Deletion of the Receptor for Advanced Glycation Endproducts Reduces Glomerulosclerosis and Preserves Renal Function in the Diabetic OVE26 Mouse

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Running title: RAGE deletion preserves function in DN in OVE26

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Objective – Previous studies showed that genetic deletion or pharmacological blockade of the Receptor for Advanced Glycation Endproducts (RAGE) prevents the early structural changes in the glomerulus associated with diabetic nephropathy (DN). To overcome limitations of mouse models that lack the progressive glomerulosclerosis observed in humans, we studied the contribution of RAGE to DN in the OVE26 type 1 mouse, a model of progressive glomerulosclerosis and decline of renal function.

Research Design and Methods – We bred OVE26 mice with homozygous RAGE knock out (RKO) mice and examined structural changes associated with DN and used inulin clearance studies and albumin:creatinine measurements to assess renal function. Transcriptional changes in the Tgf-β1 and Plasminogen activator inhibitor 1 gene products were measured to investigate mechanisms underlying accumulation of mesangial matrix in OVE26 mice.

Results - Deletion of RAGE in OVE26 mice reduced nephromegaly, mesangial sclerosis, cast formation, glomerular basement membrane thickening, podocyte effacement, and albuminuria. The significant 29% reduction in glomerular filtration rate observed in OVE26 mice was completely prevented by deletion of RAGE. Increased transcription of the genes for Plasminogen activator inhibitor 1, Tgf-β1, Tgf-β induced, α1- (IV) collagen observed in OVE26 renal cortex was significantly reduced in OVE26 RKO kidney cortex. ROCK1 activity was significantly lower in OVE26 RKO compared to OVE26 kidney cortex.

Conclusions - These data provide compelling evidence for critical roles for RAGE in the pathogenesis of DN and suggest that strategies targeting RAGE in long-term diabetes may prevent loss of renal function.

The Receptor for Advanced Glycation Endproducts (RAGE), a member of the immunoglobulin superfamily, is upregulated in tissues subjected to the long-term impact of diabetes.(1; 2) The ligands of RAGE, including advanced glycation endproducts (AGEs), S100/calgranulins and High mobility group box-1 (HMGB1) display elevated expression in diabetic tissues.(1; 2) In diabetic nephropathy (DN), RAGE is upregulated in cells such as glomerular podocytes and endothelial cells in both humans and mice.(2; 3) Previous studies provided evidence for roles for RAGE and its ligands in mouse models of early DN. Overexpression of RAGE in vascular endothelial cells of hypo-insulinemic mice led to increased mesangial matrix expansion and glomerulosclerosis.(4) Pharmacological blockade of RAGE, using the soluble extracellular ligand binding domain of RAGE (sRAGE), in type 2 insulin-resistant db/db diabetic mice, protected against glomerulosclerosis and other classical lesions of early DN.(3) In addition, the kidneys of streptozotocin (STZ)-injected RAGE knockout (RKO) mice were protected from early mesangial matrix expansion and thickening of the glomerular basement membrane (GBM) seen in wild-type diabetic mice.(3) Myint and colleagues demonstrated protection from DN in RKO mice crossed with transgenic mice expressing iNOS under the control of the insulin promoter.(5) Others
have shown that blocking antibodies to RAGE suppressed DN in mouse models of type 1 and type 2 diabetes.(6; 7)

Until recently, very few mouse models of DN progress beyond the early disease stages of microalbuminuria and mild mesangial expansion. Transgenic over-expression of calmodulin specifically in pancreatic β cells, the OVE26 model of type 1 diabetes(8), resulted in nephromegaly, albuminuria, glomerulosclerosis, tubulointerstitial fibrosis, occasional occurrence of arteriolar hyalinosis, and the suggestion of decreased glomerular filtration rate (GFR).(9-11). Hence, the OVE26 mouse is considered one of the most human-relevant models of DN studied to date. We tested the hypothesis that RAGE contributes to advanced glomerulosclerosis and loss of renal function in long-term diabetes by crossing OVE26 mice with homozygous RAGE null mice to investigate the role of RAGE in this robust model of DN.

RESEARCH DESIGN AND METHODS
Additional information is also available in online supplemental information section at http://diabetes.diabetesjournals.org.

Mice: OVE26 mice (strain FVB(Cg)-Tg(Ins2-CALM1)26Ove Tg(Cryaa-TAg)1Ove/PneJ), and FVB controls (strain FVB/NJ) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house. FVB RKO mice, backcrossed >10 generations into FVB, were bred in house and crossed with OVE26 resulting in OVE26 RKO mice. Changes associated with DN were studied in male mice at 7 months based on our identification of this time point as one when moderate to severe histologic renal changes were observed in most male OVE26 mice. Mice were sacrificed at 1 month to test RAGE expression.

Polyclonal chicken anti-mouse RAGE IgY antibody production: Soluble (s)RAGE was produced as previously described.(1) Anti-RAGE Chicken IgY was generated and purified from serum and specificity for RAGE was confirmed.

Fluorescence Microscopy: Frozen kidney sections were obtained from 1 month old (for RAGE staining) and 7 months old mice (for Glyoxalase1 (Glo1) staining) and stained with polyclonal chicken anti-mouse RAGE IgY or Rat monoclonal [6F10] to Glo1 (Abcam, Cambridge, MA). Fluorescence microscopy was performed using a Lasersharp 2000 BioRad scanning confocal microscope (Bio-Rad, Hercules, CA).

