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Advanced glycation end-products activate the renin-angiotensin system through the RAGE/PI3-K signaling pathway in podocytes

Abstract

Purpose: The purpose of this study was to investigate the effects of advanced glycation end-products (AGEs) on the components of the renin-angiotensin system (RAS) in podocytes and to understand the mechanism of these effects.

Methods: Immortalized mouse podocytes were exposed to various concentrations of AGEs for different time intervals. The expression levels of angiotensinogen (AGT), angiotensin II type 1 and 2 receptors (AT1R and AT2R) and renin were examined by real-time PCR and western blot; the receptor for AGEs (RAGE) and both Akt and phosphorylated Akt were examined by western blot; levels of angiotensin II (Ang II) were assayed by ELISA, and the activity of angiotensin-converting enzyme (ACE) was evaluated by measuring the production of hippuric acid in vitro.

Results: Treatment with AGEs resulted in significant increases in the expression of AGT (62%, $P=0.002$) and AT1R (59%, $P=0.01$). Moreover, Ang II levels increased significantly in both cell lysates (70%, $P=0.018$) and conditioned media (65%, $P=0.01$). ACE activity was also significantly higher in cell lysates (68%, $P=0.035$) and conditioned media (65%, $P=0.023$). There were no changes in renin or AT2R expression ($P>0.05$). AGEs did increase the expression of RAGE by 50% ($P=0.012$) and the phosphorylation of Akt by 100% ($P=0.001$). When podocytes were pretreated with anti-RAGE antibody (50 μg/ml) or the phosphoinositide 3-kinase (PI3-K) inhibitor, LY294002 (10 μM), the AGEs-induced increases in AGT and AT1R expression were reduced. Likewise, Ang II levels and ACE activity decreased significantly.

Conclusion: AGEs activate the RAS in podocytes through the RAGE-PI3-K/Akt-dependent pathway and lead to an increase in podocyte apoptosis.
Diabetic nephropathy (DN) is a serious complication of diabetes and, ultimately, it progresses to glomerular sclerosis and end-stage renal disease (ESRD). Current evidence indicates that AGEs increase at an accelerated rate in diabetes and are associated with the development of severe diabetic complications. Since the glomerular capillary wall functions as an efficient and selective barrier, podocytes are critically involved in maintaining the glomerular filtration barrier; damage to podocytes can lead to proteinuria and initiate glomerulosclerosis, which can result in the progressive loss of kidney function.

Accumulating evidence supports the idea that the intrarenal RAS plays an important role in progressive kidney disease; suppression of the RAS with either ACE inhibitors or AT1R antagonists reduces proteinuria and retards the progression of DN. Sing et al. found an increased Ang II and renin content in glomerular extracts obtained from streptozotocin-induced diabetic rats. Local RAS is activated in mesangial cells and proximal tubular cells by high glucose and in podocytes by mechanical stress. However, the effects of AGEs on the RAS in podocytes are still uncertain and a clear understanding of the mechanism of how AGEs activate the RAS in podocytes is lacking.

Phosphoinositide 3-kinase (PI3-K) and its downstream mediator, Akt, play a central role in a diverse range of cellular responses including cell growth, survival, proteolysis and malignant transformation. While recent studies have shown that the activation of PI3-K may play a part in diabetic complications, its role in diabetic nephropathy, especially in the context of activated RAS, is relatively unstudied.

The purpose of this investigation was to characterize the effects of AGEs on the components of the RAS. How AGEs activated the RAS was examined with anti-RAGE antibody and PI3-K inhibitor to identify the underlying pathway involved.

Methods

Podocytes culture

A conditionally-immortalized mouse podocyte cell line was generously provided by Dr. Peter Mundel (Harvard Medical School, Boston, MA, USA). Cells were harvested as previously described. Cells were cultured in a non-permissive condition in 10% fetal bull serum with 10 U/ml INF-γ in RPMI 1640 media at 33°C. When podocytes were 60-80% confluent, they were transferred to 37°C and incubated for 7-10 days to allow differentiation. Passages 8–14 were used for all experiments. Before treatment, differentiated podocytes were cultured in media containing 1% fetal bull serum for 24 hours. LY294002 was purchased from Sigma-Aldrich.

