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Site-specific AGE modifications in the ECM: a role for glyoxal in protein damage in diabetes

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Abstract

Non-enzymatic modification of proteins in hyperglycemia is a major proposed mechanism of diabetic complications. Specifically, advanced glycation end products (AGEs) derived from hyperglycemia-induced reactive carbonyl species (RCS) can have pathogenic consequences when they target functionally critical protein residues. Modification of a small number of these critical residues, often undetectable by the methodologies relying on measurements of total AGE levels, can cause significant functional damage. Therefore, detection of specific sites of protein damage in diabetes is central to understanding molecular basis of diabetic complications and for identification of biomarkers which are mechanistically linked to the disease.

The current paradigm of RCS-derived protein damage places the major focus on methylglyoxal (MGO), an intermediate of cellular glycolysis. We propose that glyoxal (GO) is a major contributor to extracellular matrix (ECM) damage in diabetes. Here, we review the current knowledge and provide new data about GO-derived site-specific ECM modification in experimental diabetes.

Keywords

diabetes; advanced glycation end products; glyoxal; methylglyoxal; extracellular matrix; collagen IV; mass spectrometry; pyridoxamine

Diabetes is one of the major human diseases, which leads to serious complications such as neuropathy, retinopathy, microvascular disease, atherosclerosis, and nephropathy. Proposed unifying mechanism of the pathobiology of diabetic complications considers several pathogenic pathways including a rise in the levels of intracellular methylglyoxal (MGO) due to inhibition of glycolysis, and the resulting increase in MGO-derived protein modifications ((1) and Fig. 1). Unlike glucose which modifies primarily lysine residues, MGO and other reactive carbonyl species (RCS) react preferentially with arginine residues with high reaction rates (2), and may play important role in pathogenesis because of high abundance of

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arginine residues within protein active sites (3). Indeed, in hyperglycemia, metabolically derived MGO has been implicated in modification and functional impairment of a number of proteins (1, 4–6). However, in the extracellular diabetic milieu other mechanisms including autooxidation of glucose and peroxidation of lipids may also contribute to specific RCS-derived protein damage (Fig. 1). In this case, glyoxal (GO) may play a key role in protein damage since it is a major product of glucose and lipid oxidation (7–10).

GO-derived AGEs and their impact on protein structure and function

Glyoxal in the tissues can originate from both exogenous and endogenous sources. The role of the exogenous sources, which include GO-rich foods and environmental pollutants, in modulation of GO tissue levels and GO-derived tissue damage is unknown (11). Endogenous glyoxal can derive from such diverse pathways as peroxidation of lipids (10), oxidation of alpha-amino acids by myeloperoxidase system (12) and photo-degradation of retinoid compounds (13). In diabetes, autooxidation of glucose and oxidation of glucose-protein adducts are the important sources of endogenous GO production with glucose autooxidation being the major pathway (7, 14–15).

Glyoxal can modify proteins via adduction of lysine, arginine and cysteine residues as well as formation of lysine-lysine and lysine-arginine cross-links (14, 16–18). The levels of GO-derived modifications such as N^ε-(carboxymethyl)lysine (CML) and N₈-(5-hydro-4-imidazolone-2-yl) (G-H1) have been shown to correlate with diabetic complications suggesting their potential contribution to pathogenesis (19). The mechanisms of such contribution are still unknown but several studies demonstrated the detrimental impact of GO-derived modifications on function of proteins involved in antioxidant defenses, neurodegenerative disorders, and cell-matrix interactions (20–22).

Site-specific protein modification by GO: difference between modification and functional damage

It is yet unclear whether GO-derived protein modifications that accumulate in diabetes have mechanistic significance in the pathogenesis or simply indicate secondary glycoxidative damage. The measurements of total levels of protein-GO adducts cannot provide significant insights into the pathogenic mechanisms since only a small fraction of amino acids susceptible to these modifications is located within functionally important protein sites. Therefore, depending on microenvironment and reactivity of specific amino acid side chains, the levels of GO adducts may not always reflect functional protein damage as schematically illustrated in Fig. 2. Indeed, the total levels of specific protein adducts such as CML are often not reliable as disease biomarkers (23–24). In contrast, determination of site-specific modifications within functionally important protein sites may offer direct insight into early pathogenic events. These site-specific AGE modifications could also be utilized as more reliable disease biomarkers which are directly linked to pathogenic mechanisms.

