Chronic Aminoguanidine Attenuates Renal Dysfunction and Injury in Aging Rats

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We have previously shown that aging is associated with increased lipid peroxidation, reductions in renal function, and increased glomerular sclerosis. The mechanism(s) responsible for these age-related changes are not clear. The purpose of the present studies was to determine if there was an increase in inducible nitric oxide synthase (iNOS) with aging, and if so, whether inhibition of iNOS would prevent aging injury by preventing free radical-mediated lipid peroxidation. iNOS protein expression in the kidney increased by approximately 90% by 24 months. Inhibition of iNOS by aminoguanidine (0.1% in drinking water) for 9 months, beginning at 13 months of age, reduced blood pressure, improved glomerular filtration rate by 70%, and renal plasma flow by 40%, whereas glomerular sclerosis was considerably reduced. Renal F$_2$-isoprostanes and malondialdehyde levels, markers of oxidative stress and lipid peroxidation, were not reduced by aminoguanidine. Aminoguanidine also did not attenuate immunostaining for advanced glycosylation end products (AGE) in the kidneys. These findings suggest that aminoguanidine attenuates aging renal dysfunction by inhibiting a pathophysiologic function of iNOS that is independent of free radical-mediated lipid peroxidation or significant effects on AGE deposition.

KEY WORDS: F$_2$-isoprostanes, glomerular filtration rate, renal plasma flow, advanced glycosylation end products, lipid peroxidation, free radicals.

Aging in humans and rats is characterized by a decrease in renal function and glomerular sclerosis.$^{1-3}$ The mechanism(s) responsible for age-related renal injury are not clear. Various hypotheses have been put forth. One hypothesis that has received considerable attention is the possibility that free radical generation and associated lipid peroxidation are important factors in causing age-related injury.$^4$ Lipid peroxidation could play a particularly important role in the age-related injury of the glomerular capillary by promoting sclerosis.$^{5,6}$ We have previously found that F$_2$-isoprostanes, a reliable index of tissue lipid peroxidation,$^7$ are elevated in the kidneys of aging rats.$^8$ We have also

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demonstrated that antioxidant treatment with vitamin E can prevent this age-related increase in renal F₂-isoprostanes and improve renal function.⁸

In line with the oxidative injury hypothesis of aging is the role that nitric oxide (NO), a free radical, may play in promoting age-related oxidative stress and subsequent renal injury. Of the three types of NO synthase isoforms, the inducible isoform of NO synthase (iNOS) produces large (nanomolar) amounts of NO for prolonged periods of time.⁹ NO produced by iNOS is known to have important actions in cellular cytotoxicity⁹,¹⁰ and may also play an important role in mediating the lipid peroxidation associated with renal aging. Treatment of aging rats with aminoguanidine, an inhibitor of iNOS,¹¹ has been shown to prevent the cardiovascular and renal morphologic changes associated with aging.¹² However, in that study the authors proposed that the mechanism for protection was the effect of aminoguanidine to prevent production of advanced glycosylation end products (AGE), and therefore they did not measure any indices of oxidative stress.¹²

The present studies were performed to determine whether iNOS protein levels are increased in the kidney of aging male rats, whether long-term aminoguanidine treatment could prevent the age-related reduction in renal function, and if so, whether the protection with aminoguanidine was afforded by inhibition of lipid peroxidation or by reduction in renal AGE.

MATERIALS AND METHODS

Rats Male Sprague Dawley rats were purchased from the aging colony at Harlan SD (Indianapolis, IN), and allowed to equilibrate for at least 1 week before study. All rats were maintained on standard rat diet (Teklad, Harlan, SD) and tap water in a 12-h/12-h light/dark cycle.

Detection of Renal iNOS Protein by Western Blots

For Western blot analyses, the kidneys from young (aged 5 months, n = 6) and old rats (aged 12 and 24 months, n = 5–7 each age) were homogenized 20% (w/v) in 20 mmol/L HEPES, pH 7.5, containing 100 μmol/L peptatin A, 100 μg/mL aprotinin, 10 mmol/L EDTA, 100 μg/mL leupeptin, 1 mmol/L phenanthroline, and 1 mmol/L E-64 (Sigma Chemical Company, St. Louis, MO). Western blots for iNOS were performed as previously described for endothelial NO synthase,¹³ using a commercially available mouse monoclonal anti-bNOS antibody (1:1250) (Transduction Laboratories, Lexington, KY). The bound antibody was detected by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL) on x-ray film. Densities of bands on films were scanned and quantified.

