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# Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria

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## ABSTRACT

**Introduction** Advanced glycation end-products (AGEs) are implicated in the pathogenesis of diabetic nephropathy (DN). Previous studies have shown that AGEs contribute to glomerulosclerosis and proteinuria. Podocytes, terminally differentiated epithelial cells of the glomerulus and the critical component of the glomerular filtration barrier, express the receptor for AGEs (RAGE). Podocytes are susceptible to severe injury during DN. In this study, we investigated the mechanism by which AGEs contribute to podocyte injury.

**Research design and methods** Glucose-derived AGEs were prepared in vitro. Reactivation of Notch signaling was examined in AGE-treated human podocytes (in vitro) and glomeruli from AGE-injected mice (in vivo) by quantitative reverse transcription-PCR, western blot analysis, ELISA and immunohistochemical staining. Further, the effects of AGEs on epithelial to mesenchymal transition (EMT) of podocytes and expression of fibrotic markers were evaluated.

**Results** Using human podocytes and a mouse model, we demonstrated that AGEs activate Notch1 signaling in podocytes and provoke EMT. Inhibition of RAGE and Notch1 by FPS-ZM1 (N-Benzyl-4-chloro-N-cyclohexylbenzamide) and DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester), respectively, abrogates AGE-induced Notch activation and EMT. Inhibition of RAGE and Notch1 prevents AGE-induced glomerular fibrosis, thickening of the glomerular basement membrane, foot process effacement, and proteinuria. Furthermore, kidney biopsy sections from people with DN revealed the accumulation of AGEs in the glomerulus with elevated RAGE expression and activated Notch signaling.

**Conclusion** The data suggest that AGEs activate Notch signaling in the glomerular podocytes. Pharmacological inhibition of Notch signaling by DAPT ameliorates AGE-induced podocytopathy and fibrosis. Our observations suggest that AGE-induced Notch reactivation in mature podocytes could be a novel mechanism in glomerular disease and thus could represent a novel therapeutic target.

## Significance of this study

### What is already known about this subject?

- Diabetes is presented with elevated advanced glycation end-products (AGEs) in serum and tissues including the kidney.

### What are the new findings?

- AGEs induce Notch activation in glomerular podocytes.
- Notch activation resulted in epithelial to mesenchymal transition of podocytes.
- Administration of AGEs resulted in glomerulosclerosis and proteinuria.
- Inhibition of receptor for AGEs or Notch activation abrogates AGE-induced proteinuria.

### How might these results change the focus of research or clinical practice?

- Inhibitors of γ-secretase, a key enzyme that triggers Notch activation, could ameliorate AGE-induced Notch activation to prevent proteinuria in diabetic conditions.

## INTRODUCTION

Glomerular podocytes are terminally differentiated visceral cells and provide epithelial coverage to the glomerular capillaries. Podocytes, owing to their unique structure and localization, regulate glomerular permselectivity, contribute to the glomerular basement membrane (GBM), and counteract intracapillary hydrostatic pressure. Therefore, podocytes are considered instrumental in regulating the normal function of the glomerulus and are indispensable for the ultrafiltration of blood and the formation of primary urine. Platelet-derived growth factor and vascular endothelial growth factor derived from podocytes are required for the maintenance of parietal epithelial cells and endothelial cells, respectively.[1 2] Podocyte injury and loss are the early cellular changes in glomerular diseases that are clinically evidenced by proteinuria and renal failure due to glomerulosclerosis.[3] The number of podocytes was found to decline in diabetic nephropathy (DN).[4] Since intact podocytes were identified from the urine of patients with proteinuria, it was proposed that podocytes could detach from underlying GBM.[5] The transition of podocytes from epithelial to highly motile

mesenchymal phenotype or abnormalities in proteins (integrins or dystroglycans) that help podocytes to adhere to GBM might cause podocyte dehiscence and loss into the urine.[6 7] De-differentiation of podocytes and reactivation of embryologically active signaling events elicit podocyte injury and contribute to proteinuria.[8–10]

Notch signaling is highly conserved and helps in the transmission of short-range signals between adjacent cells. Notch signaling regulates podocyte fate determination during the early development of the kidney.[11 12] The Notch pathway comprises five ligands (Delta-like 1, 3, and 4, and Jagged 1 and 2) and four membrane receptors (Notch 1–4). Interaction of Notch ligand from a donor cell with the Notch receptor of the receiving cell results in cleavage of Notch receptor by ADAM (a distintegrin and metalloprotease) and γ-secretase, thus resulting in the release of the Notch intracellular domain (NICD1). NICD1 forms a ternary complex with retinol-binding protein-j and mastermind-like protein in the nucleus and activates expression of target genes.[13] Blockage of Notch signaling during the early development of mouse kidney results in podocyte depletion.[11 14] Although active Notch signaling is crucial during the development, it remains inactive in adult kidneys. Ectopic expression of Notch in podocytes is associated with focal segmental glomerulosclerosis and apoptosis.[15] Recently, we and others have shown that Notch signaling is reactivated in podocytes from subjects with DN.[10 15] Nevertheless, the mechanism of Notch reactivation in podocytes from diabetic kidney disease remains to be elucidated.

A large body of evidence suggests that advanced glycation end-products (AGEs) play a significant role in the pathogenesis of macrovascular and microvascular diabetic complications. AGEs accumulate in the glomerulus not only during hyperglycemia, but also due to aging and uremia. Accumulation of AGEs is associated with glomerular hyperfiltration, thickening of GBM, glomerulosclerosis, and tubulointerstitial fibrosis in diabetic settings, thus deteriorating renal function.[16] Carboxymethyl lysine, one of the best characterized AGEs, was shown to elicit epithelial to mesenchymal transition (EMT) and proteinuria.[17] Renal AGEs contribute to the progressive damage of the renal architecture, thus impairing renal function in patients with DN. Subjects with diabetes and end-stage renal disease (ESRD) had twice as many tissue AGEs as patients with diabetes without ESRD, suggesting that the severity of DN and impaired renal function are parallel to elevated AGE levels.[18] Indeed, serum levels of AGEs and degraded AGEs get elevated in subjects with diabetes with renal dysfunction.[19] AGE-modified skin collagen predicts the severity of renal dysfunction in people with type 1 diabetes.[20] Since elevated levels of AGEs and reactivation of Notch signaling temporally contributed to the pathophysiology of nephropathy, in this study we investigated whether AGEs induce Notch signaling in podocytes. We found that AGEs induced reactivation of Notch signaling in podocytes and induced cytoskeletal abnormalities. We also found that Notch activation is parallel to EMT of podocytes in vitro and AGEs induced glomerulosclerosis, altered podocyte morphology, and proteinuria in vivo.

