

Prevention of Diabetes-Increased Aging Effect on Rat Collagen-Linked Fluorescence by Aminoguanidine and Rutin

PATRIZIO R. ODETTI, ANTONELLA BORGOGGIO, ANGELO DE PASCALE, RANIERI ROLANDI, AND LUCIANO ADEZATI

Products from the advanced Maillard reaction, which increase during aging and diabetes, may contribute to the development of the typical pathology of aging and diabetes. These compounds are detectable only by their characteristic fluorescence, and few data based on long-term studies are available. For this reason, we studied subcutaneous skin collagen fluorescence in 57 nondiabetic (10- to 110-wk-old) and 74 streptozocin-induced diabetic (10- to 22-wk-old) rats. An exponential increase ($r = 0.969$, $P < 0.001$) of collagen-linked fluorescence (excitation at 370 nm, emission at 440 nm) was observed with aging; after a lag, diabetes induced an earlier dramatic elevation of the fluorescence, suggesting a more complicated phenomenon than simple accumulation. To prevent such increases, the effects of $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ aminoguanidine, suggested to be an inhibitor of the advanced glycosylation reaction, and $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ rutin, an aldose reductase inhibitor, in drinking water were tested. Both treatments had a significant lowering effect on collagen fluorescence in diabetic rats. The mechanisms by which aminoguanidine and rutin prevent the accumulation of fluorescence are unknown, but these observations raise the question of whether they could be identical. If fluorescence is a marker for age-related pathologies and diabetic sequelae, aminoguanidine and rutin could have therapeutic effects in their prevention. *Diabetes* 39:796–801, 1990

Diabetes mellitus accelerates aging phenomena by a mechanism likely to be multifactorial. However, hyperglycemia can be considered the most important factor (1–5).

The detrimental effects of glucose have been attributed

From the Departments of Internal Medicine and Physics, University of Genoa, Genoa, Italy.

Address correspondence and reprint requests to P. Odetti, Department of Internal Medicine, Viale Benedetto XV 6, 16132 Genoa, Italy.

Received for publication 26 September 1989 and accepted in revised form 25 January 1990.

to nonenzymatic glycosylation (6,7). Nonenzymatic glycosylation end products may form in vivo and play an important role in the pathogenesis of diabetic sequelae (8).

These products are derived from the so-called Maillard reaction that occurs between reducing sugars and amino groups on proteins. The reaction begins with the early well-known and extensively studied glycosylation reaction (Amadori compounds), which proceeds over a long period through several chemical rearrangements to form irreversible pigmented and fluorescent products with a characteristic spectrum (browning products; 9–11).

The presence of such fluorescent products bound to skin collagen was reported in subjects with type I (insulin-dependent) diabetes mellitus (12). Their accumulation rate was elevated twofold over that of nondiabetic subjects, and there was an overall correlation with the severity of complications. Studies suggest that excess formation of collagen-linked fluorescence in diabetes might be preventable by aminoguanidine (13–15) or even aldose reductase inhibition (16).

This study evaluated the effects of age and diabetes duration in rats on tissue fluorescence accumulation and tested the ability of aminoguanidine and rutin, an aldose reductase inhibitor (17), to prevent fluorescent product accumulation. Although preliminary data are available on tissue fluorescence in experimental diabetes, little information is available on long-term accumulation of fluorescence in aging and diabetes.

RESEARCH DESIGN AND METHODS

Fifty-seven Wistar rats, weighing $\sim 200 \text{ g}$ (8–10 wk old), were used for the aging study. Every week, up to wk 40 and every 4–6 wk until the end of the study (wk 110), two rats were killed by guillotine after anesthesia. Blood and skin samples were collected for the determination of blood glucose, glycosylated hemoglobin, and tissue collagen fluorescence.

Diabetes was induced with streptozocin (50 mg/kg i.p.; Upjohn, Kalamazoo, MI) in 74 Wistar rats weighing $\sim 200 \text{ g}$; none of the diabetic rats received insulin treatment. Aminoguanidine hydrochloride (Sigma, St. Louis, MO) was added to the drinking water (1 g/L) of a randomized group

of diabetic rats ($n = 24$); a similar group ($n = 22$) received rutin (Sigma) added to the drinking water (1 g/L). The remaining diabetic rats ($n = 28$) were untreated controls.

Two rats in each diabetic group (untreated, aminoguanidine treated, and rutin treated) were killed by guillotine approximately every week from wk 10 to 20. Blood samples were collected for determination of glucose (enzymatic colorimetric methods, GOD-PAP kit, Boehringer Mannheim, Mannheim, FRG) and glycosylated hemoglobin (affinity chromatographic column, Glycaffin Isolab kit, Mascia Brunelli, Milan, Italy).