Experimental Design for Aminoguanidine Treatment

Rats were divided into two groups: Group 1, control group of old untreated rats, aged 13 months (n = 6), which was allowed to age for 9 months; and Group 2, old rats, aged 13 months, treated with aminoguanidine (0.1%) in the drinking water for 9 months (n = 6). Water intake was measured daily. The resulting dose of aminoguanidine was approximately 50 to 70 mg/kg/day. To verify that the dose of aminoguanidine used did not inhibit endothelial NOS (eNOS),¹⁴ intravenous infusion of aminoguanidine at 60 mg/kg/h was performed in young rats. This dose had no effect on blood pressure, which would be expected if eNOS were inhibited, and also caused renal vasculature dilation with slight increases in GFR and renal plasma flow (data not shown).

Before the administration of the aminoguanidine, and at 2, 6, and 9 months during the aminoguanidine treatment, rats were placed in metabolism cages and urine was collected for 24 h for measurement of urinary nitrate/nitrite excretion, as previously described.¹⁵

At 22 months of age, control and aminoguanidine-treated rats were anesthetized by intraperitoneal (IP) injection of inactin (100 to 110 mg/kg body weight; RBI, Natick, MA), and placed on a heat-regulated surgery table to maintain rectal temperature at 36° to 38°C. The following catheters were placed: femoral arterial (for continuous monitoring of blood pressure and for blood sampling); femoral venous for infusion of isoncotic artificial rat plasma (2.5 g/dL bovine immunoglobulin, 2.5 g/dL bovine serum albumin in Ringer’s solution) at 12.5 mL/kg/h for 45 min during the preparatory surgery and thereafter at 1.5 mL/kg/h throughout the experimental period, to maintain a euvoletic preparation¹⁶,¹⁷; left jugular venous for infusion of 0.9% saline with or without ³H-inulin (15 to 20 μCi/mL 0.9% saline; New England Nuclear, Wilmington, DE) at 1 mL/h; and left ureteral for collections of urine samples into oil in graduated glass tubes. A tracheostomy was also performed. The left renal vein was cannulated in the retrograde position with a 23-g needle connected to PE-50 tubing to be used for renal venous blood sampling and calculation of renal plasma flow.

After a 40-min equilibration period for ³H-inulin infusion, two 30-min urine collections were made and midpoint arterial and renal venous blood samples were also taken. After the experiment the left kidney was removed, weighed, and placed in 10% buffered formalin for morphologic and immunohistochemical studies. The right kidney was snap-frozen in liquid nitrogen for determination of malondialdehyde and F₂-isoprostane levels.
Analysis of Urine and Plasma Samples  Urine (1 μL) and plasma (5 μL) samples were counted by liquid scintillation and used to calculate GFR, renal plasma flow, filtration fraction, and renal vascular resistance, as previously described by us.18

Measurements of Indices of Lipid Peroxidation

Kidney Malondialdehyde  Malondialdehyde, an index of lipid peroxidation, in renal homogenates was measured by colorimetric thiobarbituric acid reaction according to the method of Ohakawa, as described previously.19

Renal F2-Isoprostanes  Levels of renal F2-isoprostanes were measured using gas chromatography/negative ion-chemical ionization-mass spectrometry as free F2-isoprostanes after extraction of lipids from the kidney homogenate, saponification, purification, and derivatization, as described previously.20,21

Detection of Advanced Glycosylation End Products  Immunohistochemical detection of AGE was performed using affinity purified anti-AGE IgG, as we have previously described.8,22,23 Briefly, the antibodies were prepared by injecting animals with AGE-modified keyhole limpet hemocyanin and affinity purifying the IgG population on an AGE albumin column. These antibodies have no reaction in normal human kidneys, but recognize AGE in tissues and plasma from diabetic individuals.22 Using this antibody, we have previously shown that aging in the rat kidney is associated with an increase in the immunostaining for AGE.8 Sites of binding of primary antibody were visualized using the avidin-biotin peroxidase method according to the manufacturer’s instructions (Sigma Chemical Company).

 Morphologic Studies  Kidneys were formalin-fixed, embedded in paraffin, cut into 3-μm serial sections, and placed on slides. Slides were stained with periodic acid Schiff (PAS) reagent. More than 200 glomeruli per section were evaluated by a blinded observer for the presence of sclerosis and graded according to the percentage of each glomerulus undergoing sclerosis (0, 1–25%, 26–50%, 51–75%, 76–100%).

Statistical Analyses  Statistical differences in densitometric scans of bands on Western blots from the three groups of aging rats were determined by analyses of variance (ANOVA), with \( P < .05 \) defined as significant. The functional, biochemical, and morphologic data from old rats treated with aminoguanidine and old untreated rats were analyzed by unpaired \( t \) test using Statview 512 software for the Macintosh. Significance was defined as \( P < .05 \). All data values are expressed as mean ± SEM.

