

Interactions between Renin Angiotensin System and Advanced Glycation in the Kidney

Merlin C. Thomas, Christos Tikellis, Wendy M. Burns, Katarzyna Bialkowski, Zemin Cao, Melinda T. Coughlan, Karen Jandeleit-Dahm, Mark E. Cooper, and Josephine M. Forbes
Baker Medical Research Institute, Melbourne, Victoria, Australia

Although hemodynamic and metabolic factors are individually implicated in the development of diabetic nephropathy, their interaction has not been defined clearly. In this study, the effects of angiotensin II (Ang II) and advanced glycation end products (AGE) both individually on each other are explored and compared. In the first study arm, Sprague-Dawley rats received a continuous infusion of AGE-modified rat serum albumin (RSA) or unmodified RSA for 4 wk with or without the angiotensin receptor type 1 antagonist valsartan. In the second arm, animals received a continuous infusion of Ang II (58.3 ng/kg per min) with or without the AGE inhibitor pyridoxamine. Components of the intrarenal renin-angiotensin system were measured using real time reverse transcription-PCR, immunohistochemistry, and standard angiotensin-converting enzyme (ACE) activity assays. Renal and serum AGE were quantified by immunohistochemistry, ELISA, and AGE-fluorescence. After an infusion of AGE-RSA, renal expression of angiotensinogen, ACE, renin, and angiotensin receptor type 1 were increased significantly (all $P < 0.01$), and ACE activity was elevated. This was associated with tubular and glomerular hypertrophy and AGE accumulation, which could be antagonized by valsartan. However, valsartan had no effect on increased filtration fraction associated with an AGE-RSA infusion. At the same time, an infusion of Ang II increased the serum and renal accumulation of AGE and advanced oxidation protein products and induced renal hypertrophy and salt retention that could be antagonized by pyridoxamine. However, pyridoxamine had no effect on renal vasoconstriction manifested by reduced renal blood flow. AGE and Ang II have overlapping activities in the kidney. The beneficial effects of blockade of either pathway underline the importance of this interaction in diabetic renal disease and the aging kidney.

J Am Soc Nephrol 16: 2976–2984, 2005. doi: 10.1681/ASN.2005010013

Several metabolic and hemodynamic factors contribute to the development and progression of diabetic nephropathy (1). Activation of the intrarenal renin angiotensin system (iRAS) and the subsequent generation of angiotensin II (Ang II) seem to be among the most important mediators of hemodynamic changes in diabetic nephropathy (2). Advanced glycation end products (AGE), formed after prolonged hyperglycemia and oxidative (carbonyl) stress, also have a wide range of chemical, cellular, and tissue effects implicated in diabetic nephropathy (3). However, neither metabolic nor hemodynamic stimuli act in isolation (1). Although hyperglycemia is fundamental for the initiation of renal injury in diabetes, altered renal hemodynamics including glomerular hypertension may also be required for the development of kidney damage (4). In addition, recent studies demonstrate that Ang II-dependent renal injury in the mRen-2 rat after the induction of diabetes can be ameliorated after inhibition of AGE formation (5). Furthermore, it has been demonstrated that blockade of the iRAS significantly attenuates the formation and accumulation of renal AGE in experimental diabetes (6,7). This study charac-

terizes the interactions between AGE and the iRAS that occur in the kidney *in vivo* and demonstrates important effects of each stimulus on the other pathway of direct relevance to the pathogenesis of diabetic nephropathy and age-related reductions in renal function.

Materials and Methods

Animals

Male Sprague-Dawley rats (Biologic Resource Laboratory, Perth, Australia) that were aged between 8 and 9 wk and weighed between 200 and 250 g were used in this study. The protocols for animal experimentation and the handling of animals were in accordance with the principles established by the Animal Welfare Committee of the Baker Heart Research Institute.

AGE Infusion Model

Previous studies have demonstrated that repeated infusion of AGE-modified albumin results in albuminuria and glomerular changes similar to that observed in diabetic nephropathy (8). For examining the early changes in this model, either rat serum albumin (RSA; $n = 10$) or AGE-modified RSA ($n = 10$) was infused into 20 male Sprague-Dawley rats at a dose of 50 mg/kg per d for 28 d using Alzet osmotic minipumps (Model 2002; Alzet Corp., Cupertino, CA) implanted subcutaneously in the midscapular region. Animals then were randomized further to receive an angiotensin receptor type 1 (AT₁) antagonist (valsartan 30 mg/kg by gavage) in a dose previously demonstrated to inhibit the iRAS and attenuate renal injury in diabetic nephropathy (2).

AGE-RSA was prepared by incubation of RSA (Sigma Chemical Co.,

Received January 5, 2005. Accepted July 7, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Assoc. Prof. Merlin C. Thomas, Baker Medical Research Institute, P.O. Box 6492, Melbourne, Victoria 8008, Australia. Phone: +61-3-8532-1280; Fax: +61-3-8532-1480; E-mail: mthomas@baker.edu.au

St. Louis, MO) with 0.5 M glucose in phosphate buffer, as described previously (9). RSA was handled in the same way without the inclusion of glucose. After incubation for 90 d at 37°C, samples were dialyzed thoroughly against PBS for 48 h. AGE-RSA and RSA then were passed over an Affiblu column (Bio-Rad Laboratories Inc., Hercules, CA), a heparin Sepharose column (Pharmacia, Uppsala, Sweden), and an endotoxin column (Detoxigel; Pierce, Rockford, IL) to remove possible contaminants. Samples finally were filtered through a 200- μ m filter to remove aggregates and placed into osmotic minipumps in a sterile fume hood. The resulting AGE-RSA contained 8 μ mol of carboxymethyl-lysine (CML) per mmol of lysine as assessed by GC-MS (Suzanne Thorpe, University of South Carolina, personal communication, April 5, 2004).

Ang II Infusion Model

Chronic infusion of Ang II into normal rats results in tubulointerstitial injury associated with moderate systolic hypertension (10). In a second protocol, either human Ang II (Auspep, Parkville, VIC, Australia) at a dose of 58.3 ng/kg per min ($n = 10$) or vehicle (0.15 mol/L sodium chloride and 1 mmol/L acetic acid, $n = 10$) was infused into 20 male Sprague-Dawley rats for 14 d using an osmotic mini-pump (Alzet Corp.) implanted subcutaneously in the midscapular region as described above. Animals were randomized further to receive the AGE inhibitor pyridoxamine (1 g/L in drinking water) in a dose previously shown to inhibit the development of diabetic nephropathy in experimental animals (11).