Subcutaneous collagen from the inner layer of abdominal skin was assayed for fluorescence according to Monnier et al. (18). A small sample of connective tissue was minced into pieces, washed with saline solution, and homogenized for 60 s with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in phosphate-buffered saline, pH 7.4. Lipids were extracted from the pellet with chloroform/methanol (2:1) with mild shaking overnight. The pellet was washed with methanol, water, and HEPES buffer (0.02 M, pH 7.5) containing 0.01 M CaCl_2 (buffer H) and then stored overnight at 4°C in 1 M NaCl in 0.05 M Tris-HCl buffer (pH 7.4). The precipitate was suspended in buffer H with 10% (wt/wt) purified collagenase (type VII, Sigma). Digestion was carried out for 24 h at 37°C with mild shaking. The clear supernatant was used for determination of hydroxyproline content and fluorescence. The remaining undigested collagen represented <15% of the total amount.

Fluorescence was determined in the digest at 440 nm on excitation at 370 nm; the spectra were recorded with an Aminco-Bowman spectrofluorometer, and the results were expressed in arbitrary units of fluorescence (AUF) per milligram hydroxyproline. The hydroxyproline assay was performed according to the method of Stegemann and Stalder (19). Collagen content was calculated assuming a hydroxyproline content of 14% collagen by weight (20).

Statistical analysis included determination of regression lines, confidence limits, and relative correlation coefficients for comparison of regression curves. Analysis of variance and Student's *t* test for unpaired data were also used (21). Data are expressed as means \pm SD.

RESULTS

Effect of age.

Healthy control rats increased their body weight progressively from 180 ± 10 g at 8 wk to 450 ± 25 g at 110 wk. Blood glucose ranged between 2.6 and 5.2 mM but did not show any changes. Similarly, glycosylated hemoglobin, which ranged between 2.2 and 5.6%, showed steady levels with advancing age (Fig. 1, A and B).

Intensity of fluorescence in the insoluble connective tissue increased progressively from 71.7 AUF at 10 wk to 137 AUF at 20 wk to 1110 AUF at 110 wk. The line of best fit was an exponential curve ($y = 67.79 \cdot e^{0.028x}$, $r = 0.969$, $P < 0.001$; Fig. 1C). Blood glucose and glycosylated hemoglobin did not correlate with tissue fluorescence.

Effect of diabetes. All treated and untreated diabetic groups showed very low weight gain (270 ± 40 g) during the study compared with nondiabetic control rats of the same age (380 ± 15 g).

Blood glucose concentration increased to 14 mM within 1 wk after streptozocin injection in all the rats. In the untreated