## RESEARCH DESIGN AND METHODS

### Materials

The anti-AGE antibody was generated inhouse (NIN, Hyderabad). Anti-RAGE (#3611), anti-activated Notch1 (#8925), anti-WT-1 (#212951), anti-SIRT1 (#110304), anti-histone H2B (#177430), cleaved caspase 3 (#2302) and Bax (#32503) were obtained from Abcam (Cambridge, Massachusetts). Anti-E-cadherin (#3195), anti-N-cadherin (#13116), anti-α-SMA (#19245), anti-Notch1 (#3608) and anti-β-actin (#4970) were purchased from Cell Signaling Technology (Danvers, Massachusetts). Anti-fibronectin (#PAA037Hu01), anti-vimentin (#PAB040Hu01), anti-Col4 (#PAA180Hu01), and anti-JAG1 (#PAB807Hu01) were procured from Cloud-Clone (Houston, Texas). Anti-Hes1 (SC166410) and anti-Zeb2 (#PA520980) were purchased from Santa Cruz Biotechnology (Dallas, Texas) and Thermo Fisher Scientific (Waltham, Massachusetts), respectively. Fetal bovine serum, RPMI 1640, TRIzol reagent, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester (DAPT) (#D5942), N-Benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) (#553030), phalloidin fluorescein isothiocyanate labeled (P5282) and glutaraldehyde solution (#G5882) were obtained from Sigma-Aldrich (St Louis, Missouri). ProLong Diamond Antifade Mountant (#P36961) was purchased from Molecular Probes Life Technologies, and Alexa Fluor 488 or 594 conjugated anti-mouse or anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). Alexa Fluor 568 preadsorbed (ab175692) was purchased from Abcam. Mouse/Rabbit PolyDetector DAB HRP Brown Detection Kit was procured from Bio SB (Santa Barbara, California). Primers were from Integrated DNA Technologies (Coralville, Iowa). Protein marker, cDNA reverse transcription kit, and quantitative reverse transcription-PCR (qRT-PCR) reagents were from Bio-Rad Laboratories (Hercules, California). Scrambled RNA and small interfering RNA (siRNA) specific to receptor for AGEs (RAGE) and Notch1 were purchased from Kaneka Eurogentec (Belgium).

### Preparation of glucose-derived AGEs

Glucose-derived AGEs were prepared as reported earlier with minor modifications.[21 22] Briefly, sterile preparations of bovine serum albumin (BSA) (100 mg/mL) were mixed with D-glucose (90 mg/mL) and 1 mM sodium azide in 0.4M phosphate buffer at pH 7.6 and incubated for 2 weeks at 37°C. Dialysis of D-glucose-BSA preparation was performed against phosphate buffer to eliminate the unreacted D-glucose. BSA and D-glucose were separately incubated in the same conditions. Formation of glucose-derived AGEs was confirmed using non-tryptophan AGE fluorescence (λex: 370 nm and λem: 400–500 nm) and by western blotting with AGE-specific antibody. The

extent of glycation of BSA was assessed by measuring free amino groups in the D-glucose-BSA preparation using 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay as a recommended protocol from the manufacturer (#BC86). Briefly, 50–200 µg/mL samples were prepared in reaction buffer (0.1M $NaHCO_3$) and 0.1% TNBS added to 500 µL of each sample. Further, samples were incubated and absorbance measured at 335 nm.

### Human podocyte culture and experimentation

Immortalized human podocytes were maintained and differentiated essentially as detailed earlier.[10] Differentiated podocytes were treated with variable concentration of AGEs (50–200 µg/mL) for different time intervals (0–72 hours) with or without chemical inhibitors: DAPT (5 µg/mL) and FPS-ZM1 (1 µg/mL). Protein lysate and RNA were prepared from these podocytes and used for western blotting and qRT-PCR. A wound-healing assay was also performed with podocytes essentially as described earlier.[10] Phalloidin staining for F-actin was performed to visualize the distribution of stress fibers in differentiated podocytes as described previously.[23] The γ-secretase activity was measured as recommended by the manufacturer's protocol (ImmunoTag, G-Bioscience, St Louis, Missouri). Albumin influx assay was performed as described earlier.[10]

### Protein isolation and immunoblotting

Protein was extracted from human podocytes (HPC) and isolated glomerular podocytes with the radio immuno precipitation assay (RIPA) buffer consisting of protease inhibitor cocktail (Roche Diagnostics). Nuclear extract was prepared using the CelLytic NuCLEAR Extraction Kit (Sigma Aldrich). The homogenate was centrifuged at 12 000 × *g* for 25 min at 4°C and supernatant collected. An equal amount of protein from the supernatant was electrophoresed through 8%–12% gradient polyacrylamide gels and blotted on nitrocellulose membrane. The blot was probed with the corresponding primary and secondary antibodies and then developed by chemiluminescence substrate (#1705060, Bio-Rad) and visualized by ChemiDoc XRS System (Bio-Rad).

### RNA interference and transfection

siRNA sequences directed against RAGE and Notch1 or a non-silencing control sequence (20 nM) was transfected transiently with Lipofectamine RNAiMax Reagent (Life Technologies), following the manufacturer's instructions. Sixteen hours after the transfection, HPC cells were exposed with or without AGEs for 48 hours.

### Immunofluorescence

Immunofluorescence analysis was performed as described earlier.[10] Briefly, human podocytes were cultured on coverslips and treated with or without AGEs. These cells are fixed with paraformaldehyde (4%) and probed with primary antibodies overnight. The next day, the samples were incubated with Alexa Fluor-conjugated secondary antibody and DAPI for 1 hour at room temperature. Images were acquired using Upright Microscopes Leica DM4 B and DM6 B (Leica Microsystems trinocular).

### Animals and tissues

C57 black/6J male mice (6–8 weeks old, 30±5 g) were used in this study. These mice were randomly distributed into four groups, viz control, AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treatment groups (six mice, each group). Mice in the control group received an equal volume of phosphate buffer as a vehicle, whereas the experimental group received intraperitoneal injections of in vitro prepared AGEs (10 mg/kg body weight), AGEs and inhibitors DAPT (10 mg/kg body weight), and AGEs and FPS-ZM1 (1 mg/kg body weight) on a daily basis for 4 weeks. At the end of the experimental period, 24-hour urine was collected to measure glomerular filtration rate (GFR), albumin, and creatinine levels as detailed earlier.[10] Additionally, urine was subjected to Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and stained with silver nitrate to visualize the proteins in urine. Animals were perfused and kidneys were harvested as described previously.[10] Glomeruli isolation was carried out as reported earlier and the glomerular lysate was used for immunoblotting.[24] Kidney sections (4 µm) from paraffin-embedded tissues were stained for WT1, NICD1, podocin, RAGE, α-SMA, Col IV and fibronectin, using routine protocol.[10]

### Assessment of podocyte apoptosis

Formalin-fixed, 4 µm thin mice kidney tissue sections were stained using a TUNEL staining kit (#ab66110) according to the manufacturer's instructions. Further, human podocyte apoptosis was detected in vitro by DAPI staining. Human podocyte cells were grown on the coverslip and treated with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1. Next cells were fixed with 4% paraformaldehyde-phosphate buffer saline (PBS) and washed twice with ice-cold PBS. Following these, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at 37°C and stained with 1 mg/mL DAPI dissolved in PBS for 30 min at 37°C. The cells were rinsed twice with ice-cold PBS and fluorescent images were captured from the images acquired using Upright Microscopes Leica DM4 B and DM6 B (Leica Microsystems trinocular). The percentage of condensed nuclei was calculated as the ratio of condensed nuclei to total cells counted, and a minimum of 100 cells/field and at least six fields in each well were counted.

### Morphometric analysis and TEM imaging

The extent of renal injury was evaluated by performing Masson's trichrome (MT) and periodic acid-Schiff (PAS) staining, and glomerular damage score was performed as described earlier.[25] Transmission of electron microscopy (TEM) imaging was performed to visualize the changes in podocyte foot processes and GBM essentially as described earlier.[10]

### Human patient samples

Kidney specimens were collected without patient identifiers from archived kidney biopsies from Guntur Medical College, Guntur, Andhra Pradesh, India. A total of 12 kidney tissue sections were collected from patients with DN (n=12) and significant proteinuria, while 10 kidney sections were collected from non-diabetic individuals from archived kidney biopsies. Kidney biopsy serial sections (4 µm) from paraffin-embedded tissues were stained for AGEs, RAGE, NICD1, and HES1. To investigate the association of glycation parameters (hemoglobin A1c (HbA1c) and glycated albumin) with proteinuria, we have recruited subjects with DN (n=42) and healthy volunteers (n=34) and performed clinical characteristics (online supplementary table S1).