Measurement of Physiologic and Biochemical Parameters

The following parameters were measured serially in all groups: Body weight; blood glucose, measured using a glucometer (Accutrend; Boehringer Mannheim GmbH Biochemica, Mannheim, Germany); systolic BP, measured by tail-cuff plethysmography in conscious, warmed rats (12); and glycated hemoglobin, measured by HPLC (CLC330 GHb Analyzer; Primus, Kansas City, MO) (13). Urine was collected from animals placed in individual metabolic cages (Iffa Credo, L'Arbresle, France) for 24-h measurement of sodium excretion by flame photometry and albumin excretion rate by RIA (14). GFR was measured by a single-injection isotopic technique (^{99}Tc -diethylenetriaminepenta-acetic acid) developed in our laboratory and expressed as milliliters per minute corrected for body surface area (15).

Quantification and Localization of AGE

The level of circulating AGE in circulating plasma proteins was estimated using an ELISA with a monoclonal AGE antibody that recognizes the nonfluorescent AGE, CML at its primary epitope (16). Incomplete digestion of AGE-modified proteins (*e.g.*, those infused in this experiment) results in the production of low molecular weight fluorophores (LMWF) that accumulate in the serum (17) and correlate with the level of tissue AGE-modification in diabetes (6,7). LMWF present in plasma samples were assayed using on-line spectrofluorometric detection in a flow system as described previously (6,17). Results were normalized to the values for AGE-modified albumin hydrolyzed with proteinase K.

AGE-fluorescence (370/440 nm) was determined in enzymatically hydrolyzed renal tissue, using the same flow injection assay as described above. Results were expressed normalized for the protein content of the supernatant determined before hydrolysis using the BCA assay (BioRad). Neither fluorescence assay was performed in pyridoxamine-treated animals because of interference from the intrinsic fluorescence of the pyridoxamine. Localization of renal AGE was deter-

mined by immunohistochemistry using a polyclonal AGE antibody as described previously (18).

Activity of *i*RAS

Renal gene expression of mRNA for component mediators of the RAS (angiotensin-converting enzyme [ACE], ACE-2, angiotensinogen, renin, AT₁, and AT₂) was assessed by real-time quantitative reverse transcription-PCR. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer Inc., PE Biosystems, Foster City, CA) as previously used by our group (6,7,9,10).

Renal and serum ACE activity was determined after incubation with the synthetic ACE-specific substrate hippuryl histidylleucine, as adapted from Freidland *et al.* (19). The presence of activated ACE was assessed in the AGE infusion model using *in vitro* quantitative autoradiography (20). Renal expression of AT₁ and AT₂ proteins was quantified by Western blotting using specific polyclonal antibodies (both 1:250; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Intrarenal localization of ACE, ACE-2, AT₁, and AT₂ were characterized further by immunohistochemistry in Bouin's fixed tissue. Staining for ACE was performed using a polyclonal anti-ACE antibody (1:500; Chemicon, Temecula, CA) (21). Staining for ACE-2 was performed using a polyclonal ACE-2 antibody donated by Millennium Pharmaceuticals (1:200; Cambridge, Boston, MA) (22). Staining for AT₁ and AT₂ was performed using specific polyclonal antibodies (both 1:250; Santa Cruz Biotechnology Inc.).

Estimation of Renal Plasma Flow

Effective renal plasma flow (ERPF) was estimated from the clearance of endogenous hippuric acid clearance in conscious animals as described by Baunack *et al.* (23). Serum samples were taken in the morning, at the time of constant low hippurate excretion. Urine samples were collected in metabolic cages as detailed above and analyzed in a 1:50 dilution. Hippuric acid was measured in serum and urine samples using liquid chromatography after Kubota *et al.* (24). ERPF was calculated as the urinary volume multiplied by the area under the urine hippurate curve divided by that of plasma hippurate and expressed as ml/min per m² body surface area. Interassay variability was 2% and, interday variability was 5%.

Morphometry and Markers of Renal Hypertrophy and Injury

The volume of glomeruli in the outer cortex of each kidney was determined in periodic acid-Schiff sections, using the point-counting method described by Weibel and Gomez (25). The presence of early tubular hypertrophy was estimated by the measurement of mean least tubular diameter as described by Okada and Takahashi (26).

Ang II-mediated hypertrophy is thought to occur in the G₁-phase of the cell cycle and depends on the induction of the cyclin-dependent kinase inhibitor protein p27^{kip1} *via* stimulated production of oxygen radicals (27). Expression of p27^{kip1} was estimated by Western blotting in whole kidney samples using cortical preparations. Tubular expression of the proliferating cell nuclear antigen (PCNA) was estimated using immunohistochemistry.

The renal expression of early markers of tubular injury in diabetes, vimentin and α -smooth muscle actin (α -SMA), were determined by Western blotting. Cortical homogenates were obtained as above and probed using antibodies directed against vimentin (monoclonal, mouse anti-human [L-6 ascites fluid]; 1:500 dilution in 1% milk in TBST; Sigma) and α -SMA (monoclonal, mouse anti-human [clone 1A4], 1:1000 dilution in 0.5% milk in TBST; Dako, Carpinteria, CA). The level of

expression was determined by analysis of staining using the Optimax image system. Results are expressed relative to control animals, which were given the arbitrary value of 1. Constitutive expression of β -tubulin is shown as loading control.

Induction of oxidative stress is a key component to Ang II- and AGE-mediated cellular injury (3,6). As a marker of oxidative stress, advanced oxidation protein products (AOPP) were measured by spectrophotometry in renal homogenates as described by Witko-Sarsat *et al.* (28). Data are expressed calibrated with results obtained from chloramine-T standard.

Statistical Analyses

Continuous data are expressed as mean \pm SEM except where otherwise specified. Differences in continuous variables were compared using *t* test (two groups) or one-way ANOVA (three or more groups). Spearman rank order correlation was used to analyze associations between continuous variables. Differences in categorical variables were compared using the Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant.

Results

Physiologic Parameters

AGE Infusion. AGE infusion did not significantly influence glycemic control or systolic BP levels over the study period (Table 1). Animals that were treated with AGE-RSA had slightly greater weight gain during the 4-wk study period ($36 \pm 1\%$) compared with RSA control animals ($32 \pm 1\%$) and animals that were treated with valsartan ($31 \pm 2\%$, respectively, both *versus* AGE-RSA; $P < 0.01$). Neither urine output nor water or food intake was significantly different among the various treatment groups (data not shown). However, urinary sodium excretion was significantly reduced in animals that received infusions of AGE-RSA compared with animals that received RSA alone (Table 2). Furthermore, treatment with valsartan increased the urinary salt excretion in animals that received AGE-RSA (*versus* AGE-RSA, $P < 0.01$).

Ang II Infusion. An infusion of Ang II resulted in systemic hypertension and an increase in albuminuria ($P < 0.01$) but had no significant effect on glycemic control as measured by HbA_{1c} or fasting plasma glucose levels (Table 1). Pyridoxamine had no significant effect on either systolic BP levels or albuminuria in

this model. However, mean urinary salt excretion was reduced in animals that were treated with pyridoxamine (*versus* Ang II, $P = 0.05$).