Blood glucose and glycosylated hemoglobin measurements: Blood glucose measurements were made from non-fasted mice at 4-week intervals and prior to sacrifice at 7 months using a Freestyle Blood Glucose Meter (Alameda, CA). Levels above 499 mg/dl (“HI” reading), the maximum reading for the glucometer, were denoted as 500 mg/dl for calculation purposes. Blood Hgb A1c was measured using Cholestech GDX A1c test cartridges for human Hgb because the last 7 amino acids in the N terminus are identical between human and mice (Cholestech, Hayward, California).

Methylglyoxal measurement: Levels of methylglyoxal were determined in frozen kidney cortex tissue obtained from six female 7 month-old mice, as previously described.(12)

Glyoxalase 1 Western: 30μg of kidney cortex lysate was run on a 12% NuPAGE gel using MES running buffer (Invitrogen, Carlsbad, CA) under reducing conditions. After transfer to nitrocellulose membrane (Invitrogen, Carlsbad, CA), staining was performed using Rat monoclonal [6F10] to Glo1 (1:1000) (Abcam, Cambridge, MA) followed by chicken anti-rat (1:1000) (Santa Cruz Biotechnology, Santa Cruz, Ca) Blots were then stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and probed with anti-β actin antibody (1:2500) (Becton Dickinson, Franklin Lakes, NJ).
Bands were quantified using Alpha imager software (Alpha Innotech, San Leandro, CA).

**Morphometry:** Kidneys were harvested and bisected longitudinally. One half kidney was fixed in 10% formalin overnight then dehydrated, embedded in paraffin, sectioned at 3 μm, mounted on 3-aminopropyltriethoxy silane-coated glass slides (Sigma, St Louis, MO), stained with periodic acid Schiff (PAS) (Sigma) and analyzed by light microscopy.

**Pathology scoring:** Pathology was scored in a semi-quantitative manner by a renal pathologist (V D’A), blinded to the genotypes of the animals as described in the supplemental information.

**Glomerular basement membrane (GBM) and podocyte effacement measurements:** Glutaraldehyde fixed kidney cortex tissue was analyzed by electron microscopy. The thickness of the GBM of multiple capillaries was measured in 6 – 8 glomeruli per mouse (N=5 per group). A mean of 55 measurements was taken per mouse (from podocyte to endothelial cell membranes) at random sites where the GBM was displayed in best cross-section. The same glomeruli were scored for degree of podocyte effacement, defined as the percentage of total glomerular capillary surface area over which the podocyte foot processes were effaced.

**Urine collection and measurement of albuminuria:** Urine was collected over 24 hours from each mouse in individual mouse metabolic cages (Nalgene, Rochester, NY) and frozen at -80°C for subsequent analyses. Urinary albumin and creatinine levels were measured using the murine specific Albuwell ELISA kit and the Creatinine Companion kit (Exocell, Inc, Philadelphia, PA) according to manufacturer’s instructions.

**Measurement of glomerular filtration rate (GFR) and effective renal plasma flow (RPF):** Standard renal inulin clearance studies were performed during continuous hemodynamic monitoring as published,(13; 14) in male mice at 7 months of age. Details are provided in supplemental methods.

**Glomerular isolation:** Glomeruli were isolated by perfusing anesthetized mice with Dynabeads (Invitrogen, Carlsbad, CA), digesting the kidney tissue and performing a series of sieving steps. Glomeruli were picked under the microscope using a pipette and placed in RNAlater (Ambion, Austin, TX).

**RNA isolation, reverse transcription and real time:** RNA was isolated from ~20-30mg of kidney cortex tissue (for all real time with the exception of Glyoxalase 1) or ~1000 isolated glomeruli (for Glyoxalase 1 real time and for microarray sample preparation) per mouse using the RNAqueous kit (Ambion) and reverse transcription was performed with the Superscript III kit (Invitrogen) according to manufacturers’ instructions. Real time primers and probes for murine 18s, Glyoxalase1, Tgfbl, Tgfb1, Serpine1, and α1-(IV) collagen were purchased from PE Applied Biosystems (Foster City, CA). Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System with TaqMan PCR Master Mix (PE Applied Biosystems).

**ROCK1 activity assays:** Activation of ROCK1 was evaluated on lysates of kidneys isolated from OVE26 or OVE26 RKO mice, as previously described.(15) Details provided in supplemental information.

**Microarray experimental methods:** RNA was isolated from ~1000 isolated glomeruli per mouse using the RNAqueous kit (Ambion, Austin, TX). Total RNA concentration was assessed using a ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and quality was assessed on a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA) using the Agilent RNA 6000 Nano kit. The RNA was then amplified, biotin labeled and fragmented using the Gene Chip 3’IVT Express Kit (Affymetrix, Santa Clara, CA). 10 μg of fragmented RNA was hybridized to Mouse
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Genome 430. 2.0 GeneChips (Affymetrix). All samples were prepared with the two-cycle protocol recommended by the manufacturer. One array per mouse was used. 3 arrays (mice) for each genotype were used.

Microarray data analysis: Microarray data was analyzed as described in the supplemental information section. All microarray data has been deposited in Gene Expression Omnibus. The GEO accession number is GSE208444.

