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

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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 g · kg^{-1} · day^{-1} aminoguanidine, suggested to be an inhibitor of the advanced glycosylation reaction, and 1 g · kg^{-1} · 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 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 ~200 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 ~200 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

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

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 spectrophotofluorometer, 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 ± SD.

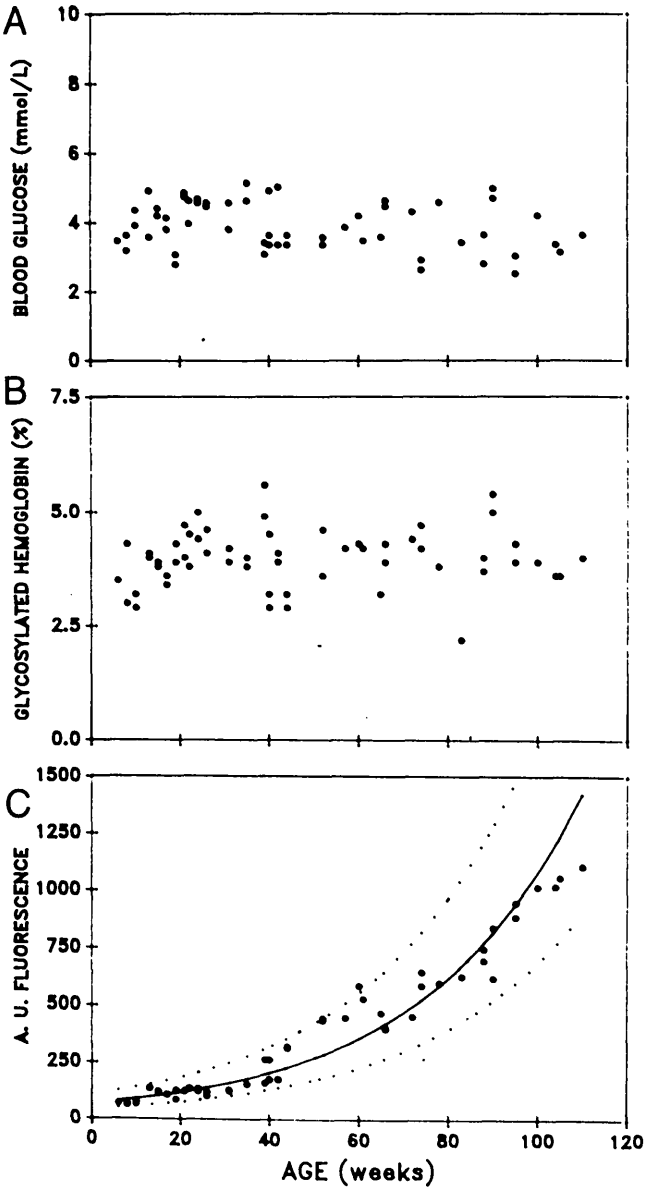

FIG. 1. Relationship between blood glucose (*A*), glycosylated hemoglobin (*B*), and arbitrary units (AU) of fluorescence (per mg hydroxyproline; C) and age in nondiabetic Wistar rats. Fluorescence, recorded at 440 nm with excitation at 370 nm, was measured in subcutaneous collagen-digested samples (see RESEARCH DESIGN AND METHODS). *Dotted lines*, 95% confidence limits. n = 57 in each panel. For AU fluorescence regression line, $y = 67.79 \cdot e^{0.28x}$, r = 0.969, $P <$ 0.001.

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 diabetic group, blood glucose fluctuated at ~16.5 mM (range 15.7–29.1 mM, mean 19.9 ± 5.99 mM; Fig. 2*A*). 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 ± 4.9%; Fig. 2*B*). 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.