Renal Hemodynamics. The mean GFR was lower in animals after a 4-wk AGE-RSA infusion compared with those that received RSA alone. In addition, ERPF was reduced and filtration fraction was elevated in animals after an AGE infusion (Table 2). Treatment with valsartan normalized the GFR but not the ERPF in AGE-RSA-treated animals. A 2-wk Ang II infusion had no significant effect on GFR, although ERPF was significantly reduced. This was not significantly modified by pyridoxamine.

Estimation of AGE Exposure

An infusion of AGE-RSA resulted in a significant increase in circulating LMWF levels compared with animals that received RSA alone (Figure 1A). Although the AGE-modified RSA contained a significant number of CML modifications, there was no significant difference in the level of CML-AGE in circulating plasma proteins by ELISA. Nonetheless, renal immunostaining with a polyclonal anti-CML-AGE antibody was increased in animals after an infusion of AGE-RSA (Figure 2). In addition, tissue AGE fluorescence was significantly increased in these animals (Figure 1B), correlating with serum LMWF levels ($R = 0.54$, $P < 0.01$). Treatment with valsartan reduced both tissue AGE and serum LMWF levels in animals that were treated with AGE-RSA.

Ang II infusion also produced an elevation in levels of circulating LMWF, to levels similar to that achieved with an infusion of AGE-RSA (Figure 1A). Similar changes were seen with respect to renal AGE fluorescence (Figure 1B) and on immunohistochemistry in response to Ang II infusion (Figure 2). Again, there was a good correlation between renal AGE fluorescence and serum LMWF ($R = 0.42$, $P < 0.01$). Owing to the intrinsic fluorescence of pyridoxamine, AGE fluorescence could not be assessed accurately in the serum of pyridoxamine-treated animals. However, consistent with its known effects as an AGE inhibitor, pyridoxamine was asso-

Table 1. Biophysical parameters of the two study models^a

	Glucose (mmol/L)	HbA _{1c} (%)	Systolic BP (mmHg)	Weight (g)
AGE infusion				
sham	6.1 \pm 0.2	3.1 \pm 0.2	122 \pm 5	439 \pm 6
RSA	6.2 \pm 0.3	3.1 \pm 0.2	120 \pm 2	436 \pm 7
AGE-RSA	6.8 \pm 0.3	3.0 \pm 0.2	119 \pm 3	452 \pm 9 ^b
AGE-RSA + valsartan	6.9 \pm 0.3	3.0 \pm 0.2	110 \pm 4 ^{b,c}	426 \pm 10 ^c
Ang II infusion				
vehicle	6.4 \pm 0.1	3.0 \pm 0.2	125 \pm 5	318 \pm 11
Ang II	6.1 \pm 0.3	3.1 \pm 0.2	175 \pm 7 ^b	302 \pm 9
Ang II + pyridoxamine	6.1 \pm 0.3	3.1 \pm 0.2	165 \pm 9 ^b	310 \pm 6

^aData are shown as mean \pm SEM. AGE, advanced glycation end products; RSA, rat serum albumin; Ang II, angiotensin II. $P < 0.05$ ^b*versus* model control, ^c*versus* AGE-RSA.

Table 2. Renal parameters of the two study models^a

	Renal Mass (g/m ²)	Glomerular Volume (mm ³ × 10 ⁶)	Proximal Tubular Diameter (μm)	GFR (ml/min per m ²)	ERPF (ml/min per m ²)	Na Excretion (mmol/d)	AER (mg/d)
4-wk AGE infusion							
sham	47.4 ± 0.6	0.8 ± 0.1	46 ± 1	62 ± 3	242 ± 23	1.2 ± 0.1	0.5 ± 0.1
RSA	47.6 ± 0.6	0.9 ± 0.1	47 ± 1	60 ± 2	236 ± 22	1.1 ± 0.1	0.5 ± 0.1
AGE-RSA	49.8 ± 0.9 ^b	1.2 ± 0.1 ^b	52 ± 1 ^b	55 ± 2 ^b	158 ± 13 ^b	0.8 ± 0.1 ^b	0.6 ± 0.1
AGE-RSA + valsartan	47.0 ± 0.8 ^c	0.9 ± 0.1 ^c	48 ± 1 ^c	60 ± 1 ^d	160 ± 9 ^b	1.7 ± 0.1 ^{b,c}	0.5 ± 0.1
2-wk Ang II infusion							
vehicle	48.4 ± 1.0	0.8 ± 0.1	46 ± 1	75 ± 2	255 ± 9	1.3 ± 0.1	0.3 ± 0.1
Ang II	55.5 ± 1.7 ^b	0.9 ± 0.1	55 ± 2 ^b	77 ± 2	182 ± 9 ^b	2.1 ± 0.2	2.6 ± 0.6 ^b
Ang II + pyridoxamine	46.8 ± 0.8 ^{b,d}	0.9 ± 0.1	48 ± 1 ^c	75 ± 2	171 ± 13 ^b	1.4 ± 0.2 ^{b,d}	2.8 ± 0.5 ^b

^aData are shown as mean ± SEM. ERPF, effective renal plasma flow; AER, albumin excretion rate.

^b*P* < 0.05 versus model control, ^cversus AGE-RSA, ^dversus Ang II.

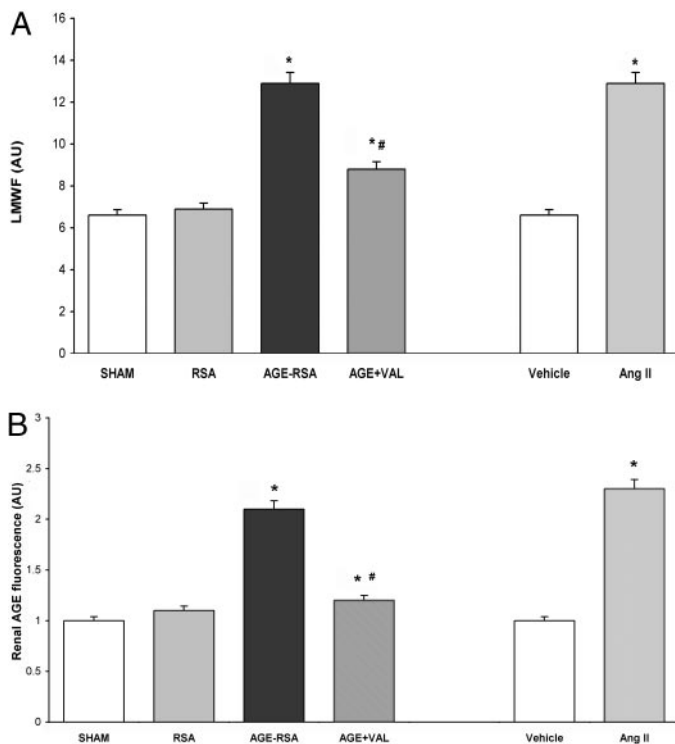


Figure 1. (A) Serum low molecular weight fluorescence (LMWF) and (B) renal advanced glycation end product (AGE)-fluorescence in the two models. Mean ± SEM; **P* < 0.01 versus vehicle; #*P* < 0.01 versus AGE-rat serum albumin (RSA).

ciated with a reduction in AGE immunostaining in Ang II-infused animals (Figure 2).