Statistical analysis: For the statistical analysis of all the data sets comparing 4 groups, a one-way ANOVA model followed by paired comparisons was performed. Bonferroni correction due to multiple comparisons was used to control the overall type one error rate. Log transformation was used for the urine albumin:creatinine analysis (Fig 5). For Fig 3d&e, the comparison was done only between OVE26 and OVE26 RKO groups because the values for other groups were 0. The severity of mesangial sclerosis data was compared using a Fisher’s exact test for contingency table (Fig 3e). All other data comparing 2 groups were analyzed using an independent two-sample t-test. Analyses were performed in SAS version 9.1 (SAS Institute Inc., Cary, NC), with the exception of the ANOVA and t-tests, which were performed using Stat View (Adept Scientific, Acton, MA).

RESULTS

RAGE is expressed in glomeruli of OVE26 mice. Previous studies reported that RAGE is expressed in human and murine glomeruli(2), but expression had not been studied in the OVE26 model. RAGE was detected in the glomeruli of kidney cortex from male OVE26 mice at 1 month of age and a baseline level of RAGE in the FVB glomerulus is provided for comparison (Fig 1a). This time point was chosen in order to determine whether RAGE expression was present in the OVE26 mouse prior to observable structural changes in the kidney, thus correlating changes in RAGE with the timing of early stages of DN development.

Precursor to RAGE ligands is reduced by RKO in OVE26 mice despite persistent hyperglycemia. OVE26 mice displayed significant increases in blood glucose levels compared to the non-diabetic FVB mice (498 ± 6 vs. 138 ± 36 mg/dl) (P<0.001) (Fig 1b). RKO did not alter the degree of hyperglycemia in OVE26 mice (494 ± 11 mg/dl) (P=0.7438). Blood glucose levels measured at 4-week intervals beginning at 8 weeks of age showed the same trends among groups as the measurements at 7 months (data not shown).

Hemoglobin (Hgb) A1c was significantly increased in OVE26 mice versus FVB controls (8.87 ± 0.33 vs. 5.35 ± 0.25 %) (P<0.0001) (Fig 1c), confirming sustained hyperglycemia (Fig 1b). Hgb A1c was normal in FVB mice, with or without RAGE (5.35 ± 0.25% vs. 6.03 ± 0.57%) (Fig 1c). Importantly, Hgb A1c was comparably elevated among OVE26 mice with or without RAGE (8.87 ± 0.33 vs. 8.95 ± 0.41 %) (Fig 1c), confirming that RKO did not impact glycemic control.

We studied the effect of RAGE deletion in the context of hyperglycemia on the levels of methylglyoxal (MG), a key precursor of AGEs. Levels of MG were 3.25 fold higher in OVE26 kidney cortex compared to FVB (P<0.05), but were unaffected by RKO in FVB mice. However, levels of MG were 5.93 fold lower in OVE26 RKO cortex compared to OVE26 cortex (P<0.05), despite equivalent degrees of hyperglycemia in both groups (Fig 1d).

Glyoxalase 1 levels are increased by RKO in OVE26 mice. To further probe the differences in MG levels among OVE26 and OVE26 RKO cortex, we assessed the levels of mRNA and protein for glyoxalase 1, an important regulator of MG levels in vivo. Our data revealed that both mRNA transcript
levels (P<0.05) (Fig 1e) and protein levels for glyoxalase 1 were higher in OVE26 RKO compared to OVE26 (P<0.05) (Fig 1f). Immunofluorescence staining localized glyoxalase 1 particularly to the glomeruli of OVE26 and OVE26 RKO mice (Fig 1g).

**OVE26 RKO Mice are Partially Protected from Glomerulosclerosis.** To establish the optimal time point for testing the role of RAGE in DN in the OVE26 model in mice raised in our facility, we monitored the pathological changes in the kidney at 5, 6 and 7 months of age. At 5 months of age, mesangial sclerosis was mild in most OVE26 mice. At 6 months of age, more OVE26 mice revealed moderate pathology. At 7 months of age, we consistently observed a range of moderate to severe mesangial sclerosis including the formation of mesangial nodules causing capillary luminal narrowing (Fig 2a), focal tubular atrophy, interstitial fibrosis and chronic inflammation (Fig 2b), and progression to segmental (Fig 2c) and global glomerulosclerosis (Fig 2d). Glomeruli of OVE26 mice appeared enlarged compared to FVB controls (Fig 3a,b), owing to diffuse and global mesangial sclerosis (Fig 3b, right panel). The cortical tubules contained focal proteinaceous casts (Fig 3b, left panel). Thus, to investigate the role of RAGE in advanced DN, we studied OVE26 mice crossed with FVB RKO mice at age 7 months. OVE26 RKO mice were partially protected from these pathological alterations including significantly fewer casts (Fig 3c,d), and reduced mesangial matrix expansion (Fig 3c,e) (P<0.05). Tubular atrophy that was present in OVE26 mice (Fig 2b) was completely prevented in OVE26 RKO mice (data not shown). The histology of FVB RKO mice did not differ significantly from that of FVB mice (data not shown).