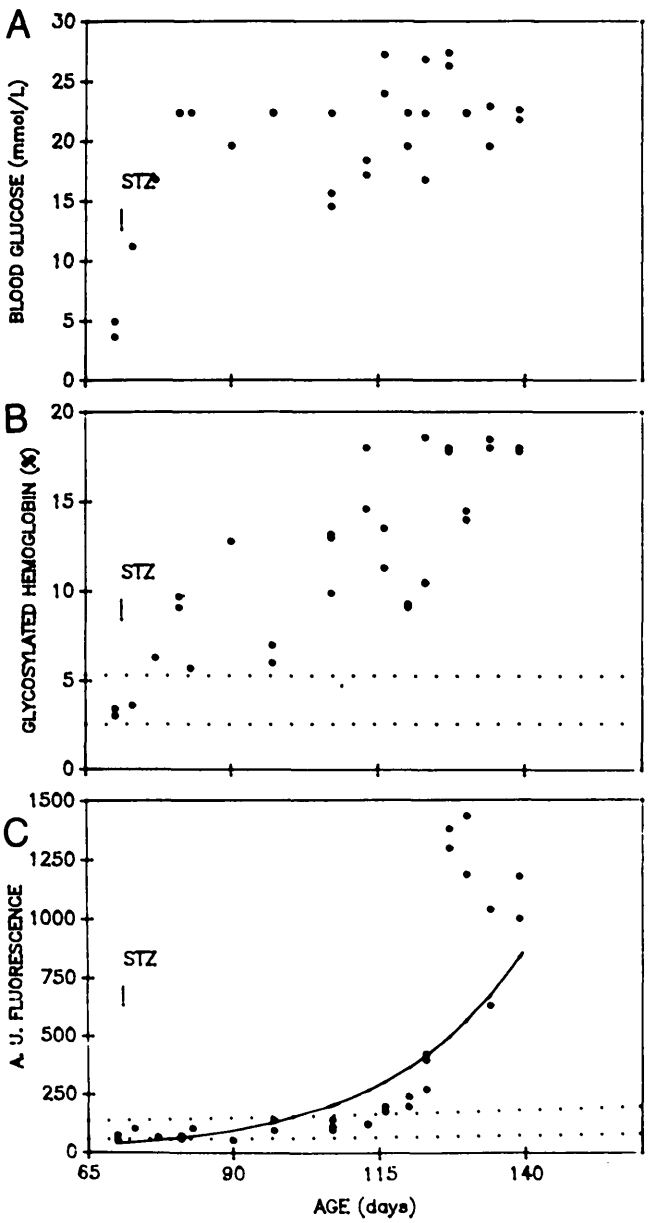

FIG. 2. Relationship between blood glucose (*A*), glycosylated hemoglobin (*B*), and arbitrary units (AU) of fluorescence (per mg hydroxyproline; *C*) and age in streptozocin (STZ)-induced (50 mg/kg i.p. at 10 wk of age) diabetic Wistar rats. Fluorescence was measured as described in RESEARCH DESIGN 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 ± 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. 3*C*).

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. 4*C*). Levels of blood glucose (19.1 ± 7.2 mM) and glycosylated hemoglobin (11.3 ± 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