Activation of iRAS

Intrarenal expression of components of the RAS were significantly modified after infusion of AGE-RSA. Notably, the expression of ACE-2 and AT₂ were reduced in animals that received an infusion of AGE-RSA compared with RSA alone, whereas the renal expression of ACE, angiotensinogen, renin,

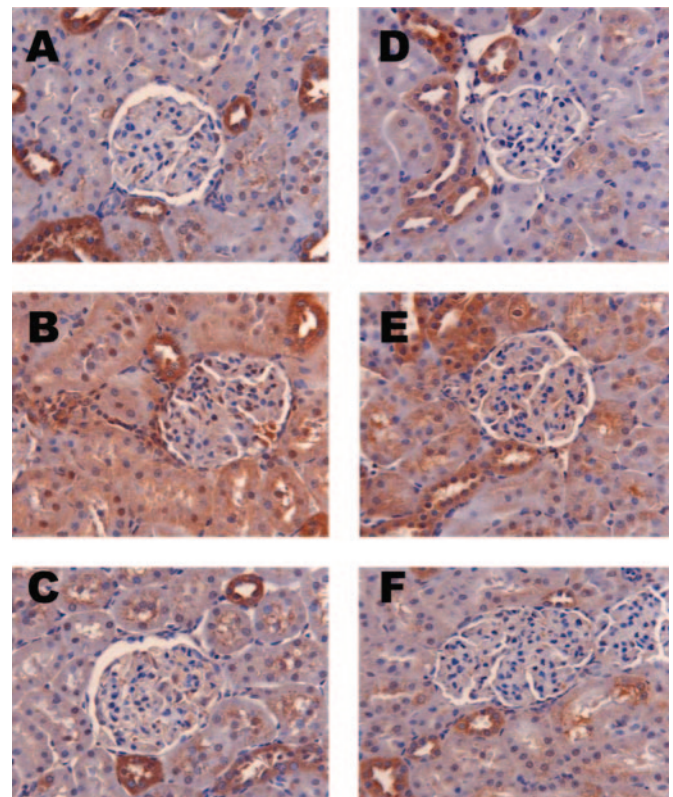


Figure 2. (A) Immunohistochemical staining for AGE in the kidney in animals that received infusions of RSA (A), AGE-RSA (B), AGE-RSA + valsartan (C), vehicle alone (D), angiotensin II (Ang II; E), and Ang II + pyridoxamine (F).

and AT₁ were elevated in animals after an infusion of AGE-RSA, compared with animals that received RSA alone or sham (Figures 3 and 4). This was associated at the tissue level with an increase in renal ACE binding sites and a concomitant increase in ACE activity (Figure 5). By contrast, serum ACE activity was reduced in animals after an infusion of AGE-RSA compared with animals that received RSA or sham infusions (both *P* < 0.01).

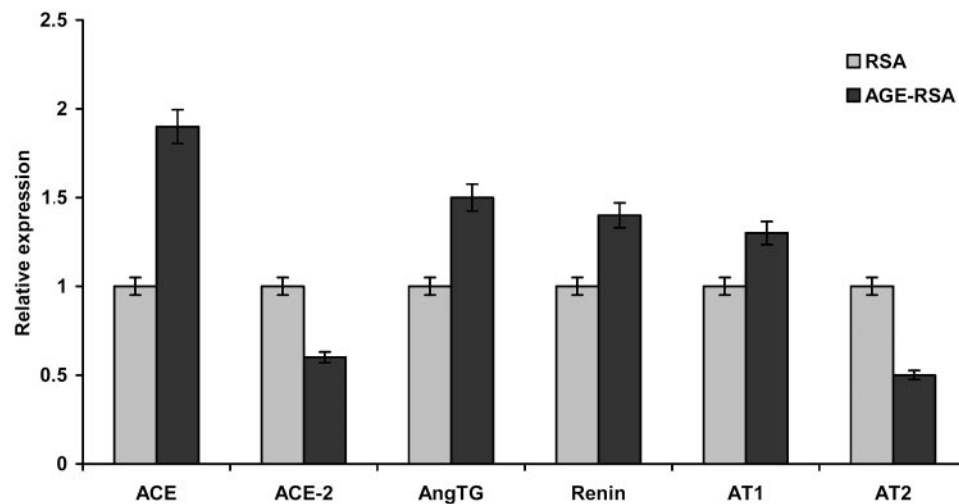


Figure 3. Renal gene expression of components of the intrarenal renin-angiotensin system (iRAS) as measured by real-time reverse transcription-PCR. Mean \pm SEM; * $P < 0.05$ versus RSA.

An infusion of Ang II was associated with increased renal expression of ACE mRNA (1.9 ± 0.5 AU) when compared with animals that received vehicle alone (1.0 ± 0.2 AU). Treatment with pyridoxamine abrogated these changes (0.8 ± 0.1 AU). Ang II infusion has no significant effect on serum ACE activity (data not shown).

Renal Hypertrophy

Body weight-adjusted renal mass was increased after a 4-wk infusion of AGE-RSA compared with animals that received RSA alone or sham controls. In addition, there was a significant increase in both glomerular volume and mean tubular diameter seen in animals that received AGE-RSA compared with animals that received RSA alone or sham controls (Table 2). Tubular hypertrophy was associated with increased immunostaining for the proliferative marker PCNA in the proximal tubule in AGE-RSA-infused animals (AGE-RSA 0.68 ± 0.04 versus RSA 0.38 ± 0.05 AU; $P < 0.05$). The increase in glomerular volume, tubular mass, and proliferation response to AGE-RSA was abolished with valsartan treatment (tubular PCNA in AGE-RSA + Val 0.41 ± 0.06 versus AGE-RSA; $P < 0.01$).

Body weight-adjusted renal mass was also increased after an infusion of Ang II (Table 2). This was attributable to an increase in tubular mass. As previously reported in this model, there was no significant change in glomerular volume. An infusion of Ang II also increased PCNA staining in the cortical tubules, compared with animals that received vehicle alone (Ang II 0.56 ± 0.09 versus vehicle 0.33 ± 0.04 ; $P < 0.01$). Notably, this proliferative response was attenuated by pyridoxamine (Ang II + pyridoxamine 0.38 ± 0.06 versus Ang II; $P < 0.05$). However, the increase in tubular mass was unaffected by pyridoxamine. Neither pyridoxamine nor valsartan had significant effects on renal mass in the absence of Ang II or AGE-RSA infusion (data not shown).

