Mechanistic Studies of Advanced Glycosylation End Product Inhibition by Aminoguanidine

DIANE EDELSTEIN AND MICHAEL BROWNLEE

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/41/1/26/358713/41-1-26.pdf by guest on 23 January 2022

Aminoguanidine-HCl inhibits the formation of advanced glycosylation end products (AGEs) in vitro and in vivo, but the mechanism by which this occurs has not been determined. Aminoguanidine inhibited glucose-derived AGE formation on RNase A by 67–85% at aminoguanidine-glucose molar ratios of 1:5 to 1:50 without affecting the concentration of Amadori products. Fast-atom-bombardment mass spectrometry of RNase peptides incubated with glucose alone or with glucose plus aminoguanidine showed that aminoguanidine inhibited the formation of AGEs without forming an adduct with glycosylated peptide. These data suggest that the primary mechanism of aminoguanidine action is reaction with Amadori-derived fragmentation products in solution. These findings are relevant to the potential clinical use of aminoguanidine in the prevention of diabetic complications. ***Diabetes*** **41:26–29, 1992**

Nonenzymatic carbonyl-amine addition reactions occur between glucose and amino groups on proteins in vivo, and the tight correlation between ketoamine (Amadori product) formation on hemoglobin and time-averaged plasma glucose concentration in diabetic patients has led to widespread clinical use of this measurement (1,2). On longer-lived proteins such as collagen, some of these early glycosylation products formed in vivo also serve as precursors of advanced glycosylation end products (AGEs), complex glucose-derived protein cross-links that accumulate over time (3,4).

From the Department of Medicine and Diabetes Research Center, Albert Einstein College of Medicine, Bronx, New York.

Address correspondence and reprint requests to Dr. Michael Brownlee, Department of Medicine and Diabetes Research Center, Albert Einstein College of Medicine, Bronx, NY 10461.

Received for publication 5 July 1990 and accepted in revised form 27 September 1991.

To date, evidence has been obtained for two general types of glucose-derived AGE cross-links (5,6). One type resembles the heterocyclic imidazole derivative 2-furoyl-4(5)-(2-furanyl) 1-*H*-imidazole and appears to form from the condensation of two Amadori products (7). The other type of AGE cross-link appears to form from Amadori-derived fragmentation compounds such as 3-deoxyglucosone (8,9). These highly reactive dicarbonyl compounds cyclize to form electrophilic pyrrole intermediates with reactive OH groups in benzylic positions, which then react with amino groups to form pyrrole-based cross-links. Formation of other AGEs may involve generation of glycolaldehyde from Schiff bases via a reverse aldol condensation reaction (10).

Because AGE formation in vivo has been hypothesized to play a role in the development of the characteristic tissue pathology of diabetes, pharmacological agents were sought that could inhibit this process by selectively blocking reactive carbonyls on ketoamine products and on their derivatives such as 3-deoxyglucosone and glycolaldehyde. The nucleophilic hydrazine compound aminoguanidine-HCl has been the most extensively investigated compound of this type and effectively inhibits the formation of AGEs in vitro and in vivo (11,12).

The primary mechanism by which aminoguanidine inhibits the formation of AGE has not been determined, however. In this study, we obtained evidence that aminoguanidine acts primarily by reacting with Amadori-derived fragmentation products such as 3-deoxyglucosone in solution, thereby preventing subsequent AGE formation on susceptible proteins.

RESEARCH DESIGN AND METHODS

Bovine pancreatic RNase A (type XII) and human serum albumin were obtained from Sigma (St. Louis, MO). Sodium phosphate (monobasic) and anhydrous glucose were obtained from Fisher (Springfield, NJ). Aminoguanidine-HCl was obtained from Aldrich (Milwaukee, WI). [U-^{14}C]glucose (3 mCi/mmol) was obtained from DuPont-

NEN (Boston, MA). Sephadex-PD10 columns were obtained from Pharmacia (Piscataway, NJ). Guanidine-HCl was obtained from Boehringer Mannheim (Indianapolis, IN), and Centricon 10 columns were obtained from Amicon (Danver, MA).