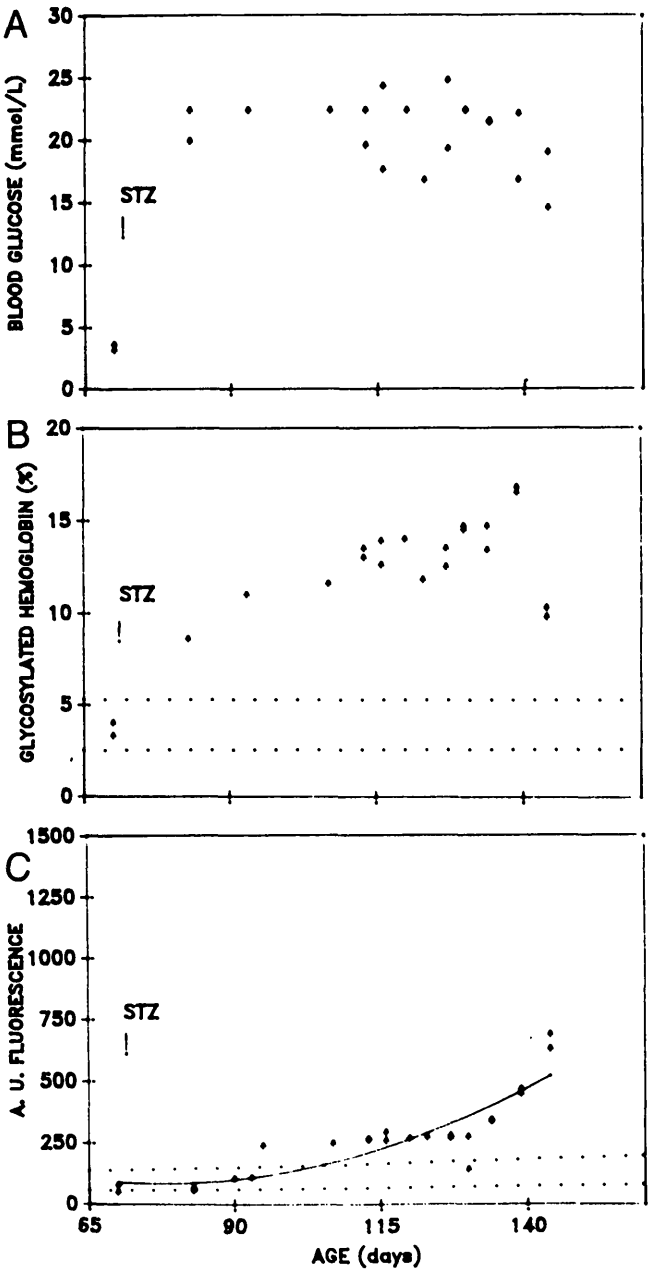

FIG. 3. Relationship between blood glucose (n = 20; *A*), glycosylated hemoglobin (n = 19; *B*), and arbitrary units (AU) of fluorescence (per mg hydroxyproline, n = 22; *C*) and age in streptozocin (STZ)-induced (50 mg/kg i.p. at 10 wk of age) diabetic Wistar rats treated with 1 g · kg^{-1} · day^{-1} 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, $y = 651 - 14.78x + 0.097x^2$, $r = 0.881$, $P < 0.001$.