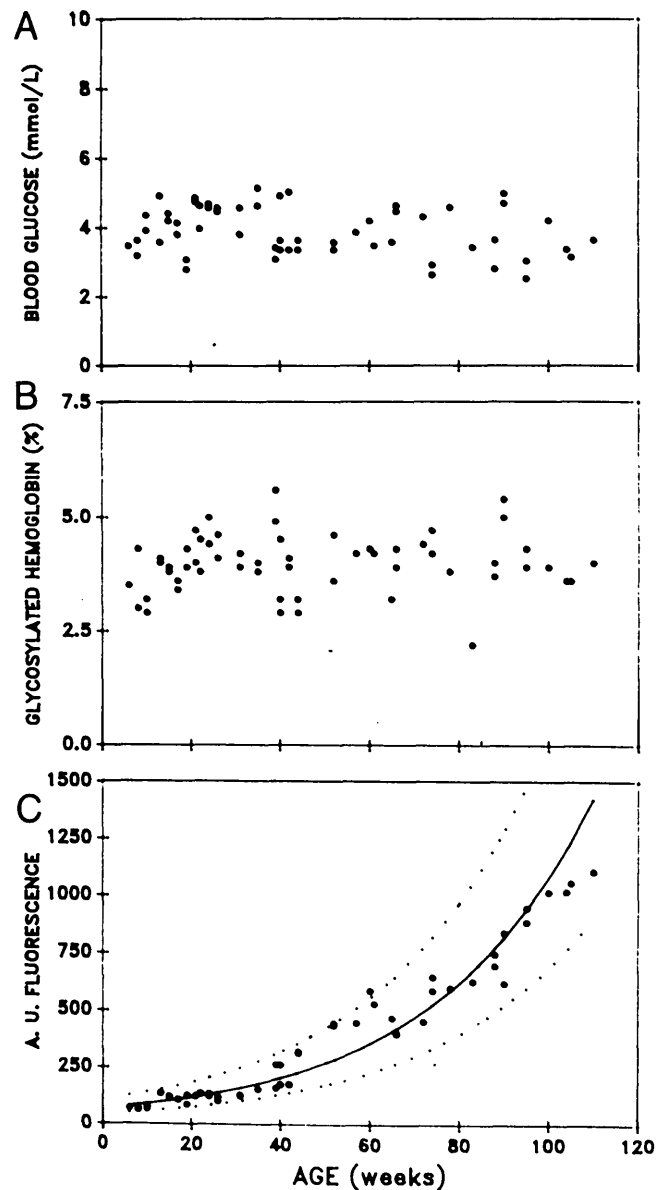


FIG. 1. Relationship between blood glucose (A), glycosylated hemoglobin (B), and arbitrary units (AU) of fluorescence (per mg hydroxyproline; C) and age in nondiabetic Wistar rats. Fluorescence, recorded at 440 nm with excitation at 370 nm, was measured in subcutaneous collagen-digested samples (see RESEARCH DESIGN AND METHODS). Dotted lines, 95% confidence limits. $n = 57$ in each panel. For AU fluorescence regression line, $y = 67.79 \cdot e^{0.028x}$, $r = 0.969$, $P < 0.001$.

diabetic group, blood glucose fluctuated at ~ 16.5 mM (range 15.7–29.1 mM, mean 19.9 ± 5.99 mM; Fig. 2A). We also found increasing levels of glycosylated hemoglobin within 3 wk after streptozocin injection; as expected, it remained elevated for the duration of the experiment (range 6–18.6%; mean $11.8 \pm 4.9\%$; Fig. 2B). In the untreated diabetic group, the emission spectrum of subcutaneous tissues was qualitatively similar to the nondiabetic controls (data not shown).

After a lag of 6–7 wk, the fluorescence increased in an exponential fashion until wk 20 ($y = 1.914 \cdot e^{0.019x}$, $r = 0.864$, $P < 0.01$, $n = 28$; Fig. 2C). Mean fluorescence in this group was 417 ± 471 AUF, and the last values were >1000 AUF.