### Statistical analysis

Data are presented as mean±SD. For the calculation of the significance of the difference between the two groups of the data, Student's t-test was used. A level of $p \leq 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism V.6.0.

## RESULTS

### Glucose-derived AGEs induce Notch signaling in podocytes in vitro

Glucose-derived AGEs were prepared as described in the 'Research design and methods' section by incubating D-glucose and BSA. We have noticed browning in D-glucose-BSA preparation after 2 weeks, and the extent of AGE formation was demonstrated by measuring non-tryptophan AGE fluorescence (figure 1A). We assessed the degree of glycation of BSA during incubation with D-glucose by measuring its free amino groups. There was about a 50% reduction in free amino groups in D-glucose-BSA preparation (figure 1B). High-molecular-weight aggregates were observed in D-glucose-BSA preparation (figure 1C). Free amino groups of BSA can react with the carbonyl group of glucose and form AGEs via the Maillard reaction. Therefore, we assessed the formation of AGEs in the D-glucose-BSA preparation by immunoblotting with the anti-AGE antibody. The data suggest AGEs were selectively noticed in D-glucose-BSA preparation (figure 1D). Since we confirmed the formation of glucose-derived AGEs in D-glucose-BSA preparation, hereafter they are referred to as AGEs. Then, we investigated the effect of AGEs on Notch signaling components in human podocytes. We found that AGEs induced RAGE and NICD1 expression in a dose-dependent manner (figure 1E), whereas BSA naïve to AGE modification failed to elicit NICD1 expression (figure 1F). We have also observed that AGEs induced Notch ligand (JAG1), NICD1, and Notch downstream transcription factor HES1 in podocytes (figure 1G). Since γ-secretase cleaves Notch1 to release NICD1, we measured γ-secretase activity in cells treated with AGEs. We observed a dose-dependent increase in γ-secretase activity with AGE treatment (figure 1H). All these data suggest that Notch signaling is activated in human podocytes exposed to AGEs.

### Both RAGE and γ-secretase are required for AGE-activated Notch signaling

To confirm the role of γ-secretase in AGE-induced Notch1 activation, we next treated the human podocytes with AGEs in the absence or presence of a well-established γ-secretase inhibitor, DAPT. As expected, DAPT treatment to the AGE-exposed human podocytes decreased the γ-secretase activity (online supplementary figure S1A). Further, we noticed reduced γ-secretase activity in podocytes that were treated simultaneously with AGEs and FPS-ZM1, a RAGE inhibitor (online supplementary figure S1A). The ability of AGEs to induce NOTCH1, JAG1, or HES1 is ameliorated in the presence of FPS-ZM1 as measured by qRT-PCR (figure 2A–C) and western blotting (figure 2D). Furthermore, DAPT also prevented HES1 expression in AGE-treated human podocytes as measured by qRT-PCR (figure 2C) and immunoblotting (figure 2D). Interestingly, NICD1 levels were decreased following treatment with DAPT (figure 2D). As anticipated, DAPT treatment does not affect the expression of both NOTCH1 (figure 2A) and its ligand JAG1 (figure 2B,D). Next, we studied the essential role of RAGE and NOTCH1 in AGE-induced Notch1 signaling by siRNA-mediated knocking down of RAGE and NOTCH1 expression, respectively. Knockdown of RAGE expression resulted in blunting of AGE-induced expression of NOTCH1, NICD1, JAG1, and HES1 (figure 2E), while knockdown of NOTCH1 expression resulted in blunting of AGE-induced expression of NICD1, JAG1, and HES1 expression (figure 2E). Furthermore, AGE-induced γ-secretase activity is also ameliorated by the knocking down of RAGE and NOTCH1 expression, respectively (online supplementary figure S1B).

Activation of Notch1 signaling was shown to enhance the migratory properties of podocytes via EMT.[10] Furthermore, in our earlier study, podocytes exposed to N(ε)-carboxymethyl lysine were shown to possess enhanced migration properties and undergo phenotypic switch via EMT.[17] Therefore, we assessed the migratory property of podocytes treated with AGEs in the presence and absence of DAPT and FPS-ZM1. Both DAPT and FPS-ZM1 attenuated AGE-induced podocyte motility (online supplementary figure S1C). As we could see the enhanced migration of podocytes treated with AGEs, we assessed the expression of epithelial marker (E-cadherin) and mesenchymal markers (N-cadherin and vimentin). Loss of E-cadherin and increased N-cadherin, that is, cadherin switch, are hallmark features of EMT. Exposure of podocytes to AGEs manifested in cadherin switch and increased expression of vimentin (figure 2F,G), whereas DAPT and FPS-ZM1 prevented AGE-induced cadherin switch and vimentin expression (figure 2F,G). EMT is accompanied by dramatic changes in cytoskeleton remodeling.[26] Therefore, to assess the cytoskeletal abnormalities induced by AGEs, we stained podocytes with phalloidin,