The expression of p27^{kip1} was increased significantly after an infusion of either Ang II or AGE-RSA (Figure 6). The effect of AGE-RSA on p27^{kip1} was prevented after blockade of AT₁ with

valsartan. Treatment with pyridoxamine attenuated the increase in p27^{kip1} in Ang II-treated animals, although it did not completely normalize this parameter.

Markers and Mediators of Tubular Injury

After an infusion of AGE-RSA, protein expression of markers of tubular injury (vimentin and α -SMA) were increased compared with animals that had received RSA alone. Treatment with valsartan partly attenuated these changes (Figure 6). Equally, a 2-wk infusion of Ang II increased the cortical expression of α -SMA and vimentin. Treatment with pyridoxamine reduced the expression of α -SMA and vimentin (Figure 6).

Serum and renal AOPP were significantly increased after an infusion of AGE-RSA (Figure 7). Treatment with valsartan partly attenuated these changes. Similarly, a 2-wk infusion of Ang II increased the renal and serum AOPP, which were normalized after treatment with the pyridoxamine.

Discussion

AGE and the activation of the iRAS both are important mediators of early diabetic kidney disease, as the blockade of either pathway individually is capable of slowing the progression of experimental nephropathy, in the absence of changes in glycemic control (5–7). In addition, a close interaction between hyperglycemia and altered renal hemodynamics in diabetes has long been established. For example, glomerular hypertension modifies the threshold for and the severity of renal injury associated with hyperglycemia (29). Our data demonstrate that an infusion of AGE-RSA is able to modify key components of the iRAS and that an infusion of Ang II is able to increase the accumulation of AGE, both in the serum and in the kidney. Moreover, blockade of AT₁ is able to prevent some of the pathogenic changes induced by AGE, suggesting that activation of the iRAS represents a key pathogenic mechanism by which AGE are able to induce tissue and cellular dysfunction.

The clinical relevance of these findings remains to be determined. The AGE-RSA used in this experiment was highly

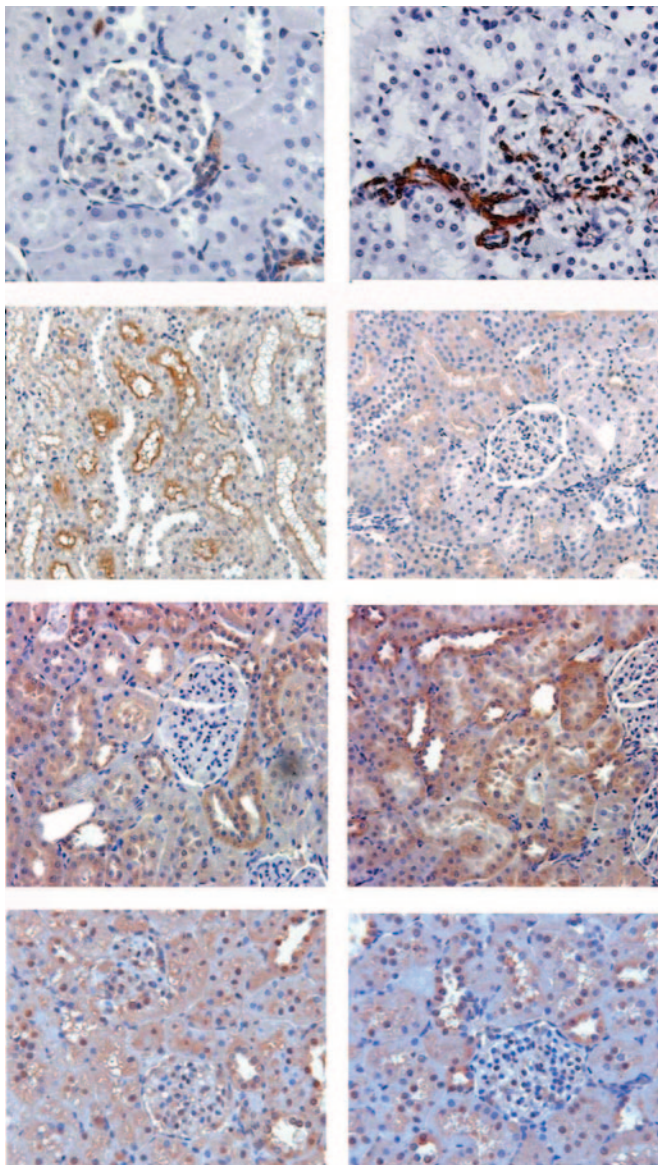


Figure 4. Protein expression of components of the iRAS in RSA (left) and AGE-RSA (right) infusion models.

modified with 0.5 M glucose, unlike the minimally modified protein found *in vivo*. However, intact AGE-RSA was not recovered from the plasma protein, whereas an increase in AGE fluorescence in the LMW fraction was demonstrated in both models. This suggests that LMW fragments of incompletely digested AGE proteins were most likely the mediators of renal dysfunction in our model, reaching the tubule after filtration. Notably, the degree of modification of these LMW fragments may not be dissimilar to fragments derived from senescent protein *in vivo*. The proximal tubule is known to be the main site for reabsorption of filtered AGE (30) (as well as Ang II synthesis) and therefore may be considered uniquely sensitive to the effects of circulating AGE, even in the absence of diabetes.

Patients with poor glycemic control have a greater reduction in intrarenal vascular resistance after blockade of the RAS than

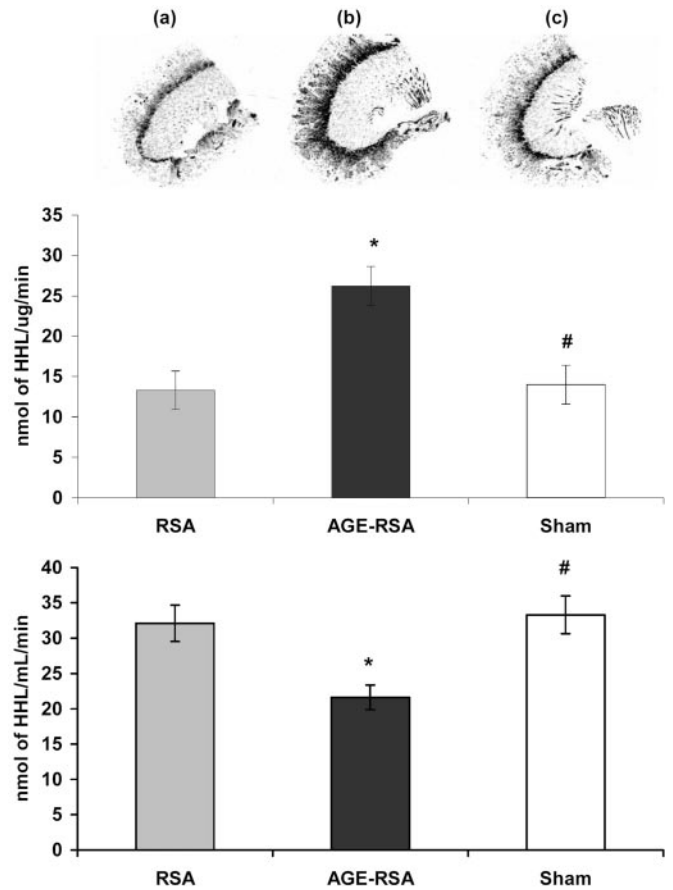


Figure 5. Active renal angiotensin-converting enzyme binding (top), renal activity (middle), and serum activity (bottom) in animals that were treated with RSA (a), AGE-RSA (b), or SHAM (c). Mean ± SEM; **P* < 0.05 versus RSA; #*P* < 0.05 versus AGE-RSA.

