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Glucose Autoxidation Induces Functional Damage to Proteins via Modification of Critical Arginine Residues

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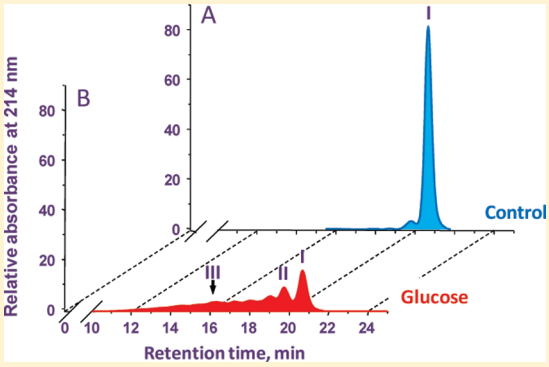
Supporting Information

ABSTRACT: Nonenzymatic modification of proteins in hyperglycemia is a major mechanism causing diabetic complications. These modifications can have pathogenic consequences when they target active site residues, thus affecting protein function. In the present study, we examined the role of glucose autoxidation in functional protein damage using lysozyme and RGD-α3NC1 domain of collagen IV as model proteins *in vitro*. We demonstrated that glucose autoxidation induced inhibition of lysozyme activity as well as NC1 domain binding to $\alpha_V\beta_3$ integrin receptor via modification of critical arginine residues by reactive carbonyl species (RCS) glyoxal (GO) and methylglyoxal while nonoxidative glucose adduction to the protein did not affect protein function. The role of RCS in protein damage was confirmed using pyridoxamine which blocked glucose autoxidation and RCS production, thus protecting protein function, even in the presence of high concentrations of glucose. Glucose autoxidation may cause protein damage *in vivo* since increased levels of GO-derived modifications of arginine residues were detected within the assembly interface of collagen IV NC1 domains isolated from renal ECM of diabetic rats. Since arginine residues are frequently present within protein active sites, glucose autoxidation may be a common mechanism contributing to ECM protein functional damage in hyperglycemia and oxidative environment. Our data also point out the pitfalls in functional studies, particularly in cell culture experiments, that involve glucose treatment but do not take into account toxic effects of RCS derived from glucose autoxidation.



Diabetes is one of the major human diseases, now frequently referred to as the diabetes epidemic.[1] Chronic diabetes leads to serious complications such as neuropathy, retinopathy, microvascular disease, atherosclerosis, and diabetic nephropathy.[2−5] In susceptible individuals, the severity of diabetic complications correlates with the severity of hyperglycemia, suggesting that these complications are triggered by the elevation in glucose levels.[6] The mechanisms underlying susceptibility and tissue damage are unknown.

A widely accepted hypothesis is that hyperglycemia accelerates the nonenzymatic chemical modification of proteins, termed advanced glycation end products (AGEs), which perturb protein function, thus contributing to diabetic complications. In hyperglycemia, levels of reactive carbonyl species (RCS) such as glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG) can be elevated by several potential mechanisms including glucose autoxidation, glycoxidation of glucose−protein adducts, and impaired glucose metabolism in the cell.[7,8] Unlike glucose which modifies lysine residues, RCS react preferentially with arginine as well as lysine residues with high reaction rates.[9] Physiological RCS such as GO and MGO may play an important role in pathogenesis because of high abundance of arginine residues within protein active sites.[10] Long-lived extracellular matrix (ECM) proteins would be particularly susceptible to RSC-induced functional damage through modification of the active site arginine residues in the ECM.[11,12]

The rate of MGO formation from intracellular metabolism is very high; however, most of metabolically derived MGO is metabolized by the cellular carbonyl detoxification mechanisms.[10] In hyperglycemia, this metabolically derived MGO has been implicated in modification and functional impairment of a number of proteins.[12−15] In the extracellular diabetic milieu other mechanisms, including autoxidation of glucose and protein glycation intermediates, may also contribute to specific RCS-derived protein damage (see Scheme 1). In this case, GO may play an important role in protein damage since it is a major product of glucose autoxidation.[7,16]

The focus of previous studies involving functional protein damage from glucose autoxidation has been on free radical mechanisms.[17−20] In the present study, we examined the role

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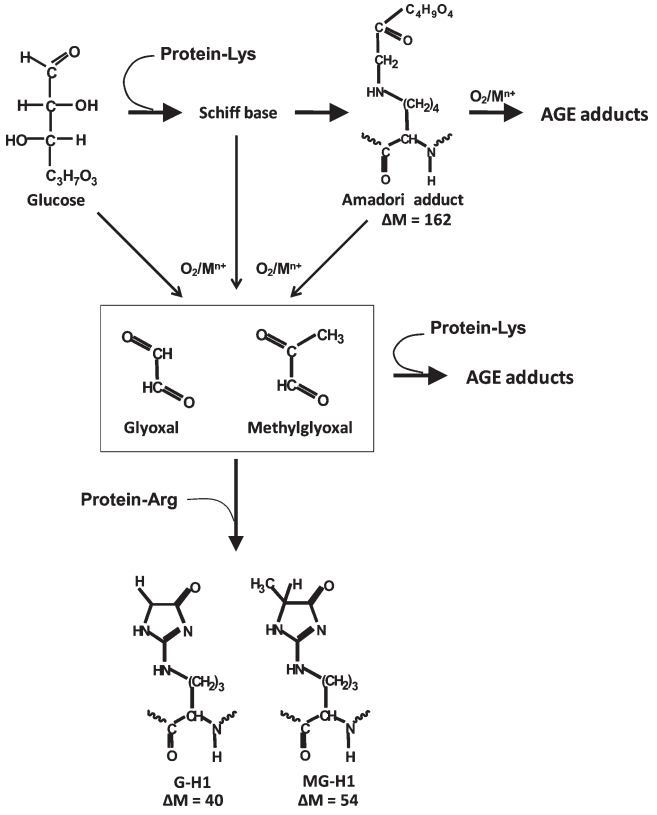
Scheme 1. Schematic Presentation of RCS Pathways Originated from Glucose and Glycated Protein Intermediates[a]



[a] Glucose can directly modify proteins whereby its aldehyde group reacts with the ε-amino groups of lysine residues and N-terminal α-amino groups of proteins, forming a Schiff base that rearranges to an Amadori intermediate. This intermediate undergoes further rearrangements to yield heterogeneous AGEs. Furthermore, hyperglycemia elevates the levels of reactive carbonyl species, such as methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglycosone (not shown), which preferentially modify protein arginine residues.

of GO and MGO in the mechanism of functional protein damage induced by glucose autoxidation. Using lysozyme and recombinant RGD-α3NC1 domain of collagen IV as model proteins, we demonstrated that glucose inhibited lysozyme activity and NC1 domain binding to integrin receptor. The inhibition was due to modification of critical arginine residues by GO and MGO which were continuously generated from glucose under oxidative conditions. This glucose autoxidation was required for functional damage since nonoxidative glucose adduction did not affect protein functionality. Because arginine residues are present at high frequencies within active sites of a variety of proteins, this may be a common mechanism of ECM protein functional damage which operates in hyperglycemia accompanied by oxidative conditions.

