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

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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 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 α3β1 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, α3β1 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

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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 α,β, and α,β-integrins were purchased from Chemicon (Temecula, CA); α2- and β1-integrin monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA); fibronectin, pyridoxamine hydrochloride, GLA, and MGO were obtained from Sigma-Aldrich (St. Louis, MO); α-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.

**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
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 electrophile 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 NH2-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. 3A, inset). However, this modification did not affect endothelial cell adhesion (Fig. 3A). Similarly, modification of full-length collagen IV in the presence of glucose, as indicated by the extensive conversion of lysine residues to CML (Fig. 3B, inset), had no effect on cell adhesion (Fig. 3B).

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. 4A). 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. 4A). These results suggest that the arginine residue of RGD motif within

![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.](image1)

![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.](image2)
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. 4B). 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: Arg78, Arg101, and Arg202 (data not shown). However, because all of these residues are located within α3NC1 domain, their modification by MGO had no effect on cell adhesion (Fig. 4A). No modifications were found on the rest of arginines: Arg6, Arg12, Arg41, Arg68, Arg109, Arg171, Arg181, and Arg205, probably because of steric hindrance in the context of natively folded RGD-α3NC1 protein (data not shown). Proteolytic peptides containing Arg202 have not been detected (data not shown).

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 αβ3 is a member of an extended family of integrins, cell surface receptors that mediate cell-matrix interactions. As with a number of other integrins, αβ3 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, αβ3 demonstrated strong binding to RGD-α3NC1, whereas virtually no binding to α3NC1 domain was detected (Fig. 6A, inset). Thus, any effect on αβ3 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. 6A). In fact, after a 20-day incubation, a significant effect was already apparent at 1 μmol/L MGO (Fig. 6A), 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

TABLE 1
Effect of BSA-Amadori on endothelial cell adhesion

<table>
<thead>
<tr>
<th>Additive</th>
<th>Cell adhesion (A295)</th>
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<tr>
<td></td>
<td>10 days</td>
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<tr>
<td>Buffer</td>
<td>1.080 ± 0.089</td>
</tr>
<tr>
<td>BSA</td>
<td>1.019 ± 0.032</td>
</tr>
<tr>
<td>BSA-Amadori</td>
<td>1.139 ± 0.103</td>
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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.
protein intermediates, glyoxal and GLA, also inhibited αβ3 binding, albeit less efficiently (Fig. 6A, inset).

The interaction of collagen IV with the various cell types is mediated mainly via binding of the integrins α1β1 and α2β1 to the specific site in the triple-helical part of the molecule (49). Moreover, it has been demonstrated that Arg461 within the α2 chain is essential for binding of α1β1 integrin (50). Modification of EHS collagen IV by MGO inhibited binding of purified α1β1 integrin to collagen IV (Fig. 6B). 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 αβ3 binding (Fig. 7A and B). This protection depended on pyridoxamine concentration and was already significant at equimolar concentrations of MGO and pyridoxamine (Fig. 7B).

**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 α1β1 and α2β1 integrins (55). Subsequently, Asp461 in
the \(\alpha_1(IV)\) chain and an Arg\(^{461}\) in the \(\alpha 2(IV)\) 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 \(\alpha_1\beta_1\) integrin–mediated cell adhesion to collagen IV (56). Moreover, the same sequence exists in \(\alpha 1\) chain of collagen IV (residues 385–390), where it may serve as a binding site for \(\alpha_1\beta_1\) 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 \(\alpha 5\) 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-\(\alpha 3\)NC1 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. 6.** Binding of \(\alpha_1\beta_3\) integrin to RGD-\(\alpha 3\)NC1 and \(\alpha_1\beta_1\) integrin to mouse collagen IV. A: Plates coated with RGD-\(\alpha 3\)NC1 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 \(\alpha_1\beta_1\) 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-\(\alpha 3\)NC1 were preincubated alone (C) or with MGO, glyoxal, or GLA (0.5 mmol/l) at 37°C for 24 h, and binding of \(\alpha_1\beta_1\) integrin was determined. Binding of \(\alpha_1\beta_3\) integrin to \(\alpha 3\)NC1 was also determined. \(\ast P<0.05\), glyoxal, GLA, or MGO vs. control; \(\ast\ast P<0.05\), glyoxal or GLA vs. MGO.

**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-\(\alpha 3\)NC1 were preincubated with or without MGO (0.5 mmol/l) or with MGO and different concentrations of pyridoxamine. Binding of \(\alpha_1\beta_3\) integrin was determined in a solid-phase binding assay. \(\ast P<0.05\), MGO ± pyridoxamine vs. control; \(\ast\ast P<0.05\), MGO + pyridoxamine vs. MGO.

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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 constraints 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.

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