At the ultrastructural level, the mesangial areas in OVE26 glomeruli were expanded by increased matrix and hyalinosis (Fig 2e), in some cases obliterating capillary lumens (Fig 2h). In OVE26 mice, the podocyte foot processes appeared effaced (Fig 2f-h & 4b) and there was significant GBM thickening (Fig 2f and 4e). In contrast, OVE26 RKO demonstrated less podocyte effacement compared to the OVE26 (Fig 4c,d) and no significant increase in GBM thickness compared to FVB mice (Fig 4c,e) (P<0.05). The glomeruli of FVB RKO mice were indistinguishable ultrastructurally from those of FVB mice (Fig 4d,e, image not shown).

**Albuminuria in OVE26 mice is markedly ameliorated by RKO.** Urinary albumin and creatinine were measured at 7 months of age. OVE26 mice displayed significantly increased albumin:creatinine ratios compared to FVB mice (822 ± 159 mg/mg vs. 46 ± 14 mg/mg) (P<0.001) (Fig 5). OVE26 RKO mice demonstrated significantly lower albumin:creatinine ratios (359 ± 152 mg/mg), reduced by ~56%, compared to OVE26 mice (Fig 5) (P=0.0085). FVB RKO albumin:creatinine ratios were not significantly different from FVB ratios (P=0.9793).

**Renal insufficiency is present in OVE26 mice but prevented by RKO.** (a) Metabolic, hematocrit (Hct), hemodynamic, and urine flow data in mice subjected to inulin clearance studies - Hct was comparable among the 4 groups, from the beginning to the end of the experiment (Table 1), indicating negligible blood loss. In response to body weight-based mild saline infusion (to prevent volume depletion, especially in diabetic mice), all groups experienced a small but similar decline in Hct (Table 1). Throughout the 3-hour experiment, Hct, averaged from 5 serial readings per mouse, was also comparable (Table 1), suggesting similar degrees of volume expansion and stability of blood volume. Mean systolic blood pressure (SBP) was comparable among the 4 groups (Table 1), specifically without hypotension in any mice. Mean urine flow rates were similar between the FVB and OVE26 mice, without
or with RKO (Table 1). Urine flow rate, however, was lower in the FVB RKO group vs. the OVE26 RKO group (P= 0.0073). Despite this decrease in flow rate, their absolute urine volumes (>75 µl per period) posed no technical problems for counting inulin or quantification by mass. Overall, these hemodynamic parameters suggested good stability of the surgical preparations during anesthesia. Body weight (BW) was comparable among the 4 groups (Table 1). Weights of both kidneys (KW) in OVE26 mice were markedly increased (0.82 ± 0.03 vs. 0.49 ± 0.02 g in FVB controls, P<0.0001) (Fig 6a), consistent with the well-known nephromegaly in diabetes. Furthermore, KW/BW was also markedly increased in OVE26 vs. FVB controls (2.82 ± 0.11 versus 1.54 ± 0.08 %, respectively; P<0.0001) (Fig 6b). Although nephromegaly was prominent in OVE26 diabetic mice, this was partially attenuated by RAGE deletion (0.71 ± 0.02 g, P <0.005, Δ=14% reduction) (Fig 6a). KW/BW of OVE26 mice was reduced by RKO (2.35 ± 0.05 versus 2.82 ± 0.11 %, p< 0.001, Δ=17 % reduction) (Fig 6b).

(b) Absence of impaired renal functional phenotypes in FVB RKO mice - While RKO imparted major structural (Figs 3 & 4) and functional effects in OVE26 mice (Fig 5 & 6, vide infra), we could not detect any significant alterations in BW, Hct, SBP, KW, or renal function in FVB RKO controls. More specifically, GFR was essentially unchanged by RKO in FVB mice whether expressed in units per mouse (Fig 6c), per 100 g BW (Fig 6d), or per g KW (Fig 6e).

(c) Renal Insufficiency in OVE26 versus non-diabetic controls - Compared to age- and sex-matched FVB controls, 7 month-old OVE26 showed a significant reduction in GFR (456 ± 18 vs. 325 ± 24 µl/min/mouse, P<0.0003, Δ=29% reduction, Fig 6c). The decline in GFR remained significant whether factored for BW (1106 vs. 1443 µl/min/100 g BW, P<0.003, Δ=24% reduction, Fig 6d) or factored for KW (401 vs. 940 µl/min/g KW, p<0.001, Δ=57% reduction, Fig 6e).

(d) Preservation of renal function in OVE26 mice by RKO - In OVE26 mice, RKO prevented loss of GFR, which otherwise developed in OVE26 mice (436 ± 37 vs. 325 ± 24 µl/min per mouse, P<0.005, Δ=34% increase, Fig 6c). Further, in OVE26 mice, RKO preserved GFR, as levels were comparable to those of FVB and FVB RKO mice (456 ± 18 and 404 ± 21 µl/min per mouse, respectively, Fig 6c). Likewise, reduction in renal function was prevented in OVE26 RKO mice versus OVE26 mice, whether GFR was factored by BW (1457 ± 134 vs. 1106 ± 78 µl/min/100 g BW, P<0.006, Δ=32% increase, Fig 6d) or factored by KW (620 ± 60 vs. 401 ± 35 µl/min/g KW, P<0.004, Δ=55% increase, Fig 6e). Significantly, in OVE26 RKO mice, GFR was preserved when factored for BW at levels similar to those of FVB controls, with or without RAGE (1457 ± 134 vs. 1443 ± 56 and 1287 ± 64 µl/min/100 g BW, Fig 6d). Although OVE26 RKO mice did not appear to have fully normalized GFR when factored by KW compared to levels of FVB controls (Fig 6e), this was primarily a mathematical aberration, and due to the marked nephromegaly (Fig 6a). Although RKO significantly reduced KW of diabetic mice, the attenuation was only 14 % (Fig 6a). Thus, RKO is reno-protective in OVE26 mice. The prevention of renal insufficiency conferred by RKO is independent of glycemic control and systemic hemodynamic alterations.