EXPERIMENTAL PROCEDURES

Materials. D(+)-Glucose was purchased from Gibco. Lysozyme, pyridoxamine dihydrochloride, DTPA, glyoxal, and methylglyoxal were purchased from Sigma-Aldrich. 2,3-Diaminonaphthalene (DAN) was purchased from Acros. 3-Deoxyglucosone and D-[1-^{13}C]glucose (99 atom % ^{13}C) was kindly provided by Dr. Anthony Serianni (University of Notre Dame). Bacterial collagenase (CLSPA) was purchased from Worthington (Lakewood, NJ). All other chemicals were purchased from Sigma-Aldrich and Fisher.

Quantitation of Reactive Carbonyl Species (RCS). Glucose alone (30 and 100 mM) or in the presence of pyridoxamine (1 mM) or DTPA (1 mM) was incubated in the dark in 0.15 M sodium phosphate buffer in aseptic conditions at 37 °C for up to 40 days. Control samples were immediately snap-frozen in liquid nitrogen without incubation and stored at −70 °C. Other samples were frozen upon completion of incubation and stored at −70 °C. Three major products of glucose autoxidation—methylglyoxal, glyoxal, and 3-deoxyglucosone—were assayed by reverse-phase HPLC with precolumn derivatization with 2,3-diaminonaphthalene (DAN) as previously described with some changes.[21,22] Briefly, 0.1 mL of RCS-containing sample was added to 1 mL of PBS followed by 0.1 mL of 0.1% DAN solution in methanol. The derivatization reactions were carried out overnight at 4 °C followed by the injection of a 20 μL aliquot onto Symmetry C_{18} column (Waters, Milford, MA). The LC solvents were (A) 0.5% formic acid and (B) 0.25% formic acid in acetonitrile. The column was eluted with a gradient (20% B for 1 min; 20−80% B in 7 min; 80% B for 4 min; 80−20% B in 1 min; 20% B for 10 min) at a flow rate of 1 mL/min. The LC effluent was monitored with fluorescent detector (excitation at 271 nm and emission at 503 nm). The identity of dicarbonyl compounds was determined by coelution with GO-, MGO-, and 3DG-derived quinoxaline standards and confirmed by mass spectrometry using selected reaction monitoring mode (Figures S1−S3). Glucose did not interfere with measurements of GO and MGO. However, significant amounts of 3-DG were detected in control samples indicating 3-DG formation during analytical procedures. Therefore, 3-DG results were excluded from further quantitative analyses. Concentrations of GO and MGO in the reaction mixture were calculated using calibration standards; for GO and MGO standards, calibration was linear in the range of 0−100 μM. Limits of detection for GO and MGO calculated using signal-to-noise method according to the ICH Q2B guidelines were 96.5 and 11.5 pmol, respectively. Interbatch coefficient of variation was 18% (n = 3) and 14% (n = 6) for GO and MGO, respectively.

Modification of Lysozyme by Glucose or RCS *in Vitro*. All incubations were carried out in 0.15 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide to prevent bacterial growth. Solutions were incubated in the dark at 37 °C for 40 days. Lysozyme (8 mg/mL) was incubated with various amounts of D-glucose in the presence or absence of either PM or DTPA. Incubations with either MGO or GO were carried out under the same conditions. Aliquots were removed at 0, 20, and 40 days and stored at −80 °C until analysis.

Analysis of Glucose-Modified Lysozyme by FPLC and ESI-MS. Glucose-modified lysozyme was analyzed using Mono S 5/50 GL column (GE Lifescience). To exchange the buffer and to remove the excess of glucose and PM from lysozyme, samples were desalted using PD-10 columns (GE Lifescience). Protein concentration was assayed with BCA (Pierce), and samples were diluted with 20 mM sodium phosphate buffer, pH 6.8 (buffer A), to a final protein concentration of 1 mg/mL prior to injection. Sample aliquots (20 μL) were injected onto Mono S column and eluted with linear gradient of NaCl in the same buffer (buffer B:

0.4 M NaCl in 20 mM sodium phosphate buffer, pH 6.8). The elution profile was monitored at 214 and 280 nm.

Electrospray ionization mass spectrometry (ESI-MS) was performed by direct infusion of lysozyme samples at 10 μL/min flow rate using a built-in syringe pump of Thermo LTQ ion trap mass spectrometer. Lysozyme samples were diluted in 50% methanol supplemented with 0.5% acetic acid.

Analysis of Glucose-Modified Lysozyme Using Phenylboronate−UPLC. Lysozyme (8 mg/mL) was incubated with glucose in the presence of 1 mM DTPA to inhibit metal-ion-catalyzed oxidative reactions. After 15 day incubation, free glucose was removed by five rounds of centrifugal ultrafiltration (Amicon Ultra, 10K cutoff) at 4 °C. The resulting samples were analyzed using phenylboronate−UPLC and the previously described protocol with some modifications.[23] A Tricon HPLC column (5 mm × 100 mm) was packed with 1 mL of boronate affinity gel (Pierce). Glycated lysozyme (0.1 mg) was analyzed using the packed boronate affinity column and Acquity UPLC system (Waters). The solvents were (A) 250 mM ammonium acetate and 50 mM $MgCl_2$, pH 8.1, and (B) 0.1 M acetic acid. A gradient (0% B for 5 min; 0−100% B in 0.1 min; 100% B for 10 min; 100−0% B in 0.1 min; 0% B for 10 min) was used to separate glycated from non-glycated lysozyme at a flow rate of 0.5 mL/min. The elution profile was monitored at 280 nm with a UV detector.

Following multiple chromatographic runs, the corresponding native lysozyme and lysozyme−Amadori fractions were combined, concentrated by centrifugal ultrafiltration at 4 °C, and diluted with water for activity measurements. In the preliminary experiments, exposure of lysozyme to 0.1 M acetic acid for several hours followed by concentration and dilution with water did not affect enzymatic activity (data not shown).

Determination of Lysozyme Activity. The enzymatic activity of lysozyme was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* cells according to Shugar.[24] Samples containing lysozyme were diluted to 17 μg/mL in distilled water and kept on ice. An aliquot of 100 μL of diluted lysozyme was mixed with a 900 μL aliquot of a fresh suspension of 0.3 mg/mL *Micrococcus lysodeikticus* cells in 0.1 M potassium phosphate buffer, pH 7.0. The rate of lysis was determined by measuring the change in absorbance at 450 nm every 15 s for 2 min (ΔA_{450}).

