Effect of Chronic Aminoguanidine Treatment on Age-Related Glycation, Glycoxidation, and Collagen Cross-linking in the Fischer 344 Rat

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Aminoguanidine (AG) is an inhibitor of protein modification by the advanced Maillard reaction. We evaluated its effects in preventing age-related collagen cross-linking, glycation, and glycoxidation in Fischer 344 rats by administering the drug in their drinking water at 1 g/l from the time they were 6 months until they were 24 months of age. Body weight and food and water consumption were consistently recorded throughout the study. Plasma glucose was measured by the glucose oxidase method, and collagen cross-linking was assessed by tail tendon break time (TBT) in urea. Glycation (furosine) and glycoxidation (pentosidine and carboxymethyllysine) were assessed by high-performance liquid chromatography in acid hydrolysates of skin and tendon collagen. Water consumption dramatically increased ($p < .0001$) after 20 months of age and was accelerated in the control versus AG-treated rats ($p < .0001$). Plasma glucose increased approximately 20% at age 19 months in both groups ($p < .0001$). TBT, glycation, and glycoxidation all increased significantly ($p < .0001$) with age. However, except for a modest decrease of TBT at all ages that approached significance ($p = .077$), AG had no effect on collagen glycation or glycoxidation. These results are important because they suggest that $\alpha,\beta$-dicarbonyl compounds that can be trapped by aminoguanidine do not play a major role in collagen aging in the rat. Instead, post-Amadori pathways involving oxidative or nonoxidative fragmentation of the Amadori product emerge as the more likely mechanism of collagen cross-linking in aging.

CERTAIN aspects of diabetic complications resemble accelerated aging, such as increased rate of cardiovascular disease, increased incidence of cataract formation, and limitations in joint mobility. Because these sequelae occur in the population as a whole during aging, a pragmatic interest is manifest in the discovery and development of pharmaceutical interventions for the prevention or treatment of age-related diseases and disabilities. One such intervention proposed and presently under scrutiny is the use of aminoguanidine (1).

Many studies have shown beneficial effects of aminoguanidine (AG) use in ameliorating or preventing complications caused by experimental diabetes and aging. At the organ level, these have included the inhibition of experimental diabetic retinopathy (2), nephropathy (3), and neuropathy (4). At the tissue level, ameliorations of diabetes-induced glomerular and retinal basement membrane thickening (5), motor nerve conduction velocity (6), retinal pericyte loss (2), cataract formation (7), and a host of vascular and collagen changes (8–10) have been observed. However, the potential usefulness of AG goes beyond the treatment of diabetes. In animal studies, AG use is able to either prevent or retard age-related diseases such as glomerular sclerosis (11) and cardiovascular disease, including arterial stiffening (12), atherogenesis (13), and cardiac hypertrophy (11,12). It is also able to ameliorate cerebral damage from experimentally induced arterial occlusion as a model of stroke (14).

Many hypotheses have been proposed to explain the mechanisms and mode of action of AG. It has been found to inhibit various enzymes such as nitric oxide synthase (15), aldose reductase (16), diamine oxidase (17), and semicarbazide-sensitive amine oxidase (18). Alternatively, a hypothesis that bears significance for the present study has to do with its ability to block the Maillard reaction in the formation of advanced glycosylation end products (AGEs), thus preventing nitric oxide quenching (19) and collagen cross-linking by AGEs (1).

In the Maillard reaction (20), AG has been proposed to react with the Amadori product, thus blocking further reaction (21). However, this hypothesis has not been substantiated (5,22–24). A more likely mechanism has to do with the ability of AG to trap highly reactive carbonyl intermediates of the Maillard pathways as triazine compounds (25–27). More recent in vitro studies have shown that AG inhibits only AGEs formed through $\alpha,\beta$-dicarbonyl fragments originating from these pathways, but it may have little effect on AGEs formed through intermediates of post-Amadori pathways where oxidative decomposition and fragmentation may not occur (26,28). Examples of $\alpha,\beta$-dicarbonyl com-
pounds trapped by AG include glyoxal, methylglyoxal, and 3-deoxyglucosone (26,27,29).

Glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG) are potent protein cross-linking agents (20,26,27,30,31). In addition, glyoxal and 3-DG are precursors of glycoxidation products, in particular carboxymethyllysine (CML) and pentosidine, respectively (26,32,33). Their formation rate is accelerated in diabetes (34,35) and is expected to increase with advancing age (34–37) as a result of the age-related decrease in glucose tolerance that has been observed both in humans and rodents (38,39). Conversely, because caloric restriction is associated with a decrease in mean glycemia (40,41), one would expect their plasma levels and therefore the age-related tissue AGE product formation to decrease in calorically restricted rodents. The latter association was already reported for the glycoxidation products CML and pentosidine (37,42).

Here, we have tested the hypothesis that chronic treatment with AG from 6 to 24 months of age should prevent the age-related collagen cross-linking and glycoxidation as reflected by the tendon break time (TBT), pentosidine, and CML assays. If indeed the α,β-dicarbonyl compounds that are being trapped by AG play an important role in collagen aging, one would then expect AG to inhibit collagen aging as previously reported in diabetic rats (43).

METHODS

Animal and Dietary Procedures

A total of 45, male Fischer 344 (F344) rats were obtained at 6 months of age from Charles River Laboratories (Wilmington, MA) and housed individually in microisolator cages in the animal facility at the University of Texas, San Antonio. Other housing procedures including health surveillance have been previously described (44). Nine of these rats were immediately killed, and tissues including blood were collected as described below. The remaining 36 rats were divided into two groups and housed 3 rats/cage. One group of 18 rats was fed a normal chow diet (Teklad LM-485 Autoclavable Diet 012, Harland Teklad, Madison, WI) and given AG hydrochloride (Alteon, Northvale, NJ) in the drinking water at a dosage of 1 g/l. The other group was fed the same diet without AG. Body weights and food and water consumption were recorded weekly throughout the study. Because rats were housed in groups, food consumption was determined by subtracting the final weight from the original weight of food placed in the hopper divided by the time interval between measures and the number of rats per cage. Similarly, water consumption was determined from the disappearance of water in the bottle placed in each cage as measured by weight. In turn, weight was converted to volume by using the density of water (Figure 1).

Over the time interval of this study, a total of three rats were lost as a result of death from unknown causes—one from the control group and two from the AG group.

Tissue and Plasma Collection Procedures

When the rats were ages 10, 12, and 19 months, their blood was collected in heparinized tubes from a nick in their tail veins. As a way to avoid stress effects on glucose levels, samples were obtained within 1 minute of initial cage disturbance. Six rats from each group at ages 12 and 19 months, and the remaining rats at 24 months, were killed by CO₂ narcosis. Tissues consisting of abdominal skin and whole tails were immediately excised. Skin was frozen in small plastic bags at −70°C. Tendon bundles were dissected from individual tails and similarly stored frozen in 1-ml microcentrifuge tubes containing saline.

Measurements of Plasma Glucose

Plasma was prepared by centrifugation of blood samples for 20 minutes at 4°C and then stored frozen at −70°C. Glucose levels were measured by the glucose oxidase method as previously described by Masoro and colleagues (40).

Measurement of TBT, Furosine, Pentosidine, and CML

Skin and tendon samples were shipped to Cleveland on dry ice and stored frozen at −70°C. TBT was determined as previously detailed (45). Samples of tendon and skin were also extracted and processed by methods described elsewhere (45). Pentosidine and furosine were determined in acid hydrolysates by high-performance liquid chromatography (HPLC) as described earlier (37,45,46). Similarly, CML was determined by HPLC with a two-step injection technique with postcolumn derivatization using phthalaldehyde reagent (OPA) as described elsewhere (47).

Statistics

Homogeneity of variance among treatments and normality within treatment groups were tested at α = .05 by the Burr-Foster Q Test and the Wilk-Shapiro W Test adjusted for unequal sample sizes, respectively (48,49). Data were transformed by either the square root or logarithmic transformation for statistical comparison of treatments (49). The outlier test that was conducted at α = .05 and the test for
differences in slopes were made by methods described by Snedecor and Cochran (50). Pearson correlations, regression analysis and equations, and analysis of variance (ANOVA) were computed by SPSS (Chicago, IL). The comparison of means in Figure 2 was by the Newman-Keuls/SNK test (49).