Increases in PAI-1, Tgfb1, Tgfb induced, and α1- (IV) collagen transcripts are found in OVE26 kidney cortex but are prevented by RAGE deletion. To explore the pathogenic mechanisms linked to DN in OVE26 mice, we identified significant changes in Serpine1 expression (the gene for Plasminogen activator inhibitor 1 (PAI-1)) through a microarray study comparing expression of genes in isolated glomeruli
from FVB and OVE26 mouse kidneys at 2 months of age. Serpine1 levels were significantly increased by 1.46 fold in OVE26 glomeruli (False discovery rate (FDR)=0.02) (Table S1, see supplemental information). To determine whether PAI-1 could be a downstream effector of RAGE in the diabetic kidney, we performed real time PCR on kidney cortex RNA from mice at 7 months of age and found that levels of PAI-1 mRNA were increased 4.3 fold in OVE26 compared to FVB kidney cortex (P<0.0001), whereas the levels in PAI-1 mRNA in OVE26 RKO were significantly lower than those in OVE26 (P<0.0005), and were not significantly different from FVB cortex (P=0.3723) (Fig 7a).

One of the central pathways that has been implicated in the pathogenesis of DN is the Tgf-β pathway. Previous studies illustrated that PAI-1 expression may be induced by Tgf-β, leading to inhibition of extracellular matrix degradation.(16-18) PAI-1 can also directly stimulate Tgf-β expression leading to increased synthesis of extracellular matrix.(17) We thus performed real time PCR on kidney cortex RNA from mice for Tgfb1 at 7 months of age and found that levels of Tgfb1 were increased 1.9 fold in OVE26 compared to FVB cortex (P<0.01), whereas levels in OVE26 RKO were much lower and not significantly different from FVB cortex (P=0.8904) (Fig 7B).

To further investigate the impact of RAGE on the Tgf-β1 pathway in the OVE26 and OVE26 RKO cortex, we performed real time PCR on TGFβ induced transcript as a measure of activity level and found significantly lower levels in OVE26 RKO compared to OVE26 cortex (P<0.0005) (Fig 7C).

In parallel, to explore the effects of RAGE on extracellular matrix deposition, we performed real time PCR for α1-(IV) collagen, one of the collagen IV species secreted by podocytes of the glomeruli,(19) and found significantly lower levels in OVE26 RKO compared to OVE26 cortex (P<0.0001) (Fig 7D).

The type I Tgf-β receptor may interact with a number of different molecules including RhoA which activates ROCK1.(20; 21) ROCK1 has recently been shown to be potentially important in DN(22; 23) and has been shown to be downstream of RAGE signaling in the diabetic vasculature. (15) We thus measured ROCK1 activity in OVE26 and OVE26 RKO kidney cortex and found that ROCK1 activity was significantly lower in OVE26 RKO compared to OVE26 cortex lysates, N=3 per group (P< 0.005) (Fig 7E).

**DISCUSSION**

In this study, we employed OVE26 mice to investigate the role of RAGE for the first time in a model displaying progressive and advanced features that closely resemble human DN, including loss of GFR.(10; 11; 24; 25) Several previous models used to study RAGE, such as STZ injection and db/db mice,(3) do not progress beyond microalbuminuria and mild mesangial expansion. Diabetic mice due to the iNOS transgene display advanced glomerulosclerosis, but no tubulointerstitial fibrosis or arteriolar hyalinosis, (4; 26) features that are present in OVE26 mice.(9) Here, we have illustrated that several of these advanced features of DN in the OVE26 model are impacted by deletion of RAGE, such as the occurrence of segmental and global glomerular sclerosis, nodule formation, tubular atrophy, podocyte effacement and thickening of the GBM. Importantly, the decline in GFR suggested previously in OVE26 mice,(9) and validated in our study, was completely prevented by RAGE deletion. In the previous study, however, although a 17% decrease in GFR in OVE26 vs. non-diabetic control mice was demonstrated, this occurred in the presence of hypotension, thus supporting that the decline in GFR in OVE26 mice was due to the structural pathology.
RAGE deletion preserves function in DN in OVE26

A recent study reported increased serum creatinine in OVE26 mice but paradoxically increased creatinine clearance compared to FVB mice at ages 3, 6 and 9 months.(11) This disparity underscores the limitations of using creatinine clearance to assess GFR, which could be inaccurate due to artifacts from urine collection or due to the use of non-HPLC methods for determination of serum creatinine in diabetic animals.(24; 26; 27) To overcome these limitations, we used inulin clearance, the gold standard for measuring GFR (28). Beyond confirming previous findings of segmental and global sclerosis, nodule formation, and tubular atrophy, the observed decline in GFR in OVE26 mice in the absence of hypotension in our studies suggests a causal relationship with the pathology associated with DN. This inference is supported by the degree of mesangial matrix expansion and the accompanying reduction in capillary lumen diameter, which would be expected to decrease ultrafiltration surface area and hence GFR. In addition, the focal tubular atrophy and interstitial fibrosis, which are known to correlate with declining renal function,(29) may have contributed to renal insufficiency. This close functional-pathologic correlation helps establish the utility and validity of OVE26 mice to study clinical DN, providing a basis to test the hypothesis of renoprotection by RKO.

