Original Article

Mechanism of Perturbation of Integrin-Mediated Cell-Matrix Interactions by Reactive Carbonyl Compounds and Its Implication for Pathogenesis of Diabetic Nephropathy

Vadim K. Pedchenko,[1] Sergei V. Chetyrkin,[1] Peale Chuang,[1] Amy-Joan L. Ham,[2,3] Moin A. Saleem,[4] Peter W. Mathieson,[4] Billy G. Hudson,[1,2] and Paul A. Voziyan[1]

Perturbation of interactions between cells and the extracellular matrix (ECM) of renal glomeruli may contribute to characteristic histopathological lesions found in the kidneys of patients with diabetic nephropathy. However, the mechanism by which the diabetic conditions may affect cell-ECM interactions is unknown. Existing hypotheses suggest a role of glucose in direct modification of ECM. Here, we have demonstrated that carbonyl compound methylglyoxal (MGO) completely inhibited endothelial cell adhesion to recombinant α3 noncollagenous 1 domain of type IV collagen mediated via a short collagenous region containing RGD (Arg-Gly-Asp) sequence as well as binding of purified $\alpha_v\beta_3$ integrin to this protein. Specific MGO adducts of the arginine residue were detected within RGD sequence using mass spectrometry. Modification by carbonyl compounds glyoxal or glycolaldehyde had similar but smaller effects. MGO strongly inhibited adhesion of renal glomerular cells, podocytes, and mesangial cells to native collagen IV and laminin-1 as well as binding of collagen IV to its major receptor in glomerular cells, $\alpha_1\beta_1$ integrin. In contrast, modification of these proteins by glucose had no effect on cell adhesion. Pyridoxamine, a promising drug for treatment of diabetic nephropathy, protected cell adhesion and integrin binding from inhibition by MGO. We suggest that in diabetes, perturbation of integrin-mediated cell-matrix interactions occurs via the modification of critical arginine residues in renal ECM by reactive carbonyl compounds. This mechanism may contribute to the development of diabetic nephropathy. *Diabetes* 54:2952–2960, 2005

One of the proposed pathogenic mechanisms of diabetic nephropathy, a major complication of diabetes, is modification of renal extracellular matrix (ECM) by nonenzymatic reactions. ECM proteins are prominent targets for such modification because of their direct accessibility to elevated levels of circulating glucose and very slow turnover rates, which allow for accumulation of these modifications. Diminished cell interactions with modified proteins of glomerular ECM may provide a link between diabetes and diabetic nephropathy characterized, among other features, by marked morphological changes in the glomerular matrix as well as by shedding of glomerular epithelial cells (podocytes) from the underlying glomerular basement membrane (GBM), which may compromise glomerular filtration (1,2).

Type IV collagen and laminin are the major constituents of mesangial matrix and GBM, renal ECMs that support, respectively, mesangial cells and both endothelial cells and podocytes (3). Nonenzymatic modification of collagen IV and laminin in the presence of glucose has been reported to inhibit their interaction with endothelial and mesangial cells (4,5). The observed effects were relatively modest (20–50%), even at 500 mmol/l glucose, a very high supraphysiological concentration (4,5). On the other hand, modification of ECM proteins with glucose-6-phosphate, a more reactive sugar compared with glucose, actually increased endothelial cell adhesion when used at lower, more physiologically relevant concentrations (6). The proposed hypotheses for the mechanism of inhibition are the modification of lysine residues in ECM proteins by glucose, protein cross-linking, and conformational changes due to glycation (4,5). However, the experimental support for these hypotheses is lacking; thus, the molecular mechanism remains unknown.

In diabetes, hyperglycemia is always accompanied by a rise in the levels of low–molecular weight carbonyl compounds, a phenomenon known as "carbonyl stress" (7). Concentrations of carbonyl compounds glyoxal, methylglyoxal (MGO), and 3-deoxyglucosone (3-DG) are significantly elevated above the normal levels in serum and certain tissues of diabetic patients and in experimental animal models of diabetes (8–12). Several mechanisms have been suggested to cause this increase. Carbonyl species glyoxal, MGO, and glycolaldehyde (GLA) can

From the [1]Department of Medicine, Division of Nephrology, Vanderbilt University Medical Center, Nashville, Tennessee; the [2]Department of Biochemistry, Vanderbilt University Medical Center, Nashville, Tennessee; the [3]Mass Spectrometry Research Center, Vanderbilt University Medical Center, Nashville, Tennessee; and the [4]Children's and Academic Renal Unit, University of Bristol, Bristol, U.K.

Address correspondence and reprint requests to Paul A. Voziyan, Division of Nephrology, Vanderbilt University Medical Center, S-3223 MCN, 1161 21st Ave. S., Nashville, Tennessee 37232-2372. E-mail: paul.voziyan@vanderbilt.edu.

Received for publication 11 May 2005 and accepted in revised form 30 June 2005.

CML, carboxymethyl lysine; 3-DG, 3-deoxyglucosone; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membrane; GLA, glycolaldehyde; MGO, methylglyoxal; MS/MS, tandem mass spectrometry; NC1, noncollagenous 1.

derive from autoxidation of glucose or Schiff base intermediate formed during the reaction of glucose with protein amino groups as well as from oxidation of lipids (13–16). A protein-Amadori adduct, another glycation intermediate, is a major source of 3-DG (17). MGO can also originate from either spontaneous or enzymatic degradation of triose phosphates derived from glucose (18). The glyoxalase system, which normally scavenges excessive glyoxal and MGO, is weakened by oxidative stress of diabetes, which could further increase the levels of these α-oxoaldehydes (19). One of the pathogenic consequences of the elevated levels of reactive dicarbonyl compounds may be the acceleration of nonenzymatic modification of proteins. Nonenzymatic protein modifications by reactive carbonyl compounds include lysine and arginine adducts as well as arginine-lysine and lysine-lysine cross-links (20). The elevated levels of protein modifications derived specifically from glyoxal, MGO, 3-DG, and GLA have been found in diabetic patients and in animal models of diabetes using mass spectrometry or immunostaining techniques; these modifications were present in plasma proteins as well as in the kidney and retina organs, which are commonly injured in diabetic patients (21–25). Interestingly, administration of MGO to mice caused pathological changes in kidneys characteristic of diabetic complications (26).

Modification of arginine residues of matrix proteins by nonenzymatic reactions is a plausible mechanism for inhibition of cell-ECM interactions because arginine is present in a number of integrin binding sites, which interact with multiple members of integrin superfamily mediating cell adhesion (27,28). However, the effects on additional cell adhesion motifs lacking arginine could not be excluded because of the presence of other nucleophilic residues susceptible to nonenzymatic glycation (28,29). Here, we used cultured endothelial, glomerular epithelial, and mesangial cells, along with recombinant noncollagenous (NC1) domains of collagen IV and different purified native matrix proteins to address the mechanism of inhibition of cell-matrix interactions in the context of diabetic conditions and renal cell adhesion to ECM. Our results show that this mechanism does not operate through modification of ECM proteins and, specifically, lysine residues by glucose. Instead, we demonstrate that MGO and other reactive carbonyl compounds can modify critical arginine residues within integrin-binding sites of ECM proteins and perturb cell-ECM interactions, thus suggesting a mechanism that may underlie the linkage between carbonyl stress and diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Materials. Purified $\alpha_v\beta_3$ and $\alpha_1\beta_1$ integrins were purchased from Chemicon (Temecula, CA); α_v- and β_1-integrin monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA); fibronectin, pyridoxamine hydrochloride, glyoxal, GLA, and MGO were obtained from Sigma-Aldrich (St. Louis, MO); D-glucose, mouse type IV collagen, and laminin-1 isolated from EHS tumor were purchased from Invitrogen (Carlsbad, CA); vitronectin was obtained from TaKaRa Biomedicals (Shiga, Japan).