In the in vitro model systems, analysis of GO adducts at specific protein sites allowed for a more precise understanding of the impact of these modifications on protein structure and function. In RNase treated with purified glyoxal, formation of GO-arginine adducts was

shown to be a highly site-specific process (25). Primary sites of the adduct formation were Arg³⁹ and Arg⁸⁵, the arginine residues closest to the active site of the RNase (25). When lysozyme was incubated with glucose under oxidative conditions, there was a significant increase in the levels of arginine G-H1 adducts (9). One of the primary modification sites was located at Arg¹¹⁴, a residue critical for lysozyme activity (26). This residue was effectively protected from GO modification when glucose autooxidation was inhibited under non-oxidative solution conditions (9). These results demonstrated that GO produced during autooxidation of glucose can modify critical arginine residues, thus causing functional damage to proteins.

Modification of ECM proteins by GO

ECM proteins are significantly more susceptible to glycoxidative damage compared to both circulating and intracellular proteins because they have relatively long life times. For example, human skin and cartilage collagens have half-lives of ~15 and ~117 years, respectively (27). ECM proteins of renal glomerular basement membrane are also relatively long-lived with an average half-life of more than 100 days (28). Slow ECM turnover rates allow modifications to accumulate to higher levels so they are more likely to interfere with protein function (27). 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 and deglycation (29–30).

Consequently, GO-derived modifications are significantly increased in ECM subjected to chronic hyperglycemia and carbonyl stress of diabetes. CML adducts were detected in diabetic experimental models and clinical renal specimens taken from diabetic patients (31–33). High level of CML was found in diabetic renal GBM and in ECM of human retina (34–35). Similarly, elevated GO-derived arginine adducts were found in ECM proteins isolated from diabetic animal model and from diabetic patients (36–37).

In the ECM, there are potentially three types of functionally critical sites that could be damaged upon modification by GO: 1) the cellular receptor binding sites conferring cell-ECM interactions; 2) the proteolytic cleavage sites important in ECM turnover, and 3) the subunit interface sites critical for ECM assembly.

Cell-ECM interactions

Mechanistic in vitro studies have suggested that modification of arginine residues within integrin binding motifs using GO can perturb integrin-dependent cell-ECM adhesion (9, 22). The disruption of these integrin-ECM interactions may be important in the pathogenesis as mice lacking integrin $\alpha_1\beta_1$, a principal collagen IV receptor, developed more severe expansion of mesangial ECM than their wild type counterparts (33). On the other hand, modification of lysine residues in collagen IV to CML had no effect on cell adhesion to this ECM protein, but specifically inhibited cell proliferation (38). This observation suggests that specific modifications of ECM by GO derived from glucose autooxidation may affect distinct cell functions by interfering with binding of different cellular receptors.

Functioning of one of such receptors, receptor for advanced glycation end products (RAGE), may also be affected by GO since modification of lysine residues to CML is required for RAGE binding (39). However, the importance of circulating CML-modified proteins as RAGE ligands has been questioned based on relatively low degree of modification of circulating proteins under physiological conditions (40). Compared to circulating proteins, long-lived ECM proteins can accumulate much higher levels of CML under pathophysiologic conditions *in vivo* (36). This could make glycoxidized ECM a better RAGE ligand capable of providing multiple binding sites necessary for RAGE dimerization and activation (41). Modification of collagen in the presence of glucose has been shown to modulate neutrophil adhesion and migration via RAGE-dependent PI3K signaling pathway (42). Since CML can derive from glucose and lipids via glyoxal route (10, 14–15), GO is likely to play a role in this RAGE-dependent modulation. Identification of CML clusters with potential of binding RAGE in diabetic ECM will help to determine the mechanism of RAGE-ECM interactions.