Modification of Collagen IV RGD-α3NC1 Domain and Integrin Binding Assay. Modification of collagen IV with glucose was performed as previously described.[25] The 96-well plates were coated with recombinant RGD-α3NC1 overnight at 4 °C. Plates were washed with 150 mM sodium phosphate buffer and incubated in the same buffer with or without D-glucose alone or D-glucose plus either PM or DTPA. Incubations were carried out for 30 days in the dark at 37 °C in the wet chamber to prevent evaporation.

For solid-phase integrin binding assay, plates were washed and blocked with 1% BSA/TBS for 2 h at 30 °C as previously described.[26] Purified $\alpha_V\beta_3$ integrin was overlaid in binding buffer (TBS, 0.1% BSA, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, 5 mM octylglucoside) and incubated for 2 h at 30 °C. The plates were washed three times with washing buffer (TBS, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.01% Tween 20) and incubated with anti-α_v integrin antibodies (P2W7, 1:500) for 1 h. After extensive washing, the bound antibodies were detected using alkaline phosphatase-conjugated antimouse IgG antibodies. *p*-Nitrophenyl phosphate substrate (Sigma) was added to the wells, and absorbance was measured at 410 nm. Nonspecific binding obtained by preincubation of purified integrin with 10 mM EDTA for 30 min at 4 °C was subtracted from all of the obtained values.

Animal Experiments. The principles of laboratory animal care were followed according to institutional IACUC guidelines. Male Sprague−Dawley rats (Charles River, Wilmington, MA) were housed in a 12 h light: dark cycle and temperature controlled animal facility. Standard food and water were available *ad libitum*. Animals were randomly assigned to either control or diabetic groups. Diabetes was induced with intravenous administration of streptozotocin (Sigma, St. Louis, MO), 60 mg/kg of body weight, in a saline solution. Control rats were injected only with saline solution. Diabetes was confirmed if serum glucose concentration was at least 250 mg/dL on two consecutive days, with glucose testing beginning on the second day post STZ administration. Thereafter, in the diabetic animals, serum glucose was measured three times per week for 12 weeks using the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Palo Alto, CA).

Isolation of ECM Preparations Enriched in Collagen IV NC1 Domains. Extracellular matrix proteins were isolated from the kidneys of control rats, STZ-diabetic rats, and STZ-diabetic rats treated with PM as previously described,[27] with minor modifications. Rat kidneys were homogenized in protease inhibitor cocktail with a homogenizer, and washed three times with inhibitors (precipitated by centrifugation at 8000*g* for 10 min). The pellet was washed three times with the same solution and then suspended in 0.05% sodium azide and shaken for 1 h on ice and precipitated by centrifugation at 8000*g* for 10 min. The pellet was washed with the same solution and then suspended in 1 M NaCl containing 200 Kunitz units/mL DNase, 2 mM phenylmethylsulfonyl fluoride, and 0.1% protease inhibitors and gently shaken for 90 min at room temperature. The solution was precipitated by centrifugation at 8000*g* for 10 min, and the pellet resuspended in 1% sodium deoxycholate and gently shaken for 1 h on ice. The resulting pellet was washed three times with distilled water. Detergent-prepared basement membranes from rat kidneys were solubilized by digestion with bacterial collagenase as previously described.[28] Collagenase digest was dialyzed against 50 mM Tris-HCl, pH 7.5, and passed through a DEAE column, and a pass-through fraction was collected. The presence of NC1 domains was confirmed using SDS-PAGE with highly purified NC1 domains as standards.

LC-MS/MS Analysis of Protein Modifications *in Vitro* and *in Vivo*. The site-specific modifications in lysozyme or in partially purified collagen IV NC1 domains from rat kidneys were analyzed by tandem mass spectrometry. The samples were prepared using the spin filter protocol for proteomic analysis.[29] Lysozyme samples were digested with chymotrypsin; renal collagen IV NC1 domain preparations were digested with trypsin, chymotrypsin, or elastase to improve sequence coverage. The data-dependent scanning was performed using a LTQ Orbitrap (Thermo Fischer Scientific, San Jose, CA) mass spectrometer equipped with an Eksigent AS1 autosampler and an Eksigent 1D+ HPLC pump attached directly to the instrument's nanospray source. The peptides were separated on a capillary tip, 100 μm × 18 cm, packed with C_{18} resin (Jupiter C_{18}, 3 μm, 300 Å, Phenomenex, Torrance, CA) using an inline vented trapping column that was 100 μm × 6 cm. The flow rate during the solid phase extraction phase of the gradient was 2.5 μL/min, and during the separation phase it was 500 nL/min. Mobile phase A was 0.1% formic acid, while mobile phase B was acetonitrile with 0.1% formic acid. A 95 min gradient was performed with a 15 min washing period (100% A for the

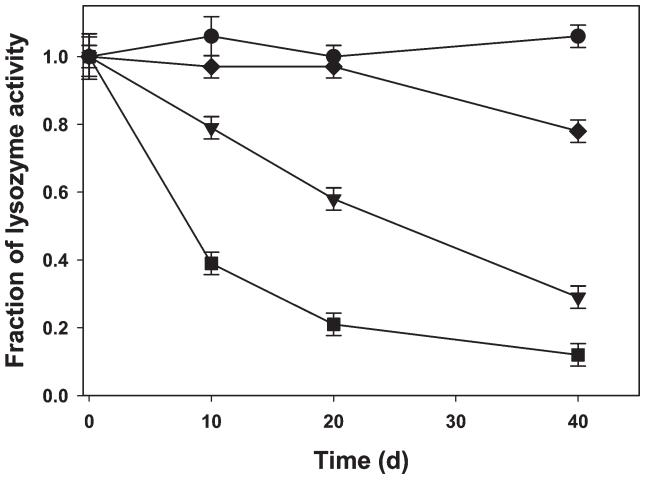


Figure 1. Inhibition of lysozyme activity in the presence of glucose. Lysozyme was incubated alone (circles), with 5 mM glucose (diamonds), 30 mM glucose (triangles), or 100 mM glucose (squares) in 0.15 M Na-phosphate buffer at 37°C for 40 days. Lysozyme activity was determined as described under Experimental Procedures.