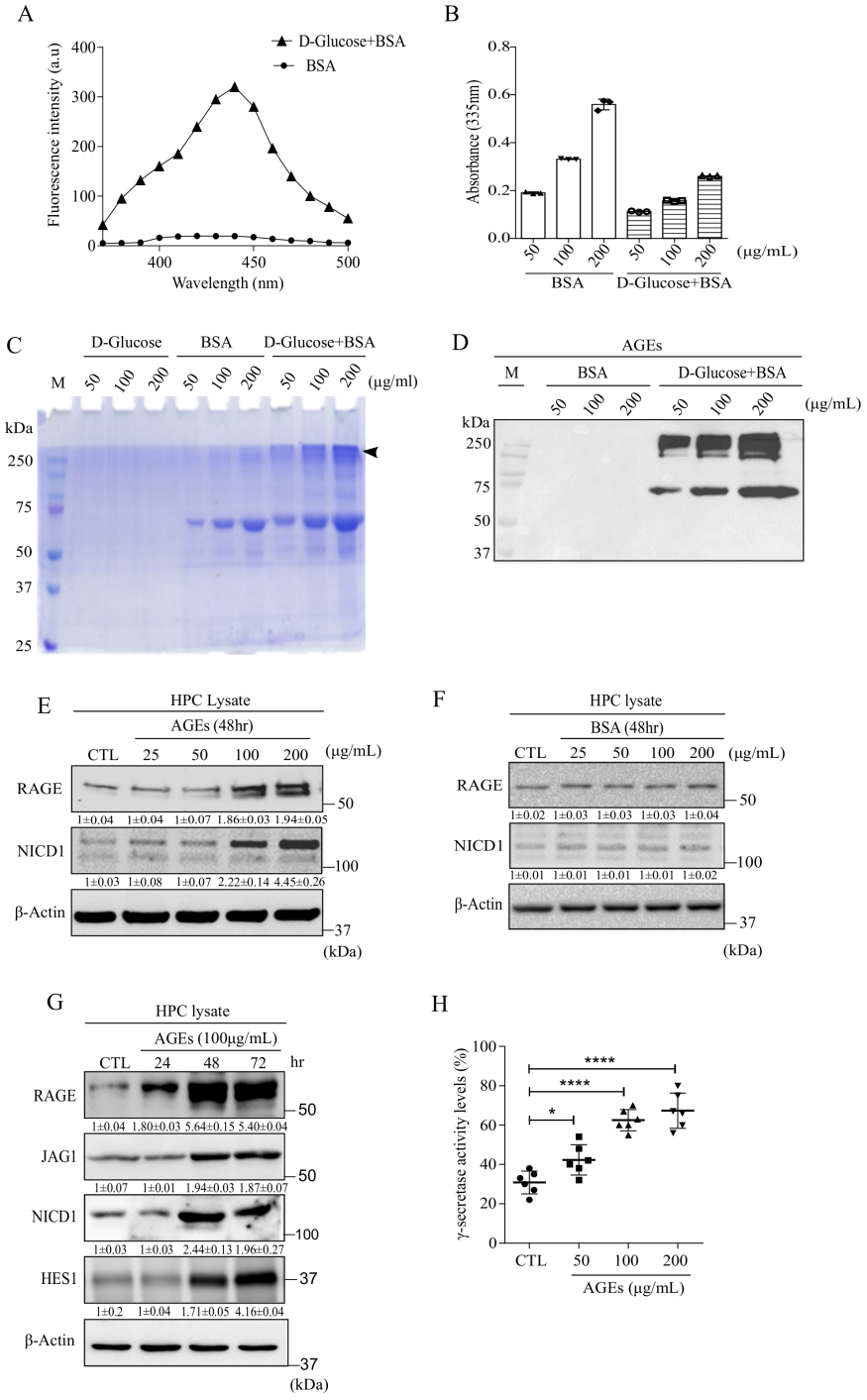


**Figure 1** AGEs induce Notch signaling in podocytes. (A) Non-tryptophan AGE fluorescence was measured to demonstrate the formation of AGEs in D-glucose+BSA preparations at Ex: 370 nm and Em: 400–500 nm. (B) Quantification of free amines in BSA and D-glucose+BSA preparations of various concentrations (50–200 μg/mL of BSA) by TNBS assay. (C) BSA and D-glucose+BSA preparations were subjected to SDS-PAGE and stained with Coomassie blue. The arrowhead indicates high-molecular-weight aggregates in the stacking region of the gel. (D) Immunoblots showing the presence of AGEs in D-glucose+BSA preparations. M indicates the standard protein marker (D,E). (E) Immunoblots showing the expression of RAGE, NICD1, and β-actin in HPC cells treated with AGEs (25–200 μg/mL) for 48 hours. (F) Immunoblots showing the expression of RAGE, NICD1, and β-actin in HPC treated with BSA alone (25–200 μg/mL) for 48 hours. (G) Immunoblots showing the expression of RAGE, JAG1, NICD1, HES1, and β-actin in HPC treated with AGEs (100 μg/mL) for indicated time intervals (24–72 hours). (E–G) The fold expression was presented after normalizing with β-actin. (H) γ-secretase activity in HPC treated with (50–200 μg/mL) or without AGEs for 48 hours (n=6). *P<0.05, ****P<0.0001. Data are presented as mean±SD (n=3). AGEs, advanced glycation end-products; a.u, arbitrary unit; BSA, bovine serum albumin; CTL, control; Em, emission; Ex, excitation; HES1, hairy and enhancer of split homolog1; HPC, human podocyte; JAG1, jagged1; NICD1, Notch intracellular domain; RAGE, receptor for AGEs; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; TNBS, trinitrobenzene sulfonic acid.

