patients with diabetes, providing an important link between the deposition of AGEs and the activation of a chymase-dependent Ang-generating system in the development of diabetic vascular complications. AGEs are key mediators in diabetic vascular complications, including diabetic nephropathy, retinopathy, neuropathy, and atherosclerosis.[31] Administration of AGEs in vivo promotes atherosclerosis[19]; inhibition of AGEs formation decreases microvascular and macrovascular diabetic complications in humans and animal models.[20]
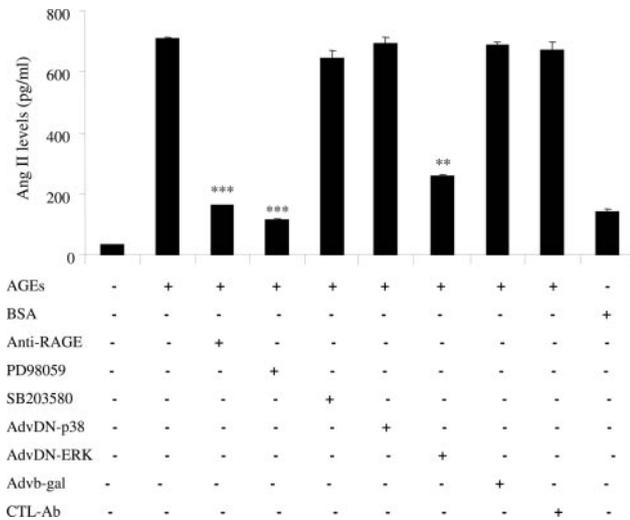
Figure 8. Blockade of RAGE and ERK1/2 kinase inhibits AGE-induced Ang II generation. Enzyme immunoassay analysis demonstrates that AGE (50 μg/mL)–induced Ang II generation at 24 hours is blocked by the addition of a neutralizing rabbit anti-RAGE antibody (10 μg/mL) and an ERK1/2 MAP kinase inhibitor (PD98059, 10 μmol/L) and by overexpression of Adv-DN–ERK MAP kinase but not by blockade of p38 MAP kinase with addition of an inhibitor (SB203580, 10 μmol/L) and Adv-DN-p38 MAP kinase. The specificity of the anti-RAGE antibody, Adv-DN-ERK, and Adv-DN-p38 MAP kinases is confirmed by the inability to block AGE-induced Ang II generation by the isotype control rabbit IgG (10 μg/mL) and control Adv-β-gal construct. Results are shown as mean±SEM for 3 independent experiments. **$P<0.01$, ***$P<0.001$ vs AGE stimulation and/or Adv-β-gal or control antibody, respectively.

Second, the most significant finding in this study is that AGEs were able to induce vascular chymase expression and chymase-dependent Ang II generation via the RAGE-ERK1/2 MAP kinase signaling pathway. This finding was supported by the following: (1) AGEs were able to activate both p38 and ERK1/2 MAP kinase after binding to its receptor RAGE, which was blocked by a neutralizing RAGE antibody or specific inhibitors to ERK1/2 or p38 MAP kinase and by overexpressing Adv-DN-ERK1/2 or Adv-DN-p38 MAP kinase; and (2) AGEs were capable of inducing vascular chymase expression and subsequently chymase-dependent Ang II generation via the RAGE-ERK MAP kinase signaling pathway that could be abolished by addition of a neutralizing anti-RAGE antibody and a specific ERK1/2 inhibitor but not p38 inhibitor or by overexpressing an Adv-DN-ERK1/2 but not Adv-DN-p38 MAP kinase. Indeed, it is known that the engagement of RAGE by AGEs is a key mechanism for AGE-mediated diabetic complications.[32,33] RAGE is known to mediate AGE-induced cell activation via multiple signaling pathways, including p38, ERK1/2 MAP kinases, NF-kB, and activating protein-1.[33] We have previously shown that AGEs signal through the RAGE to induce tubular epithelial-myofibroblast transdifferentiation directly through the MEK1-ERK1/2 MAP kinase pathway[23] and that the AGE-RAGE engagement is capable of mediating renal and vascular fibrosis via the ERK1/2 and p38 MAP kinase-Smad signaling cross-talk pathway.[34] In the present study, we provide new evidence for the importance of the RAGE-

ERK1/2 MAP kinase signaling pathway in AGE-induced vascular chymase expression and chymase-dependent Ang II generation. These in vitro findings support the notion that deposition of AGEs in the vascular wall may act as a key mediator of vascular chymase expression via upregulation of RAGE and activation of the ERK1/2 MAP kinase pathway, as demonstrated in Figures 1 and 2.

Finally, a key observation is that our in vitro system suggests that chymase may be quantitatively more important in Ang II generation than ACE in some pathological states in diabetes and may be a major pathway for vascular Ang II generation in response to AGEs. This was supported by the ability of chymostatin, a chymase blocker, to reduce 70% of AGE-induced Ang II generation, whereas inhibition of ACE with an ACE inhibitor produced only a 40% inhibition of Ang II generation in response to AGEs. This is consistent with our earlier report demonstrating a more intense upregulation of vascular chymase than ACE in arteriopathy in diabetic nephropathy, particularly in the setting of the diabetes with hypertension,[11] and the notion that chymase contributes largely to the Ang II generation in chronic cardiovascular diseases and is an important alternative Ang II–generating pathway in the kidney in ACE-knockout mice.[9] It is unlikely that there is any confounding from Ang II breakdown because, to actually confound the results, the rates of breakdown would have to be different across various experimental conditions, ie, chymostatin treatment versus captopril treatment.

In summary, we demonstrated that upregulation of vascular chymase is associated with the deposition of AGEs, the upregulation of RAGE, and the activation of ERK1/2 MAP kinase in coronary and renal arteriopathy in patients with diabetes. In vitro, AGEs are able to induce chymase expression and chymase-dependent Ang II generation in human VSMCs via the RAGE-ERK1/2 MAP kinase–dependent mechanism. Furthermore, compared with ACE, chymase contributes to the majority of Ang II production in response to AGEs. Thus, chymase may represent an important alternative Ang II–generating pathway in diabetes, particularly in diabetic vascular complications. These findings imply that dual blockade of Ang II with an ACE inhibitor and a receptor blocker may be necessary to prevent progression of diabetic vascular complications.

Acknowledgments

Disclosures

None.

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CLINICAL PERSPECTIVE

Diabetes, ever increasing in prevalence, is a major cause of vascular disease. Diabetic vascular complications are mediated in part by generation of angiotensin (Ang) II, which leads to both hypertension and progression of vascular disease at least in part independently of its hypertensive effects. Although traditionally it has been thought that Ang II is generated by angiotensin-converting enzyme (ACE), it is now recognized that there are alternative Ang II–generating pathways, the most important of which is chymase. Another hallmark of diabetes is the generation of advanced glycation end products, a result of hyperglycemia-induced nonenzymatic glycation of proteins, as well as lipids. We observed that advanced glycation end products upregulate chymase expression and chymase-dependent Ang II generation, accounting for nearly 70% of local Ang II generation in vascular tissues. Because chymase cannot be inhibited by ACE inhibitors, our study suggests we should consider dual renin-angiotensin system blockade with ACE inhibitors and Ang II receptor blockers early in the treatment and prevention of diabetic vascular complications.

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