Cell culture. Human umbilical vein endothelial cells were obtained from BioWhittaker (Charlotte, NC). Cells were grown in EGM-2 MV medium (BioWhittaker) and used between passages 4 and 7. Conditionally immortalized mouse mesangial cells were provided by Dr. Ambra Pozzi (Vanderbilt University Medical Center, Nashville, TN) (30). Conditionally immortalized human podocytes were derived as previously described (31). Mesangial cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 units/ml interferon-γ at 33°C; podocytes were propagated in RPMI medium with the same supplements but without interferon-γ. For differentiation, cells were cultured in the above medium without interferon-γ at 37°C. Low passages (<20) of the immortalized cells were used to ensure the preservation of the specific properties characteristic for differentiated state (30,31).

Proteins. Recombinant human α3NC1 and RGD-α3NC1 domains of type IV collagen with the NH_2-terminal FLAG sequence were stably expressed in HEK 293 cells and purified from conditioned medium by affinity chromatography on anti-FLAG agarose (Sigma) as previously described (29).

Modification of the proteins. Proteins were immobilized on 96-well plates (Nunc, Rochester, NY) in Tris-buffered saline at 4°C overnight. Plates were washed twice with 200 mmol/l sodium phosphate buffer, pH 7.5, and incubated in the same buffer with or without different additives in the wet chamber to prevent changes in sample volume due to evaporation; 0.02% sodium azide was added to suppress bacterial growth. All incubations were carried out in the dark at 37°C. No protein desorption was detected after different treatments as determined by alkaline phosphatase competition assay (32) and enzyme-linked immunosorbent assay (ELISA) using anti-FLAG antibody (data not shown).

Physiologically relevant Amadori-BSA was prepared with diabetic concentration of glucose (30 mmol/l) using a previously described procedure (33).

Cell adhesion and integrin binding assays. Cell adhesion was quantified using crystal violet staining as previously described (29). Binding of integrins to different matrix proteins was determined in a solid-phase binding assay and quantified using corresponding integrin antibodies and ELISA as previously described (29).

Detection of protein-carboxymethyllysine. Modification of protein lysine residues to carboxymethyl lysine (CML) were measured by ELISA using polyclonal anti-CML antibody R618 as described previously (34).

Mass-spectrometry analysis of arginine modifications. Human recombinant RGD-α3NC1 (30 μg) was incubated in 0.2 mol/l sodium phosphate buffer, pH 7.5, with or without 2 mmol/l MGO for 20 h at 37°C. After the removal of unreacted MGO, protein was reduced, alkylated with iodoacetamide, and digested with chymotrypsin (1:100 enzyme-to-protein ratio, wt/wt) in 0.1 mol/l NH_4HCO_3 buffer, pH 8, for 4 h at room temperature. Resulting peptide mixtures were snap-frozen in liquid nitrogen and stored at −70°C until mass-spectrometry analysis.

The liquid chromatography–tandem mass spectrometry (liquid chromatography-MS/MS) analysis was performed on a ThermoFinnigan LTQ linear ion trap mass spectrometer using previously described protocol (35). Protein was identified using the SEQUEST 2.0 algorithm (36) and the SEQUEST Browser software. A list of peptides was created and each peptide was run through P-Mod software to check for possible chemical modifications (37).

Statistical analysis. Data were expressed as mean ± SD, and statistical analysis was performed using Student's *t* test for unpaired samples or ANOVA followed by post hoc Student-Newman-Keuls comparisons. Differences were considered statistically significant if *P* values were less than 0.05.

RESULTS

We have recently reported that endothelial cell adhesion to COOH-terminal fragment of human α3 chain of type IV collagen is mediated through both non-RGD regions of α3NC1 domain and adjacent RGD motif of collagenous domain using purified recombinant proteins with preserved native structure (29). In this paper, we used the same model to elucidate the mechanism by which diabetic conditions may affect cell-matrix interactions, particularly in renal glomerulus. The use of both RGD-α3NC1, a fragment of the human α3(IV) chain, and EHS collagen, purified α1(IV) and α2(IV) chains, allowed us to more accurately model specific protein components of native glomerular matrices.

Modification of major glomerular ECM proteins by MGO inhibits cell adhesion to these proteins. In choosing the experimental conditions for MGO treatment, we were guided by the finding that the incubation of human albumin with 0.5 mmol/l MGO for 24 h resulted in modification of ~1 Arg residue per molecule (38). Modification by MGO strongly inhibited endothelial cell adhesion to RGD-α3NC1 with maximum effect achieved at 2 mmol/l MGO after 24 h (Fig. 1). Similar experimental conditions were then applied to determine the effect of MGO on glomerular cell adhesion. Kidney glomerulus is characterized by specific distribution of collagen IV α-chains (39). In

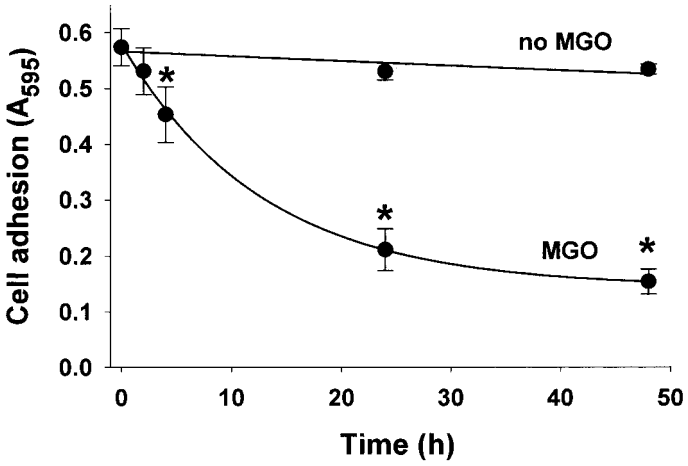

FIG. 1. Effect of MGO modification of RGD-α3NC1 protein on endothelial cell adhesion. Plates coated with RGD-α3NC1 (20 μg/ml) were preincubated with or without 2 mmol/l MGO at 37°C for the indicated times. Cell adhesion was determined as described in RESEARCH DESIGN AND METHODS. In all figures, the error bars represent the SD (n = 4); *P < 0.05, MGO vs. control.

particular, the α3 chain is localized specifically to the GBM, which supports podocytes and endothelial cells, whereas α1 and α2 chains form the mesangial matrix supporting mesangial cells (39). Mesangial cells also interact with GBM in paramesangial regions (40). Along with collagen IV, laminin-1 is another major constituent of mesangial matrix (41). In the GBM, laminin-1 is present only during kidney development and is replaced by laminin-11 in the mature kidney (42). Because the purified laminin-11 is not available commercially, we modeled podocyte adhesion using laminin-1. Both α1 chain of laminin-1 and α5 chain of laminin-11 interact with integrins via RGD site (43,44). As shown in Fig. 2, MGO-modification of RGD-α3NC1 domain, full-length collagen IV, or laminin-1 resulted in inhibition of both mesangial cell and podocyte adhesion to these matrix proteins.