TBT, glycation and glycoxidation data (Figures 3 and 4) were tested for homogeneity of variance among treatments and normality within treatment groups. In these tests, all rejections occurred in the untreated control groups. Both homogeneity and normality were rejected once for TBT (Figure 3) as a result of the data point at \([x, y]\) coordinates \([19, 196]\), where \(x = \text{age}\) and \(y = \text{level}\). Normality was further rejected four times as a result of the following data points: skin furosine, \([19, 643]\); tendon pentosidine, \([6, 0.76]\); and twice for skin CML, \([6, 131]\) and \([19, 119]\). In a further analysis, four of these five data points were found to be significant \((p < .001)\) outliers. The one exception was that for pentosidine. Thus, in the results, these data points are excluded from computations and analyses of treatment means shown in Figures 3 and 4.

### Results

Male F344 rats were treated with AG in their drinking water at 1 g/l from 6 to 24 months of age. Initial ANOVA analyses showed significant \((p < .0001)\) main effects caused by age when data were pooled across treatments for body weight, food consumption, and water consumption (Figures 1A–1C). Mean body weights of rats significantly \((p < .0001)\) increased until age 17 months and thereafter significantly \((p < .0001)\) declined at an accelerated rate, especially beyond 20 months of age (Figure 1A). As determined by ANOVA across all ages, AG treatment compared with the control treatment (Figures 1A–1C) was found to significantly affect both food \((p = .003)\) and water \((p < .0001)\) consumption, but not body weight \((p = .996)\). However, in further analyses across limited ages, these effects from food and water became significant only after 20 months \((p = .013\) and \(p < .0001)\), and not before this age \((p = .8\) and \(p = .2)\). Regression analyses showed a slight but non-significant \((p = .6)\) increase in food consumption in the control rats after age 20 months. In contrast, mean food consumption in AG-treated rats significantly \((p = .003)\) decreased after this age (Figure 1B). In comparison, mean water consumption after the age of 20 months increased in both treatments (Figure 1C). This observation was significant and more accelerated in the control group (slope = +4.3, \(p = .005)\) than it was in the AG group (slope = +2.0, \(p = .055)\). Subsequently, a slope test showed these two slopes (i.e., 4.3 vs 2.0) were significantly \((p < .0001)\) different.

Because water consumption began to increase in both control and treated rats after the age of 20 months (Figure 1C), but food consumption showed opposing trends during this time (Figure 1B), the data of Figures 1B and 1C were reexpressed as water consumed per food intake as shown in Figure 1D. This reexpression was necessary to show that the age-related increase in water intakes was not simply due to increased food intakes occurring in control rats during this time. Interestingly, differences between the control versus treated rats now appeared after the age of 18 months (Figure 1D). However, the results were still the same. An ANOVA showed significant \((p < .0001)\) main effects for both age and treatment. Likewise, a slope analysis showed that the age-related increase occurred more rapidly (Figure 1D) and...
significantly ($p < .0001$) in the control versus treated rats (i.e., slope = 0.077 vs 0.064).

As a preliminary to the assessment of glycation and glycoxidation, glucose was measured at ages 10, 12, and 19 months in the plasma of these rats (Figure 2). As previously explained, great care was taken not to stress the rats at the time of bleeding. Results showed significant effects for age ($p < .0001$) and treatment ($p = .011$). Results from a multiple comparison test (SNK) showed that glucose was moderately but significantly ($p < .01$) elevated in control versus AG-treated rats at ages 10 and 19 months, but not at 12 months (Figure 2).

Collagen glycation (furosine), glycoxidation (pentosidine, CML), and cross-linking (TBT) were determined in skin and tendon of Fischer 344 rats. Each point is mean level $\pm$ SD at the indicated age. The age effect is significant ($p < .0001$) whereas that for aminoguanidine is nonsignificant ($p > .05$) for all parameters by analysis of variance. (see Figure 1 for the legend).