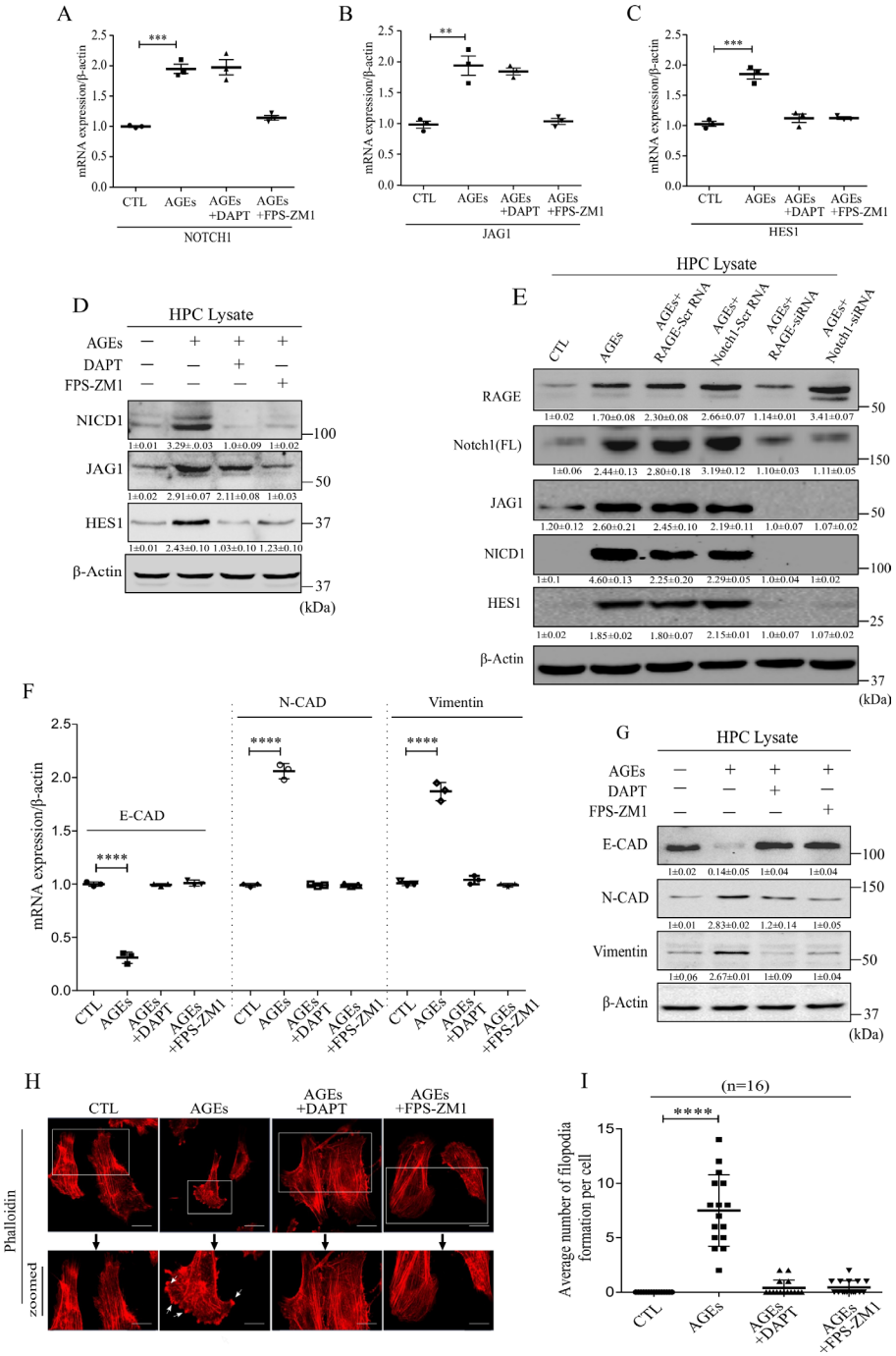


**Figure 2** AGE-activated Notch signaling promotes EMT in podocytes. (A–C) qRT-PCR analysis showing the expression of (A) NOTCH1, (B) JAG1, and (C) HES1 in HPC treated with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1. β-actin was used as an internal control. **P<0.01, ***P<0.0001. (D) Immunoblots showing the expression of NICD1, JAG1, HES1, and β-actin in HPC treated with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 (48 hours). The fold change values were presented after normalizing with β-actin. (E) HPC cells transfected with specific siRNA targeting RAGE and Notch1 or scramble RNA (Scr) were subjected to immunoblotting for RAGE, NOTCH, JAG1, and NICD1. (F) Expression of E-CAD, N-CAD, and vimentin in HPC (CTL, AGEs, AGEs+DAPT, and AGEs+FPS-ZM1) was analyzed by qRT-PCR. The expression of β-actin was used as an internal control. ****P<0.0001. (G) Immunoblotting analysis of expression of E-CAD, N-CAD and vimentin in HPC (CTL, AGEs, AGEs+DAPT, and AGEs+FPS-ZM1). (E,G) The fold change values were presented after normalizing with β-actin expression. (H) Phalloidin staining of podocytes showing F-actin arrangement. The white arrows indicate filopodia formation. Scale bar=20 µm. (I) Quantification of the average number of filopodia formation observed from the phalloidin staining (n=16). ****P<0.0001. Data are presented as mean±SD (n=3). AGEs, advanced glycation end-products; CTL, control; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester; E-CAD, E-Cadherin; EMT, epithelial to mesenchymal transition; FPS-ZM1, N-Benzyl-4-chloro-N-cyclohexylbenzamide; HES1, hairy and enhancer of split gene1; HPC, human podocyte; JAG1, jagged1; N-CAD, N-Cadherin; NICD1, Notch intracellular domain; qRT-PCR, quantitative reverse transcription-PCR; RAGE, receptor for AGEs; siRNA, small interfering RNA.

which specifically stains F-actin. DAPT and FPS-ZM1 prevented AGE-induced F-actin reorganization in human podocytes (figure 2H). We measured an average number of filopodia per cell and found that DAPT and FPS-ZM1 prevented AGE-induced filopodia formation in human podocytes (figure 2I). Motonishi *et al*[27] showed that SIRT1 regulates the integrity of the podocyte actin cytoskeleton and prevents glomerular injury. Therefore, we assessed SIRT1 expression in AGE-treated podocytes and found that AGEs marginally induced SIRT1 expression (online supplementary file 1). All these data suggest that podocytes undergo EMT on treatment with AGEs, and Notch activation is required for EMT of AGE-treated podocytes in vitro.

### AGE-induced Notch1 signaling is abrogated by RAGE inhibitor in vivo

We investigated for co-localization of NICD1 and HES1 in the nucleus in response to AGE treatment. As expected there is a predominant accumulation of both NICD1 and HES1 in the nucleus of podocytes as demonstrated by immunofluorescence (figure 3A) and immunoblotting of nuclear fraction from podocytes exposed to AGEs (figure 3B). A large body of evidence reported the accumulation of AGEs in glomeruli from patients with DN or experimental animals.[26] Therefore we investigated the expression of RAGE and components of Notch signaling in mice administered with AGEs for 4 weeks. We have observed enhanced RAGE expression in glomerular sections from AGE-administered mice (figure 3C). Furthermore, elevated glomerular expression of NICD1, JAG1, and HES1 was observed in AGE-injected mice (figure 3D,E), whereas coadministration of DAPT ameliorated AGE-induced NICD1 and HES1 expression (figure 3D,E). Indeed simultaneous administration of RAGE inhibitor (FPS-ZM1) attenuated the AGE-induced expression of NICD1, JAG1, and HES1 (figure 3D,E). Furthermore, both DAPT and RAGE inhibitor blunted the AGE-induced cadherin switch and vimentin expression in glomerular lysates (figure 3F). Together the data suggest AGEs activate Notch signaling and induce EMT in glomerular podocytes.

### Notch1 signaling is required for AGE-induced glomerular fibrosis

DN is presented with progressive renal fibrosis.[28 29] AGEs were shown to induce fibrosis and contribute to the pathology of DN.[30] Therefore, we examined the paraffin-embedded sections of AGE-treated mice for fibrosis. We noticed renal fibrosis in the mice administered with AGEs, as analyzed by PAS and MT staining (figure 4A). Glomerular damage score was found elevated in AGE-treated mice (figure 4B). It is noteworthy that both DAPT and RAGE inhibitor had prevented AGE-induced fibrosis and glomerular damage (figure 4A,B). Next, we measured the expression of fibrotic markers in AGE-treated mice and mice treated with DAPT and RAGE inhibitor. Indeed, DAPT and FPS-ZM1 inhibited the AGE-induced fibrotic markers: collagen IV, α-SMA, and fibronectin (figure 4C–F).

Glomerular fibrosis is presented with thickening of GBM that may manifest in the podocyte foot process effacement.[31] Since we observed elevated expression of fibrotic markers in AGE-injected mice, we next assessed the morphology of GBM and podocytes in these mice. Although TEM analysis of AGE-administered mice shows thickening of GBM and podocyte foot process effacement, co-treatment with DAPT or FPS-ZM1 abrogated this adverse effect of AGEs on glomerular architecture (figure 4G). The thickening of GBM might lead to the dehiscence of podocytes.[32] Suztak *et al* have reported that the depletion of podocytes occurs at the onset of DN.[33] Next, we assessed whether podocyte depletion occurred in AGE-administered mice. Quantification of WT1, a podocyte-specific marker, revealed the presence of detached podocyte in many glomeruli, whereas DAPT and RAGE inhibitor prevented AGE-dependent podocyte depletion (figure 4H,I). Earlier, studies have shown that podocytes undergo apoptosis in response to noxious stimuli.[34] Therefore, we assessed podocyte apoptosis in AGE-treated human podocytes (in vitro) and mice (in vivo). Glomerular sections from mice treated with AGEs showed increased TUNEL positive cells (online supplementary figure S2A), elevated cleaved caspase 3, and Bax expression (online supplementary figure S2B,C). Quantification of condensed nuclei from AGE-treated human podocyte cells revealed a 40%±15% presence of podocyte apoptotic bodies (online supplementary figure S2D). Together the data confirm that AGE-induced Notch signaling leads to glomerular fibrosis and podocyte depletion.

### Inhibiting Notch1 activation abrogates AGE-induced proteinuria

As we have stated, podocytes are instrumental in regulating glomerular permselectivity and either podocyte injury or loss elicits proteinuria. Since AGE treatment showed podocyte foot process effacement (figure 4G), we measured the permselectivity of podocytes in vitro by albumin influx assay. Exposure of podocyte monolayer to AGEs resulted in increased permeability to albumin, whereas DAPT or FPS-ZM1 prevented AGE-induced albumin leakage across podocyte monolayer (figure 5A). The expression of predominant slit-diaphragm proteins (podocin and nephrin) decreased in the glomerulus of AGE-treated mice (figure 5B,C). Since damage to the slit-diaphragm contributes to proteinuria, we measured the renal function parameters and found that the administration of AGEs to mice resulted in increased urinary albumin to creatinine ratio (UACR) and a decline in GFR (figure 5D and online supplementary figure S3A). Mice treated with AGEs had increased the amount of protein in the urine (online supplementary figure S3B), whereas co-treatment of mice with DAPT or FPS-ZM1 rescued the expression of podocin and nephrin and blocked AGE-induced proteinuria (figure 5A–D and