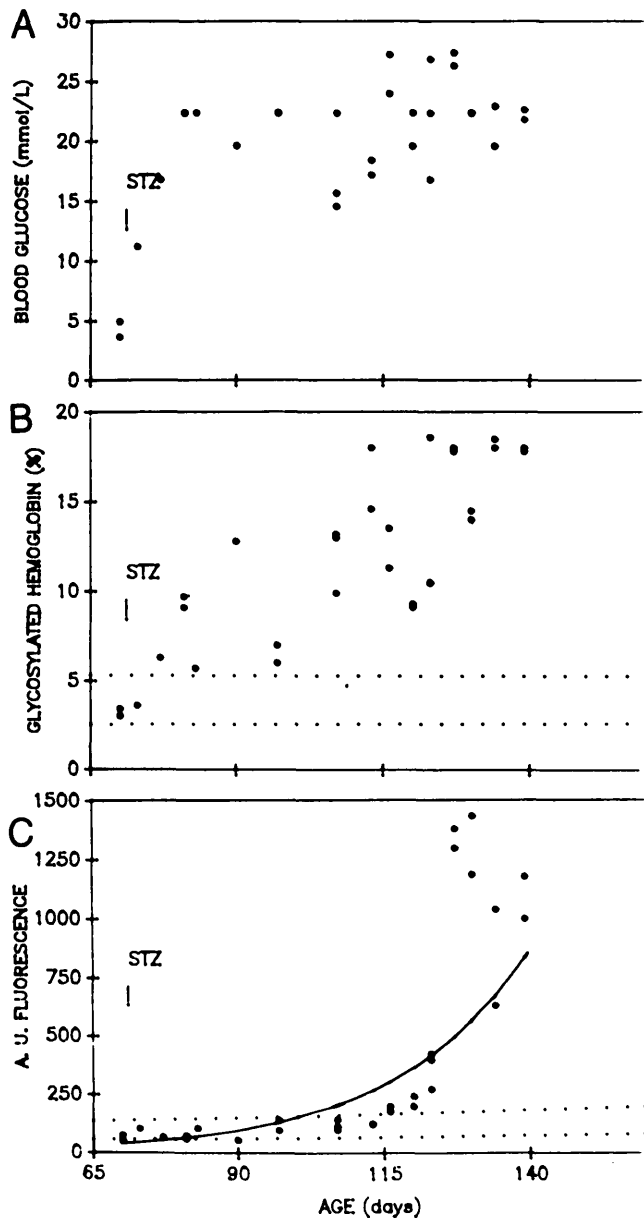


FIG. 2. Relationship between blood glucose (A), glycosylated hemoglobin (B), and arbitrary units (AU) of fluorescence (per mg hydroxyproline; C) and age in streptozocin (STZ)-induced (50 mg/kg i.p. at 10 wk of age) diabetic Wistar rats. Fluorescence was measured as described in RESEARCH DESIGN AND METHODS and Fig. 1. Dotted lines, 95% confidence intervals of nondiabetic rat values. Vertical lines, STZ injection. $n = 28$ in each panel. For AU fluorescence regression line, $y = 1.914 \cdot e^{0.0191x}$, $r = 0.864$, $P < 0.001$.

As expected, there was good correlation between blood glucose and glycosylated hemoglobin ($r = 0.553$, $P < 0.01$). **Effect of aminoguanidine and rutin.** Treatment with rutin did not modify the levels of blood glucose (19.2 ± 5.7 mM) or glycosylated hemoglobin ($12.1 \pm 3.46\%$) (Fig. 3, A and B). However, we observed a significant delay in fluorescence accumulation; overall mean fluorescence was significantly lower than in the untreated diabetic group (270 ± 166 AUF), and the highest value was just under 700 AUF (Fig. 3C).

Aminoguanidine administration induced a more pronounced reduction of connective tissue fluorescence (mean 232 ± 136 AUF, maximum value 450 AUF), and at the end

of the experiment, the levels of fluorescence obtained in this group were similar to values in the untreated diabetic rats with shorter diabetes duration and values of healthy control rats 52 wk old (Fig. 4C). Levels of blood glucose (19.1 ± 7.2 mM) and glycosylated hemoglobin ($11.3 \pm 4.29\%$) were similar to those of other diabetic groups (Fig. 4, A and B).

Analysis of variance of the mean of the last 10 values (matched for diabetes duration) of the three diabetic groups showed a significant difference for the fluorescence values