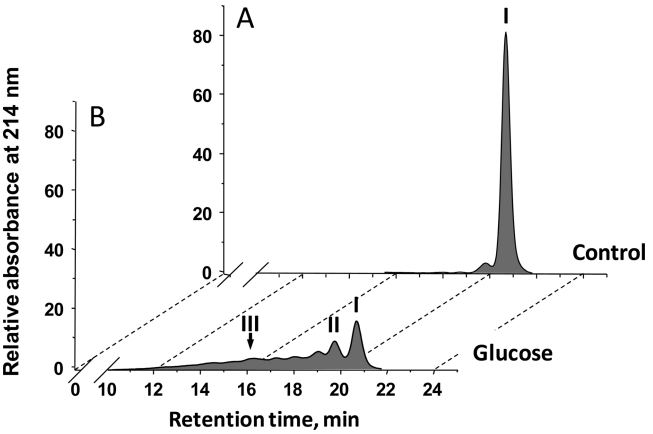


Figure 2. Ion-exchange FPLC analysis of native and glucose-modified lysozyme. Lysozyme was incubated alone (A) or with 100 mM glucose (B) in 0.15 M Na-phosphate buffer, pH 7.4, at 37 °C for 15 days and fractionated using Mono S column. Peaks I, II, and III were assayed for lysozyme activity (see Table 1) and by direct infusion electrospray mass spectrometry (see Figure 3) as described under Experimental Procedures.

first 10 min followed by a gradient to 98% A at 15 min) to allow for removal of any residual salts. After the initial washing period, a 60 min gradient was performed in which the first 35 min was a slow, linear gradient from 98% A to 75% A, followed by a faster gradient to 10% A at 65 min and an isocratic phase at 10% A at 75 min. MS/MS spectra of the peptides were obtained using data-dependent scanning in preview mode, which consisted of one full MS spectrum (mass range of 400−2000 amu) followed by five MS/MS spectra.

The data generated from the data-dependent LC-MS/MS experiments were analyzed using Sequest.[30] Peptides were matched based on the theoretical digestion of the known protein sequences. Searches were also performed for specific modifications, such as G-H1, MG-H1, CML, and Amadori intermediate; the peptide identities were confirmed by manual analysis of MS/MS spectra. For *in vitro* experiments, peptide m/z were included in the list for targeted analysis (Table S1). The whole range of LC profile was divided by several segments 2−5 min wide, and the mass spectrometer was set to monitor full m/z range followed by 10−14 (one scan in full range 400−2000 m/z) values depending on retention time for particular peptide with an isolation window of 3 m/z. The peak containing the peptide of interest was extracted from the chromatogram, and the area of the peak was determined using Xcalibur software. The area of the peak containing modified peptide was normalized to a reference peptide that did not contain any modification as previously described.[31]

^{13}C NMR Experiments. 30 mM D-[1-^{13}C]glucose was incubated in 150 mM sodium phosphate buffer (pH 7.5) containing 10% D_2O, 0.02% sodium azide, and 1% TSP either alone or with 5 mM PM at 37 °C for 40 days in the dark. Relative intensity of α-pyranose and β-pyranose signals was measured on a Bruker 400 MHz NMR spectrometer at 27 °C, collecting 1024 transients per spectrum. Prior to the initial measurement at 0 days, glucose solutions were incubated for 4 h at 37 °C to allow for anomeric equilibrium.[32]

Statistical Analyses. Data were expressed as means ± S.D., and statistical analysis was performed using Student's t test for unpaired samples or ANOVA followed by posthoc Student−Newman−Keuls comparisons. Differences were considered statistically significant if p values were less than 0.05.

RESULTS

Inactivation of Lysozyme in the Presence of Glucose. Upon incubation with either 30 or 100 mM glucose, enzymatic activity of lysozyme was significantly inhibited in time- and concentration-dependent fashion (Figure 1). To investigate the mechanisms whereby glucose inhibits lysozyme activity, we analyzed protein samples using cation-exchange FPLC (Figure 2). The native protein was eluted as a major single peak at about 21 min (over 95% of the protein) and retained full enzymatic activity (Figure 2A, peak I, and Table 1). In the samples incubated with glucose, only 27% of the protein was eluted at this retention time (Figure 2B, peak I). The multiple modified lysozyme species were characterized by the reduced retention time and reduced specific activity (Figure 2B, representative peaks II and III, and Table 1). In all samples, over 90% of the total lysozyme was eluted from the column indicating minimal protein loss upon chromatography (data not shown).

Effects of Glucose-Derived RCS and Amadori Products on Lysozyme Activity. To determine glucose-induced lysozyme modifications that may cause the observed loss of enzymatic activity, peaks I, II, and III (Figure 2) were analyzed by direct sample infusion into ESI-linear ion-trap mass spectrometer. The MS spectrum of the control sample demonstrated single predominant species corresponding to molecular weight of native lysozyme (Figure 3A). Chromatographic peak I from glucose-treated samples contained additional modified species with mass differences of +162 amu and +(2 × 162) amu, corresponding to either one or two lysozyme−Amadori adducts (Figure 3B). Nevertheless, the protein in peak I retained full enzymatic activity (Table 1). Chromatographic peak II from glucose-treated samples contained other multiple modified species in addition to Amadori adducted protein (Figure 3C). The prominent modified species with the mass differences of +40 and +54 amu were consistent with the presence of GO−protein (G-H1) and MGO−protein (MG-H1) adducts, respectively (Figure 3C). The species with

Table 1. Specific Activity of Different Lysozyme Fractions Isolated Using Ion-Exchange FPLC[a]

	peak I (U/mg ± SD × 10^{-3})	peak II (U/mg ± SD × 10^{-3})	peak III (U/mg ± SD × 10^{-3})	area of peak I (% total)
control	51.96 ± 2.73			95
glucose	55.83 ± 1.31	14.15 ± 1.07	0.36 ± 0.12	27

[a] Lysozyme was incubated in 0.15 M Na−phosphate buffer, pH 7.4, at 37 °C for 15 days: alone (control) and with 100 mM glucose (glucose). Peaks I, II, and III (see Figure 2) were assayed for specific activity of lysozyme (units per mg of protein, U/mg) as described under Experimental Procedures.

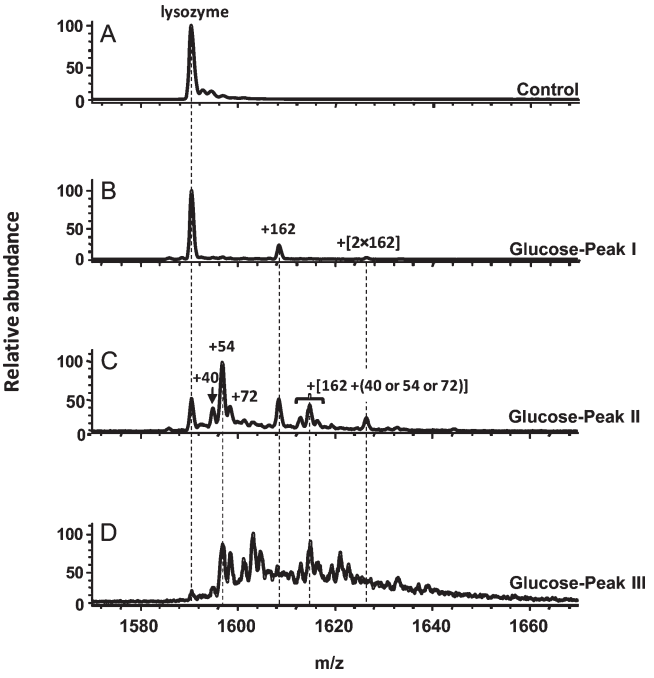


Figure 3. ESI-MS analysis of native and glucose-modified lysozyme. Lysozyme was incubated as described in Figure 2. Peaks I, II, and III (see Figure 2) were analyzed by direct infusion ESI-MS. An ion with +9 charge state was used for sample analyses. Peaks corresponding to the masses of modified protein are indicated by the numbers correspondent to characteristic mass differences of G-H1 (+40 amu), MG-H1 (+54 amu), CEL or dihydroimidazolone (+72 amu), and Amadori (+162 amu). The mass differences were rounded to the closest integer.