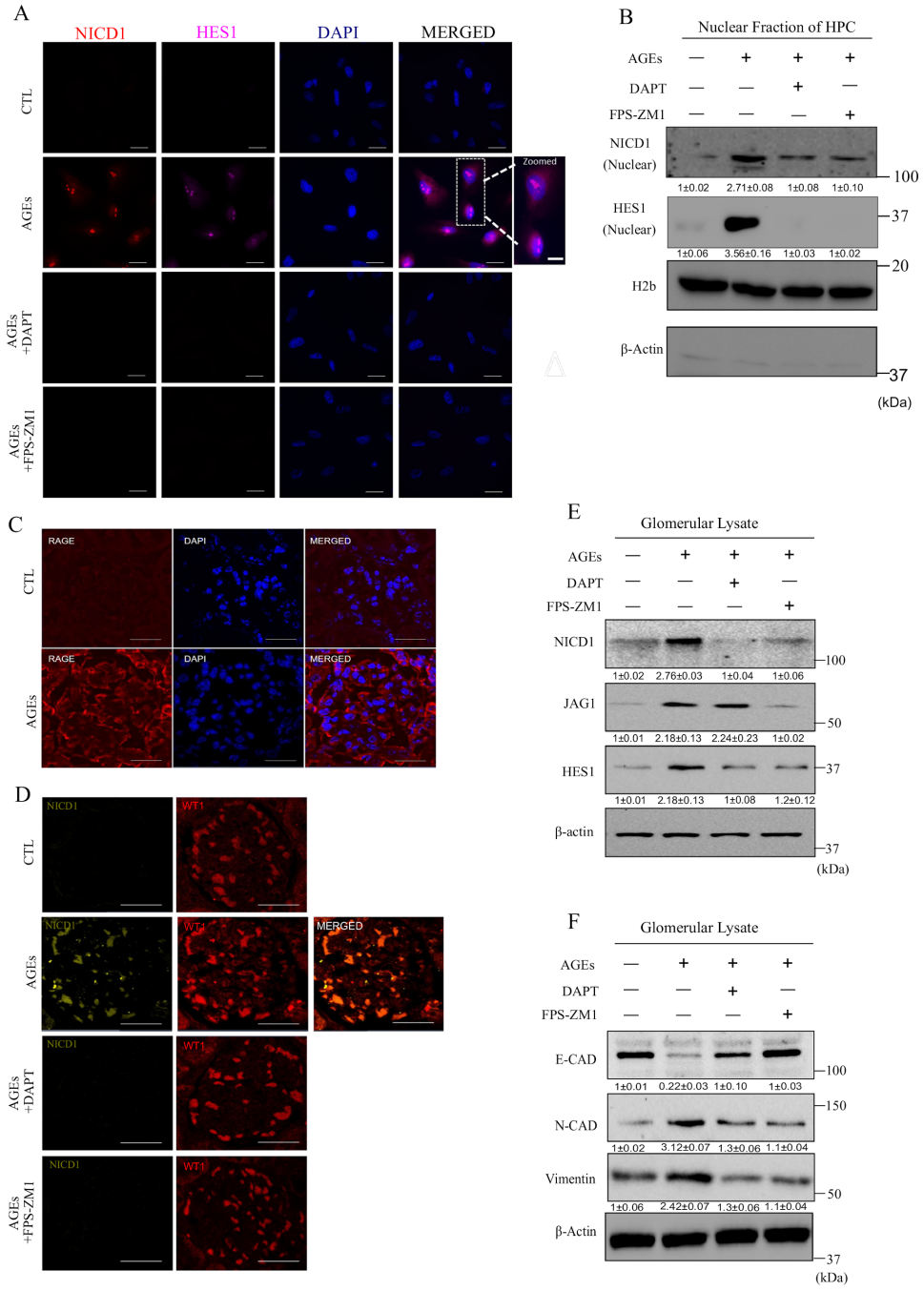


**Figure 3** AGEs induce Notch signaling in mice glomeruli and inhibition of γ-secretase and RAGE ameliorates EMT in mice kidney. (A) Immunofluorescence for the nuclear co-localization study of NICD1 (Cy3, red) and HES1 (Cy5, far-red) in HPC treated with or without (CTL) AGEs, AGEs+DAPT and AGEs+FPS-ZM1. Magnification ×630. Scale bar=20 µm. (B) Immunoblots for NICD1, HES1 and histone H2B, the nuclear extract of HPC, treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1. The fold change values were presented with the expression of the respective genes after normalizing with H2B. (C) Immunostaining for RAGE expression in mice glomeruli from with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6, each group). Magnification ×630. Scale bar=20 µm. (D) Double immunostaining with anti-NICD1 (Alexa Fluor 555) and anti-WT1 (Cy3, red) in glomerular sections from with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 treatment (n=6, each group). Magnification ×630. Scale bar=20 µm. (E) Immunoblotting analysis for NICD1, JAG1, HES1, and β-actin in mice glomerular lysates from with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 treated mice (n=6, each group). The fold change values were presented with the expression of the respective genes after normalizing with β-actin. (F) Immunoblotting analysis for E-CAD, N-CAD, vimentin, and β-actin in glomerular lysates from with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treated mice. The fold change values were presented with the expression of the respective genes after normalizing with β-actin. Data are presented as mean±SD (n=3). AGEs, advanced glycation end-products; CTL, control; DAPI, 4′,6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester; E-CAD, E-Cadherin; EMT, epithelial to mesenchymal transition; FPS-ZM1, N-Benzyl-4-chloro-N-cyclohexylbenzamide; HES1, hairy and enhancer of split gene1; HPC, human podocyte; JAG1, jagged1; N-CAD, N-Cadherin; NICD1, Notch intracellular domain; RAGE, receptor for AGEs.