All glycosylation reactions were conducted at 37°C in 0.2 M sodium phosphate buffer (pH 7.8) containing 60 mg/L penicillin-VK, 40 mg/L gentamicin, 250 μg/L fungazone, 10 mg/L polymyxin B, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. RNase (10 mg/ml) was incubated for either 7 days or 16 h with 0.5 M glucose and varying concentrations of aminoguanidine to give aminoguanidine-glucose molar ratios of 0, 1:5, 1:10, 1:20, and 1:50. After incubation, samples were dialyzed against buffer unless otherwise specified.

AGE formation was determined by diluting samples threefold with water, and specific fluorescence was determined with a Perkin-Elmer LS 3B fluorescence spectrometer at excitation-emission wavelength of 370/440 nm (13).

[U-^{14}C]glucose was purified by removing highly reactive decomposition products (14) just before use by a modification of the method of Higgins and Bunn (15). The radiolabeled glucose was dissolved in 0.02 M phosphate buffer (pH 7.8) containing 20 mg/ml bovine serum albumin, $NaBH_3CN$ (0.11 ml of a 0.2-M solution) was added, and the sample was allowed to react for 90 min at 37°C. Purified radiolabeled glucose was recovered by passing the sample through a Centricon 10 column and a Bio-Rad AG50X8 column.

Schiff base adducts were discharged from glycosylated proteins by a modification of the method of Baynes et al. (16). RNase samples were incubated in 0.5 M glucose containing 6 μCi [U-^{14}C]glucose and varying amounts of aminoguanidine for 16 h at 37°C. Aliquots were diluted with 0.97 M sodium acetate to pH 5 and incubated at 37°C for 1 h. Glycosylated protein containing only Amadori products was then eluted from Sephadex PD 10 columns equilibrated with 0.1 M sodium acetate (pH 5).

RNase samples were incubated in 0.5 M glucose containing 6 μCi [U-^{14}C]glucose and varying amounts of aminoguanidine for 7 days at 37°C. The reaction mixture was cooled on ice, and glycosylated protein was recovered from Sephadex PD 10 columns equilibrated with 0.1 M Tris-HCl (pH 7.4) in the cold to preserve Schiff base adducts.

A peptide corresponding to residues 2–10 of RNase (ETAAAKFER) was synthesized in 430A peptide synthesizer (Applied Biosystems, Foster City, CA) with t-BOC chemistry. After cleavage from the resin with hydrogen fluoride, the peptide was characterized by amino acid analysis and mass spectrometry. This lysine-containing RNase fragment (30 μmol) was then incubated with either 100 mM glucose or glucose plus 25 mM aminoguanidine for 7 days as described above. Fast–atom-bombardment (FAB) mass spectral analysis was then performed on a Finnigan MAT-90 (Bremen, Germany) to detect peptide-bound glycosylation products and glycosylation product derivatives.

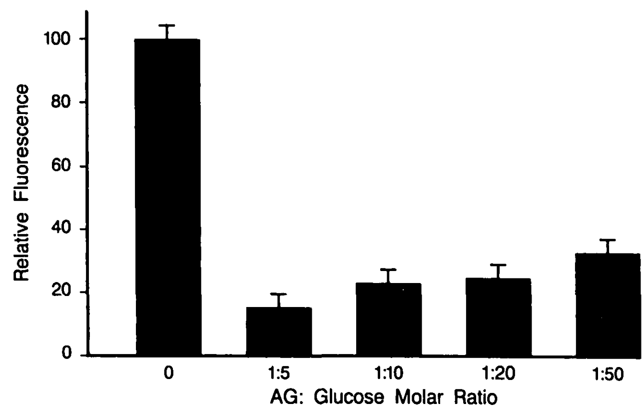

FIG. 1. Effect of aminoguanidine (AG):glucose molar ratio on advanced glycosylation end product formation. RNase (10 mg/ml) was incubated with 0.5 M glucose and AG at concentrations to give indicated molar ratios for 7 days. Advanced glycosylation end product formation was determined by specific fluorescence at excitation-emission wavelength of 370/440 nm.

RESULTS

In the absence of aminoguanidine-HCl, fluorescence characteristic of AGEs (13) was intense after a 1-wk incubation (450 U relative to blank). In contrast, the development of fluorescence characteristic of AGEs was inhibited 85% when incubated with aminoguanidine at a 1:5 ratio relative to glucose (Fig. 1). The degree of inhibition of AGE formation was not statistically different when the aminoguanidine concentration was further reduced to a 1:10 or 1:20 ratio relative to glucose. At an aminoguanidine-glucose molar ratio of 1:50, the degree of inhibition of AGE formation was still 67%.