the mass difference of +72 indicated the presence of other MGO-derived adducts, dihydroimidazolone, a product of MG-H1 hydration and/or CEL[33] (Figure 3C). Lysozyme enzymatic activity in this peak was significantly diminished (Table 1). Protein in chromatographic peak III underwent further complex modification, while retaining major RCS-derived species, and was completely inactive (Figure 3D and Table 1). Thus, RCS-derived lysozyme modifications, but not Amadori protein adducts, cause the loss of lysozyme activity.

To further investigate the role of Amadori adducts, samples of lysozyme−Amadori were prepared with either 30 or 100 mM glucose. The samples contained approximately 3% and 17% of modified protein, respectively, as determined by phenylboronate−HPLC, yet both were as active as unmodified lysozyme (Figure 4A,B). We then isolated native (Figure 4B, retention time ∼1 min) and Amadori (Figure 4B, retention time ∼12.5 min) fractions from lysozyme−Amadori sample prepared with 100 mM glucose and compared their enzymatic activities. As shown in Figure 4C, there was no significant difference in specific activity between these two fractions. These data confirmed that direct glucose adduction to lysozyme and formation of lysozyme−Amadori product does not affect enzymatic activity.

Generation of RCS from Glucose under Oxidative Conditions. We explored a possibility that RCS produced upon glucose oxidation may be responsible for enzyme inactivation observed in Figure 1. Glucose autoxidation and formation of RCS have been previously characterized in significant detail.[7,16,34,35] We confirmed formation of these species under our experimental conditions that caused enzyme inactivation (see Figure 1). Incubation of either 30 or 100 mM glucose for 40 days under oxidative conditions resulted in generation of multiple dicarbonyl compounds including three major physiologically relevant species which have been identified as GO, MGO, and 3-DG using RP-HPLC with precolumn derivatization (Figure 5A,B) and confirmed by tandem mass spectrometry (Figures S1−S3). Concentrations of GO and MGO formed upon glucose degradation under our experimental conditions are shown in Table 2. We also detected several chromatographic peaks indicating the presence of other potential dicarbonyl species generated upon glucose degradation (Figure 5). The identity of these species and their physiological relevance is yet unknown.

When glucose was incubated in the presence of either DTPA or PM, compounds which can block oxidative pathways via sequestration of catalytic metal ions,[36,37] formation of GO and MGO was dramatically inhibited (Figure 5).

Even though PM can directly scavenge GO and MGO,[38,39] it appears that sequestration of metal ions is sufficient to inhibit RCS formation from glucose since DTPA cannot scavenge RCS (data not shown). PM can also potentially interact with glucose;[40] however, this interaction is unlikely to have significant effect on glucose levels, and the rate of RCS formation since molar concentration of glucose in our experiments was 6−20-fold higher than that of PM and because of glucose rapid anomeric equilibrium (half-time ∼7 min at 37 °C[32]). To confirm that glucose levels are not significantly affected by PM, we performed ^{13}C NMR studies using D-[1-^{13}C]glucose. There was no detectable change in the amount of two major cyclic forms, α- and β-glucopyranoses, after 40 day incubation with PM (Figure S4).

Next, we determined whether RCS can inhibit lysozyme activity. When added directly to lysozyme solutions, both GO and MGO inhibited enzymatic activity but only at relatively high concentrations (Figure S5). MGO was more effective at inhibiting lysozyme activity with maximum inhibition evident after 18 h of incubation (Figure S5A). However, after 20 day incubations the degree of inhibition by GO and MGO was similar (Figure S5B) and did not change after 40 day incubations (data not shown), suggesting that low levels of added RCS are depleted by side reactions early in the incubation period before they can cause functional damage. Thus, it appears that continuous RCS production via glucose autoxidation is required for inhibition of lysozyme activity.

Glucose-Derived RCS Modify Arginine Residues in Lysozyme. In the previous studies, we and others have demonstrated that the direct treatment with purified MGO can cause impairment