those with good control (31), consistent with the hypothesis that iRAS activity may be influenced by metabolic factors. High extracellular glucose is known to stimulate directly angiotensinogen synthesis in tubular cells (32–35). Osmotic diuresis may also contribute to activation, although this does not seem to be the major cause in diabetes as blockade of glucose transport with phlorizin has different effects on the RAS to those seen in diabetes (36). Factors beyond the glucose levels therefore must contribute to activation of the RAS in diabetes, as euglycemia does not normalize the response to AT₁ blockade in patients with diabetes (37). This study demonstrates that an infusion of exogenous AGE is able to modify critical components of the iRAS, including ACE, AT₁, and angiotensinogen. In addition, renal expression of AT₂ and ACE-2 were reduced in this model, leading to increased signaling through vasoconstrictor arm of the iRAS.

Whether these changes in the RAS are a direct or indirect effect of AGE remains to be established. There is good evidence that AGE are able to induce local inflammation and oxidative stress, both of which may modify expression of the local RAS. Activation of receptors for AGE and AT stimulate a proinflammatory cascade involving the transcription factor NF-κB (9,38).

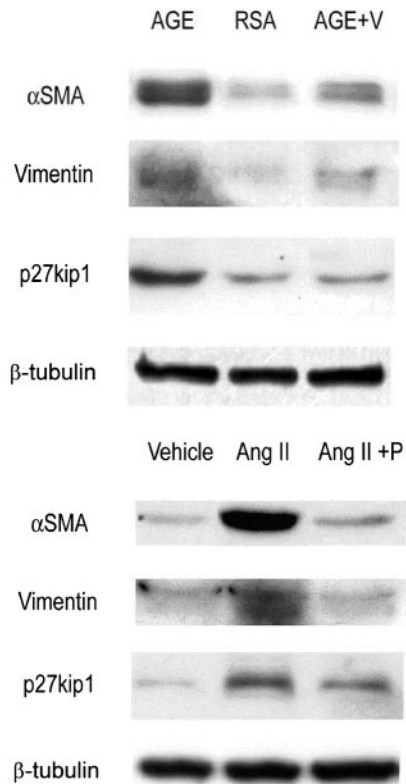


Figure 6. Expression of markers of renal injury and hypertrophy in AGE infusion and Ang II infusion models.

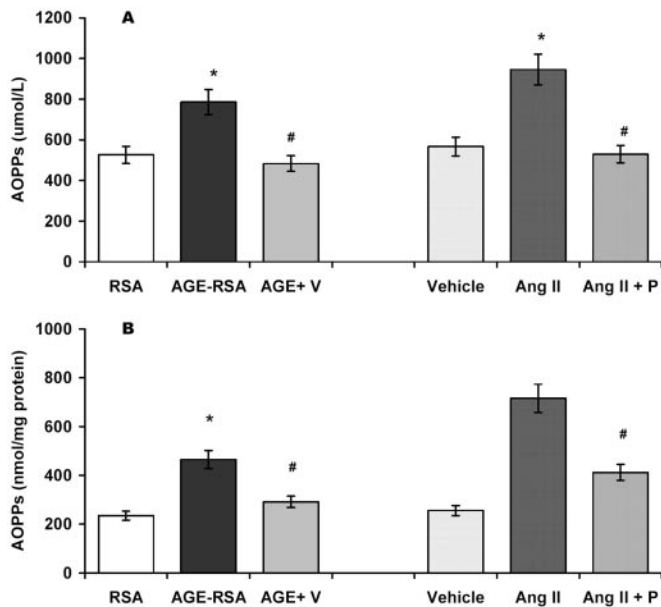


Figure 7. Advanced auto-oxidation protein products (AOPP) in serum (A) and renal tissue (B). Mean \pm SEM; * $P < 0.05$ versus RSA/vehicle; # $P < 0.05$ versus AGE-RSA/Ang II.

AOPP in both kidney and systemically were induced by both Ang II and AGE-RSA. We showed previously that tissue AGE and LMWF are reduced in experimental diabetes by the ACE inhibitor ramipril, possibly by inhibiting the formation of reac-

tive oxygen species (ROS) (6). Pyridoxamine is also known to have potent antioxidant effects (39). The importance of ROS generation in both of these pathways is illustrated further by the use of antioxidants to antagonize the effect of hyperglycemia and Ang II, respectively. For example, inhibitors of oxidation are able to block enhanced Ang II generation under hyperglycemic conditions (39). Similarly, the generation of superoxide is necessary for the renal actions of Ang II (40). As ROS can be considered part of the signaling cascade of both AGE and the RAS, it is conceivable that part of their interaction may be explained by each increasing the sensitivity to the other. For example, depletion of endogenous antioxidants increases the susceptibility of tubular cells to activation by AGE (41). Similarly, activation of the receptor for AGE (RAGE) may result in increased sensitivity to Ang II-mediated injury in vascular smooth muscle cells (42).

ROS do not act solely as downstream mediators of glucose, AGE, and the iRAS. Indeed, the generation of ROS may lead to both the activation of the RAS (36) and the formation of AGE through the formation of reactive glycooxidation and lipoxidation products such as methylglyoxal. It is conceivable that the renal AGE-accumulation seen in animals that were treated with Ang II may have occurred through an increase in tubular oxidative stress and tissue injury. Furthermore, pyridoxamine is a potent antioxidant and may have inhibited AGE-accumulation through this pathway (36). Notably, this protective effect was independent of hemodynamic changes induced by Ang II, including albuminuria, suggesting that there is a separation between the direct vasoconstrictor effects of Ang II and pathways that lead to renal hypertrophy in this model.