Our results indicate that renal insufficiency in OVE26 mice was prevented by RKO, associated with significant ameliorations of pathology and albuminuria. Their nephromegaly was only minimally improved, possibly due to other upstream factors directly consequent to hyperglycemia. Over a shorter duration of STZ-induced diabetes, we previously found normalization of KW/BW by RKO.(3) Another study found persistently increased glomerular volume despite marked podocyte loss in advanced DN of OVE26 mice,(25) making it conceivable for the coexistence of nephromegaly and preserved glomerular function.

Despite limitations of previously used DN models, it has been reported that deletion or blockade of RAGE or inhibitors of AGE consistently led to partial preservation of renal structure and/or improved serum creatinine.(3; 5-7; 30-34) The diversity of these models provides further evidence for the role of RAGE, as sRAGE,(3) anti-RAGE antibodies,(6; 7) or frank RAGE deletion exert salutary effects on the increased albuminuria and histopathology in early stages of mouse DN. Previously, in 27 week-old db/db mice exhibiting low creatinine clearance, we found significant benefits of sRAGE administration,(3) corroborating the adverse impact of RAGE on raising serum creatinine(4) and the salutary action by RKO on reducing serum creatinine in a type 1 DN model.(5) Our present studies, however, provide the first direct evidence for functional preservation in diabetic mice by RKO, as assessed by GFR.

Methylglyoxal, a highly reactive α-oxoaldehyde, is formed in cells primarily from the triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. In diabetes, hyperglycemia triggers enhanced production of methylglyoxal, one consequence of which is the rapid modification of proteins and other substrates to generate AGEs, which may trigger signaling pathways leading to structural and functional changes of DN, at least in part via the actions of TGF-β and PAI-1. The finding that methylglyoxal is significantly lower in OVE26 RKO mice, which have fewer structural changes and less functional impairment than OVE26 mice, supports this hypothesis. Notably, blood glucose levels in the OVE26 RKO did not differ significantly from the OVE26 mice. This suggests that the difference in levels of methylglyoxal did not result from a reduction.
RAGE deletion preserves function in DN in OVE26

Glyoxalase 1, a defense against glycation in vivo, detoxifies reactive α-oxoaldehydes, thereby, removing deleterious species such as methylglyoxal. Our finding that glyoxalase 1 mRNA and protein levels are higher in OVE26 RKO mouse kidney cortex suggests that reduced activation of RAGE by AGEs may occur because of enhanced removal of methylglyoxal. The importance of the link between RAGE and glyoxalase 1 is that this may set up a positive feedback loop with relevant consequences. We have shown that by deleting RAGE, glyoxalase 1 mRNA and protein levels increase significantly, which decreases the tissue concentration of MG and suppressed related AGE formation. These data suggest that disruption of RAGE signaling may be a beneficial therapeutic target in prevention of progression of DN.

To begin to explore potential molecular mechanisms accounting for the reduction in mesangial sclerosis noted in OVE26 RKO mice, we assessed mRNA levels of PAI-1, Tgf-β1, Tgf-β induced, and α1- (IV) collagen. PAI-1 has been implicated previously in the pathogenesis of DN. In PAI-1 knock out mice treated with stz or crossed with type 2 diabetic db/db mice, albuminuria was improved and glomerular injury was reduced compared to the diabetic, PAI-1- expressing animals. A link between PAI-1 induction and RAGE has been previously demonstrated, but only in cell culture. Berrou et al. measured PAI-1 protein expression in cultured mesangial cells in response to both glycated albumin and carboxymethyl lysine (CML) AGE, two RAGE ligands identified through in vitro experiments. An anti-RAGE antibody partially blocked the AGE and CML induced PAI-1 expression in these cells. The significant reduction we find in Tgf-β1, Tgf-β induced and PAI-1 in OVE26 RKO mice suggests that deletion of RAGE may both reduce mesangial matrix accumulation and facilitate matrix degradation, both factors linked to reduced glomerulosclerosis. Reduced levels of α1-(IV) collagen expression in OVE26 RKO kidney cortex confirm these findings, as they are indicative of mesangial matrix accumulation.

Here we have shown that ROCK1 activity is significantly decreased in OVE26 RKO compared to OVE26 kidney cortex lysates. The importance of this finding is highlighted not only by known roles of ROCK1 in DN, the involvement of ROCK1 in Tgf-β and PAI-1 downstream signaling, but also our recent finding that activation of the ROCK1 branch of the Tgf-β pathway contributes to RAGE-dependent acceleration of atherosclerosis in diabetic ApoE null mice. The significant changes in ROCK1 activity in the kidney in the absence of RAGE suggest a novel mechanism by which RAGE may promote pathological change in DN.