MTT assay for cell proliferation

Podocyte viability was assessed using the MTT (3-[4,5]dimethylthiazol- 2,5-diphenyltetrazolium bromide) assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. This assay is a nonradioactive cell proliferation assay that identifies living cells and is based on the cellular conversion of a tetrazolium salt into a formazan product; a chromophore that can be quantified by spectrophotometry. Briefly, podocytes were plated in a 96-well plate and were allowed to adhere overnight; then, an appropriate concentration of AGEs was added and incubated with the podocytes for 24 hours. A labeling dye included in the kit was added 4 hours prior to the end point. The stop solution was then added to each well to stop the reaction and solubilize the cells and the absorbance was read at 490 nm using a SPECTRAmax (Molecular Devices, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) assay

Confluent podocytes were cultured under control and experimental conditions. Supernatants were collected, centrifuged for 10 min at 2000 x g and were assessed for LDH release using a LDH cytotoxicity kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). The cytotoxicity of the control and experimental samples was expressed as the percentage of LDH release compared with the total intracellular LDH content. The latter was determined by lysis of representative cell monolayers using 2% Triton X-100 (v/v).

Angiotensin-converting enzyme (ACE) activity

ACE activity in the media and cell lysates was determined as previously described using a commercial kit (Navy General Hospital, Beijing, China). Hippuryl-histidyl-leucine is hydrolyzed to hippuric acid and histidyl-leucine in the presence of exogenous ACE. Hippuric acid was extracted with ethyl acetate. The extracted liquor was evaporated to dryness at 130°C for 15 min and was then dissolved in 1 M NaCl. The mixture was monitored by UV spectrophotometry at 228 nm. One unit of ACE activity was defined as the amount of enzyme required to release 1 nmol of hippuric acid per minute per milliliter of sample.
Measurement of Ang II

Ang II levels were determined in cell lysates and media. Podocytes were harvested in 1% FBS medium for 24 hours and then cultured in control and experimental conditions. The cells were washed with ice-cold PBS, scraped in extraction buffer and homogenized. Cell lysates and media were centrifuged at 12,000 x g for 10 minutes at 4°C and supernatants were collected. Ang II levels in the cell lysates and the media were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from podocytes using Trizol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. Total RNA (500 ng) from each group was reverse transcribed using the reverse transcriptase (RT) provided in a SYBRPremix Ex Taq kit (Perfect Real Time; Takara, Otsu, Shiga, Japan). Reactions were carried out at 37 °C for 15 min and then 85 °C for 5 s. The primers used in the experiments are listed in Table 1. PCR was performed by using an ABI Prism 7000 sequence detection system (AppliedBiosystems, Foster City, CA, USA) and a SYBRPremix Ex Taq Perfect Real Time kit.

Western blot

Podocytes were washed twice with cold PBS and scraped with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin (Cell Signaling Technology, Beverly, MA, USA) and protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined with the Bradford reaction. A total of 20 μg of boiled extracts was loaded on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% fat-free milk before incubation with antibody. The following primary antibodies were used: rabbit anti-AGT (1:2000, Epitomics, Burlingame, CA, USA), rabbit anti-AT1R, rabbit anti-AT2R and rabbit anti-renin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). A total of 20 μg of boiled extracts was loaded on 8% SDS-PAGE gels and incubated with rabbit anti-RAGE (1:2000, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-phospho-Akt and mouse anti-Akt (1:2000, Cell Signal Technology, Danvers, MA, USA). Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc); bands were detected using the ECL chemiluminescence system (Millipore, Billerica, MA, USA).