ECM turnover

Excessive accumulation of the ECM is a well-known lesion in diabetic complications (43). The role of matrix glycation in this phenomenon is supported by detection of AGE cross-links in tissues and by decreased activity of specific matrix metalloproteases (MMPs) towards glycated ECM proteins (44–45). The effect of GO-derived cross-links on ECM turnover is probably minimal since major physiological cross-links derive via non-oxidative pathways (45). However, there may be other mechanisms besides glucose-induced cross-linking that modulate ECM turnover. One example is engagement of cellular receptors by glycoxidized ECM. Cells grown on glucose-modified collagen expressed less of MMP-2 and more of endogenous collagen IV and MMP inhibitor TIMP-1 compared to cells grown on unmodified collagen (38, 46). Exposure of tenocytes to CML-collagen I increased intracellular activity of transglutaminase, enzyme that catalyzes formation of non-AGE cross-links in ECM (47). In addition, non cross-link GO-derived modifications such as CML, G-H1 and CMA may contribute to the lower digestibility of diabetic ECM because the consensus cleavage sites of many MMPs contain lysine and arginine residues (48). Identification of GO-derived non-cross link modifications within ECM receptor binding and protease cleavage sites may provide important clues about the role of glucose autooxidation in matrix turnover.

ECM assembly and stability

There are some indications that glycoxidation of ECM proteins may affect their conformation and assembly. For example, the conserved regions of NC1 domains of collagen IV critical in chain recognition during protomer assembly are enriched in arginine and lysine residues (49). It has been reported that collagen modified *in vitro* with either GO or glucose had greater thermal stability, mean fiber radius, and pore size compared to unmodified collagen (50–52). However, these studies were performed at supraphysiological concentrations of either glucose or GO, a condition which favors cross-link formation. Determination of GO-derived modifications within specific sites at different ECM assembly interfaces *in vivo* may prove critical for understanding how GO affects ECM network stability.

To provide initial insights into this question, we isolated collagen IV NC1 domains, which play a key role in the collagen IV network assembly, from renal ECM of control and STZ-diabetic rats. Pooled samples from three animals per treatment group were analyzed using LC-MS/MS following proteolytic digestion at sequence coverage of >97% as previously described (9). We found that GO-derived G-H1 modification of Arg¹⁶⁹ (R¹⁶⁹) in α 1NC1 domain was increased from about 7% in control animals to over 21% in diabetic animals (Table 1). In diabetic animals treated with PM, a compound which can inhibit glucose autooxidation and lipoxidation (9, 53–54), the degree of modification was attenuated to about 14% (Table 1).

The effect of this modification on NC1 domain structure was determined using molecular dynamics (MD) simulations. Analyses of MD simulations show that the side chain of unmodified R¹⁶⁹ residue is bridging the interface between A and B subdomains of the α 1NC1 monomer (Fig. 3, main panel and lower inset). In contrast, the side chain of G-H1¹⁶⁹ residue is rotated by approximately 90° relative to unmodified residue, resulting in a displacement of ~5 Å as measured from the amine nitrogen atoms of R¹⁶⁹ to equivalent positions in the imidazole ring of G-H1 (Fig. 3, lower and upper insets). MD simulations predict that protons on the imino functional groups of the R¹⁶⁹ residue participate in multiple hydrogen bonds with residues Q¹³, S⁸⁴, and Y¹⁸⁴ (Fig. 3, lower inset and table), which span the A/B subdomain interface. Hydrogen bond occupancy results indicate that G-H1¹⁶⁹ is unable to form comparable hydrogen bonds (Fig. 3, table). Therefore, our MD results predict that G-H1¹⁶⁹ reduces the enthalpic stabilization and thus consistent with the negative impact of G-H1¹⁶⁹ on conformational stability of NC1 domain. The finding that PM, a prospective drug in treatment of diabetic nephropathy (55), inhibited G-H1¹⁶⁹ formation suggests a role for stability of NC1 domains in diabetic renal pathology.

Conclusions

Reactive carbonyl species are key agents in propagation of diabetic protein damage to arginine residues within the sites critical to protein function. While the primary focus so far has been on glycolysis byproduct MGO, GO may be equally important in protein damage, especially in the ECM. In particular, stability of collagen IV networks may be compromised in diabetes due to GO-modified arginine residues within assembly interface of collagen IV NC1 domains. Since GO can potentially modify a variety of different ECM functional sites, it may be an important pathogenic intermediate whereby glucose and lipid oxidation causes damage to ECM proteins, thus contributing to complications of diabetes.