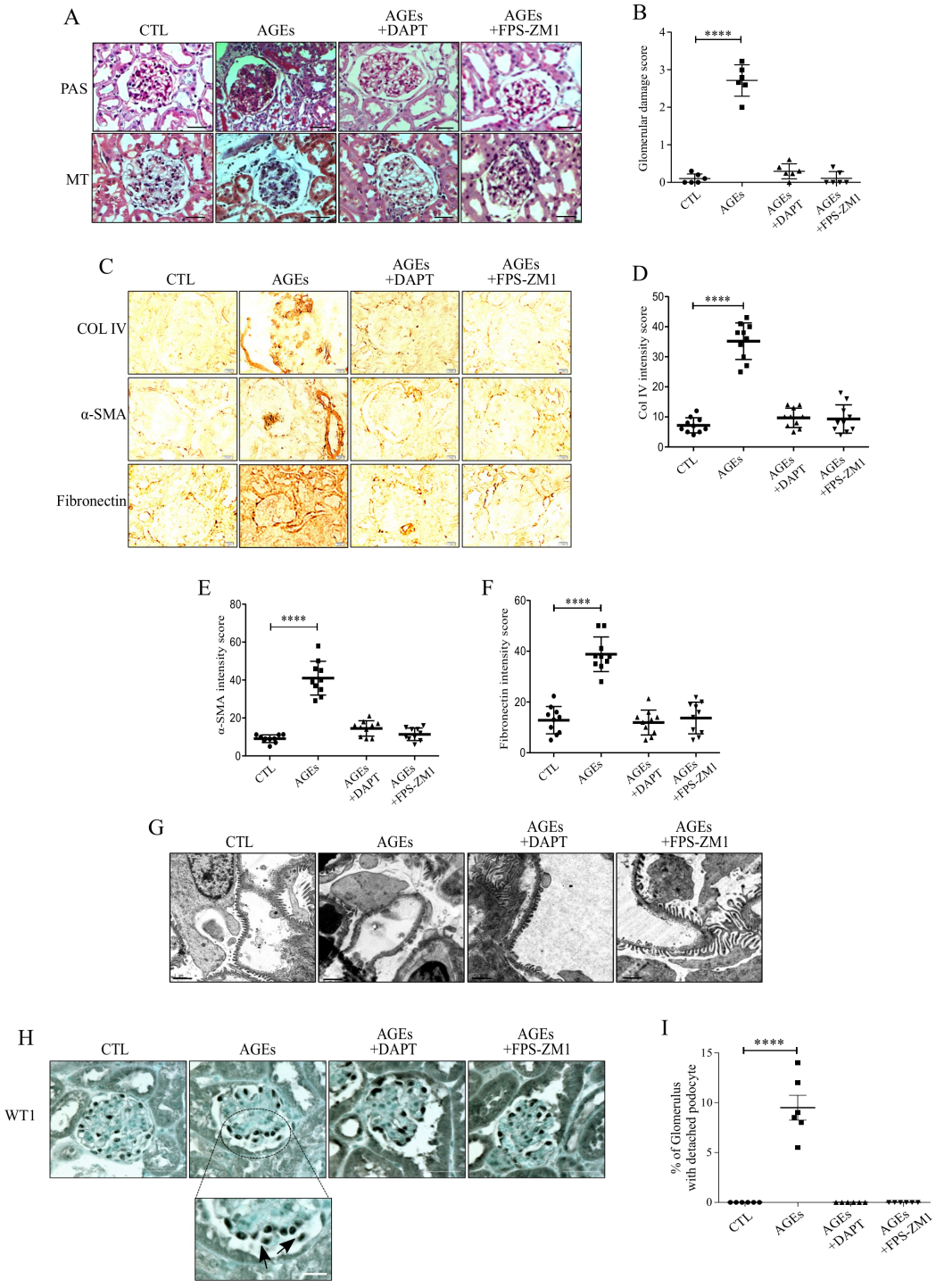


**Figure 4** AGE-induced Notch activation leads to fibrosis and podocyte foot process effacement. (A) Representative images of PAS and MT staining in mice glomeruli from with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treatment. Magnification ×400. Scale bar=50 µm. (B) The glomerular damage score was quantified as described in the 'Research design and methods' section. (C–F) Immunohistochemical staining for Col IV, α-SMA, and fibronectin in glomerular sections from mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). Magnification ×400. Scale bar=50 µm. The intensity of glomerular expression of Col IV, α-SMA, and fibronectin was quantified using ImageJ (NIH). ****P<0.0001. (G) Representative TEM images of podocytes from mice treated with or without (CTL), AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). Scale bar=1 µm. (H) Representative images of immunohistochemical staining for WT1 (podocytes) in the glomerulus from mice treated with or without (CTL) AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). Magnification ×630. The arrows indicate detached podocytes. Scale bar=20 µm. (I) The percentage of glomerulus with detached podocytes was quantified with the help of ImageJ (NIH). ****P<0.0001. Data are presented as mean±SD. AGEs, advanced glycation end-products; Col IV, collagen IV; CTL, Control; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester; FPS-ZM1, N-Benzyl-4-chloro-N-cyclohexylbenzamide; MT, Masson's trichrome; NIH, National Institutes of Health; PAS, periodic acid-Schiff; α-SMA, alpha-smooth muscle actin; TEM, transmission of electron microscopy.