When aminoguanidine-HCl was absent, each mole of RNase contained 0.654 ± 0.029 mol Amadori product after incubation for 16 h followed by removal of Schiff bases as described in METHODS (Fig. 2). This number is similar to that previously reported for proteins incubated under these conditions (16). Addition of aminoguanidine at either the highest (1:5) or lowest (1:50) molar ratio concentration used in these experiments had no statisti-

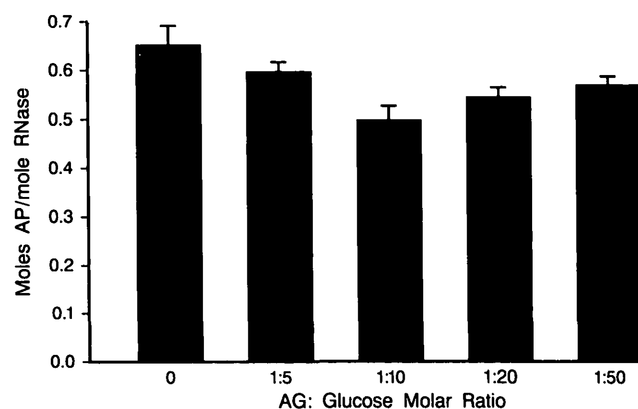

FIG. 2. Effect of aminoguanidine (AG):glucose molar ratio on Amadori product (AP; ketoamine) formation. RNase (10 mg/ml) was incubated with 0.5 M glucose containing 6 μCi [U-^{14}C]glucose and AG at concentrations to give indicated molar ratios for 16 h. AP formation was determined after discharging Schiff base adducts with acid and separating protein-bound APs by gel filtration.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/41/1/26/358713/41-1-26.pdf by guest on 23 January 2022

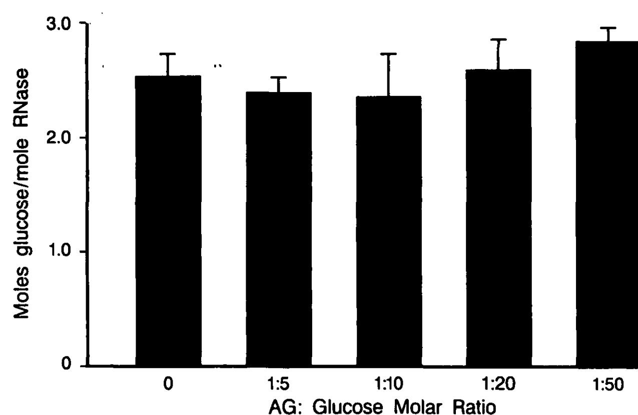

FIG. 3. Effect of aminoguanidine (AG):glucose molar ratio on [U-^{14}C]glucose incorporation. RNase (10 mg/ml) was incubated with 0.5 M glucose containing 6 μCi [U-^{14}C]glucose and AG at concentrations to give indicated molar ratios for 7 days. Moles of incorporated glucose were determined after gel filtration at 4°C to preserve Schiff base adducts.