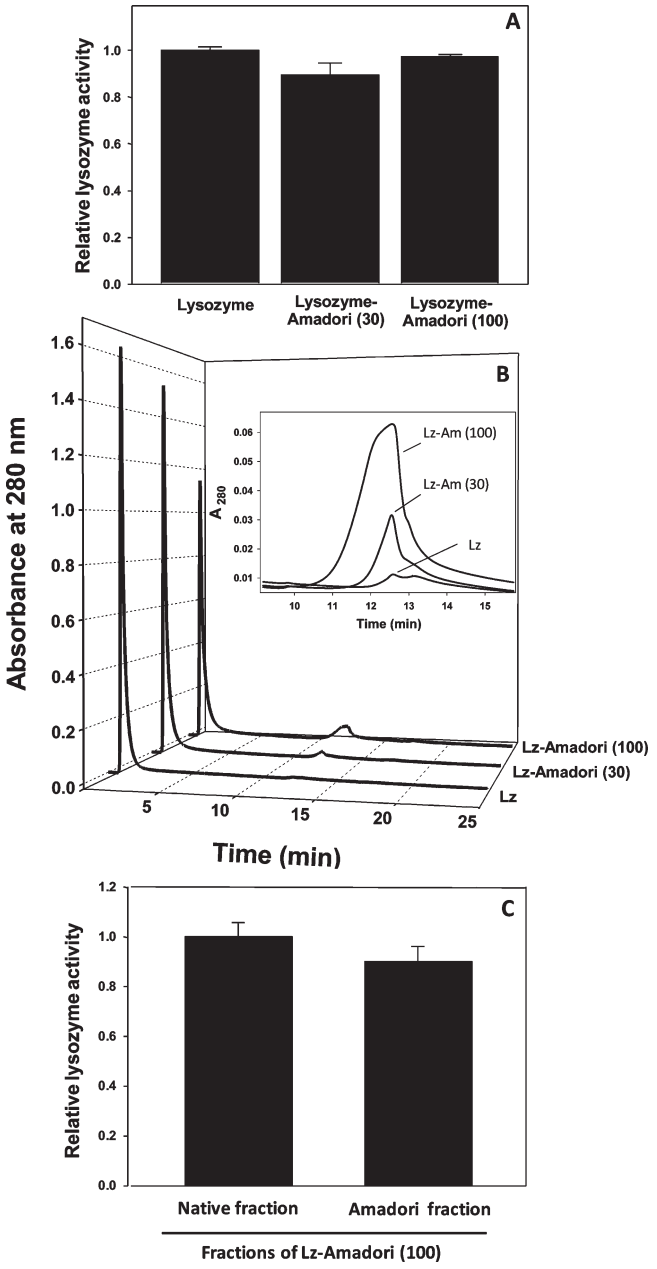


Figure 4. Enzymatic activity of lysozyme−Amadori preparations. Lysozyme−Amadori was prepared by incubation of native lysozyme with either 30 or 100 mM glucose in the presence of 1 mM DTPA for 15 days followed by removal of free glucose using centrifugal ultrafiltration as described under Experimental Procedures. (A) Enzymatic activity of native lysozyme and lysozyme−Amadori prepared with either 30 or 100 mM glucose. (B) Characterization and fractionation of lysozyme−Amadori preparations using phenylboronate HPLC. Amadori content was 0.1%, 3%, and 17% for native lysozyme, lysozyme−Amadori prepared with 30 mM glucose, and lysozyme−Amadori prepared with 100 mM glucose, respectively. Inset: a blow-up of Amadori peak. (C) Enzymatic activity of the native fraction (retention time ~1 min) and the Amadori fraction (retention time ~12.5 min) isolated from lysozyme−Amadori sample prepared with 100 mM glucose.

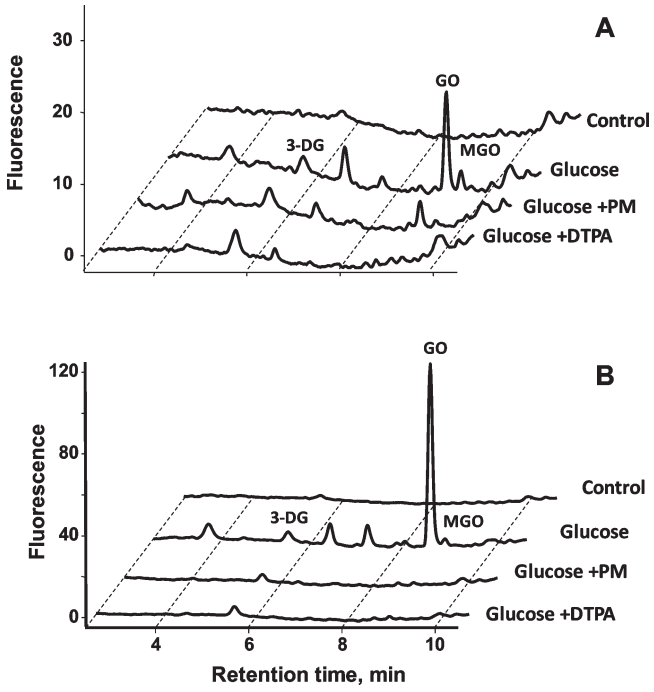


Figure 5. Glucose autoxidation and its inhibition by PM and DTPA. 30 mM (A) and 100 mM (B) glucose were incubated for 40 days at 37 °C in 0.15 M Na−phosphate buffer with or without 5 mM PM or 1 mM DTPA. Controls were immediately frozen without incubation. The identity and concentration of three major products of glucose autoxidation, methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) were determined as described under Experimental Procedures. Quantitative results for GO and MGO are shown in Table 2.

Table 2. Concentrations of Reactive Carbonyl Species Glyoxal and Methylglyoxal Generated during Autoxidation of Glucose[a]

	GO (μM)	MGO (μM)
	30 mM glucose	
control	n.d.	n.d.
G	21.74 ± 4.13	0.50 ± 0.07
G+PM	5.09 ± 0.92	0.21 ± 0.06
G+DTPA	n.d.	n.d.
	100 mM glucose	
control	n.d.	n.d.
G	237.82 ± 2.38	0.76 ± 0.11
G+PM	2.00 ± 0.06	0.28 ± 0.04
G+DTPA	n.d.	0.27 ± 0.13

[a] Glucose (30 and 100 mM) was incubated as indicated in Figure 5. Glyoxal (GO) and methylglyoxal (MGO) were quantified as described under Experimental Procedures. Data presented as the mean ± SD; n.d. = not detected.

of protein function.[11,12,14,41] To investigate mechanism of inhibition of protein function by glucose-derived GO and MGO, we determined the sites and relative abundances of RCS−arginine MG-H1 and GH-1 adducts and glucose−lysine Amadori adducts in lysozyme following glucose treatment. The identified chymotryptic peptides provided almost complete coverage of lysozyme primary structure (Figure S6). Peptides containing modifications of arginine and/or lysine residues are shown in Table S1. We found significant increase in the levels of G-H1 and MG-H1 modifications (Figure 6). Importantly, high levels of modifications

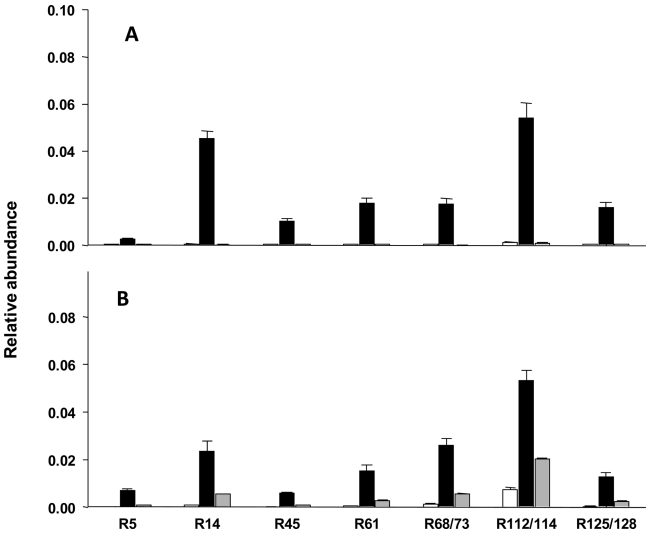


Figure 6. Quantitative analysis of site-specific arginine modifications in lysozyme. Lysozyme was incubated alone (control, open bars), with 100 mM glucose (glucose, black bars), or 100 mM glucose and 5 mM PM (glucose + PM, gray bars). G-H1 (A) and MG-H1 (B) modifications of specific arginine protein residues were quantified using mass spectrometry as described under Experimental Procedures. Lysozyme residues are numbered according to Uniprot sequence P00698 with the leader peptide removed (see Figure S6). Each bar represents the mean ± SD ($n = 3$). For all the data: $P < 0.05$, control vs glucose; $P < 0.05$, glucose vs glucose + PM.