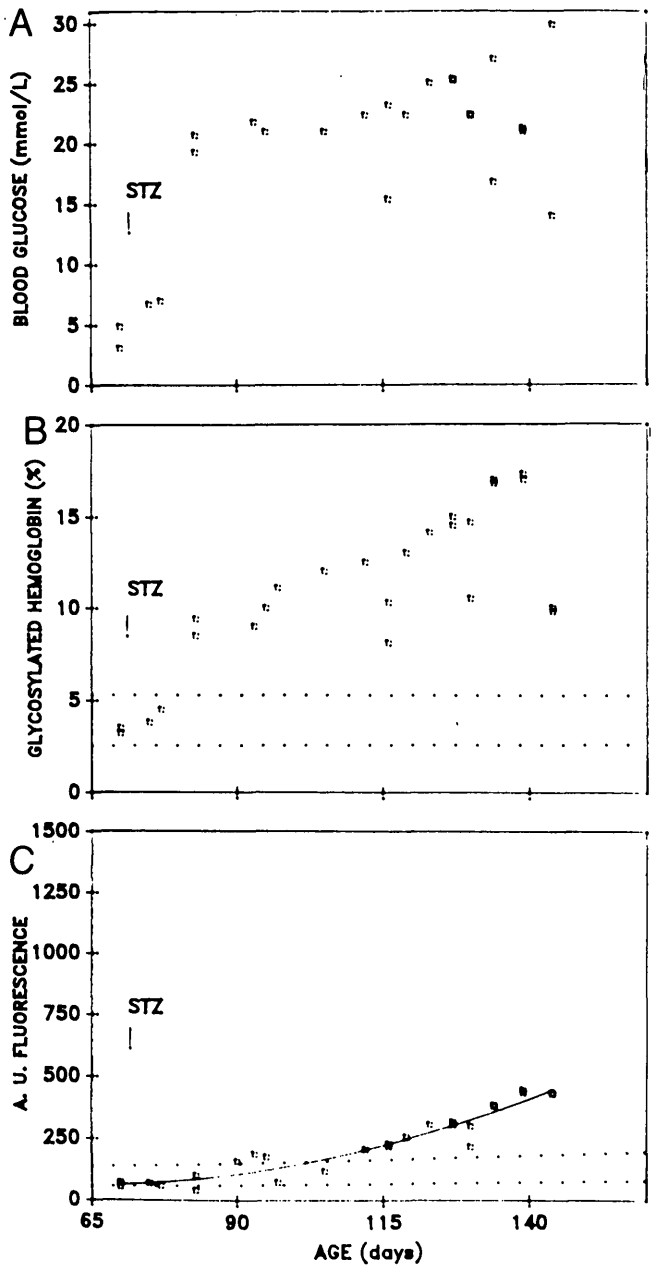

FIG. 4. Relationship between blood glucose (n = 22; *A*), glycosylated hemoglobin (n = 23; *B*), and arbitrary units (AU) of fluorescence (per mg hydroxyproline, n = 24; *C*) and age in streptozocin (STZ)-induced (50 mg/kg i.p. at 10 wk of age) diabetic Wistar rats treated with 1 g · kg^{-1} · day^{-1} 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–

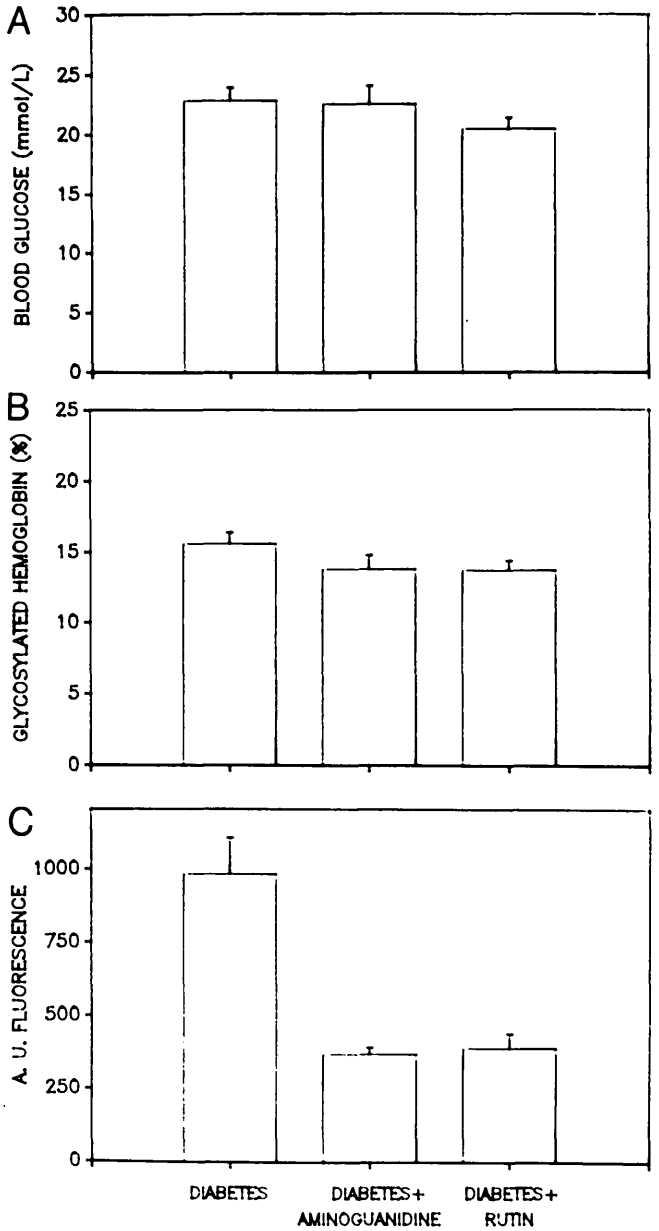

FIG. 5. Means ± SE of last 10 values (matched for diabetes duration) of blood glucose (*A*), glycosylated hemoglobin (*B*), and arbitrary units (AU) of fluorescence (*C*) in streptozocin-induced diabetic Wistar rats that were untreated, treated with (1 g · kg^{-1} · day^{-1}) aminoguanidine, or treated with 1 g · kg^{-1} · 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 aminoguanidinelike 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.

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