### Discussion

AG is a nucleophilic hydrazine that is capable of preventing cross-linking and other AGE formation in collagen during experimental diabetes as shown in vitro (24) and in vivo (8,9,51). Currently, it is being evaluated as a drug for the treatment of diabetes-related complications in clinical trials with humans (1). In these trials, despite problems with early closure of some cohorts (52), the results showed that AG was able to reduce the risk of doubling of serum creatinine in Type I diabetic patients with progressive renal disease. However, this result, which was also the study’s primary endpoint in the Phase III clinical trials, did not reach statistical significance (53).

Previously, it has been shown that AG is able to inhibit experimental diabetes-induced tail tendon cross-linking and autofluorescence of collagen in rodents (1,2,8,9,51). In the present research, we wanted to determine whether chronic AG treatment would affect the age-related increase of parameters previously measured in our laboratory for collagen cross-linking, glycation, and glycoxidation (37,45,46). A strong rationale for this study was the fact that we previously demonstrated an inverse relationship between longevity and pentosidine formation rate in skin of several mammalian species (37). Thus, the availability of a drug that would block its formation might be helpful in subsequent studies to investigate whether the processes determining longevity and tissue glycoxidation and cross-linking are linked.

The results of this study show that AG treatment of F344 rats from 6 to 24 months of age decreased the variation noted within parameters of untreated control rats somewhat. This has also been observed in another study investigating the effects of AG on glycation and glomerular capillary basement membrane thickness in birds (54). It was also found in the present study that AG had a marginal effect upon inhibiting the age-related increase of TBT, but without an effect on glyoxidative parameters measured in skin and tendon. Furthermore, even though there was a small but consistent difference between control and treated groups in TBT at each measured time point shown in Figure 3, the inhibitory effect of AG upon TBT was not progressive (Figure 3) and the difference as a whole only approached statistical significance by an ANOVA ($p = .077$).

As observed in a previous study with rodents (46), furosine progressively and dramatically increased with age at almost the same rate as that noted for pentosidine and CML (Figure 4). Interestingly, furosine is a measure of the Amadori product specific for glucose (46), whereas both pentosidine and CML are measures of AGEs and can originate from a variety of sugars (26,37,45,46). In addition, CML can originate from dicarbonyl intermediates; that is, it can originate from glyoxal and glycolaldehyde, from the degradation of lipid (33), and the metabolism of serine by myeloperoxidase (55). Furthermore, the finding in the present study that furosine most highly correlated with the pentosidine level of the same tissue suggests that pentosidine originates from glycation.

Based on previous data, AG was not expected to have an effect on furosine levels because this drug reportedly does not affect the initial step of the Maillard reaction, namely glycation (5,22–24). This was confirmed in Figure 4. How-
ever, if AG was an efficacious anti-AGE agent, it would be expected to inhibit both tissue pentosidine and CML formation. As shown in Figure 4, there was a trend for tendon CML to be progressively lower in the treated group at ages 12 and 19 months, but the trend did not hold true at 24 months. There was also no effect of AG treatment on skin CML (Figure 4).

Among various reasons for the failure of AG to block cross-links and AGE formation is the administering mode and dosage of the drug. This has been a contentious issue in the human clinical trials. For example, in one study (56), a fairly high dose (1200 mg) administered orally to diabetic patients resulted in plasma levels of only 10 μg/ml. Thus, the efficacy of the drug to produce the desired effects has been questioned (57) and previously addressed in animal studies by several other investigators (10,54,58). However, the route and dosage used in the present study, that is, 1 g/l in drinking water, have proven adequate in many studies (8,11,12,59). Regardless of the route of administration, the effect of AG on tissue glycoxidation has not always been consistent, especially in studies of experimental diabetes. For example, in the studies by Soulis-Liparota and colleagues (59), AG prevented the increase of diabetes-related collagen fluorescence in isolated glomeruli and renal tubules, but surprisingly not in the whole kidney of diabetic Sprague-Dawley (SG) rats. Likewise, in the study by Nyengaard and colleagues (10) using the same rat strain, it was found that the effects of AG on tissue glycoxidation and other diabetes-related parameters were strikingly discordant. On the one hand, it normalized aortic pentosidine, but on the other hand it surprisingly had no effect on collagen-linked fluorescence measured in aorta, kidney, and skin. Nyengaard and colleagues (10) measured AG concentrations in these tissues because of suspected dosage inadequacy. However, significant levels of this drug were detected in all tissues assayed. Thus, it may be concluded from the results of these investigators that the formation of these AGE fluorophores involved mechanisms not accessible to the action of AG, or do not involve dicarbonyl intermediates. Conversely, the ability of AG to block aortic pentosidine formation suggests that the latter may originate from a non-Amadori product precursor such as, for example, dehydroascorbic acid.