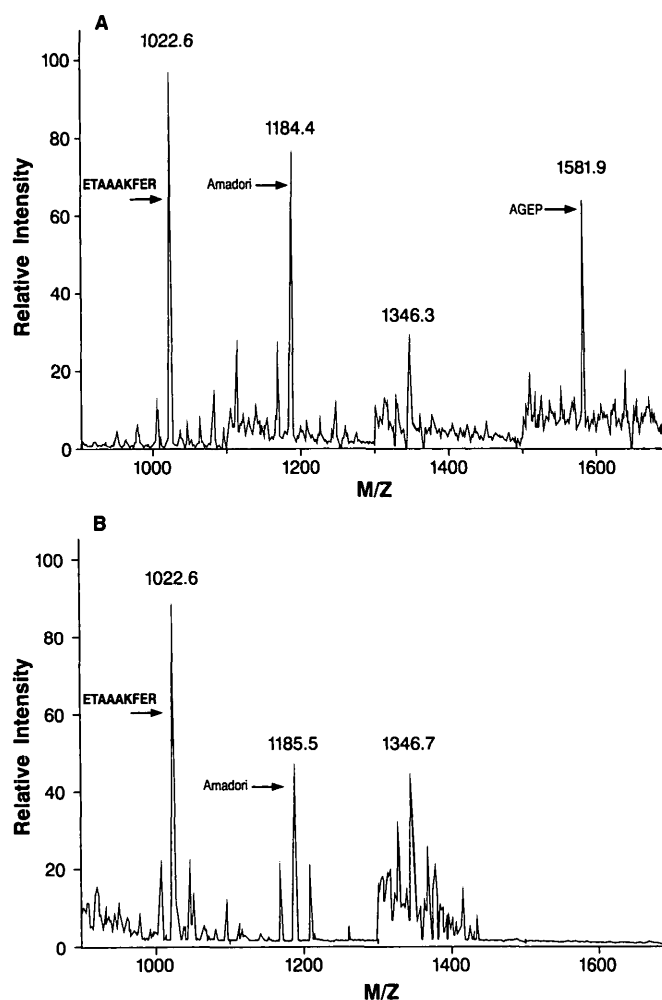

FIG. 4. Effect of aminoguanidine on glycosylated RNase peptide mass spectra. A peptide corresponding to residues 2–10 of RNase (30 μmol) was incubated with either 100 mM glucose or glucose plus 25 mM aminoguanidine for 7 days and then subjected to fast-atom-bombardment mass spectral analysis. Calculated and experimentally determined mass of peptide alone was 1022.6. Spectra show RNase peptide incubated with glucose alone (*A*) and with glucose plus aminoguanidine (*B*). AGEP, advanced glycosylation end product.

cally significant effect on the number of Amadori products formed per mole of RNase. Similar results were obtained when the effect of aminoguanidine on radiolabeled glucose incorporation was examined after incubation for 7 days (8,9). In the absence of aminoguanidine, 2.53 ± 0.16 mol glucose was incorporated per mole of RNase (Fig. 4). At aminoguanidine-glucose molar ratios that resulted in substantial inhibition of AGE formation (Fig. 3), the number of moles of glucose incorporated per mole of RNase remained unchanged.

FAB mass spectra obtained from RNase peptide incubated with glucose alone (Fig. 4*A*) showed peaks corresponding to the mass of the peptide alone (1022.6 m/z), the peptide Amadori product (1184.4 m/z), an unidentified peak having a mass equal to that of a disubstituted Amadori product (1346 m/z), and a higher-mass fragment AGE of unknown structure (1581.9 m/z). In contrast, the mass spectra obtained from RNase peptide incubated with glucose plus aminoguanidine (Fig. 4*B*) showed only the disappearance of the AGE peak. No new peak corresponding to an aminoguanidine–glycosylated peptide adduct was evident.

DISCUSSION

The data from these experiments suggest that the primary mechanism by which aminoguanidine inhibits the formation of AGEs is by reacting with Amadori-derived fragmentation products such as 3-deoxyglucosone in solution.

It has been proposed that aminoguanidine inhibits AGE formation by reacting with glucose in solution and thus reducing the effective concentration of the glycosylating agent (17). The data presented in Fig. 1 are inconsistent with this hypothesis, however, because in all cases, the concentration of aminoguanidine was significantly less than that of glucose. At an aminoguanidine-glucose ratio of 1:5, e.g., it would be predicted that aminoguanidine could reduce AGE formation by up to 20% and at a ratio of 1:20, up to 5%. Because the observed inhibitions at these concentrations are well over 80%, however, this mechanism cannot account for the action of aminoguanidine. However, these results do not exclude the possibility of low-affinity interactions between glucose and aminoguanidine in solution.

The data presented in Figs. 2 and 3 similarly rule out a mechanism of aminoguanidine action involving destabilization of the Amadori rearrangement, because aminoguanidine had no effect on Amadori-product formation. Inhibition of AGE formation occurring with undetectable differences in the level of Amadori product is consistent with the relatively small number of .amino groups involved in AGE formation (18).