Modification of matrix proteins by glucose does not affect cell adhesion. To investigate how other factors of diabetic milieu may affect cell-matrix interactions, we addressed the hypothesis that a direct modification of ECM proteins by glucose, particularly of lysine residues, may inhibit cell adhesion (5). Unlike MGO, which is a highly active electrofile that can react with both arginine and lysine protein side chains (20), glucose reacts preferentially with ε-amino group of lysine or α-amino group of NH_2-terminal amino acid forming advanced glycation end products via lysyl-Amadori intermediate (45). Incubation of RGD-α3NC1 with high (up to 1 mol/l) concentrations of glucose for 10 days at 37°C resulted in a dramatic increase in CML, a glucose-derived modification of lysine residues (45), as was determined using ELISA (Fig. 3*A*, inset). However, this modification did not affect endothelial cell adhesion (Fig. 3*A*). Similarly, modification of full-length collagen IV in the presence of glucose, as indicated by the extensive conversion of lysine residues to CML (Fig. 3*B*, inset), had no effect on cell adhesion (Fig. 3*B*).

We then investigated another possible mechanism of glucose-induced inhibition of cell adhesion, i.e., through formation of lysyl-Amadori intermediate on circulating proteins followed by cross-linking with arginine residues of matrix proteins. To this end, we prepared a physiologically relevant albumin-Amadori intermediate that mimics closely the degree of modification of serum albumin in diabetes (33). Preincubation of this glycated albumin with RGD-α3NC1 for up to 20 days did not affect endothelial cell adhesion to RGD-α3NC1 (Table 1). Similarly, endothelial cell adhesion to full-length collagen IV was not affected after preincubation with BSA and very high (500 mmol/l) concentration of glucose (data not shown).

MGO inhibits cell adhesion via modification of critical arginine residues in ECM proteins. To investigate the mechanism of inhibition of cell-matrix interaction by MGO, we used recombinant proteins α3NC1 or RGD-α3NC1, which represent either NC1 domain of α3 chain of human collagen IV or a larger fragment of α3 chain also including the adjacent collagenous region containing RGD sequence. MGO strongly inhibited endothelial cell adhesion to RGD-α3NC1 in a concentration-dependent manner (Fig. 4*A*). Modification with MGO inhibited cell adhesion to RGD-α3NC1 to the level of α3NC1 but had no effect on cell adhesion to α3NC1 itself (Fig. 4*A*). These results suggest that the arginine residue of RGD motif within

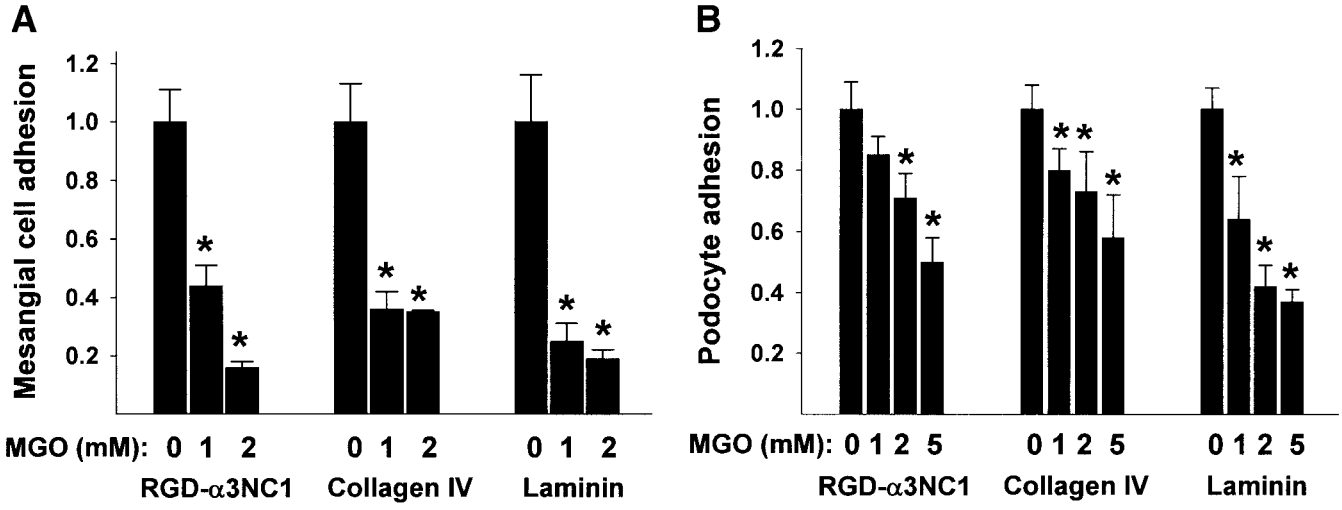

FIG. 2. Adhesion of mesangial cells and podocytes to different matrix proteins modified by MGO. Plates coated with RGD-α3NC1, collagen IV, or laminin (all at 20 μg/ml) were preincubated with or without the indicated concentrations of MGO at 37°C for 24 h, and adhesion of mesangial cells (*A*) or podocytes (*B*) was determined. *P < 0.05, MGO vs. control.

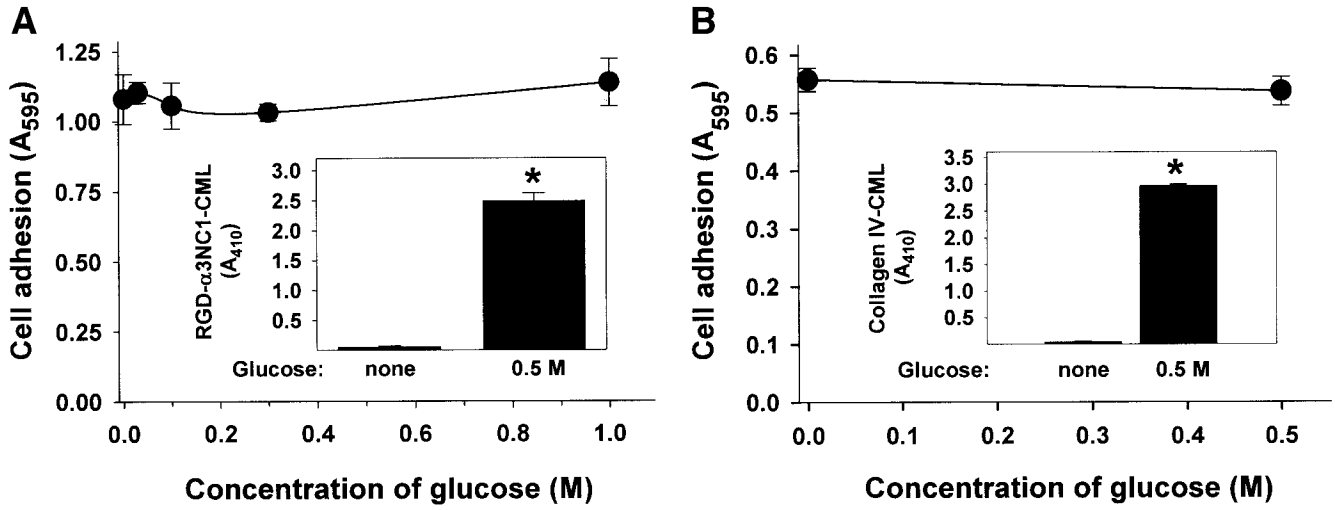

FIG. 3. Effect of glucose modifications of matrix proteins on endothelial cell adhesion. Plates coated with either RGD-α3NC1 (*A*) or collagen IV (*B*) (20 μg/ml) were preincubated with the indicated concentrations of D-glucose at 37°C for 10 days, and cell adhesion was measured (●). *Insets*: The level of lysine modifications to CML was determined in the samples incubated with 0.5 mol/l glucose for 10 days using ELISA. *$P < 0.05$, glucose vs. control.