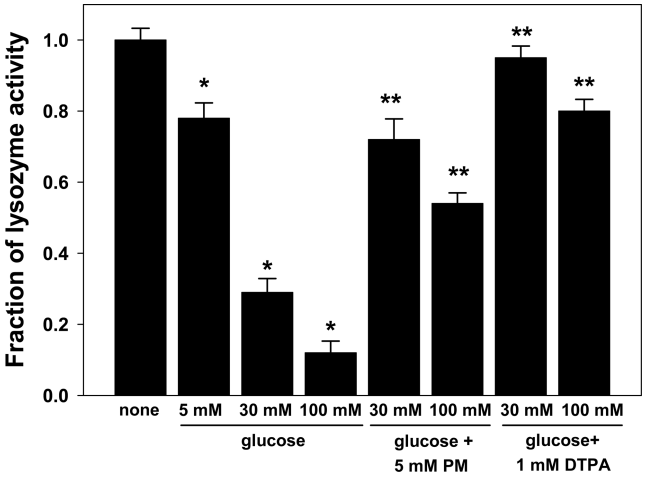


Figure 7. Protective effect of PM and DTPA on lysozyme activity. Lysozyme was incubated with either 30 or 100 mM glucose without or with 5 mM PM or 1 mM DTPA in 0.15 M Na−phosphate buffer at 37 °C for 40 days. Lysozyme activity was determined as described under Experimental Procedures.

were detected at Arg^{114}, a residue critical for lysozyme activity.[42] These RCS-derived arginine modifications were effectively inhibited by PM (Figure 6). Both PM and DTPA protected lysozyme activity in the presentence of glucose (Figure 7). In contrast to MGH-1 and GH-1 adducts, PM treatment increased the levels of Amadori adducts (Figure 8), as expected because PM prevents degradation of protein−Amadori adducts.[37,43]

These findings are consistent with the change in abundances of corresponding unmodified lysozyme peptides. In glucose-treated

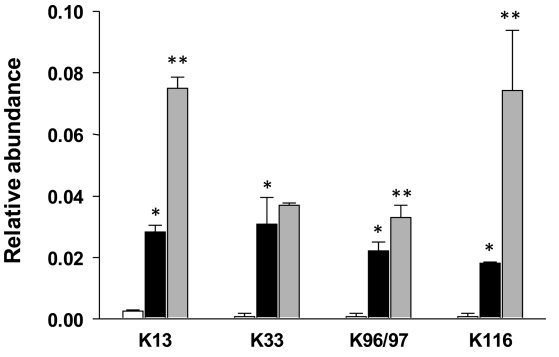


Figure 8. Quantitative analysis of site-specific Amadori adducts in lysozyme. Lysozyme was incubated alone (control, open bars), with 100 mM glucose (glucose, black bars), or 100 mM glucose and 5 mM PM (glucose + PM, gray bars). Amadori adducts to specific lysine protein residues were quantified using mass spectrometry as described under Experimental Procedures. Each bar represents the mean ± SD ($n = 3$). *$P < 0.05$, control vs glucose; **$P < 0.05$, glucose vs glucose + PM.

sample, we observed a decrease in the abundances of unmodified peptides containing R14, R68/73, and R112/114 (Table S3), which are major sites of modification by GO and MGO (Figure 6). Inhibition by PM of the decrease in these unmodified peptides was also consistent with PM inhibition of site-specific GO- and MGO-derived modifications (Table 3 and Figure 6).

Glucose-Derived RCS Inhibit Integrin−Collagen IV Interactions. To determine whether glucose autoxidation can cause functional damage to ECM proteins, we investigated integrin binding to recombinant RGD-α3 NC1 domain of collagen IV, which possesses functionally critical arginine residue within RGD motif that can be modified by RCS.[11] We reasoned that RCS produced upon glucose autoxidation would modify critical arginine and inhibit integrin binding. Indeed, when RGD-α3NC1 domain was preincubated with either 30 or 100 mM glucose followed by glucose removal, binding of integrin $\alpha_v\beta_3$ to this modified domain was significantly inhibited (Figure 9). This inhibition was either absent or significantly diminished when generation of RCS from glucose was blocked with either PM or DTPA (Figure 9).

RCS-Derived Adducts Accumulate in Renal ECM in Experimental Hyperglycemia. We have previously demonstrated an increase in total CML modifications, which can derive from GO, in renal collagen IV in human diabetes.[44] Here, we isolated NC1 domains of collagen IV from renal ECM of control and STZ-diabetic rats to determine whether RCS-derived modifications occur in collagen IV sites in hyperglycemia. Pooled samples from three animals per treatment group were analyzed using LC-MS/MS following proteolytic digestion as described under Experimental Procedures. Several modifications of specific lysine and arginine residues were found within α1NC1 and α5NC1 domains of collagen IV at sequence coverage of 99% and 97%, respectively. A 1.7−1.8-fold increase in modification levels at K^{56} site of α1NC1 domain and K^{40}/R^{41} site of α5NC1domain was observed in diabetic samples vs controls (Table 3). However, the magnitude of this increase was only about 2 times greater than typical coefficient of variation values in LC/MS-based label-free protein quantitation experiments.[45] More significantly, we found that GO-derived G-H1 modification of Arg^{169} in α1NC1 and α5NC1 domains was increased by 4.9-fold in diabetic animals compared to controls (Table 3 and Figure S7). Consistent with

Table 3. Sites and Relative Levels of Glucose- and/or Carbonyl-Derived Modifications in Collagen IV NC1 Domains Isolated from Kidneys of Control and STZ-Diabetic Rats[a]

peptide sequence	fold change diabetic/control	collagen IV chain
(R^{55})**K^{CML}**FSTMPFLFC*NINNVC*NFASR(N^{77})	1.8	α1
(L^{33})LYVQGN**K^{Am}R$^{G\text{-}H1}$**AHGQDLGTAG(S^{52})	1.7	α5
(S^{163})CLEEF**R$^{G\text{-}H1}$**SAPFIEC*HG(R^{179})	4.9	α1 and α5

[a] Modified lysine and arginine residues within peptide sequences are shown in bold. Modifications are indicated as follows: CML, N^{ϵ}-carboxymethyllysine, ΔM = +58.0055; Am, fructosyllysine (Amadori product), ΔM = +162.0528; G-H1, 5-hydroimidazolone, ΔM = +39.9949; *, carbamidomethylcysteine, ΔM = +57.0215. Underlined are the NC1 domain interface contact residues.[69]