Acknowledgments

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Abbreviations

LC-MS/MS	liquid chromatography-tandem mass spectrometry
AGE	advanced glycation end-product

GO	glyoxal
MGO	methylglyoxal
G-H1	N ₈ -(5-hydro-4-imidazolone-2-yl) ornithine
CML	N ^ε -(carboxymethyl) lysine
CMA	carboxymethyl arginine
ECM	extracellular matrix
PM	pyridoxamine
ROS	reactive oxygen species
RCS	reactive carbonyl species

References

1. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*. 2005; 54(6):1615–1625. [PubMed: 15919781]
2. 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*. 1994; 269(51):32299–32305. [PubMed: 7798230]
3. Rabbani N, Thornalley PJ. Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. *Amino Acids*. 2010
4. Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, et al. High Glucose Increases Angiopoietin-2 Transcription in Microvascular Endothelial Cells through Methylglyoxal Modification of mSin3A. *J Biol Chem*. 2007; 282(42):31038–31045. [PubMed: 17670746]
5. Queisser MA, Yao D, Geisler S, Hammes HP, Lochner G, Schleicher ED, et al. Hyperglycemia impairs proteasome function by methylglyoxal. *Diabetes*. 2010; 59(3):670–678. [PubMed: 20009088]
6. Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes*. 2006; 55(7):1961–1969. [PubMed: 16804064]
7. 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*. 1995; 34(11):3702–3709. [PubMed: 7893666]
8. Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J*. 1999; 344(Pt 1):109–116. [PubMed: 10548540]
9. Chetyrkin S, Mathis M, Pedchenko V, Sanchez OA, McDonald WH, Hachey DL, et al. Glucose Autooxidation Induces Functional Damage to Proteins via Modification of Critical Arginine Residues. *Biochemistry*. 2011
10. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem*. 1996; 271(17):9982–9986. [PubMed: 8626637]
11. Lange JN, Wood KD, Knight J, Assimos DG, Holmes RP. Glyoxal formation and its role in endogenous oxalate synthesis. *Adv Urol*. 2012; 2012:819202. [PubMed: 22567004]
12. Hazen SL, d'Avignon A, Anderson MM, Hsu FF, Heinecke JW. Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to oxidize alpha-amino acids to a family of reactive aldehydes. Mechanistic studies identifying labile intermediates along the reaction pathway. *J Biol Chem*. 1998; 273(9):4997–5005. [PubMed: 9478947]