RGD-α3NC1 is a target for the inhibition of cell adhesion by MGO.

In the competition experiments using free amino acids, only arginine protected from MGO-induced inhibition of cell adhesion to RGD-α3NC1, whereas lysine had no effect (Fig. 4*B*). This finding further indicates that the inhibition of cell adhesion to RGD-α3NC1 by MGO resulted from modification of an arginine residue within RGD sequence. Inability of the excess of free lysine to compete out the MGO effect suggests that modification of lysine residues within RGD-α3NC1 protein is not involved in inhibition of cell adhesion by MGO, a notion consistent with the results of the glucose experiments (Fig. 3).

Modification of an arginine residue within RGD sequence was directly demonstrated by mass-spectrometric analysis of MGO-modified RGD-α3NC1. Several modified proteolytic peptides containing RGD motif were detected in MGO-treated sample, whereas no modified peptides were found in untreated control (Table 2). The analysis of the MS/MS data using P-Mod algorithm (37), as shown for one of the peptides in Fig. 5, assigned the modifications to the arginine residue with the molecular masses of modifications corresponding to two known MGO derivatives of arginine, 5-hydro-5-methyl-4-imidazolone and 4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine (20,46). The same two MGO-derived modifications were also found on several other arginine residues of RGD-α3NC1 protein: Arg^{78}, Arg^{101}, and Arg^{202} (data not shown) [see (29) for amino acid positions]. However, because all of these residues are located within α3NC1 domain, their modification by MGO had no effect on cell adhesion (Fig. 4*A*). No modifications were found on the rest of arginines: Arg^{6}, Arg^{12}, Arg^{43}, Arg^{58}, Arg^{109}, Arg^{171}, Arg^{181}, and Arg^{205}, probably because of steric hindrance in the context of natively folded RGD-α3NC1 protein (data not shown). Proteolytic peptides containing Arg^{231} have not been detected (data not shown).

TABLE 1
Effect of BSA-Amadori on endothelial cell adhesion

Additive	Cell adhesion (A_{295}) 10 days	20 days
Buffer	1.080 ± 0.089	1.267 ± 0.044
BSA	1.019 ± 0.032	1.221 ± 0.034
BSA-Amadori	1.139 ± 0.103	1.198 ± 0.033

Plates coated with RGD-α3NC1 were incubated with buffer alone, unmodified BSA, or Amadori-BSA (25 mg/ml each) at 37°C for indicated times. Cell adhesion was measured as described in RESEARCH DESIGN AND METHODS.

Similar to results obtained with RGD-α3NC1 protein, free arginine but not lysine protected cell adhesion to full-length collagen IV upon modification by MGO (data not shown). Modification by MGO also strongly inhibited cell adhesion to fibronectin and vitronectin (data not shown), in agreement with a known critical role of RGD motifs in mediating cell adhesion to these ECM proteins (47,48). These data are consistent with MGO-induced inhibition of cell adhesion in major ECM proteins via modification of specific arginine residues involved in integrin-mediated cell-matrix interactions.

Modification of RGD-α3NC1 and collagen IV by MGO and other reactive carbonyl compounds inhibits integrin binding to these proteins. Integrin $\alpha_v\beta_3$ is a member of an extended family of integrins, cell surface receptors that mediate cell-matrix interactions. As with a number of other integrins, $\alpha_v\beta_3$ interacts with matrix proteins predominantly via specific binding to RGD sequence (27) and, as shown in our previous study, is a major endothelial cell receptor for RGD-α3NC1 (29). In the solid-phase binding assay, $\alpha_v\beta_3$ demonstrated strong binding to RGD-α3NC1, whereas virtually no binding to α3NC1 domain was detected (Fig. 6*A*, inset). Thus, any effect on $\alpha_v\beta_3$ binding would be due to modification of an arginine residue within RGD sequence of RGD-α3NC1 protein. Integrin binding to MGO-treated RGD-α3NC1 was strongly inhibited with half-maximal inhibitory concentration decreasing dramatically (from 160 to 5 μmol/l) as incubation time increased from 24 h to 20 days (Fig. 6*A*). In fact, after a 20-day incubation, a significant effect was already apparent at 1 μmol/l MGO (Fig. 6*A*), an MGO concentration close to that found in diabetic plasma (9,11). Similar to MGO, two other reactive carbonyl compounds that can be generated during autoxidation of glucose and glycated

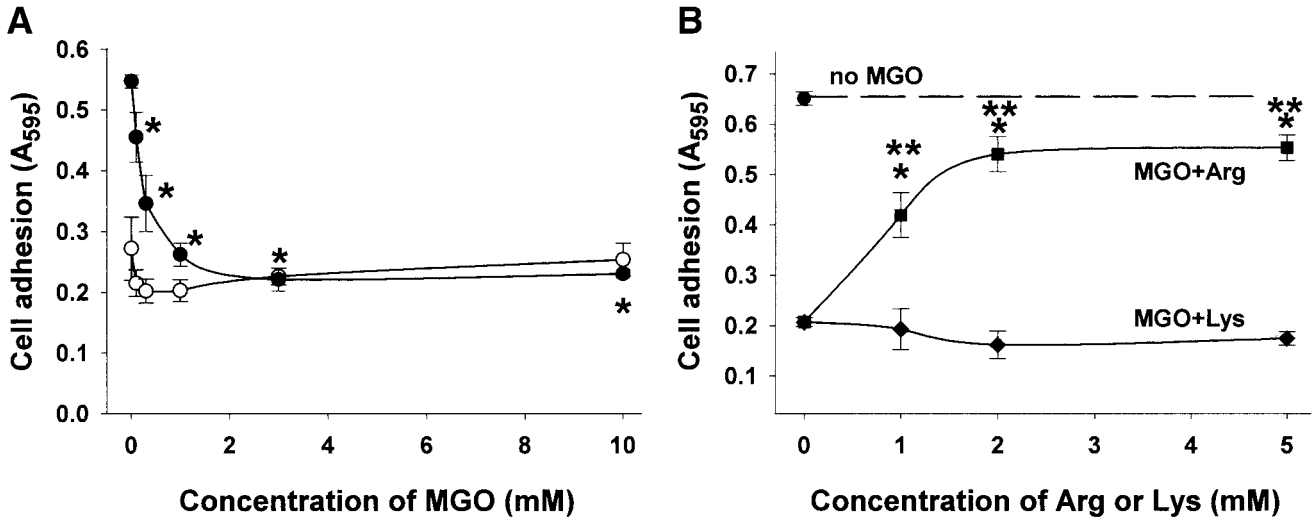

FIG. 4. Effect of arginine modification on cell adhesion. *A*: Recombinant RGD-α3NC1 (●) or α3NC1 (○) were preincubated with various concentrations of MGO at 37°C for 24 h. **P* < 0.05, MGO vs. control. *B*: Plates coated with RGD-α3NC1 were preincubated without MGO (●) or with 2 mmol/l MGO and the indicated concentrations of lysine (♦) or arginine (■) at 37°C for 24 h, and endothelial cell adhesion was determined. **P* < 0.05, MGO+Arg vs. MGO; ***P* < 0.05, MGO+Arg vs. MGO+Lys.

protein intermediates, glyoxal and GLA, also inhibited $\alpha_v\beta_3$ binding, albeit less efficiently (Fig. 6*A*, inset).