In addition to demonstrating that the primary mechanism of aminoguanidine action is reaction with Amadori-derived fragmentation products in solution, the mass spectra data show that aminoguanidine does not form stable adducts with peptide-bound early glycosylation products. This suggests that formation of the previously characterized imidazole-type fluorescent cross-link

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/41/1/26/358713/41-1-26.pdf by guest on 23 January 2022

occurs by a different pathway than the proposed condensation of two Amadori products, although aminoguanidine-ketoamine adducts catalyzing fragmentation product formation could form transiently. These findings are relevant to the potential clinical use of aminoguanidine in the prevention of diabetic complications (19,20), because neither stabilization of potentially harmful Amadori products by aminoguanidine nor formation of potentially antigenic aminoguanidine-glycosylated peptide adducts appears to occur. Thus, any macromolecular dysfunction caused by excessive Amadori product formation alone could be reversed with normoglycemia, whereas irreversible dysfunction caused by formation of AGEs would be prevented by aminoguanidine treatment.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant RO1-DK-33861.

We thank Edward Nieves for invaluable help with the FAB mass spectometry analysis.

REFERENCES

1. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glucosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101: 527–37, 1984
2. Baynes JW, Thorpe SR, Murtiashaw MH: Nonenzymatic glucosylation of lysine residues in albumin. In *Methods in Enzymology: Posttranslational Modifications*. Vol. 106. New York, Academic, 1984, p. 88–98
3. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318:1315–21, 1988
4. Monnier VM, Cerami A: Nonenzymatic glycosylation and browning of proteins in vivo. In *The Maillard Reaction in Foods and Nutrition*. Waller GR, Feather MS, Eds. Washington, DC, Am. Chem. Soc., 1983, p. 431–39 (Symp. Ser.)
5. Baynes JW, Monnier VM (Eds.): *The NIH Conference on the Maillard Reaction in Aging, Diabetes and Nutrition*. New York, Liss, 1989, p. 1–410
6. Beck J, Ledl F, Sengl M, Severin T: Formation of acids, lactones and esters through the Maillard reaction. *Z Lebensm Unters Forsch* 190:212–16, 1990
7. Pongor S, Ulrich PC, Bencsath FA, Cerami A: Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc Natl Acad Sci USA* 81:2684–88, 1984
8. Njoroge FG, Sayre LM, Monnier VM: Detection of D-glucose derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr Res* 167:211–20, 1987
9. Farmar J, Ulrich P, Cerami A: Novel pyrroles from sulfite-inhibited Maillard reactions: insight into the mechanism of inhibition. *J Org Chem* 53:2346–49, 1988
10. Hayashi TW, Namiki M: Role of sugar fragmentation in the Maillard reaction. In *Proceedings of the 3rd International Symposium on the Maillard Reaction*. Fujimaki M, Namiki M, Kato H, Eds. New York, Elsevier, 1986
11. Brownlee M, Vlassara H, Kooney T, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein crosslinking. *Science* 232:1629–32, 1986
12. Nicholls K, Mandel TE: Advanced glycosylation end products in experimental murine diabetic nephropathy: effect of islet isografting and of aminoguanidine. *Lab Invest* 60:486–93, 1989
13. Monnier VM, Kohn RR, Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus. *Proc Natl Acad Sci USA* 81:583–87, 1984
14. Trueb B, Holenstein CG, Fischer RW, Winterhalter KH: Nonenzymatic glycosylation of proteins: a warning. *J Biol Chem* 255:6717–20, 1980
15. Higgins PJ, Bunn HF: Kinetic analysis of the nonenzymatic glycosylation of hemoglobin. *J Biol Chem* 256:5204–208, 1981
16. Watkins NG, Neglia-Fisher CI, Dyer DG, Thorpe SR, Baynes JW: Effect of phosphate on the kinetics and specificity of glycation of protein. *J Biol Chem* 262:7207–12, 1987
17. Khatami M, Suldan Z, David I, Li W, Rockey JH: Inhibitory effects of pyridoxal phosphates, ascorbate and aminoguanidine on nonenzymatic glycosylation. *Life Sci* 43:1725–31, 1988
18. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–12, 1991
19. Brownlee M: Glycosylation products as toxic-mediators of diabetic complications. *Annu Rev Med* 42:159–66, 1991
20. Soulis-Liparata T, Cooper M, Papazoglou D, Jerums G: Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes* 40:1328–34, 1991

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/41/1/26/358713/41-1-26.pdf by guest on 23 January 2022