13. Yoon KD, Yamamoto K, Ueda K, Zhou J, Sparrow JR. A novel source of methylglyoxal and glyoxal in retina: implications for age-related macular degeneration. *PLoS One*. 2012; 7(7):e41309. [PubMed: 22829938]
14. Glomb MA, Monnier VM. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem*. 1995; 270(17):10017–10026. [PubMed: 7730303]
15. Ferreira AE, Ponces Freire AM, Voit EO. A quantitative model of the generation of N(epsilon)-(carboxymethyl)lysine in the Maillard reaction between collagen and glucose. *Biochem J*. 2003; 376(Pt 1):109–121. [PubMed: 12911334]
16. Thorpe SR, Baynes JW. Maillard reaction products in tissue proteins: new products and new perspectives. *Amino Acids*. 2003; 25(3–4):275–281. [PubMed: 14661090]
17. 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*. 2002; 277(28):24907–24915. [PubMed: 11978796]
18. Ahmed N, Thornalley PJ. Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and intrinsic fluorescence. *Biochem J*. 2002; 364(Pt 1):15–24. [PubMed: 11988071]
19. Perkins BA, Rabbani N, Weston A, Ficociello LH, Adaikalakoteswari A, Niewczas M, et al. Serum levels of advanced glycation endproducts and other markers of protein damage in early diabetic nephropathy in type 1 diabetes. *PLoS One*. 2012; 7(4):e35655. [PubMed: 22558190]
20. Jabeen R, Saleemuddin M, Petersen J, Mohammad A. Inactivation and modification of superoxide dismutase by glyoxal: prevention by antibodies. *Biochimie*. 2007; 89(3):311–318. [PubMed: 17175088]
21. Kawaguchi M, Shibata N, Horiuchi S, Kobayashi M. Glyoxal inactivates glutamate transporter-1 in cultured rat astrocytes. *Neuropathology*. 2005; 25(1):27–36. [PubMed: 15822816]
22. Pedchenko VK, Chetyrkin SV, Chuang P, Ham AJ, Saleem MA, Mathieson PW, et al. Mechanism of perturbation of integrin-mediated cell-matrix interactions by reactive carbonyl compounds and its implication for pathogenesis of diabetic nephropathy. *Diabetes*. 2005; 54(10):2952–2960. [PubMed: 16186398]
23. Senolt L, Braun M, Vencovsky J, Sedova L, Pavelka K. Advanced glycation end-product pentosidine is not a relevant marker of disease activity in patients with rheumatoid arthritis. *Physiol Res*. 2007; 56(6):771–777. [PubMed: 17298211]
24. Busch M, Franke S, Wolf G, Brandstadt A, Ott U, Gerth J, et al. The advanced glycation end product N(epsilon)-carboxymethyllysine is not a predictor of cardiovascular events and renal outcomes in patients with type 2 diabetic kidney disease and hypertension. *Am J Kidney Dis*. 2006; 48(4):571–579. [PubMed: 16997053]
25. Cotham WE, Metz TO, Ferguson PL, Brock JW, Hinton DJ, Thorpe SR, et al. Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease. *Mol Cell Proteomics*. 2004; 3(12):1145–1153. [PubMed: 15377717]
26. Kawamura S, Chijiwa Y, Minematsu T, Fukamizo T, Varum KM, Torikata T. The role of Arg114 at subsites E and F in reactions catalyzed by hen egg-white lysozyme. *Biosci Biotechnol Biochem*. 2008; 72(3):823–832. [PubMed: 18323666]
27. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*. 2000; 275(50):39027–39031. [PubMed: 10976109]
28. Price RG, Spiro RG. Studies on the metabolism of the renal glomerular basement membrane. Turnover measurements in the rat with the use of radiolabeled amino acids. *J Biol Chem*. 1977; 252(23):8597–8602. [PubMed: 925014]
29. Thornalley PJ. Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. *Biochem Soc Trans*. 2003; 31(Pt 6):1343–1348. [PubMed: 14641060]
30. Szwergold BS, Howell S, Beisswenger PJ. Human fructosamine-3-kinase: purification, sequencing, substrate specificity, and evidence of activity in vivo. *Diabetes*. 2001; 50(9):2139–2147. [PubMed: 11522682]