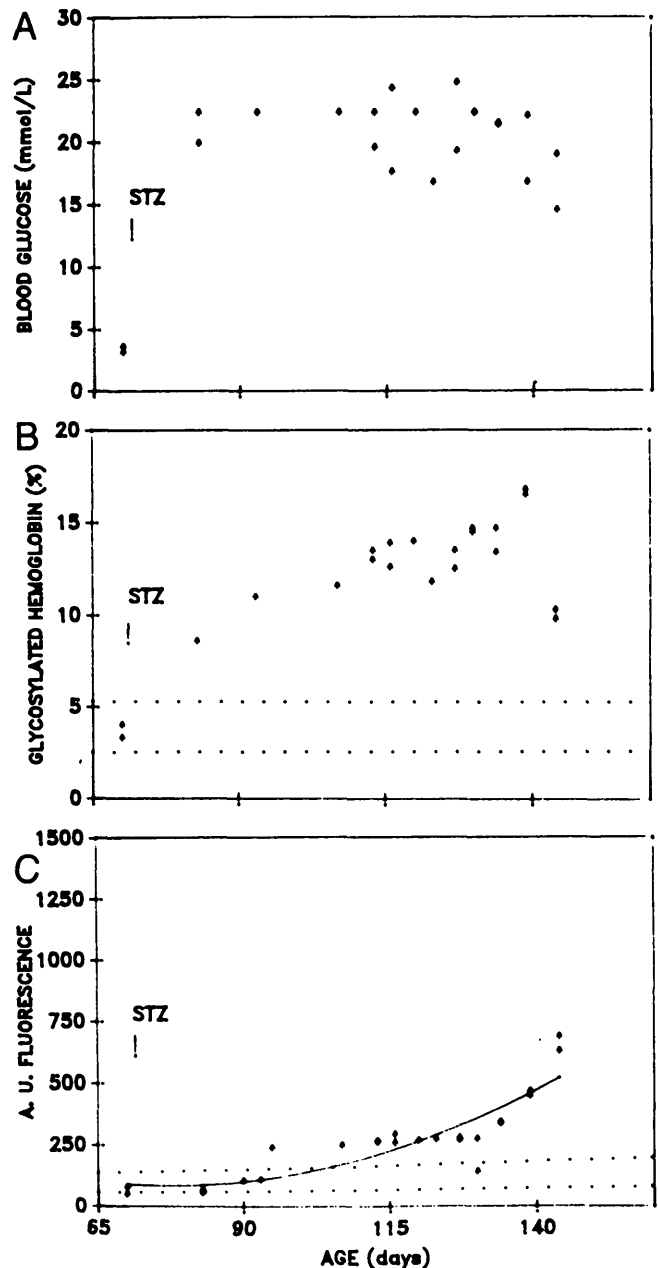


FIG. 3. Relationship between blood glucose ($n = 20$; A), glycosylated hemoglobin ($n = 19$; B), and arbitrary units (AU) of fluorescence (per mg hydroxyproline, $n = 22$; C) and age in streptozocin (STZ)-induced (50 mg/kg i.p. at 10 wk of age) diabetic Wistar rats treated with $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ rutin in drinking water, starting same day as STZ injection (vertical lines). Dotted lines, 95% confidence intervals of nondiabetic rat values. Fluorescence was measured as described in RESEARCH DESIGN AND METHODS and Fig. 1. For AU fluorescence regression line, $y = 651 - 14.78x + 0.097x^2$, $r = 0.881$, $P < 0.001$.

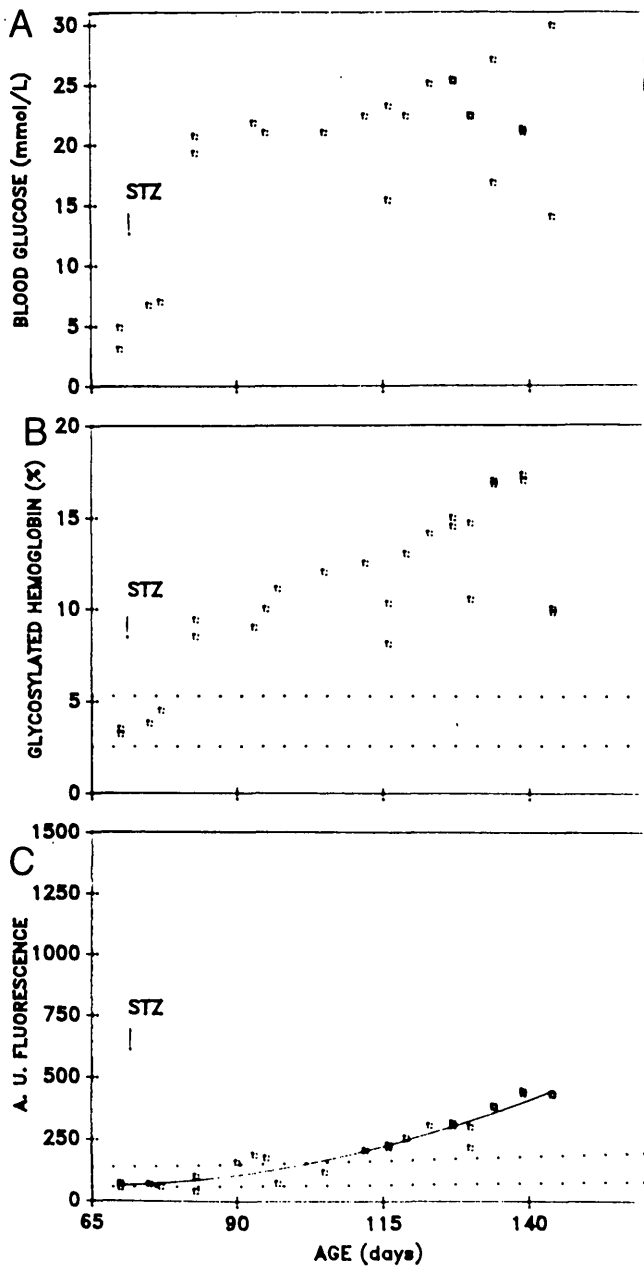


FIG. 4. Relationship between blood glucose ($n = 22$; A), glycosylated hemoglobin ($n = 23$; B), and arbitrary units (AU) of fluorescence (per mg hydroxyproline, $n = 24$; C) and age in streptozocin (STZ)-induced ($50 \text{ mg/kg i.p. at } 10 \text{ wk of age}$) diabetic Wistar rats treated with $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ aminoguanidine in drinking water, starting same day as STZ injection (vertical lines). Dotted lines, 95% confidence intervals of nondiabetic rat values. Fluorescence was measured as described in RESEARCH DESIGN AND METHODS and Fig. 1. For AU fluorescence regression line, $y = 271 - 6.90x + 0.57x^2$, $r = 0.957$, $P < 0.01$.

($F = 31.77$, $P < 0.01$) but not for glucose or glycosylated hemoglobin (Fig. 5).