Renal hypertrophy may be considered a key event in the development of diabetic nephropathy (43). However, continuous florid hyperglycemia is not required for tubular growth to occur, and hypertrophy may persist in patients with diabetes even after sustained euglycemia (44). Previous studies have shown that a chronic infusion of AGE is able to induce a $>50\%$ increase in glomerular volume (8). Our study replicates these findings and, in addition, demonstrates that AGE may also stimulate tubular hypertrophy and proliferation *in vivo*. Because the AT_1 antagonist valsartan was able to prevent changes induced by AGE, one may speculate that activation of the iRAS may play an important role in AGE-mediated renal hypertrophy. Certainly, Ang II is an important stimulus for renal hypertrophy in diabetes (25,45). In addition, recent studies suggest that AGE-induced activation in TGF- β -Smad signaling could be mediated mainly by autocrine production of Ang II (46). However, tubular injury that arises from exposure to AGE may also result in the production of growth mediators and cytokines. Notably, treatment with valsartan was also able to reduce the expression of markers of tubular injury, including α -SMA and vimentin, suggesting that the effects of AT_1 antagonism is not merely antitrophic but also modulates other pathways that are linked to progressive renal injury.

The mechanism by which valsartan blocks AGE-mediated renal hypertrophy need not be a direct one. Basal ROS production in renal tissue may exert a tonic regulatory action on tubular function, and its reduction, after blockade of the RAS or

inhibition of glycation, may act to modify the threshold required for activation of pathogenic pathways. This synergistic mechanism has previously been demonstrated for other anti-oxidants (43). This hypothesis is supported further by the recent finding that the combination of an agent that interrupts the iRAS with one that inhibits AGE formation provides superior renoprotection, in a model of experimental diabetes, than either agent alone (7).

Unlike diabetes, which is generally associated with early hyperfiltration, GFR was reduced in animals after an infusion of AGE-RSA. This effect is opposite to that seen after an infusion of glycated albumin (47) and more consistent with changes seen in advanced disease or with severe hyperglycemia (48). Notably, both of these states are associated with high levels of circulating AGE. The hemodynamic effects of AGE are thought to be mediated by a variety of pathways. Although the experiments performed in this research point to the activation of the iRAS, tonic vasoregulatory effects of ROS production and nitric oxide synthase activity may also be influenced by AGE (49). In addition, aging is associated with a progressive reduction in ERPF associated with activation of the iRAS, and these age-related changes can be attenuated by treatment with aminoguanidine, an inhibitor of advanced glycation (50).

In summary, this study demonstrates a fundamental interaction between AGE and the iRAS that occurs *in vivo*, with both stimuli activating the other pathway. As both pathways and their effects could be partly blocked by inhibitors of the other, this reinforces the potential utility of combination therapy in a diabetic context. Furthermore, these findings provide an important insight into the synergistic effects of both metabolic and hemodynamic pathways in the development and progression of diabetic kidney disease.

Acknowledgments

M.C.T. is supported by the Don and Lorraine Jacquot RACP Fellowship. J.M.F. is supported by a Juvenile Diabetes Research Foundation career development award. W.M.B. is supported by a postgraduate scholarship from Kidney Health Australia. K.J.-D. is supported by a postdoctoral fellowship from the National Heart Foundation of Australia. The Danielle Alberti Memorial Centre for Diabetes Complications is supported by grants from the Juvenile Diabetes Research Foundation and the National Health and Medical Research Council of Australia.

References

- Cooper ME: Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy. *Diabetologia* 44: 1957–1972, 2001
- Allen TJ, Cao Z, Youssef S, Hulthen UL, Cooper ME: Role of angiotensin II and bradykinin in experimental diabetic nephropathy. Functional and structural studies. *Diabetes* 46: 1612–1618, 1997
- Forbes JM, Cooper ME, Oldfield MD, Thomas MC: Role of advanced glycation end products in diabetic nephropathy. *J Am Soc Nephrol* 14[Suppl 3]: S254–S258, 2003
- Zatz R, Dunn BR, Meyer TW, Anderson S, Rennke HG, Brenner BM: 1986. Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 77: 1925–1930, 1986
- Wilkinson-Berka JL, Kelly DJ, Koerner SM, Jaworski K, Davis B, Thallas V, Cooper ME: ALT-946 and aminoguanidine, inhibitors of advanced glycation, improve severe nephropathy in the diabetic transgenic (mREN-2)27 rat. *Diabetes* 51: 3283–3289, 2002
- Forbes JM, Cooper ME, Thallas V, Burns W, Thomas MC, Brammar GC, Lee F, Grant SL, Burrell LA, Jerums G, Osicka TM: Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy. *Diabetes* 51: 3274–3282, 2002
- Davis BJ, Forbes JM, Thomas MC, Jerums G, Burns WC, Kawachi H, Allen TJ, Cooper ME: Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat. *Diabetologia* 47: 89–97, 2004
- Vlassara H, Striker LJ, Teichberg S, Fuh H, Li YM, Steffes M: Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats. *Proc Natl Acad Sci U S A* 91: 11704–11708, 1994
- Oldfield MD, Bach LA, Forbes JM, Nikolic-Paterson D, McRobert A, Thallas V, Atkins RC, Osicka T, Jerums G, Cooper ME: Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). *J Clin Invest* 108: 1853–1863, 2001
- Thomas MC, Tikellis C, Burns WC, Thallas V, Forbes JM, Cao Z, Osicka TM, Russo LM, Jerums G, Ghabrial H, Cooper ME, Kantharidis P: Reduced tubular cation transport in diabetes: Prevented by ACE inhibition. *Kidney Int* 63: 2152–2161, 2003
- Stitt A, Gardiner TA, Alderson NL, Canning P, Frizzell N, Duffy N, Boyle C, Januszewski AS, Chachich M Baynes JW, Thorpe SR, Anderson NL: The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes* 51: 2826–2832, 2002
- Buñag RD: Validation in awake rat of a tail-cuff method for measurement of systolic blood pressure. *J Appl Physiol* 34: 279–282, 1973
- Cefalu WT, Wang ZQ, Bell-Farrow A, Kiger F, Izlar C: Glycohemoglobin measured by automated affinity HPLC correlates with both short-term and long-term antecedent glycemia. *Clin Chem* 40: 1317–1321, 1994
- Soulis-Liparota T, Cooper ME, Dunlop M, Jerums G: The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague-Dawley rat. *Diabetologia* 38: 387–394, 1995
- Cooper ME, Allen TJ, O'Brien RC, Macmillan PA, Clarke B, Jerums G, Doyle AE: Effects of genetic hypertension on diabetic nephropathy in the rat—Functional and structural characteristics. *J Hypertens* 6: 1009–1016, 1988
- Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW: N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34: 10872–10878, 1995
- Thomas MC, Tsalamandris C, MacIsaac R, Medley T, Kingwell B, Cooper ME, Jerums G: Low-molecular-weight AGEs are associated with GFR and anemia in patients with type 2 diabetes. *Kidney Int*. 66: 1167–1172, 2004
- Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam B,