Evidence for the existence of mechanisms that are not blocked by AG in AGE formation was suggested initially by the work of Edelstein and Brownlee (22). AG was found to bind to fragments of glycated peptide, but surprisingly failed to bind to intact glycated peptide. In the one study by Glomb and Monnier (26), AG was totally ineffective in preventing the formation of CML when starting from a model Amadori product. In contrast, it was effective when glycation was started in the presence of the free sugar. Likewise, Booth and colleagues (28) reported that AG at different concentrations had negligible effects on the post-Amadori formation of AGEs from ribosylated serum albumin. Thus, these results suggest that AG is ineffective in the prevention of AGE formation in post-Amadori pathways not involving fragmentation products.

There is evidence that rodent strains may vary in their responsiveness to AG treatment. This is clearly shown in the results of Li and colleagues (11), in which AG provided to nondiabetic SG and F344 rats was able to ameliorate the age-related increases of albuminuria and proteinuria in both strains. However, it was found that F344 rats had less striking age-related structural changes in renal pathology and thus were in general less influenced by AG treatment compared with the SG strain. Interestingly, this study has comparative relevance to the present one in that both used F344 rats treated with AG at 1 g/l in their drinking water from ages 6 to 24 months. In the study by Li and colleagues (11), AG in F344 rats significantly reduced AGE formation measured by enzyme-linked immunosorbent assay in heart tissue, but had no effect on that of aorta and kidney during aging. In contrast, AG significantly reduced AGE formation in all three tissues in the SG rat. It was concluded that the SG rat was more responsive to the effects of AG because of this strain’s known susceptibility to renal disease at old age. However, a more important factor in explaining strain differences may relate to differences in drug half-life, which were not studied.

In summary, we found that AG, when supplied to F344 rats in their drinking water from 6 to 24 months of age, had a modest effect on inhibiting the age-related increase in TBT, a parameter of cross-linking, and no effect on skin and tendon glycoxidation as measured by pentosidine and CML. These mechanisms most probably involve post-Amadori pathways where intermediates do not undergo decomposition and fragmentation side reactions. In support, Degenhardt and colleagues (60) reported no affect of AG’s inhibiting the increase of pentosidine and CML in skin collagen of Lewis rats with streptozotocin-induced diabetes. Similarly, these investigators have also reached the same conclusion concerning the mechanism of formation of pentosidine and CML in skin. A secondary finding in the present study is that we observed an age-related increase in water intake in old rats greater than age 18 months. This also has been previously observed for F344 and SG strains of rats by Rowland and colleagues (61). The observation that AG was able to significantly ameliorate the increased water intake (Figures 1C and 1D), together with the noted age-related increase in albuminuria in this and other rat strains (11,60), may reflect a beneficial effect of the drug on age-related nephropathy as reported by Li and colleagues (11).

Conclusions

From this study, one can conclude that AG is a poor inhibitor of the advanced Maillard reaction in collagen-rich tissues. It is likely that the strong effects of AG on diabetic nephropathy and renal immunoreactive AGE formation result from a biological reduction of AGE precursors rather than from its ability to chemically trap reactive intermediates of the Maillard reaction. Alternatively, the fact that AG did not have any effect on glycoxidation and cross-linking can be interpreted in terms of its inability to trap post-Amadori, non-oxidative intermediates of the Maillard reaction in vivo. In contrast, AG as an inhibitor of the reaction in vitro is well documented (1,2,20–24,27–29). As previously mentioned, AG inhibits several enzymatic activities (15–18), so the possibility exists that its main mechanism of action in vivo is indirectly related to the Maillard reaction.
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Received January 18, 2001
Accepted April 23, 2001
Decision Editor: John Faulkner, PhD


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