The interaction of collagen IV with the various cell types is mediated mainly via binding of the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to the specific site in the triple-helical part of the molecule (49). Moreover, it has been demonstrated that Arg^{461} within the α2 chain is essential for binding of $\alpha_1\beta_1$ integrin (50). Modification of EHS collagen IV by MGO inhibited binding of purified $\alpha_1\beta_1$ integrin to collagen IV (Fig. 6*B*). Like with RGD-α3NC1, the effective MGO concentration significantly decreased as incubation time increased.

Scavenging of MGO by pyridoxamine protected both cell adhesion and integrin binding. Pyridoxamine, a drug that has shown promise in treatment of diabetic nephropathy (51) and is capable of scavenging reactive carbonyl compounds (34,52), prevented MGO-induced inhibition of cell adhesion to collagen IV and $\alpha_v\beta_3$ binding (Fig. 7*A* and *B*). This protection depended on pyridoxamine concentration and was already significant at equimolar concentrations of MGO and pyridoxamine (Fig. 7*B*).

DISCUSSION

Our results suggest that the weakening of cell-matrix interactions under diabetic conditions may occur because of modification of specific arginine residues within integrin-binding sites of matrix proteins. These arginine modifications are derived from reactive carbonyl compounds, most importantly MGO, that are elevated in diabetes (8–12). Inhibition of cell adhesion to major ECM proteins (collagen IV, laminin, fibronectin, and vitronectin) was also consistent with this mechanism. Furthermore, the mechanism was addressed in a context of cell-ECM interactions in renal glomerulus as demonstrated by a significant decrease in adhesion of both mesangial cells and podocytes to several ECM proteins modified by MGO.

Arginine residues are critical structural determinants in many binding sites of matrix proteins, which mediate ECM interactions with multiple members of integrin superfamily (27,28). For example, studies of collagen IV proteolytic fragments demonstrated that cell adhesion is mediated by several binding sites within triple helical and NC1 domains of the molecule (53,54), with the major cellular receptors being $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (55). Subsequently, Asp^{461} in

TABLE 2
Mass-spectrometry analysis of arginine modifications by MGO within RGD region of RGD-α3NC1 domain of collagen IV

Detected peptides containing RGD region	Detected peptide mass differences	
	MGO treated	Untreated
KGK**R**GDSGSPATW	54.5* (0.0326)†	ND
GK**R**GDSGSPATW	54.7 (0.0224)	ND
K**R**GDSGSPATW	54.9 (0.0090); 144.0 (0.0230)	ND
RGDSGSPATW	54.5 (0.0003); 144.3 (0.0032)	ND

*Theoretical mass differences and corresponding MGO-Arg derivatives: 54.05 atomic mass units, hydroimidazolone; 144.12 atomic mass units, tetrahydropyrimidine (16,45). †The numbers in parentheses represent *P* values for the matches by p-Mod algorithm. ND, not detected.

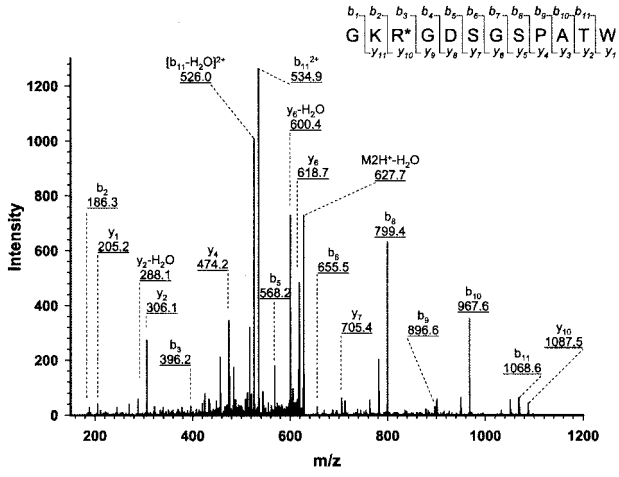

FIG. 5. The MS/MS spectrum of one of the MGO-modified RGD-α3NC1 chymotryptic peptides containing RGD motif (see Table 2). The modification was detected and assigned to specific arginine residue based on MS/MS peptide sequence data using pMod algorithm (37). R* indicates MGO-modified arginine residue; the molecular mass of the adduct equals 54.1 mass units. The precursor molecular ion ($M2H^+$) m/z = 636.8; the molecular mass of the peptide is 1,271.6 Da.

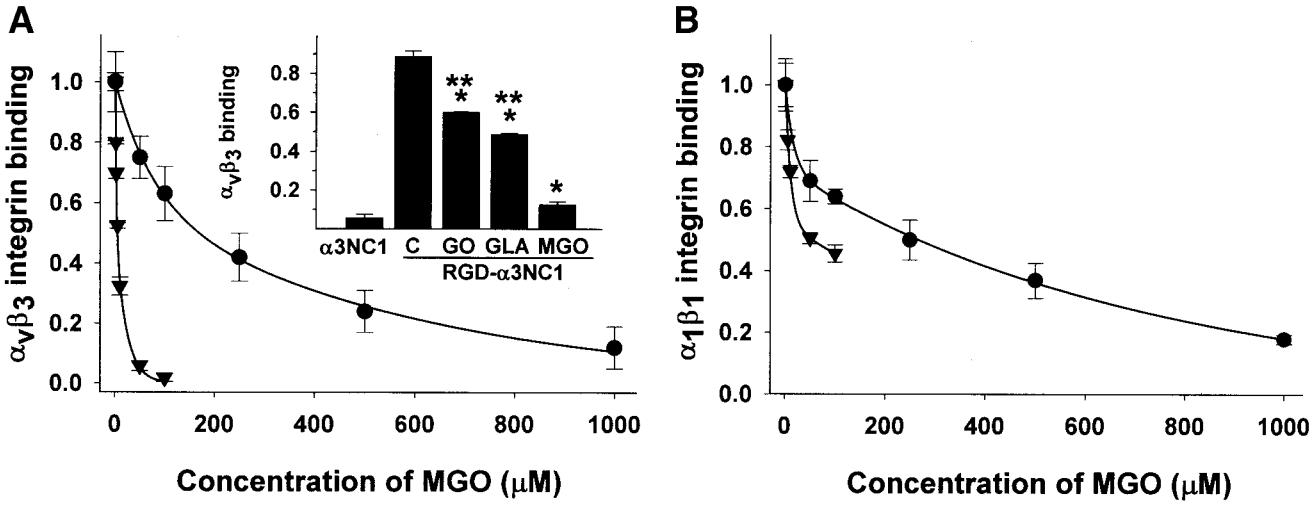

FIG. 6. Binding of $\alpha_v\beta_3$ integrin to RGD-α3NC1 and $\alpha_1\beta_1$ integrin to mouse collagen IV. *A*: Plates coated with RGD-α3NC1 were preincubated with indicated concentrations of MGO at 37°C for 24 h (●) and 20 days (▲). For 20-day incubations, MGO solutions were replaced every 24 h to maintain constant MGO concentrations. Binding of $\alpha_v\beta_3$ integrin was determined in a solid-phase binding assay. *B*: Plates coated with collagen IV were incubated with MGO as described above. Binding of $\alpha_1\beta_1$ integrin was determined. In *A* and *B*, differences for all the samples were significant compared with control ($P < 0.05$). *Inset*: Plates coated with RGD-α3NC1 were preincubated alone (*C*) or with MGO, glyoxal, or GLA (0.5 mmol/l) at 37°C for 24 h, and binding of $\alpha_v\beta_3$ integrin was determined. Binding of $\alpha_v\beta_3$ integrin to α3NC1 was also determined. *$P < 0.05$, glyoxal, GLA, or MGO vs. control; **$P < 0.05$, glyoxal or GLA vs. MGO.

the α1(IV) chain and an Arg^{461} in the α2(IV) chain have been identified as critical residues for $\alpha_1\beta_1$ binding to the triple helical domain (50). Yet another integrin binding site containing critical arginine residue, GFOGER (in which O is hydroxyproline), was found in collagen I (56). Interestingly, triple-helical GFOGER peptide potently inhibited $\alpha_2\beta_1$ integrin–mediated cell adhesion to collagen IV (56). Moreover, the same sequence exists in α1 chain of collagen IV (residues 385–390), where it may serve as a binding site for $\alpha_2\beta_1$ integrin.