- Murray-McIntosh RP, Cooper ME: Advanced glycation end products and the receptor for advanced glycation end products co-localise in organs susceptible to diabetic microvascular injury: Immunohistochemical studies. *Diabetologia* 40: 619–628, 1997
19. Friedland J, Silverstein E: A sensitive fluorimetric assay for serum angiotensin-converting enzyme. *Am J Clin Pathol* 66: 416–424, 1976
 20. Jackson B, Cubela R, Johnston C: Angiotensin converting enzyme (ACE), characterization by 125I-MK351A binding studies of plasma and tissue ACE during variation of salt status in the rat. *J Hypertens* 4: 759–765, 1986
 21. Candido R, Jandeleit-Dahm KA, Cao Z, Nesteroff SP, Burns WC, Twigg SM, Dilley RJ, Cooper ME, Allen TJ: Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. *Circulation* 106: 246–253, 2002
 22. Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME: Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* 41: 392–397, 2003
 23. Baunack E, Gartner K: Determination of renal plasma flow by endogenous hippuric acid clearance in the conscious rat. *Contrib Nephrol* 19: 211–216, 1980
 24. Igarashi P, Gulyassy P, Stanfel L, Depner T: Plasma hippurate in renal failure: High-performance liquid chromatography method and clinical application. *Nephron* 47: 290–294, 1987
 25. Weibel ER: *Stereological Methods: Practical Methods for Biological Morphometry*, Vol. 1, London, Academic Press, 1979, pp 40–160
 26. Okada K, Takahashi S: Measurement of proximal tubular diameter for evaluation of tubular hypertrophy. *Nephron* 70: 122, 1995
 27. Wolf G, Jablonski K, Schroeder R, Reinking R, Shankland SJ, Stahl RA: Angiotensin II-induced hypertrophy of proximal tubular cells requires p27Kip1. *Kidney Int* 64: 71–81, 2003
 28. Capeillere-Blandin C, Gausson V, Descamps-Latscha B, Witko-Sarsat V: Biochemical and spectrophotometric significance of advanced oxidized protein products. *Biochim Biophys Acta* 1689: 91–102, 2004
 29. Miller JA, Thai K, Scholey JW: Angiotensin II type 1 receptor gene polymorphism and the response to hyperglycemia in early type 1 diabetes. *Diabetes* 49: 1585–1589, 2000
 30. Asano M, Fujita Y, Ueda Y, Suzuki D, Miyata T, Sakai H, Saito A: Renal proximal tubular metabolism of protein-linked pentosidine, an advanced glycation end product. *Nephron* 91: 688–694, 2002
 31. Taniwaki H, Ishimura E, Kawagishi T, Matsumoto N, Hosoi M, Emoto M, Shoji T, Shoji S, Nakatani T, Inaba M, Nishizawa Y: Intrarenal hemodynamic changes after captopril test in patients with type 2 diabetes: A duplex Doppler sonography study. *Diabetes Care* 26: 132–137, 2003
 32. Zhang SL, Filep JG, Hohman TC, Tang SS, Ingelfinger JR, Chan JSD: Molecular mechanisms of glucose action on angiotensinogen gene expression in rat proximal tubular cells. *Kidney Int* 55: 454–464, 1999
 33. Gabriely I, Yang XM, Cases JA, Ma XH, Rossetti L, Barzilay N: Hyperglycemia modulates angiotensinogen gene expression. *Am J Physiol Regul Integr Comp Physiol* 281: R795–R802, 2001
 34. Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Chan JS: High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. *Endocrinology* 143: 2975–2985, 2002
 35. Choi KC, Kim NH, An MR, Kang DG, Kim SW, Lee J: Alterations of intrarenal renin-angiotensin and nitric oxide systems in streptozotocin-induced diabetic rats. *Kidney Int Suppl* 60: S23–S27, 1997
 36. Christlieb AR, Long R: Renin-angiotensin system in phlorrhizin compared with alloxan diabetes in the rat. *Diabetes* 28: 106–109, 1979
 37. Miller JA: Impact of hyperglycemia on the renin angiotensin system in early human type 1 diabetes mellitus. *J Am Soc Nephrol* 10: 1778–1785, 1999
 38. Mezzano S, Droguett A, Burgos ME, Ardiles LG, Flores CA, Aros CA, Caorsi I, Vio CP, Ruiz-Ortega M, Egido J: Renin-angiotensin system activation and interstitial inflammation in human diabetic nephropathy. *Kidney Int Suppl* 86: S64–S70, 2003
 39. Alderson NL, Chachich ME, Frizzell N, Canning P, Metz TO, Januszewski AS, Youssef NN, Stitt AW, Baynes JW, Thorpe SR: Effect of antioxidants and ACE inhibition on chemical modification of proteins and progression of nephropathy in the streptozotocin diabetic rat. *Diabetologia* 47: 1385–1395, 2004
 40. Lopez B, Salom MG, Arregui B, Valero F, Fenoy FJ: Role of superoxide in modulating the renal effects of angiotensin II. *Hypertension* 42: 1150–1156, 2003
 41. Lal MA, Brismar H, Eklof AC, Aperia A: Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. *Kidney Int* 61: 2006–2014, 2002
 42. Shaw SS, Schmidt AM, Baner AK, Wang X, Stern DM, Marero MB: S100B-RAGE-mediated augmentation of angiotensin II-induced activation of JAK2 in vascular smooth muscle cells is dependent on PLD2. *Diabetes* 52: 2381–2388, 2003
 43. Thomson SC, Vallon V, Blantz RC: Kidney function in early diabetes: The tubular hypothesis of glomerular filtration. *Am J Physiol Renal Physiol* 286: F8–F15, 2004
 44. Wiseman MJ, Saunders AJ, Keen H, Viberti G: Effect of blood glucose control on increased glomerular filtration rate and kidney size in insulin-dependent diabetes. *N Engl J Med* 312: 617–621, 1985
 45. Wolf G: Angiotensin II as a renal growth factor. *Contrib Nephrol* 135: 92–111, 2001
 46. Fukami K, Ueda S, Yamagishi S, Kato S, Inagaki Y, Takeuchi M, Motomiya Y, Bucala R, Iida S, Tamaki K, Imaizumi T, Cooper ME, Okuda S: AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. *Kidney Int* 66: 2137–2147, 2004
 47. Sabbatini M, Sansone G, Uccello F, Giliberti A, Conte G, Andreucci VE: Early glycosylation products induce glomerular hyperfiltration in normal rats. *Kidney Int* 42: 875–881, 1992
 48. Hostetter TH, Troy JL, Brenner BM: Glomerular hemodynamics in experimental diabetes mellitus. *Kidney Int* 19: 410–415, 1981
 49. Rashid G, Benchetrit S, Fishman D, Bernheim J: Effect of advanced glycation end-products on gene expression and synthesis of TNF-alpha and endothelial nitric oxide synthase by endothelial cells. *Kidney Int* 66: 1099–1106, 2004
 50. Reckelhoff JF, Hennington BS, Kanji V, Racusen LC, Schmidt AM, Yan SD, Morrow J, Roberts LJ 2nd, Salahudeen AK: Chronic aminoguanidine attenuates renal dysfunction and injury in aging rats. *Am J Hypertens* 12: 492–498, 1999