Measuring of apoptosis

Apoptosis of podocytes was measured at 24 hours by flow cytometry (Annexin-FITC apoptosis detection Kit, BD Biosciences Pharmingen, city, country). Cells were washed twice with cold PBS, were scraped into 1×binding buffer to make a cell suspension at a concentration of 1×10⁶ cells/ml and then 100 μL of the cell suspension were transferred to a 5 mL tube. With 5 μL of Annexin V-FITC and 5 μL of propidium iodide, the cells were gently mixed and incubated for 15 min at room tem-

<table>
<thead>
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<th>Table 1. Sequences of primers</th>
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<td><strong>Sequence (5’→3’)</strong></td>
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<tr>
<td><strong>AGT</strong></td>
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<tr>
<td>Sense</td>
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<tr>
<td>TGACCCAGTTCTTGCCACTGAG</td>
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<td>Antisense</td>
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<tr>
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<td><strong>renin</strong></td>
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<tr>
<td>Sense</td>
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<td>CTCCTGGCAGATCAGATGAAG</td>
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<tr>
<td>Antisense</td>
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<tr>
<td>GGAGCTCGTAGGAGCCGAGATA</td>
</tr>
<tr>
<td><strong>AT1R</strong></td>
</tr>
<tr>
<td>Sense</td>
</tr>
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</tr>
<tr>
<td>Antisense</td>
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<tr>
<td>TGAGTTGGTGTCGACACTGTTCAA</td>
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<tr>
<td><strong>AT2R</strong></td>
</tr>
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<tr>
<td>Antisense</td>
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<td>ATTTGGTGCAGTTGCGTTGA</td>
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AGT: angiotensinogen; AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor
Temperature in the dark and then 400 μL 1× binding buffer were added to each tube. Samples were analyzed by flow cytometry (BD FACSCalibur, San Jose, CA, USA) within 1 h, with excitation at 488 nm and emission collected at 525 nm (FITC-conjugated AV-labeled cells) and 620 nm (PI-labeled cells). Single labeling was used to gate and control for bleed-through. The cell population was characterized according to whether it was labeled with neither AV nor PI (viable), PI alone (necrotic), AV alone (early apoptotic), or both PI and AV (late apoptotic).

The apoptosis rate of podocytes were summarized as early apoptotic plus late apoptotic.

Statistical analysis

The results are presented as the mean ± standard deviation. The statistical significance was assessed by a nonparametric Kruskal–Wallis ANOVA analysis or a Student’s t-test. P values <0.05 were considered significant.

Results

AGEs did not affect podocyte proliferation and metabolic activity

Cell proliferation and viability was assessed by the MTT assay and LDH release. To determine the optimal concentration of AGEs to be applied to podocytes, the differentiated podocytes were incubated with concentrations of AGEs ranging from 0 to 160 μg/ml for 24 hours. Cell proliferation and viability were unaffected at concentrations of AGEs of 20-80 μg/ml but higher concentrations (160 μg/ml) inhibited cell proliferation (Figure 1A). Measurements of LDH release confirmed that 80 μg/ml of AGEs was not cytotoxic to podocytes (Figure 1B); therefore, this dose was used in all subsequent experiments.

AGEs increased the expression of AGT and AT1R in podocytes, but did not alter the expression of renin or AT2R

Incubation of podocytes in media containing 80 μg/ml AGEs produced a significant increase in both AGT and AT1R mRNA and protein levels compared with controls (P<0.05); however, there were no changes in the levels of renin or AT2R (P>0.05). In contrast, incubation in 80 μg/ml BSA did not increase either AGT or AT1R expression (Figure 2A, 2B). The increase in AGT and AT1R expressions was observed within 24 hours and was maintained for up to 48 hours (P<0.05) (Figure 2C).

AGEs increased ACE activity

ACE in podocytes is present as a membrane-bound ectoenzyme and in a secreted form; therefore, both cell lysates and conditioned media were analyzed for ACE activity. After a 24 hour incubation period in various concentration of AGEs, the activity of ACE in cell lysates and media increased in a dose-dependent manner (Figure 3A). ACE activity increased at 6 hours and the increase was sustained for up to 48 hours (Figure 3B).
3B). No significant increase in ACE activity was observed in either the control or the BSA-treated group.