31. Schleicher ED, Wagner E, Nerlich AG. Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest.* 1997; 99(3):457–468. [PubMed: 9022079]
32. Nakamura S, Tachikawa T, Tobita K, Aoyama I, Takayama F, Enomoto A, et al. An inhibitor of advanced glycation end product formation reduces N epsilon-(carboxymethyl)lysine accumulation in glomeruli of diabetic rats. *Am J Kidney Dis.* 2003; 41 Suppl 1(3):S68–S71. [PubMed: 12612956]
33. Zent R, Yan X, Su Y, Hudson BG, Borza DB, Moeckel GW, et al. Glomerular injury is exacerbated in diabetic integrin alpha1-null mice. *Kidney Int.* 2006; 70(3):460–470. [PubMed: 16775606]
34. Wendt T, Tanji N, Guo J, Hudson BI, Bierhaus A, Ramasamy R, et al. Glucose, glycation, and RAGE: implications for amplification of cellular dysfunction in diabetic nephropathy. *J Am Soc Nephrol.* 2003; 14(5):1383–1395. [PubMed: 12707408]
35. Glenn JV, Stitt AW. The role of advanced glycation end products in retinal ageing and disease. *Biochim Biophys Acta.* 2009; 1790(10):1109–1116. [PubMed: 19409449]
36. Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, et al. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J.* 2003; 375(Pt 3):581–592. [PubMed: 12885296]
37. Duran-Jimenez B, Dobler D, Moffatt S, Rabbani N, Streuli CH, Thornalley PJ, et al. Advanced glycation end products in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes. *Diabetes.* 2009; 58(12):2893–2903. [PubMed: 19720799]
38. Pozzi A, Zent R, Chetyrkin S, Borza C, Bulus N, Chuang P, et al. Modification of collagen IV by glucose or methylglyoxal alters distinct mesangial cell functions. *J Am Soc Nephrol.* 2009; 20(10):2119–2125. [PubMed: 19608705]
39. Kislinger T, Fu C, Huber B, Qu W, Taguchi A, Du Yan S, et al. N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem.* 1999; 274(44):31740–31749. [PubMed: 10531386]
40. Heizmann CW. The mechanism by which dietary AGEs are a risk to human health is via their interaction with RAGE: arguing against the motion. *Mol Nutr Food Res.* 2007; 51(9):1116–1119. [PubMed: 17854011]
41. Ostendorp T, Leclerc E, Galichet A, Koch M, Demling N, Weigle B, et al. Structural and functional insights into RAGE activation by multimeric S100B. *Embo J.* 2007; 26(16):3868–3878. [PubMed: 17660747]
42. Toure F, Zahm JM, Garnotel R, Lambert E, Bonnet N, Schmidt AM, et al. Receptor for advanced glycation end-products (RAGE) modulates neutrophil adhesion and migration on glycoxidated extracellular matrix. *Biochem J.* 2008; 416(2):255–261. [PubMed: 18643777]
43. Ban CR, Twigg SM. Fibrosis in diabetes complications: pathogenic mechanisms and circulating and urinary markers. *Vasc Health Risk Manag.* 2008; 4(3):575–596. [PubMed: 18827908]
44. Mott JD, Khalifah RG, Nagase H, Shield CF 3rd, Hudson JK, Hudson BG. Nonenzymatic glycation of type IV collagen and matrix metalloproteinase susceptibility. *Kidney Int.* 1997; 52(5):1302–1312. [PubMed: 9350653]
45. Sell DR, Biemel KM, Reihl O, Lederer MO, Strauch CM, Monnier VM. Glucosepane is a major protein cross-link of the senescent human extracellular matrix. Relationship with diabetes. *J Biol Chem.* 2005; 280(13):12310–12315. [PubMed: 15677467]
46. Anderson SS, Wu K, Nagase H, Stettler-Stevenson WG, Kim Y, Tsilibary EC. Effect of matrix glycation on expression of type IV collagen, MMP-2, MMP-9 and TIMP-1 by human mesangial cells. *Cell Adhes Commun.* 1996; 4(2):89–101. [PubMed: 8937745]
47. Rosenthal AK, Gohr CM, Mitton E, Monnier V, Burner T. Advanced glycation end products increase transglutaminase activity in primary porcine tenocytes. *J Invest Med.* 2009; 57(2):460–466.
48. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat Biotechnol.* 2001; 19(7):661–667. [PubMed: 11433279]

49. Khoshnoodi J, Sigmundsson K, Cartailier JP, Bondar O, Sundaramoorthy M, Hudson BG. Mechanism of chain selection in the assembly of collagen IV: a prominent role for the alpha2 chain. *J Biol Chem*. 2006; 281(9):6058–6069. [PubMed: 16373348]
50. Howard EW, Benton R, Ahern-Moore J, Tomasek JJ. Cellular contraction of collagen lattices is inhibited by nonenzymatic glycation. *Exp Cell Res*. 1996; 228(1):132–137. [PubMed: 8892980]
51. Makino H, Shikata K, Hironaka K, Kushiro M, Yamasaki Y, Sugimoto H, et al. Ultrastructure of nonenzymatically glycated mesangial matrix in diabetic nephropathy. *Kidney Int*. 1995; 48(2): 517–526. [PubMed: 7564121]
52. Fathima NN, Madhan B, Rao JR, Nair BU, Ramasami T. Interaction of aldehydes with collagen: effect on thermal, enzymatic and conformational stability. *Int J Biol Macromol*. 2004; 34(4):241–247. [PubMed: 15374680]
53. Metz TO, Alderson NL, Chachich ME, Thorpe SR, Baynes JW. Pyridoxamine traps intermediates in lipid peroxidation reactions in vivo: evidence on the role of lipids in chemical modification of protein and development of diabetic complications. *J Biol Chem*. 2003; 278(43):42012–42019. [PubMed: 12923193]
54. Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW. Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. *J Biol Chem*. 2000; 275(28):21177–21184. [PubMed: 10801874]
55. Lewis EJ, Greene T, Spitalewiz S, Blumenthal S, Berl T, Hunsicker LG, et al. Pyridorin in type 2 diabetic nephropathy. *J Am Soc Nephrol*. 2012; 23(1):131–136. [PubMed: 22034637]