Like in collagen IV, specific arginine residues involved in mediation of cell adhesion have also been found in laminin, another major constituent of renal basement membranes and mesangial matrix (3). In addition to a cryptic RGDN motif of the A chain (43) and the YIGSR region in the B1 chain (57) of laminin-1, two RGD sites in α5 chain of laminin-10 and -11 are also involved in cell adhesion (44). Other glomerular matrix proteins fibronectin, vitronectin, and entactin appear to mediate cell adhesion predominantly via RGD motif (47,48,58). Thus, in native extracellular matrices, a set of several critical arginine residues within structurally diverse integrin binding sites would be available for targeting by reactive carbonyl species. It also implies that cell-matrix interactions in different tissues may be affected by nonenzymatic reactions via the same mechanism. Diminished adhesion of osteoblasts to MGO-modified collagen I has been reported (59).

In our experiments, glucose modification of immobilized RGD-α3NC1 domain or full-length collagen IV did not affect cell adhesion to these proteins (Fig. 3). Although glucose does not react with arginine residues directly, it may contribute as a source of reactive carbonyl species formed during glycoxidative reactions, which may, in turn, modify arginine. Interestingly, incubation of collagen I with ribose, a sugar prone to rapid oxidative degradation,

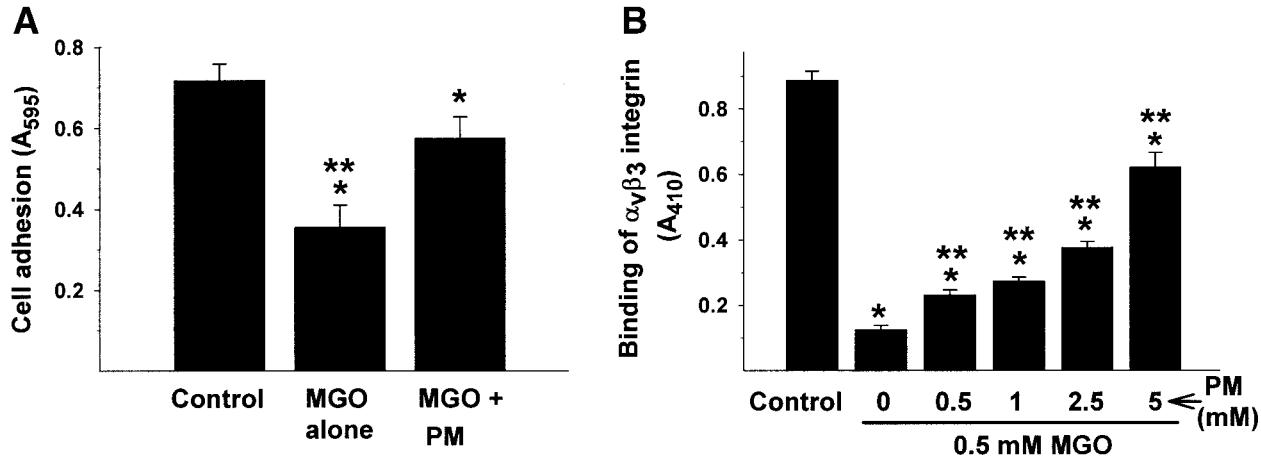

FIG. 7. Protection of cell adhesion and integrin binding by pyridoxamine (PM). *A*: Plates coated with collagen IV were preincubated with or without MGO (2 mmol/l) or with MGO and pyridoxamine (4 mmol/l) at 37°C for 24 h, and endothelial cell adhesion was determined. *B*: Plates coated with RGD-α3NC1 were preincubated with or without MGO (0.5 mmol/l) or with MGO and different concentrations of pyridoxamine. Binding of $\alpha_v\beta_3$ integrin was determined in a solid-phase binding assay. *$P < 0.05$, MGO ± pyridoxamine vs. control; **$P < 0.05$, MGO + pyridoxamine vs. MGO.

caused inhibition of osteoblast adhesion to this matrix protein (60). However, in vivo glucose autoxidation may play a secondary role in inhibition of cell-matrix interactions because glyoxal is the only α-dicarbonyl compound formed in this reaction (13). Because the concentration of glyoxal in plasma of diabetic patients and model diabetic animals is three- to sixfold lower than that of MGO (9,61) and glyoxal is significantly less active at inhibiting integrin binding (Fig. 6), MGO-producing cellular processes such as degradation of glucose-derived triose phosphates may play a more important role in perturbation of cell-matrix interactions in diabetes.

Glucose may also modify arginine residues by formation of Lys-Arg cross-links via protein-Amadori intermediate (62,63). However, this contribution may be limited by the low reactivity of glucose-Amadori intermediate compared with that of reactive carbonyl compounds and by steric constrains imposed by the matrix network structure on the formation of cross-links. In our study, albumin-Amadori intermediate, which could potentially form cross-links with arginine residues of matrix proteins, had no effect on cell adhesion (Table 1).

Glucose may also affect cell-matrix interactions by regulating the expression of cellular integrins (64). However, in our experiments, these effects were not a factor because the cells were not directly treated with either glucose or reactive carbonyl compounds.

The weakening of cell-matrix interactions due to modification of specific arginine residues in matrix proteins by reactive carbonyl species provides a mechanism by which the "carbonyl stress" of diabetes may bring about effacement of glomerular epithelium and podocyte shedding characteristic of diabetic nephropathy (2). Interestingly, podocytes found in urine of rats with experimental diabetes were viable and could attach to collagen-coated plates (65). This suggests a primary role of inhibition of cell-matrix interactions in diabetes compared with cell death, at least for podocytes. Consequently, loss of cell contact with ECM can trigger apoptotic death (66). Similarly, weakening of cell-ECM interactions could cause the loss of endothelial cells in glomerular capillaries linked to impaired blood flow, development of renal ischemia, and progression of glomerular sclerosis (67). Integrin-mediated interactions with ECM may also play a key role in the regulation of cell growth (68). Within this context, modification of matrix components by reactive dicarbonyl compounds under diabetic conditions may facilitate migration and proliferation of mesangial cells, contributing to the expansion of mesangial matrix and the increase in glomerular volume.

The steady-state levels of MGO increase significantly in plasma of diabetic patients compared with normal subjects ranging from 0.4 to 2 μmol/l (8,9). In our in vitro study, the lowest effective MGO concentration was within this physiological range, i.e., 1 μmol/l in solid-phase integrin binding experiments after a 20-day exposure. It is important to note that in diabetes, proteins of glomerular matrix would be exposed to the elevated levels of reactive carbonyl compound for years before the appearance of the initial clinical signs of nephropathy. A long time needed for development of diabetic nephropathy, very slow turnover of matrix proteins, the fact that the concentration of circulating MGO correlates positively with duration of diabetes (8), and the presence of MGO- and 3-DG–derived arginine modifications in renal glomeruli of diabetic rats and in glomerular mesangial matrix of diabetic patients (23,25,69) support the notion that our proposed mechanism may be operable under diabetic conditions in vivo.