RESULTS

The Effect of Aging on Renal iNOS Protein Expression  Figure 1 shows the effect of aging on iNOS protein as determined by Western blot analyses. There was a slight increase (14%) in renal iNOS protein in rats aged 12 months, compared with young rats (5 months). By 24 months of age renal iNOS protein was further increased by 90%.

Effect of Chronic Aminoguanidine in Aging Rats

Body Weights and Urinary Nitrate/Nitrite Excretion  Chronic aminoguanidine treatment during the 9 months had no effect on body weights measured monthly as the rats aged (data not shown). Urinary nitrate/nitrite excretion, an index of systemic NO production (Figure 2), was increased in old control rats at 15 months of age compared with 13 months, but gradually declined with further aging, as we have reported.
Treatment with aminoguanidine significantly attenuated nitrate/nitrite excretion at all time points throughout aging. In addition, the percentage of inhibition with aminoguanidine increased from 28% at 15 months of age to greater than 50% at 19 and 22 months of age.

Renal and Systemic Hemodynamics At 22 months of age, renal function was measured in the old control rats and those treated chronically for 9 months with aminoguanidine. Body and kidney weights at the time of renal function studies were not different between the two groups of rats (old untreated: 536.0 ± 20.3; old aminoguanidine: 501.5 ± 11.5 g; 2.12 ± 0.08 g). Hematocrits were also not different between the two groups (old untreated: 44.0 ± 1.1; old + aminoguanidine: 43.7 ± 0.6).

As shown in Figure 3, rats given aminoguanidine chronically had lower mean arterial pressure when compared with old untreated rats. Furthermore, as shown in Figure 4, chronic aminoguanidine improved GFR by more than 70% and renal plasma flow by more than 40%, compared with untreated old rats. In addition, renal vascular resistance was decreased by approximately 50% with aminoguanidine.

Effect of Aminoguanidine on Oxidative Stress in the Kidneys To assess the effect of aminoguanidine on the level of oxidative stress and lipid peroxidation in the aging kidneys, the sensitive method of measuring F2-isoprostanes was performed. Although numerically reduced, F2-isoprostane levels were not statistically different in the kidneys of rats given aminoguanidine (control: 10.8 ± 3.4; aminoguanidine: 6.3 ± 0.7 mg/g tissue). The other, less sensitive but commonly used method of detection of lipid peroxidation, malondialdehyde, was also performed. Kidney malondialdehyde levels were also not different between old untreated rats and those old rats treated chronically with aminoguanidine (control: 10.8 ± 1.6; aminoguanidine: 9.5 ± 0.6 nmol/mg protein).

Effect of Chronic Aminoguanidine on AGE Production in the Kidney Kidney sections were also immunostained for AGE using anti-AGE antibodies. We have previously found that there is an age-related increase in glomerular and interstitial staining for AGE in the kidney. There was also a marked increase in the adventitia of blood vessels in the aging rat kidney. However, in the present study we found that aminoguanidine treatment did not alter this staining pattern for AGE when kidney sections from untreated and treated rats were compared (data not shown).

Effect of Chronic Aminoguanidine on Glomerular Morphology As shown in Figure 5, aminoguanidine attenuated the age-related increase in glomerular sclerosis. There were more glomeruli with no sclerosis in the aminoguanidine-treated old rats than in the untreated group and the progression of glomeruli to obsolescence was also slowed with aminoguanidine.

DISCUSSION

The present study is the first evidence to our knowledge that iNOS protein, as detected by Western blots, increases in the kidney with aging. Adding further support to the hypothesis that iNOS increases with...
Aging is the finding that aminoguanidine, an inhibitor of iNOS, caused the excretion of nitrate/nitrite, metabolites of NO, to be reduced after the age of 15 months in rats. This resulted in a progressively greater increase in the percent of inhibition by aminoguanidine with aging. Because aminoguanidine has no effect on nitrate/nitrite excretion in young animals, these data suggest that the iNOS isoform plays an increasing role in the production of total body NO with aging.

Another finding of this study is that long-term aminoguanidine, an inhibitor of iNOS, attenuated the age-related reduction in renal function and protected against age-related glomerular injury. With aminoguanidine GFR was markedly improved and 75% of the glomeruli examined were without sclerosis, compared with only 60% that were normal in control rats. Thus aminoguanidine attenuated age-related structural and functional injury.