The correlation between age and fluorescence was highly significant in all three groups but with different slopes (untreated diabetic: $y = 4107.2 - 92.9x + 0.52x^2$, $r = 0.839$, $P < 0.01$; aminoguanidine treated: $y = 271.4 - 6.9x + 0.06x^2$, $r = 0.957$, $P < 0.01$; rutin treated: $y = 651.0 - 14.78x + 0.096x^2$, $r = 0.881$, $P < 0.01$ (Figs. 2C, 3C, and 4C).

DISCUSSION

Several chromophores can be found in tissues and proteins exposed to high levels of glucose in vivo or in vitro (9–11). The ability of reducing sugars to react nonenzymatically with proteins to form these products, also known as advanced glycosylation end products or browning compounds, may contribute to explanations of tissue modifications in diabetes and aging (22,23). So far, these compounds have been evaluated by their characteristic fluorescence properties (9–

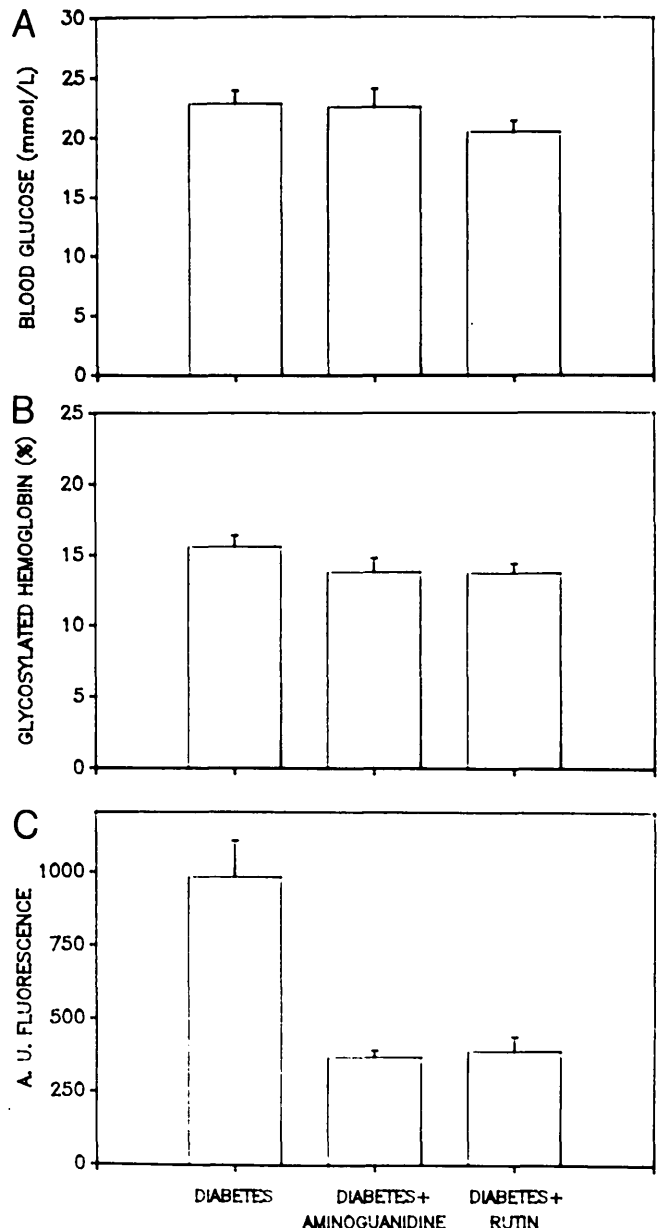


FIG. 5. Means \pm SE of last 10 values (matched for diabetes duration) of blood glucose (A), glycosylated hemoglobin (B), and arbitrary units (AU) of fluorescence (C) in streptozocin-induced diabetic Wistar rats that were untreated, treated with $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ aminoguanidine, or treated with $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ rutin. Fluorescence was measured as described in RESEARCH DESIGN AND METHODS and Fig. 1. Analysis of variance was significant only for fluorescence values ($F = 31.8$, $P < 0.01$); t test for unpaired data showed significant difference between untreated diabetic rats and diabetic rats treated with rutin ($t = 4.27$, $P < 0.01$) and diabetic rats treated with aminoguanidine ($t = 4.70$, $P < 0.01$).

11,24), even though researchers have identified some molecular structures (25–28).

Monnier et al. were the first to demonstrate that fluorescence level increases in dura mater with aging (18) and that there is an overall relationship between levels of fluorescence (excitation at 370 nm, emission at 440 nm) in skin collagen of diabetic patients and the severity of diabetic complications (12,29).

In our study, we confirmed the increase of similar tissue fluorescence with age in healthy rats. Interestingly, there was an exponential increase after 40 wk of age albeit steady levels of blood glucose and glycosylated hemoglobin (Fig. 3).