Our results also suggest that scavenging of reactive carbonyl compounds may protect integrin-mediated cell-matrix interactions, thus delaying or preventing the deterioration of kidney filtration function in diabetes. Interestingly, pyridoxamine, which protected cell adhesion and integrin binding in our experiments (Fig. 7), has also shown promising results in treatment of diabetic nephropathy. In animal studies, pyridoxamine inhibited diabetes-induced albuminuria and an increase in glomerular volume (70). In clinical trials, it significantly decreased the rate of serum creatinine accumulation in patients with overt nephropathy (71). The pyridoxamine protection of cell-matrix interactions in vivo is possible because its concentration in plasma of pyridoxamine-treated diabetic animals can be as high as 100 μmol/l (70), a significant molar excess over circulating MGO and other carbonyl compounds. Consequently, in diabetic animal models, pyridoxamine treatment decreased plasma MGO levels and specific MGO-derived protein modification (52,61). The reported therapeutic effects of pyridoxamine in diabetic patients and in animal models of diabetes further support our hypothesis that inhibition of cell adhesion by MGO and other dicarbonyl compounds may contribute to the pathogenesis of diabetic nephropathy.

ACKNOWLEDGMENTS

B.G.H. has received support from National Institute of Diabetes and Digestive and Kidney Disease Research Grant DK-065138. P.A.V. has received support from National Institute of Diabetes and Digestive and Kidney Disease Research Grant DK-066415.

We thank Dr. Ambra Pozzi for a gift of mouse glomerular mesangial cells and Anjana Dey for excellent technical assistance. We also thank Vanderbilt University for institutional support of the Proteomics Laboratory in the Mass Spectrometry Research Center through the Academic Venture Capital Fund.

REFERENCES

1. Mason RM, Wahab NA: Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol* 14:1358–1373, 2003
2. Nakamura T, Ushiyama C, Suzuki S, Hara M, Shimada N, Ebihara I, Koide H: Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol Dial Transplant* 15:1379–1383, 2000
3. Miner JH: Renal basement membrane components. *Kidney Int* 56:2016–2024, 1999
4. Haitoglou CS, Tsilibary EC, Brownlee M, Charonis AS: Altered cellular interactions between endothelial cells and nonenzymatically glucosylated laminin/type IV collagen. *J Biol Chem* 267:12404–12407, 1992
5. Anderson SS, Kim Y, Tsilibary EC: Effects of matrix glycation on mesangial cell adhesion, spreading and proliferation. *Kidney Int* 46:1359–1367, 1994
6. Bobbink IW, de Boer HC, Tekelenburg WL, Banga JD, de Groot PG: Effect of extracellular matrix glycation on endothelial cell adhesion and spreading: involvement of vitronectin. *Diabetes* 46:87–93, 1997
7. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48:1–9, 1999
8. McLellan AC, Thornalley PJ, Benn J, Sonksen PH: Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (Lond)* 87:21–29, 1994
9. Odani H, Shinzato T, Matsumoto Y, Usami J, Maeda K: Increase in three alpha,beta-dicarbonyl compound levels in human uremic plasma: specific in vivo determination of intermediates in advanced Maillard reaction. *Biochem Biophys Res Commun* 256:89–93, 1999
10. Wells-Knecht KJ, Lyons TJ, McCance DR, Thorpe SR, Feather MS, Baynes JW: 3-Deoxyfructose concentrations are increased in human plasma and urine in diabetes. *Diabetes* 43:1152–1156, 1994
11. Lapolla A, Flamini R, Dalla-Vedova A, Senesi A, Reitano R, Fedele D, Basso