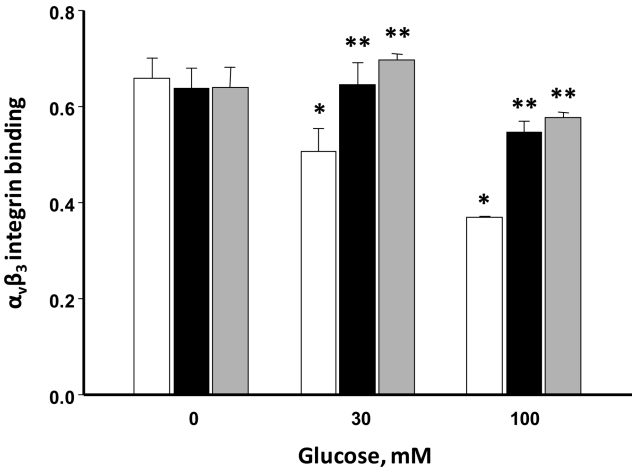


Figure 9. Binding of integrin $\alpha_v\beta_3$ to RGD-α3NC1 domain of collagen IV modified with glucose. Plates coated with RGD-α3NC1 were incubated with glucose alone (open bars), glucose and PM (black bars), or glucose and DTPA (gray bars) in aseptic conditions at 37°C for 30 days. After extensive washing, binding of integrin $\alpha_v\beta_3$ to RGD-α3NC1 was determined in a solid-phase binding assay as described under Experimental Procedures. The error bars represent the SD (n = 3). q*P < 0.05 glucose, 30 mM or glucose, 100 mM vs glucose, 0 mM; **P < 0.05, glucose vs glucose + PM or glucose + DTPA.

this finding, we also observed a decrease in corresponding unmodified peptide in diabetic animals vs controls (Table S4).

DISCUSSION

In this study, we demonstrated that GO and MGO generated from glucose under oxidative conditions can inhibit protein functions such as enzymatic activity or receptor−ligand interaction. Most importantly, this functional damage was specific to RCS and did not occur when production of RCS was blocked by inhibiting glucose autoxidation reactions, even though proteins were still exposed to high levels of glucose.

We propose that the high pathogenic potential of glucose autoxidation pathways is due to the fact that the resulting RCS, unlike glucose, preferentially target arginine residues, which occur frequently within functional sites of proteins. Analysis of over 80 000 sequences from the Swissprot database demonstrated that arginine was the most abundant residue within protein receptor binding domains.[46] Arginine was also the most frequent residue in protein nucleotide binding sites, based on the analysis of 188 3-D structures of nonhomologous protein−DNA and protein−RNA complexes from the Protein Data Bank.[47] Among the residues directly involved in catalysis in 178 nonhomologous enzyme active sites arginine was the third most frequent, behind only histidine and aspartic acid.[48] Similarly, arginine residues are ubiquitous within major integrin binding sites in ECM proteins (Table S2).[49−62] Thus, by preferential targeting of arginine residues glucose-derived RCS can potentially affect functionalities of a large number of proteins. Another factor which may contribute to higher pathogenicity of RCS compared to glucose is the overall reduced surface exposure of residues within protein active sites.[48] As a result, some of the lysine residues within these sites may be poorly accessible to bulky and slow reacting glucose molecules and will preferentially form adducts with smaller and more reactive RCS.

ECM proteins are significantly more susceptible to glycoxidative damage compared to both circulating and intracellular proteins because they have relatively long lifetimes. For example, ECM proteins of renal glomerular basement membrane such as collagen IV have a half-life of at least 100 days.[63] Slow ECM turnover rates allow AGE modifications within critical sites to accumulate to higher levels so they are more likely affect protein function. In addition, ECM proteins are much more susceptible to glycoxidative damage compared to intracellular proteins because they lack protection afforded by generally reducing cellular environment and robust cellular enzymatic defenses including carbonyl detoxification.[64] It has been suggested that binding of redox-active metal ions by specialized chaperone proteins such as transferrin is a major antioxidant defense in the extracellular environment.[65] This is consistent with our findings that in high concentration of glucose protein damage can be prevented by transition metal ion sequestration.

In our study, we detected an almost 5-fold increase in GO-derived G-H1 modification of Arg[169] in collagen IV α1 and/or α5 NC1 domains from diabetic animals compared to controls (Table 3). Unlike measurements of the total levels of RCS-derived ECM modifications,[12,41,66−68] determination of site-specific ECM modifications *in vivo* allows to infer functional damage and possible pathogenic mechanisms. For example, Arg[169] is located next to subunit interface contact regions important for collagen IV network assembly,[69] and thus, assembly or stability of the network may potentially be affected under oxidizing conditions in hyperglycemia. This finding also suggests that GO, a major RCS product of glucose autoxidation, can contribute, along with MGO, to protein functional damage within oxidizing extracellular diabetic environment.

Glucose autoxidation could be an important source of RCS in the extracellular environment (Scheme 1). While degradation of protein-bound Amadori moiety can produce RCS,[7] this contribution may be relatively small. In the present study, the molar concentration of lysozyme−Amadori adducts constituted <1% of corresponding glucose concentration in glucose-treated samples. *In vivo*, serum albumin, which is by far the most abundant circulation protein (~0.7 mM), contains only about 0.13 mM of Amadori adducts in human diabetes, i.e., only ~1% of diabetic

glucose concentration.[70] The notion that nonoxidative glucose adduction to proteins has low pathogenic potential is emphasized by the studies using the fructosamine-3-kinase knockout mice. These mice did not exhibit any pathogenic phenotype despite having significantly elevated levels of Amadori-modified tissue proteins.[71]

Our data highlight the importance of accounting for toxic effects of RCS derived from glucose autoxidation in studies which involve glucose treatment. Since RCS and glucose can potentially damage different subsets of proteins, the experimental outcomes may be different depending on the rate of glucose autoxidation. This is particularly important in cell culture studies due to pro-oxidative properties of culture media.[72]

Poor control of RCS levels may contribute to the increased susceptibility to diabetic complications in specific individuals or population groups. Interestingly, susceptibility to nephropathy in diabetic human populations correlates with circulation RCS levels.[73] Moreover, thiamine treatment has been shown to reduce circulation RCS levels in experimental diabetes and ameliorate albuminuria in diabetic patients, most likely via reversal of metabolic dysfunction in renal cells.[74] In the extracellular diabetic milieu, pharmacological inhibition of glycoxidative reactions which generate RCS may also protect against development of diabetic complications in susceptible individuals. In this study, we demonstrated that pyridoxamine, a drug candidate for treatment of diabetic nephropathy, can inhibit glucose autoxidation reactions, thus protecting proteins from RCS-induced functional damage. This mechanism may contribute to PM therapeutic effects in progressive diabetic kidney disease which have been demonstrated in clinical trials.[75]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figures S1−S7 and Tables S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AGE, advanced glycation end-product; CML, N^{ϵ}-(carboxymethyl)-lysine; CEL, N^{ϵ}-(carboxyethyl)lysine; DAN, 2,3-diaminonaphthalene; 3-DG, 3-deoxyglucosone; DTPA, diethylenetriaminepentaacetic acid; ECM, extracellular matrix; GO, glyoxal; MGO, methylglyoxal; PM, pyridoxamine; ROS, reactive oxygen species; RCS, reactive carbonyl species.

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