In contrast to the other NO synthase isoforms, iNOS produces nanomolar amounts of the free radical NO (compared with picomolar amounts by the other NO synthase isoforms) for prolonged periods of time. High concentrations of NO have been shown to be cytotoxic to cells. When Li and colleagues reported that long-term administration of aminoguanidine (18 months) attenuated age-related glomerular sclerosis in Sprague Dawley rats, we wondered if the mechanism could involve inhibition of iNOS and thus protection against free-radical–mediated lipid peroxidation and oxidative stress. In addition to its role as an inhibitor of iNOS, aminoguanidine also has antioxidant capabilities. As an antioxidant, aminoguanidine could ameliorate free-radical–mediated lipid peroxidation by binding to aldehydes and other reactive oxygen species of fatty acid peroxidation. In support of the free radical theory of aging, we have recently shown...
that lipid peroxidation, as measured by renal F2-isoprostanes, malondialdehyde, and induction of heme oxygenase-1, increases with aging.\textsuperscript{6}

With these previous data in mind and to determine the mechanism(s) by which aminoguanidine protected the kidney against aging injury, we evaluated two indices of free-radical–mediated lipid peroxidation in kidneys: measurement of renal levels of F2-isoprostane, a specific indicator of endogenous lipid peroxidation\textsuperscript{7,19,20,27,28} and the commonly used, but less sensitive, measurement of renal malondialdehyde. Unexpectedly, aminoguanidine treatment did not have a significant effect on either of these parameters. These findings are consistent with studies in which aminoguanidine protected against diabetic nephropathy in rats, but other antioxidants did not afford protection against diabetic renal injury, suggesting that another mechanism(s) was responsible.\textsuperscript{29} As in the diabetic study our data strongly suggest that the protective effects of aminoguanidine were not due to attenuation of lipid peroxidation and oxidative stress.

A change in expression of advanced glycosylation end products in the kidney was also not the mechanism by which aminoguanidine protected against aging renal injury. We found by immunohistochemistry that AGE were not attenuated with aminoguanidine treatment. Our results are in contrast to previous studies in which chronic aminoguanidine treatment of Sprague Dawley rats, but not Fisher 344 rats, resulted in a reduction in glomerular sclerosis that was associated with lower levels of AGE in the kidney, as determined by enzyme-linked immunosorbent assay.\textsuperscript{12} Despite the attenuation of glomerular sclerosis and reduction in renal AGE, aminoguanidine in these studies had no effect on creatinine clearance or blood pressure,\textsuperscript{12} which also conflicts with our findings in the present study of protection of renal function with aminoguanidine.

Aminoguanidine is also an inhibitor of diamine oxidase (DAO), which oxidizes histamine and other aliphatic amines.\textsuperscript{30} We did not measure DAO in these animals, but it has recently been shown that DAO produces superoxide radicals as an intermediate in the deamination process.\textsuperscript{25} Because we were unable to document differences in the levels of oxidative stress in these studies, we doubt that the inhibition of DAO played an important role in the aminoguanidine-derived protection of the kidney from age-related injury.

The most plausible explanation by which aminoguanidine protected against aging injury in our study is the role played by the reduction in blood pressure. The higher blood pressure in the old untreated rats would have promoted glomerular injury, thus promoting glomerular sclerosis and reducing GFR. To our knowledge, aminoguanidine treatment alone has not been shown to reduce blood pressure. In fact, Mattson and colleagues have reported that chronic (3 days) renal intramedullary infusion of aminoguanidine increases blood pressure significantly.\textsuperscript{31} In the present studies, if the blood pressure had increased during aminoguanidine treatment as Mattson and colleagues found, there would have been increased renal injury rather than the protection we found.

Whether the blood pressure was lower because of the reduction in age-related glomerular injury with aminoguanidine, or whether the lower blood pressure was a consequence of aminoguanidine cannot be determined in this study. However, the reduction in blood pressure either caused by chronic aminoguanidine directly or secondary to aminoguanidine treatment played a most important role in protection against age-related renal dysfunction and injury in the present studies.

In summary, iNOS protein increases by approximately 90% in kidneys of rats aged 24 months. Long-term aminoguanidine treatment for 9 months attenuates age-related glomerular sclerosis and loss of renal function in male rats aged 22 months. There was no effect of aminoguanidine treatment on oxidative stress nor was aminoguanidine effective in reducing the level of AGE formation in the kidney, as determined by immunohistochemistry. These findings suggest that aminoguanidine attenuates aging-related renal dysfunction not by inhibiting the oxidative stress produced by iNOS, or by significantly reducing renal AGE formation, but rather by inhibiting another pathophysiologic effect of iNOS that protected against the age-related increase in blood pressure.

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