**AGEs increased Ang II levels in podocytes**

Following a 24-hour incubation in AGEs, Ang II levels were significantly higher in both cell lysates and conditioned media compared with controls or BSA-treated groups (Figure 4A). Ang II levels increased starting at 6 hours and were sustained for at least 48 hours (Figure 4B). These results demonstrate that Ang II expression increased in podocytes in the presence of AGEs. In contrast, there was no significant difference in Ang II concentration between the control and BSA-treated groups.

**AGEs increased RAGE expression and Akt activation in podocytes**

RAGE expression and the activity of Akt in cultured podocytes were assayed by western blot. Incubation of podocytes in media containing 80 μg/ml AGEs for 24 hours resulted in a significant increase in RAGE levels compared with control and BSA-treated group (P=0.012) (Figure 5A). AGEs also in-
FIGURE 3. AGEs increased ACE activity
(A) Concentration-dependent effects of AGEs on ACE activity in media and cell lysates. AGEs (80 µg/ml) increased ACE activity by 65% in conditioned media ($P=0.023$) and by 68% in cell lysates ($P=0.035$), when compared with control. In contrast, BSA had no effect on ACE activity. (B) Time-dependent effects of AGEs on ACE activity in media and cell lysates. AGEs increased ACE activity in conditioned media and cell lysates significantly in 24 hours, and this increase persisted at 48 hours. Data were expressed as the mean ± SD of three individual experiments. Media: *$P<0.05$ vs. control, cell lysates: †$P<0.05$ vs. control.

FIGURE 4. AGEs increased Ang II levels
Ang II concentrations in media and cell lysates were measured by ELISA. (A) AGEs increased Ang II levels: AGEs (80 µg/ml) increased Ang II levels by 65% in media ($P=0.01$) and 70% in cell lysates ($P=0.018$) when compared with control cells ($P<0.05$). (B) AGEs (80 µg/ml) increased Ang II levels in a time-dependent manner in both media and cell lysates; this increase started at 12 hours and was sustained until 48 hours. Data are expressed as the mean ± SD for three individual experiments. Media: *$P<0.05$ vs. control, cell lysates: †$P<0.05$ vs. control.
creased the levels of the active form of Akt significantly (P=0.001) (Fig 5B).

**Pre-treatment with anti-RAGE antibody alleviated the activated RAS**

Podocytes were treated for 24 hours with BSA, AGEs (80 µg/ml) alone or AGEs plus pre-treatment with anti-RAGE antibody (50 µg/ml) for 60 minutes. Pretreatment with anti-RAGE antibody significantly inhibited the increase of AGT and AT1R protein expression by 37% and 34%, respectively (Figure 6A). ACE activity in cell lysates and media were reduced by 39% and 45%, respectively (Figure 6B). Ang II concentrations in cell lysates and media decreased by 52% and 53%, respectively, compared with AGES group (Figure 6C).

**PI3K inhibitor attenuated the activated RAS**

Podocytes were treated with BSA, AGEs (80 µg/ml) alone or AGEs in combination with the PI3K inhibitor LY294002 (10 µM), captopril (100 µM) or chymastatin (50 µM). The AGEs-induced increases in the expression of AGT and AT1R were decreased by 64% and 56%, respectively (Figure 7A). ACE activity in cell lysates and media were reduced by 50% and 39%, respectively (Figure 7B). Ang II concentrations in cell lysates and media were lowered by 44% and 44% in LY294002 treated group, respectively (Figure 7C). Captopril alleviated the levels of Ang II by 50% and 43% in cell lysates and conditioned media, respectively; however, chymastatin did not change the levels of Ang II concentrations in cell lysates or in conditioned media. These results indicate that the RAGE-PI3K/Akt pathway might be involved in the activated RAS in podocytes, and ACE is the main enzyme for the production of Ang II converted from Ang I.