In future studies, exploration of the relevance of the OVE26 model of DN to human DN could be explored by administering renoprotective agents that are effective in humans, such as ACE inhibitors, to RAGE-expressing and RKO OVE26 mice. AGE formation and the renin-angiotensin system have been shown to interact in the progression of renal disease. Wilkinson-Berka et al. have shown that blocking AGE formation can ameliorate angiotensin II-dependent renal injury. In addition, Thomas et al have shown that advanced glycation endproducts can activate ACE and other components of the intra-renal renin-angiotensin system. We have shown that increased levels of methylglyoxal are found in OVE26 kidney cortex and that the decreased pathology found in OVE26 RKO mice is accompanied by lower levels of methylglyoxal and increased glyoxalase 1 activity. We expect that treatment of OVE26 mice with ACE inhibitors could serve to prevent progression of renal disease in this model by potentially blocking ACE levels that may be elevated by
the presence of excess AGEs in the OVE26 mice.

In conclusion, our data underscore important roles for RAGE in the structural and functional deteriorations in advanced and progressive DN. Our findings support the notion that RAGE blockade may confer significant benefits by ameliorating severe renal histopathology and by preventing renal insufficiency in mice or patients at risk for DN.

**Author contributions:** Nina Reiniger: prepared the initial main draft of the manuscript subsequently distributed to all co-authors for editing and comments, substantial contribution to initial design of study, urine and tissue collection, blood glucose measurements, albumin:creatinine measurements, fluorescent staining imaging, glomerular isolation, RNA preparation, labeling and fragmentation of cDNA for microarray, real time PCRs, breeding and maintenance of mouse colony, some statistical analysis excluding microarray, coordination of mouse and mouse sample distribution to other coauthors. Kai Lau: GFR, Hct, BP and HgA1C measurements, contributed to manuscript, reviewed and edited manuscript. Daren McCalla: urine and tissue collection, blood glucose measurements, albumin: creatinine measurements. Bonnie Eby: GFR, Hct, BP and HgA1C measurements. Bin Cheng: statistical analyses, excluding the microarray. Yan Lu: sections of frozen kidney tissue, fluorescent staining and imaging. Wu Qu: generated the anti-RAGE antibody. Nosirudeen Quadri: methyl glyoxal measurements. Radha Ananthakrishnan: contributed to the development and validation of the assays in the laboratory for the measurement of methylglyoxal, performed methyl glyoxal measurements, reviewed and edited manuscript. Maryana Furmansky: tissue collection, glomerular isolation. Rosa Rosario: breeding, genotyping and maintenance of the mouse colony. Fei Song: breeding, genotyping and maintenance of the mouse colony. Vivek Rai: ROCK1 activity assay. Alan Weinberg: statistical analyses, excluding the microarray. Richard Friedman: data analysis on the microarray results, reviewed and edited manuscript. Ravichandran Ramasamy: contributed to initial design of study, arranged funding, supervised research, developed and validated in the laboratory the assays for measurement of methylglyoxal, reviewed all primary data on measurements of methylglyoxal and glyoxalase 1, contributed to manuscript, reviewed and edited manuscript. Vivette D’Agati: analysis of the PAS stained tissue sections and electron micrographs including scoring of mesangial sclerosis, cast formation, GBM thickness measurements, and podocyte effacement, contributed to initial design of study, contributed to manuscript, reviewed and edited manuscript. Ann Marie Schmidt: substantial contribution to initial design of study, arranged funding, supervised research, reviewed all primary data with Dr. Reiniger and determined the direction of the research, reviewed and edited the first draft of the manuscript prepared by Dr. Reiniger prior to distribution to all co-authors, reviewed and edited manuscript.

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REFERENCES

### Table 1: Metabolic, hemodynamic and urine flow data during inulin clearance studies

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<th>Groups</th>
<th>Genotypes</th>
<th>N</th>
<th>Body weight (g)</th>
<th>Hct initial (%)</th>
<th>Δ Hct (%)</th>
<th>Hct final (%)</th>
<th>Ave. of 5 Hct during clearance (%)</th>
<th>Ave. systolic BP (torr)</th>
<th>Ave. urine flow rate (μl/min)</th>
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*P < 0.01 vs. group II.
Figure Legends

Figure 1. (a) RAGE is expressed in OVE26 glomeruli: age 1 month. Original magnification: 1000x. (b) Blood glucose levels (*###P<0.0001) (N=12-15/group, except OVE26 RKO, N=6). (c) Hg A1c levels were measured at age 7 months on randomly selected N=4, 3, 9, and 6 mice used in the GFR studies in groups FVB, FVB RKO, OVE26 and OVE26 RKO, respectively (###P<0.0005, ##P<0.0001). (d) Levels of methylglyoxal (MG) and in the kidney cortex of FVB, FVB RKO, OVE26 and OVE26 RKO mice at age 7 months (*P<0.05). N=6 per group. (e) Real time PCR for Glyoxalase 1 was performed, normalized to 18s transcript levels and expressed as fold change compared to the OVE26 group (*P<0.05). N=6 per group. (f) Glyoxalase 1 protein levels measured by Western blot, normalized to Actin levels and expressed as fold change compared to the OVE26 group (*P<0.05)(N=3 per group). (g) Glyoxalase 1 staining in OVE26 and OVE26 RKO glomeruli: age 7 months. Original magnification: 1000x.