Figure 1. Intracellular and extracellular sources of reactive carbonyl species. In the cell (left), inhibition of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hyperglycemia via overproduction of mitochondrial superoxide causes accumulation of GAP and excessive intracellular formation of methylglyoxal (MGO); this MGO modifies cellular and extracellular proteins (1). Outside the cell (right), glucose, lipids and glycated proteins are oxidized to form a major product glyoxal (GO) (7–10); this GO preferentially modifies extracellular proteins.

A. Heavily modified but functionally active protein

B. Minimally modified but functionally damaged protein

Figure 2.
Schematic illustration of the impact of site-specific modification on protein functionality. Modified protein is shown in green and AGE modifications are shown in red (see text for details).

Figure 3.

Conformation of Arg¹⁶⁹ within $\alpha 1$ NC1 domain of collagen IV and impact of its modification by GO on the domain structure. Unmodified collagen IV NC1 $\alpha 1,1,2$ hexamer and a hexamer modified to G-H1 at residue R¹⁶⁹ of the first $\alpha 1$ chain were analyzed using molecular dynamic (MD) simulations with AMBER 12. Starting coordinates were derived from a crystal structure of the collagen IV hexamer (PDB 1T61). A starting structure of the G-H1-modified R¹⁶⁹ (G-H1¹⁶⁹) was constructed using PYMOL. Charge derivation of G-H1¹⁶⁹ was determined by the Restrained Electro Static Potential (RESP) method using Gaussian 03. Topology and coordinate files were generated with the xLEaP module of AMBER 12 using ff99SB parameters. The systems were solvated in a truncated octahedral box using the TIP3P water model to a distance of 8.0 Å, charge neutralized with the addition of Cl⁻ ions along a 1.0 Å Coulombic potential grid, and equilibrated under periodic boundary conditions. Production calculations were conducted at constant pressure for 40 ns; the final 30 ns of the trajectory were analyzed with the CPPTRAJ module of AMBER12 for residue specific hydrogen bonding, rms/atomic fluctuations, and water shell populations. Electrostatic interactions were treated with the particle mesh Ewald (PME) method. The SHAKE algorithm was used to constrain bond lengths involving hydrogen atoms. A non-

bonded cutoff of 9.0 Å was set during all minimization, equilibration, and production stages. Only α 1NC1 monomer is shown for simplicity (see text for details).

Table 1

Site-specific GO-derived protein damage in vivo: G-H1 modification within NC1 domain of collagen IV isolated from kidneys of control rats, STZ-diabetic rats and STZ-diabetic rats treated with PM.

Modification site	Peptide sequence	Mass accuracy (ppm)			Normalized relative abundance			Fold change relative to control	
		Control	Diabetic	Diabetic +PM	Control	Diabetic	Diabetic +PM	Diabetic	Diabetic +PM
R ¹⁶⁹	(S ¹⁶³)CLEEF ^{RS} APFIEC [§] HG(R ¹⁷⁹)	-1.0	-1.6	-2.1	6.94×10^{-2}	21.56×10^{-2}	14.09×10^{-2}	3.11	2.03
None	(S ¹⁶³)C [§] LEEFRSAPFIEC [§] HG(R ¹⁷⁹)	-1.3	-1.5	-1.2	9.31×10^{-1}	7.85×10^{-1}	8.59×10^{-1}	0.84	0.92

Normalized relative abundance = modified peptide / (unmodified peptide + modified peptide) or unmodified peptide / (unmodified peptide + modified peptide)

[§] Carbamidomethylcystein

Data represent the total relative abundance of all singly modified peptides with charge states of +2 and +3