**AGEs induced podocyte apoptosis in an Ang II-dependent fusion**

To investigate biologic significance of AGEs-induced activation of local RAS, podocyte apoptosis was measured. As shown in Figure 8, AGEs resulted in a near three-fold increase in apoptosis when compared with the control (23.4±4.6% vs. 7.8±3.8%, P=0.001). To confirm the apoptosis induced by AGEs was due to activation of RAS, Ang II receptor antagonist losartan (10-5
M) and ACE inhibitor captopril (100 μM) was added to cultured podocytes. Both losartan and captopril alleviated significantly the apoptotic podocytes induced by AGES (13.6±4.9 % vs. 23.4±4.6%, P=0.021, 17.1±5.2% vs. 23.4±4.6%, P=0.025 respectively). Chymase inhibitor chymastatin (50 μM) failed to alter the rate of apoptosis (20.8±3.6 vs. 23.4±4.6%, P>0.05). These results show that AGES-induced podocyte apoptosis occurs mainly through activation of RAS.

Discussion

AGES concentrations increased significantly in diabetes mellitus. The kidney is not only a target of AGES but also a source, because declining renal function triggers a rapid increase in plasma concentrations of AGES[16]. The intrarenal RAS plays an important role not only in the regulation of glomerular hemodynamics, but also in glomerular hypertrophy and sclerosis[17]. Several pieces of evidence have shown that high glucose and mechanical strain activate a local RAS in podocytes[8,18]. Whether AGES activate the local RAS in podocytes, and the mechanisms underlying these events, is still unclear.

The present study has demonstrated that AGES stimulated AGT and AT1R production in mouse podocytes. No significant changes in the expression of renin and AT2R were observed. Although some authors found an increase in renin content in diabetic patients[5], other authors showed low-renin state in diabetic nephropathy[19]. The levels of Ang II were higher in glomerular podocytes, which means a local RAS was activated through different pathway. The stimulatory effects of AGES on AGT and AT1R in podocytes indicate that AGES can increase both the substrate and the receptor for the generation of Ang II. Some studies have demonstrated that different-
Podocytes predominantly express AT1R (about 75%) with a lesser amount of AT2R (about 25%)[20,21]. Our data suggest that the activation of AT2R is not significantly involved in the functional responses to Ang II but that AT1R plays a more important role in mediating most of the physiological actions of Ang II. In fact, we speculate that the effects of Ang II on podocytes under physiological and pathophysiological conditions are predominantly mediated by the activation of the AT1R.

The formation of Ang II is mediated by ACE and non-ACE enzymes, and ACE has been established as the most important enzyme for the generation of Ang II[23]. Previous studies have reported ACE protein and activity in both glomerular and cultured podocytes. Our results demonstrated that AGEs increase ACE activity significantly in cell lysates and conditioned media, as compared with controls. ACE activity increased from 6 hours and persisted at 48 hours and these results are consistent with previous reports in mesangial cells[23]. Pretreatment with the ACE inhibitor, captopril, rather than the chymase inhibitor, chymastatin, ameliorated the increase of Ang II levels in AGEs-stimulated podocytes and their conditioned media.

FIGURE 7. PI3K inhibitor attenuated the activated RAS
Podocytes were treated with BSA, AGEs (80 μg/ml) alone or AGEs in combination with LY294002 (10 μM), captopril (100 μM), or chymastatin (50 μM). (A) Pre-treatment with 10 μM LY294002 led to a decrease in AGT and AT1R expression by 64% (P=0.031) and 56% (P=0.028) respectively. (B) ACE activity in the media and cell lysates were reduced by 39% (P=0.024) and 50% (P=0.021) respectively. (C) Pretreatment with LY294002 resulted in a decrease in Ang II concentrations in the media and cell lysates were reduced by 44% (P=0.031) and 44% (P=0.027) respectively. Captopril alleviated the levels of Ang II by 50% in cell lysates (P=0.025) and 43% in conditioned media (P=0.019) ; however, chymastatin did not change the levels of Ang II concentrations in cell lysates or in conditioned media (P>0.05). Media: *P<0.05 vs. control, §P<0.05 vs. AGEs group; cell lysates: †P<0.05 vs. control, ‡P<0.05 vs. AGEs group.
hibitor chymastatin did not change Ang II secretion induced by mechanical stress[18,25].