Thus, it is unlikely that this increase is due to worsening of glucose tolerance. Instead, it may be the combined result of fluorescence accumulation and progressive deterioration of connective tissue turnover, which has a very long half-life (30).

As expected, blood glucose increased quickly and sharply in diabetic rats, whereas glycosylated hemoglobin increased more slowly. In contrast, a striking lag in fluorescence accumulation was observed, suggesting that it may be the result of the Maillard reaction as a consequence of chemical degradation of glycosylated proteins. The exponential increase in fluorescence accumulation in the diabetic rats suggests the presence of unknown accelerating factors, e.g., the saturation of receptor-mediated removal of advanced glycosylation end products or the loss of endogenous aminoguanidineline compounds (31,32). Inhibition of accumulation of these products has been obtained on kidney collagen with aminoguanidine by Giambrone and Brownlee (15) and Soulis et al. (33), who also found a reduction of tissue fluorescence in healthy animals treated with aminoguanidine.

Aminoguanidine is thought to bind to glycosylated proteins, thus preventing their degradation into reactive intermediates that form fluorescent protein adducts and cross-links (8). Our results in Wistar rats confirm the efficacy of aminoguanidine in preventing the age-related accumulation of fluorescence in diabetes.

Interestingly, when we added rutin, an aldose reductase inhibitor, to the drinking water of diabetic rats, we also found a significant decrease in fluorescence compared with the untreated diabetic group (Fig. 5). This could result from a decrease in fructosylated products, which have been shown to generate fluorescence as well (16).

Suarez et al. (16) showed that addition of the aldose reductase inhibitor sorbinil to the diet decreased fluorescence accumulation in diabetic skin and proposed that protein fructosylation was mediated by the sorbitol pathway. However, Cohen and Klepser (34) were unable to find any effect of sorbinil on fluorescence in glomerular membrane collagen of diabetic rats. The nature of this discrepancy is unknown but may be related to differences in the wavelength used for fluorescence measurements or the nature of the fluorescence itself.

Our conclusion from this study is that both aminoguanidine and rutin play a similar protective role against formation of fluorescent products. The recent demonstration that peripheral nerve sorbitol levels are unaffected by aminoguanidine (35) suggests that the mechanism of action of aminoguanidine

and rutin on the formation of tissue fluorescence is different. Inhibition of the Maillard reaction *in vivo* by aminoguanidine may thus have beneficial effects on the pathogenesis of diabetic complications. However, only when specific assays are available for the individual compounds will it be possible to better understand the pathogenic role of tissue fluorescence and precisely evaluate the effectiveness of aminoguanidine as a therapeutic approach.

ACKNOWLEDGMENTS

We thank P. Bocchi for expert assistance with animal care.