E, Seraglia R, Traldi P: Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin Chem Lab Med* 41:1166–1173, 2003
12. Phillips SA, Mirrlees D, Thornalley PJ: Modification of the glyoxalase system in streptozotocin-induced diabetic rats: effect of the aldose reductase inhibitor Statil. *Biochem Pharmacol* 46:805–811, 1993
13. Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW: Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* 34:3702–3709, 1995
14. Hayashi T, Namiki M. In *Amino-Carbonyl Reactions in Food and Biological Systems*. Fujimaki M, Namiki M, Kato H, Eds. Tokyo, Kodansha, 1986, p. 29–35
15. Thornalley PJ, Langborg A, Minhas HS: Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344:109–116, 1999
16. Mlakar A, Spiteller G: Reinvestigation of lipid peroxidation of linolenic acid. *Biochim Biophys Acta* 1214:209–220, 1994
17. Wells-Knecht MC, Thorpe SR, Baynes JW: Pathways of formation of glycoxidation products during glycation of collagen. *Biochemistry* 34: 15134–15141, 1995
18. Phillips SA, Thornalley PJ: The formation of methylglyoxal from triose phosphates: investigation using a specific assay for methylglyoxal. *Eur J Biochem* 212:101–105, 1993
19. Thornalley PJ: Glyoxalase I: structure, function and a critical role in the enzymatic defence against glycation. *Biochem Soc Trans* 31:1343–1348, 2003
20. Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ: Binding and modification of proteins by methylglyoxal under physiological conditions: a kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. *J Biol Chem* 269:32299–32305, 1994
21. Chellan P, Nagaraj RH: Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes. *Arch Biochem Biophys* 368:98–104, 1999
22. Shamsi FA, Partal A, Sady C, Glomb MA, Nagaraj RH: Immunological evidence for methylglyoxal-derived modifications in vivo: determination of antigenic epitopes. *J Biol Chem* 273:6928–6936, 1998
23. Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A: Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375:581–592, 2003
24. Degenhardt TP, Thorpe SR, Baynes JW: Chemical modification of proteins by methylglyoxal. *Cell Mol Biol* 44:1139–1145, 1998
25. Niwa T, Katsuzaki T, Miyazaki S, Miyazaki T, Ishizaki Y, Hayase F, Tatemichi N, Takei Y: Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. *J Clin Invest* 99:1272–1280, 1997
26. Golej J, Hoeger H, Radner W, Unfried G, Lubec G: Oral administration of methylglyoxal leads to kidney collagen accumulation in the mouse. *Life Sci* 63:801–807, 1998
27. Ruoslahti E: RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 12:697–715, 1996
28. Plow EF, Haas TA, Zhang L, Loftus J, Smith JW: Ligand binding to integrins. *J Biol Chem* 275:21785–21788, 2000
29. Pedchenko V, Zent R, Hudson BG: Alpha(v)beta3 and alpha(v)beta5 integrins bind both the proximal RGD site and non-RGD motifs within noncollagenous (NC1) domain of the alpha3 chain of type IV collagen: implication for the mechanism of endothelia cell adhesion. *J Biol Chem* 279:2772–2780, 2004
30. Chen X, Moeckel G, Morrow JD, Cosgrove D, Harris RC, Fogo AB, Zent R, Pozzi A: Lack of integrin alpha1beta1 leads to severe glomerulosclerosis after glomerular injury. *Am J Pathol* 165:617–630, 2004
31. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 13:630–638, 2002
32. Steinitz M, Baraz L: A rapid method for estimating the binding of ligands to ELISA microwells. *J Immunol Methods* 238:143–150, 2000
33. Voziyan PA, Khalifah RG, Thibaudeau C, Yildiz A, Jacob J, Serianni AS, Hudson BG: Modification of proteins in vitro by physiological levels of glucose: pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation end-products through binding of redox metal ions. *J Biol Chem* 278:46616–46624, 2003
34. Voziyan PA, Metz TO, Baynes JW, Hudson BG: A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. *J Biol Chem* 277:3397–3403, 2002
35. Vanacore RM, Friedman DB, Ham AJ, Sundaramoorthy M, Hudson BG: Identification of S-hydroxylysyl211-methionine93 as the covalent crosslink of the noncollagenous (NC1) hexamer of the alpha1alpha1alpha2 collagen IV network: a role for the posttranslational modification of lysine211 to hydroxylysine211 in hexamer assembly. *J Biol Chem* 280:29300–29310, 2005.
36. Yates JR, Eng JK, McCormack AL, Schieltz D: Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem* 67:1426–1436, 1995
37. Hansen BT, Davey SW, Ham A-J L, Liebler DC: P-Mod: an algorithm and software to map modifications to peptide sequences using tandem MS data. *J Proteome Res* 4:358–368, 2005
38. Westwood ME, Argirov OK, Abordo EA, Thornalley PJ: Methylglyoxal-modified arginine residues: a signal for receptor-mediated endocytosis and degradation of proteins by monocytic THP-1 cells. *Biochim Biophys Acta* 1356:84–94, 1997
39. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG: Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N Engl J Med* 348:2543–2556, 2003
40. Kikkawa Y, Virtanen I, Miner JH: Mesangial cells organize the glomerular capillaries by adhering to the G domain of laminin alpha5 in the glomerular basement membrane. *J Cell Biol* 161:187–196, 2003
41. Madri JA, Roll FJ, Furthmayr H, Foidart JM: Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. *J Cell Biol* 86:682–687, 1980
42. Miner JH: Building the glomerulus: a matricentric view. *J Am Soc Nephrol* 16:857–861, 2005
43. Aumailley M, Gerl M, Sonnenberg A, Deutzmann R, Timpl R: Identification of the Arg-Gly-Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1. *FEBS Lett* 262:82–86, 1990
44. Sasaki T, Timpl R: Domain IVa of laminin alpha5 chain is cell-adhesive and binds beta1 and alphaVbeta3 integrins through Arg-Gly-Asp. *FEBS Lett* 509:181–185, 2001
45. Ahmed MU, Thorpe SR, Baynes JW: Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261:4889–4894, 1986
46. Oya T, Hattori N, Mizuno Y, Miyata S, Maeda S, Osawa T, Uchida K: Methylglyoxal modification of protein: chemical and immunochemical characterization of methylglyoxal-arginine adducts. *J Biol Chem* 274: 18492–18502, 1999
47. Pierschbacher MD, Ruoslahti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309:30–33, 1984
48. Cherny RC, Honan MA, Thiagarajan P: Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion. *J Biol Chem* 268:9725–9729, 1993
49. Vandenberg P, Kern A, Ries A, Luckenbill-Edds L, Mann K, Kuhn K: Characterization of a type IV collagen major cell binding site with affinity to the alpha 1 beta 1 and the alpha 2 beta 1 integrins. *J Cell Biol* 113:1475–1483, 1991
50. Eble JA, Golbik R, Mann K, Kuhn K: The alpha 1 beta 1 integrin recognition site of the basement membrane collagen molecule [alpha 1(IV)]2 alpha 2(IV). *EMBO J* 12:4795–4802, 1993
51. Metz TO, Alderson NL, Thorpe SR, Baynes JW: Pyridoxamine, an inhibitor of advanced glycation and lipoxidation reactions: a novel therapy for treatment of diabetic complications. *Arch Biochem Biophys* 419:41–49, 2003
52. Nagaraj RH, Sarkar P, Mally A, Biemel KM, Lederer MO, Padayatti PS: Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal. *Arch Biochem Biophys* 402:110–119, 2002
53. Chelberg MK, Tsilibary EC, Hauser AR, McCarthy JB: Type IV collagen-mediated melanoma cell adhesion and migration: involvement of multiple, distinct domains of the collagen molecule. *Cancer Res* 49:4796–4802, 1989
54. Herbst TJ, McCarthy JB, Tsilibary EC, Furcht LT: Differential effects of laminin, intact type IV collagen, and specific domains of type IV collagen on endothelial cell adhesion and migration. *J Cell Biol* 106:1365–1373, 1988
55. Kramer RH, Marks N: Identification of integrin collagen receptors on human melanoma cells. *J Biol Chem* 264:4684–4688, 1989
56. Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ: The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 275:35–40, 2000
57. Graf J, Ogle RC, Robey FA, Sasaki M, Martin GR, Yamada Y, Kleinman HK:

A pentapeptide from the laminin B1 chain mediates cell adhesion and binds the 67,000 laminin receptor. *Biochemistry* 26:6896–6900, 1987
58. Yi XY, Wayner EA, Kim Y, Fish AJ: Adhesion of cultured human kidney mesangial cells to native entactin: role of integrin receptors. *Cell Adhes Commun* 5:237–248, 1998
59. Paul RG, Bailey AJ: The effect of advanced glycation end-product formation upon cell-matrix interactions. *Int J Biochem Cell Biol* 31:653–660, 1999
60. McCarthy AD, Uemura T, Etcheverry SB, Cortizo, AM: Advanced glycation endproducts interfere with integrin-mediated osteoblastic attachment to a type-I collagen matrix. *Int J Biochem Cell Biol* 36:840–848, 2004
61. Iacovella G, Alderson NL, Chachich M, Thorpe SR, Baynes JW: Pyridoxamine decreases levels of reactive alpha-dicarbonyls in plasma of zdf diabetic rats. *Diabetes* 52:A187, 2003
62. Grandhee SK, Monnier VM: Mechanism of formation of the Maillard protein cross-link pentosidine: glucose, fructose, and ascorbate as pentosidine precursors. *J Biol Chem* 266:11649–11653, 1991
63. Biemel KM, Friedl DA, Lederer MO: Identification and quantification of major maillard cross-links in human serum albumin and lens protein: evidence for glucosepane as the dominant compound. *J Biol Chem* 277:24907–24915, 2002
64. Kitsiou PV, Tzinia AK, Stetler-Stevenson WG, Michael AF, Fan WW, Zhou B, Tsilibary EC: Glucose-induced changes in integrins and matrix-related functions in cultured human glomerular epithelial cells. *Am J Physiol Renal Physiol* 284:F671–F679, 2003
65. Petermann AT, Pippin J, Krofft R, Blonski M, Griffin S, Durvasula R, Shankland SJ: Viable podocytes detach in experimental diabetic nephropathy: potential mechanism underlying glomerulosclerosis. *Nephron Exp Nephrol* 98:e114–e123, 2004
66. Frisch SM, Ruoslahti E: Integrins and anoikis. *Curr Opin Cell Biol* 9:701–706, 1997
67. Endelmann DH, Schiffrin EL: Endothelial dysfunction. *J Am Soc Nephrol* 15:1983–1992, 2004
68. Giancotti FG, Mainiero F: Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim Biophys Acta* 1198:47–64, 1994
69. Niwa T, Katsuzaki T, Ishizaki Y, Hayase F, Miyazaki T, Uematsu T, Tatemichi N, Takei Y: Imidazolone, a novel advanced glycation end product, is present at high levels in kidneys of rats with streptozotocin-induced diabetes. *FEBS Lett* 407:297–302, 1997
70. Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR, Baynes JW: Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61:939–950, 2002
71. Williams ME, Bolton WK, Degenhardt TP, Schotzinger R: A phase 2 clinical trial of pyridoxamine (Pyridorin™) in type 1 and type 2 diabetic patients with overt nephropathy (pyr-206). *J Am Soc Nephrol* 14:7A, 2003