Figure 2. Histology and Ultrastructural Pathology in Diabetic OVE mice: age 7 months. By light microscopy, the glomeruli of 7-month-old male OVE26 mice display global mesangial sclerosis with nodularity (a), with progression in some glomeruli to segmental glomerulosclerosis (c) and global glomerulosclerosis (d). There is focal tubular atrophy, interstitial fibrosis and chronic inflammation (b). By electron microscopy, the mesangial areas are expanded by increased matrix and electron dense hyaline material, consistent with insudated plasma proteins (e). There is thickening of glomerular basement membranes with overlying effacement of foot processes (f). Some mesangial areas have marked mesangial sclerosis with a nodular aspect (g). In areas of severe mesangial sclerosis, the glomerular capillary lumina are narrowed and focally occluded by the mesangial encroachment (h, L= lumen) (Arrow indicates focal lumenal occlusion). Original magnifications are marked above each image.

Figure 3. Deletion of RAGE in OVE26 mice imparts partial protection from the structural abnormalities of DN at age 7 months. (a). No histologic abnormalities were detected in FVB RKO mice (not shown). By contrast, OVE26 mice display well developed features of diabetic nephropathy including diffuse and global mesangial sclerosis and focal hyaline casts (b). OVE26 RKO mice are markedly protected from the development of mesangial sclerosis and tubular cast formation (c). There were significant differences between OVE26 mice and OVE26 RKO mice with respect to % cortical area occupied by casts (d) and the severity of mesangial sclerosis (e), where 0=no mesangial sclerosis; 1=mild; 2=moderate; 3=severe. (*P<0.05). Original magnifications are marked above each image. Semi-quantitative scoring (d,e) was performed on N=7 OVE26 RKO and N=13 OVE26 mice.

Figure 4. Deletion of RAGE in OVE26 mice imparts partial protection from the ultrastructural abnormalities of DN at age 7 months. Electron microscopy was performed on 7 month old male kidney cortex samples. FVB mice display normal glomerular ultrastructural features with well-preserved foot processes and glomerular basement membranes of normal thickness (a). By comparison, OVE26 glomeruli display increased mesangial matrix forming focal nodules, thickened glomerular basement membranes and prominent foot process effacement (b). OVE26 RKO mice were partially protected from these changes, leading to less mesangial sclerosis and partial restoration of foot processes (FP) (c). Semi-quantitative scoring of podocyte effacement
RAGE deletion preserves function in DN in OVE26

(N=5) (d). Measurements of GBM thickness (N=5) (e). (***P<0.005, ###P<0.0001). Original magnifications are marked above each image.

Figure 5. Deletion of RAGE in OVE26 mice imparts partial protection from functional abnormalities of DN at age 7 months. Albumin:creatinine levels were measured in male FVB, FVB RKO, OVE26 and OVE26 RKO mouse urine retrieved from metabolic cages at 7 months of age. (***P<0.005, ###P<0.0001). N=7-13 per group.

Figure 6. Deletion of RAGE in OVE26 mice preserves GFR in OVE26 mice at age 7 months. Body weight and glomerular function: age 7 months. (a) Weights of both kidneys from 7-mo old male mice. (b) Ratios of kidney weight to body weight of 7-mo old mice. (c-e) Clearance of inulin (CIN) expressed as (c) CIN per mouse, (d) CIN per 100g body weight and (e) CIN per g kidney weight. (**P<0.01, ***P<0.005, ##P<0.0005, ###P<0.0001). N=6-10 per group, as indicated in Table 1.

Figure 7. PAI-1 (Serpine1), Tgfb1, Tgfb induced, α1-(IV) collagen mRNA transcripts and ROCK1 activity are lower in OVE26 RKO kidney cortex compared to OVE26 kidney cortex levels at age 7 months. Real time PCR for (a) PAI-1 (b) Tgfb1, (c) Tgfb induced, and (d) α1-(IV) collagen gene products was performed, normalized to 18s transcript levels and expressed as fold change compared to the FVB or OVE26 group (**P<0.01, ***P<0.005, ##P<0.0005, ###P<0.0001). N=6 per group. (e) ROCK1 activity was measured as the amount of phosphorylated MYPT1 compared to total ROCK1 levels by Western blot. Relative activity is expressed as fold change compared to the OVE26 group (***P<0.005)(N=3 per group).
RAGE deletion preserves function in DN in OVE26
RAGE deletion preserves function in DN in OVE26
RAGE deletion preserves function in DN in OVE26
Figure 5

![Bar graph showing Albumin/Creatinine (μg/mg) for FVB, FVB RKO, OVE26, and OVE26 RKO.](Image)

Figure 6

A. Bar graph showing Kidney weight (g) for FVB, FVB RKO, OVE26, and OVE26 RKO.

B. Bar graph showing Kidney/body weight (%) for FVB, FVB RKO, OVE26, and OVE26 RKO.

C. Bar graph showing Ccr (ml/min/1.73 body weight) for FVB, FVB RKO, OVE26, and OVE26 RKO.

D. Bar graph showing Ccr (ml/min/100g body weight) for FVB, FVB RKO, OVE26, and OVE26 RKO.

E. Bar graph showing Cr (μmol/kg kidney weight) for FVB, FVB RKO, OVE26, and OVE26 RKO.
Figure 7

A

B

C

D

E

RAGE deletion preserves function in DN in OVE26