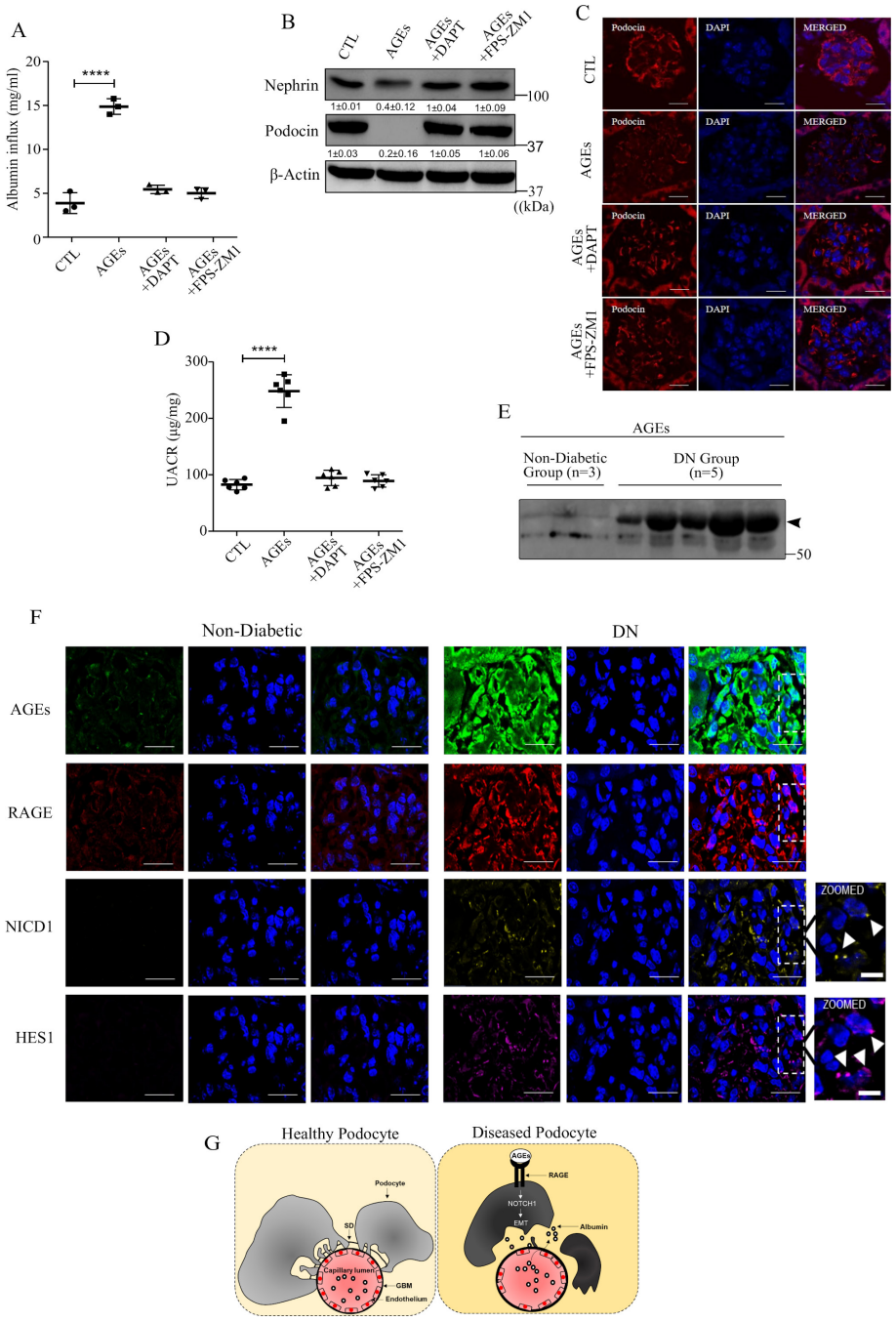


**Figure 5** Blockade of Notch and RAGE protects mice from proteinuria, and elevated levels of AGEs correlate with Notch activation in people with DN. (A) AGEs alter podocyte permeability in vitro. Albumin permeability across the podocyte monolayer was determined after 48 hours of exposure to AGEs (n=3). ****P<0.0001. (B) Immunoblotting study for podocin and nephrin expression in glomerular lysates and (C) by immunohistochemical staining for podocin in the glomerulus from mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). Magnification ×630. Scale bar=20 µm. The fold change values were presented with the expression of the respective genes after normalizing with β-actin. (D) UACR was estimated in mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). ****P<0.0001. (E) Immunoblotting analysis for AGEs in urine samples of DN (n=5) and non-diabetic group (n=3). The arrowhead indicates the positive staining for AGEs in the urine samples of a patient with DN. (F) Immunohistochemical staining of glomerular serial sections from patients with DN (n=16) and non-diabetic groups (n=10) for AGEs (DyLight488, green), RAGE (Cy3, red), NICD1 (Alexa Fluor 555), and HES1 (Cy5, far-red). Magnification ×630. Scale bar=20 µm. (G) A proposed model depicting the adverse effect of AGEs on podocytes. Activation of Notch signaling via AGE–RAGE interaction induces the thickening of GBM, EMT, and dehiscence of podocytes, which eventually result in impaired glomerular permselectivity and proteinuria. AGEs, advanced glycation end-products; CTL, control; DAPI, 4′,6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester; DN, diabetic nephropathy; EMT, epithelial to mesenchymal transition; FPS-ZM1, N-Benzyl-4-chloro-N-cyclohexylbenzamide; GBM, glomerular basement membrane; HES1, hairy and enhancer of split gene1; NICD1, Notch intracellular domain; RAGE, receptor for AGEs; SD, slit-diaphragm; UACR, urinary albumin to creatinine ratio.

online supplementary figure S3A,B). Together the data suggest that AGEs impair the podocyte function and induce proteinuria through RAGE and Notch1 signaling.

### Activated Notch signaling in subjects with DN

AGEs are elevated in plasma and accumulate in several tissues including the kidney in patients with diabetes. AGEs are considered a new therapeutic target in chronic kidney disease.[35] Elevated HbA1c and glycated albumin levels in subjects with DN were associated with decreased renal function as evidenced by increased UACR (online supplementary table S1) and increased urinary protein levels compared with control subjects (online supplementary figure S3C). Non-tryptophan AGE fluorescence also revealed the presence of AGEs in the serum from subjects with DN (online supplementary figure S3D). Indeed, AGEs were observed in urine samples from subjects diagnosed with DN (figure 5E). Notch signaling is inactive in the adult kidney, whereas activated Notch signaling is reported in glomerular diseases particularly in podocytes.[15] Therefore, to confirm our data that AGEs activate Notch1 signaling in humans, we analyzed glomerular NICD1 expression in people with DN. Immunohistochemical analysis of kidney from people with DN revealed increased expression of AGE, RAGE, NICD1, and HES1 compared with non-diabetics (figure 5). Moreover, Nephroseq analysis suggests that there is a strong correlation in the expression of RAGE, Notch1, Hes1, and EMT marker (vimentin) in non-diabetics versus DN mouse kidney data sets (online supplementary figure S3E). Together the data suggest that activated Notch signaling in subjects with DN is concomitant with EMT of glomerular podocytes.

## DISCUSSION

In the present study, we demonstrate that AGEs activate Notch1 signaling in podocytes that indeed elicit podocyte EMT and increase permeability to albumin. In vivo administration of AGEs resulted in podocyte EMT, glomerulosclerosis, GBM thickening, and podocyte foot process effacement (figure 5G). Glomerulosclerosis and albuminuria in AGE-treated mice suggest compromised renal physiology and function. RAGE inhibitor and γ-secretase inhibitor protected the mice from podocyte depletion and sclerosis. Importantly, podocyte depletion and proteinuria are common features of DN, whereas blocking of RAGE or γ-secretase successfully abrogated AGE-induced pathophysiological symptoms of DN. The data presented in our study demonstrate that AGEs that accumulate in DN activate Notch signaling in the podocytes.

Although Notch signaling is crucial during the development of glomerular podocytes, it is not requisite for differentiation of podocytes, beyond the stage of the S-shaped body formation.[11] Notch1 activity was unnoticeable in the healthy adult glomeruli; however, in patients with glomerular diseases, strong upregulation of Notch signaling was observed in podocytes.[10] Suppression of Notch signaling is considered to be a prerequisite for differentiation of renal progenitors into podocyte lineage, whereas persistent Notch activation results in a mitotic catastrophe.[11 36] Therefore, the significance of reactivation of Notch signaling in adult podocytes remains largely enigmatic. Podocyte-specific upregulation of Notch signaling is associated with progressive glomerulosclerosis, foot process effacement, and severe proteinuria.[37] Activation of Notch signaling elicited podocyte apoptosis.[15] In our recent study, we demonstrated that growth hormone (GH)-induced Notch activation resulted in podocyte foot process effacement and glomerular fibrosis, whereas inhibition of γ-secretase improved glomerular function. Similarly, in another study, it was shown that inhibition of Notch signaling in murine models improved proteinuria by reducing podocyte loss.[38] Podocyte-specific deletion of Notch1 has reduced podocyte injury and severity of proteinuria in DN murine models.[15]

Interestingly, we noticed activated Notch signaling preferentially in the glomeruli, particularly in the podocytes. Preferential localization of AGEs is observed in selective tissues particularly at the microvasculature, which includes the glomerulus, and the adverse consequences of AGEs were first described in the renal systems.[39 40] Glomerulus serves as a blood–urine barrier exposed to a large volume of serum AGEs and their reduced clearance in the diabetic milieu.[41] Indeed a progressive decline of kidney function had been demonstrated in subjects with diabetes.[42] Furthermore, elevated levels of glomerular RAGE are typically observed in experimental DN animals.[43] The exclusive podocyte distribution of RAGE and its upregulation in diabetic glomeruli possibly explain AGE-dependent Notch activation selectively in the glomerular region.[44] It is noteworthy that AGEs also stimulate the RAGE expression, and thereby enhanced AGE–RAGE interaction could also provoke adverse renal effect in diabetic conditions. A strong correlation was observed between renal AGEs and incidence of chronic kidney disease, and glomerular AGEs are considered a therapeutic target to combat complications of DN.[35]

Several master regulators that activate Notch activity in adult cells include transforming growth factor (TGF) and so on. AGEs stimulate TGF-β1 expression and NF-kB activation.[45] As Notch signaling is not warranted for the podocytes post embryonic development, and aberrant activation of Notch in podocytes is pathological, targeting podocyte Notch signaling may offer protection against diabetic renal complications. Accumulating evidence suggests that either γ-secretase inhibitor or antibodies against Notch ligands or receptors are effective in combating diabetic kidney disease.[46] It is noteworthy that people with DN showed elevated NICD1 expression in podocytes, and blocking the NICD1 expression with γ-secretase inhibitor DAPT prevented the mice from proteinuria. Taken together, we believe that the modulation of Notch signaling in DN may provide a therapeutic strategy to control the loss of kidney function.

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**Contributors** RN and AKP planned and designed the study. GBR generated an anti-AGE antibody. RN, PM, and AKS performed the experiments. RN, AKP, and GBR evaluated the data. RN, PM, and AKP wrote the manuscript. AKP is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** The study was approved by the Institutional Review Board of Guntur Medical College and Government General Hospital, Guntur, India (#GMC/IEC/120/2018) and adhered to the principles and the guidelines of the Helsinki Declaration. The animal experimental procedures were performed in adherence with the Institutional Animal Ethics Committee of the University of Hyderabad.

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