Ang II, the final effector of the RAS, plays a key role in the pathogenesis of diabetic nephropathy, especially in podocyte injury. Podocytes possess the metabolic machinery necessary for autologous synthesis of Ang II. Mouse podocytes were subjected to various concentrations of AGEs for different time intervals and the levels of Ang II in the media and cell lysates were determined using a competitive ELISA. As shown in Figure 4A and B, there was a significant increase in Ang II levels at 24 hours and these changes were still present at 48 hours. The effects of local RAS activation on podocyte apoptosis were also observed by flow cytometry. AGEs resulted in a near three-fold increase in apoptosis as compared with podocytes treated with BSA. Both losartan and captopril significantly reduced the podocyte apoptosis induced by AGEs; however, chymase inhibitor chymastatin did not significantly alter the rate of apoptosis induced by AGEs II P <0.05 vs. control, † P<0.05 vs. AGEs.

Our results show that AGEs increased the expression of RAGE in a dose-dependent manner. These results are consistent with published reports from endothelial cells[26], pericytes[27], mesangial cells[23] and podocytes[12]. RAGE is a multi-ligand, immunoglobulin superfamily, cell surface receptor that is expressed in podocytes and the glomerular endothelium in the kidney. Previous studies have shown that RAGE-overexpressing diabetic mice exhibit progressive glomerulosclerosis with renal dysfunction when compared with diabetic litter mates lacking the RAGE transgene[28]. When pre-incubated with an anti-RAGE antibody, the AGEs-induced increases in the expression of AGT, AT1R, the levels of Ang II, and ACE activity decreased significantly; therefore, the AGE-RAGE interaction in podocytes could be involved in the activated RAS. Rüster found that Ang II upregulated RAGE expression via AT2R in podocytes[29], and the upregulation of RAGE may further activate RAS. Our results show the increased levels of Ang II induce podocyte apoptosis mainly via the upregulated AT1R. These results show the increased Ang II may cause podocyte injury via both AT1R and AT2R, which aggravates the process of DN.

Our results show that AGEs induced the activation of the Akt kinase activity significantly. To determine whether the ef-

FIGURE 8. AGEs induced podocytes apoptosis in an angiotensin II-dependent fashion
Podocytes were treated with BSA, AGEs (80 μg/ml) alone or AGEs in combination losartan (10-5 M), captopril (100 μM), chymastatin (50 μM). Podocyte apoptosis was measured by flow cytometry. AGEs resulted in a near three-fold increase in apoptosis when compared with podocytes treated with BSA. Both losartan and captopril significantly reduced the podocyte apoptosis induced by AGEs; however, chymase inhibitor chymastatin did not significantly alter the rate of apoptosis induced by AGEs II P <0.05 vs. control, † P<0.05 vs. AGEs.
fects of AGEs on the RAS were PI3-kinase-dependent, the PI3-K inhibitor, LY294002, was added. LY294002 significantly reduced the effects of AGEs on the expression levels of AGT, AT1R and Ang II as well as attenuating the AGEs-induced increase in ACE activity. These results indicate that the PI3-K/Akt pathway is involved in the activated RAS induced by AGEs.

Conclusion

In summary, podocytes not only respond to Ang II by activation of AT1 receptors, but also are capable of directly producing Ang II. Stimulation of Ang II production by AGEs provides a direct link between AGES and the RAS, which may be important in the pathogenesis of DN. The following data from this study suggest that AGEs activated the RAS via the RAGE-PI3-K pathway in podocytes. First, AGEs led to the up-regulation of AGT and AT1R, the increase of Ang II production and the activity of ACE. Second, AGEs increased the expression of RAGE and the active form of Akt kinase. Third, pre-treatment with an anti-RAGE antibody or the PI3-K inhibitor LY294002 significantly inhibited the changes induced by AGEs. Thus, therapies that block the ability of AGES binding to RAGE or inhibit the PI3-K pathway may alleviate the activated RAS and delay the progression of DN. These hypotheses deserve further investigation in vivo.

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