REFERENCES

1. Pirart J: Diabetes mellitus and its degenerative complications: a prospective study of 4,400 patients observed between 1947 and 1973. *Diabetes Care* 1:168–88, 252–63, 1978
2. Godin JE: The relationship between metabolic control and vascular complications of diabetes mellitus. *Med Clin North Am* 72:1271–84, 1988
3. Alberti KGMM, Press CM: The biochemistry of the complications of diabetes mellitus. In *Complications of Diabetes*. 2nd ed. Keen H, Jarret J, Eds. London, Arnold, 1982, p. 231–70
4. Green DA, Lattimer SA, Sima AAF: Sorbitol, phosphoinositides, and sodium potassium ATPase in the pathogenesis of diabetic complications. *N Engl J Med* 316:599–606, 1987
5. Cagliero E, Maiello M, Boeri D, Roy S, Lorenzi M: Increased expression of basement membrane components in human endothelial cells cultured in high glucose. *J Clin Invest* 82:735–38, 1988
6. Cerami A, Stevens VJ, Monnier VM: Role of non-enzymatic glycosylation in the development of the sequelae of diabetes mellitus. *Metabolism* 28 (Suppl. 1):431–37, 1979
7. Kennedy L, Baynes JW: Non enzymatic glycosylation and the chronic complications of diabetes: an overview. *Diabetologia* 26:93–98, 1984
8. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetes complications. *N Engl J Med* 318:1315–21, 1988
9. Hodge JE: Chemistry of browning reactions in model systems. *Agric Food Chem* 1:928–43, 1953
10. Reynolds TM: Chemistry of non enzymatic browning. *Adv Food Res* 14:167–283, 1965
11. Ledl F, Beck J, Sengl M, Osiander H, Estendorfer S, Severin T, Huber B: Chemical pathways of the Maillard reaction. *Prog Clin Biol Res* 304:23–42, 1989
12. Monnier VM, Vishwanath V, Frank KE, Elmets GA, Dauchot P, Khon RR: Relation between complication of type I diabetes mellitus and collagen linked fluorescence. *N Engl J Med* 314:403–408, 1986
13. Brownlee M, Vlassara H, Kooney T, Ulrich P, Cerami A: Aminoguanidine prevents diabetes induced arterial wall protein cross-linking. *Science* 232:1629–32, 1986
14. Brownlee M, Vlassara H, Cerami A: Aminoguanidine prevents hyperglycemia-induced defect in binding of heparin by matrix molecules (Abstract). *Diabetes* 36 (Suppl. 1):85A, 1987
15. Giambrone MA, Brownlee M: Aminoguanidine treatment normalizes increased steady-state levels of laminin B1 mRNA in kidneys of long-term streptozotocin-diabetic rats (Abstract). *Diabetes* 38 (Suppl. 1):83A, 1989
16. Suarez G, Rajarani R, Bhurjan KC, Oronsky L, Goidi JA: Administration of aldose reductase inhibitor induces a decrease of collagen fluorescence in diabetic rats. *J Clin Invest* 82:624–27, 1988
17. Varma DS, Kinoshita JH: Inhibition of lens aldose reductase by flavonoids—their possible role in the prevention of diabetic cataracts. *Biochem Pharmacol* 25:2505–13, 1976
18. 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
19. Stegemann H, Stalder K: Determination of hydroxyproline. *Clin Chim Acta* 18:267–73, 1967
20. Hamlin CR, Kohn RR: Evidence for progressive, age related structural changes in postmature human collagen. *Biochim Biophys Acta* 236:458–67, 1971
21. Armitage P: *Statistical Methods in Medical Research*. New York, Wiley, 1971
22. Cerami A, Vlassara H, Brownlee M: Role of advanced glycosylation products in complications of diabetes. *Diabetes Care* 11 (Suppl. 1):73–79, 1988
23. Monnier VM: Toward a Maillard reaction theory of aging. *Prog Clin Biol Res* 304:1–22, 1989
24. Monnier VM, Cerami A: Nonenzymatic browning *in vivo*: possible process for aging of long-lived protein. *Science* 211:491–93, 1981
25. Njoroge FG, Sayre LM, Monnier VM: Detection of D-glucose derived pyrole compounds during Maillard reaction under physiological conditions.

- Carbohydr Res* 167:211–20, 1987
26. Hayase F, Ramanakoppa HN, Miyata S, Njoroge FG, Monnier VM: Aging of protein: immunological detection of a glucose-derived pyrrole formed during Maillard reaction in vivo. *J Biol Chem* 263:3758–61, 1989
 27. 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
 28. Sell DR, Monnier VM: Isolation, purification and partial characterization of fluorophores from aging human extracellular matrix. *Connect Tiss Res* 19:77–92, 1989
 29. Monnier VM, Elmet CA, Frank KE, Vishwanath V, Yamashita T: Age related normalization of the browning rate of collagen in diabetic subjects without retinopathy. *J Clin Invest* 78:832–35, 1986
 30. Uitto J, Ryhanen L, Tan EML: Collagen: its structure, function and pathology. In *Progress in Diseases of the Skin*. Vol. 1. Fleishmajer R, Ed. New York, Grime & Stratton, 1981, p. 103–41
 31. Vlassara H, Brownlee M, Cerami A: Novel macrophage receptor for glucose modified protein is distinct from previously described scavenger receptors. *J Exp Med* 164:1301–309, 1986
 32. Monnier VM, Sell DR, Miyata S, Nagaraj RH: The Maillard reaction as a basis for a theory of aging. In *Proc Int Symp Maillard Reaction, 4th*. Finot PA, Ed. Basel, Birkhäuser. In press
 33. Soulis T, Cooper ME, Layton G, Allen TS, Jerums G: Aminoguanidine reduces tissue fluorescence but not albuminuria in diabetic rats (Abstract). In *The Maillard Reaction in Aging, Diabetes and Nutrition. Proc Natl Inst Health Conf, Bethesda, MD, 22–23 September 1988*, p. 30
 34. Cohen MP, Klepser H: Aldose reductase inhibition does not prevent increased collagen fluorescence in diabetic rats (Abstract). *Diabetes* 38 (Suppl. 1):29A, 1989
 35. Williamson JR, Chang K, Ido Y, Ostrow E, Allison W, Harlow J, Tilton KJ: Aminoguanidine prevents diabetes (streptozotocin)-induced increase in vascular albumin permeation in rat sciatic nerve. *Diabete Metab*. In press