Aminoguanidine Ameliorates Overexpression of Prosclerotic Growth Factors and Collagen Deposition in Experimental Diabetic Nephropathy

DARREN J. KELLY,* RICHARD E. GILBERT,* ALISON J. COX,* TINA SOULIS,† GEORGE JERUMS,† and MARK E. COOPER†
*University of Melbourne, Department of Medicine, St. Vincent’s Hospital, Victoria, Australia; and †Department of Medicine, Austin and Repatriation Medical Centre, Victoria, Australia.

Abstract. Profibrotic cytokines and the formation of advanced-glycation end products (AGE) have both been implicated in the pathogenesis of glomerulosclerosis in diabetic kidney disease. However, tubulointerstitial pathology is also an important determinant of progressive renal dysfunction in diabetic nephropathy. This study sought to investigate the expression of profibrotic growth factors and matrix deposition in the glomerulus and the tubulointerstitium and to examine the effect of blocking AGE formation in experimental diabetic nephropathy. Thirty-six male Sprague-Dawley rats were randomized into control and diabetic groups. Diabetes was induced in 24 rats by streptozotocin. Twelve diabetic rats were further randomized to receive the inhibitor of AGE formation, aminoguanidine (1 g/l drinking water). At 6 mo, experimental diabetes was associated with a three-fold increase in expression of transforming growth factor (TGF)-β1 (P < 0.01 versus control) and five-fold increase in platelet-derived growth factor (PDGF)-B gene expression (P < 0.01 versus control) in the tubulointerstitium. In situ hybridization demonstrated a diffuse increase in both TGF-β1 and PDGF-B mRNA in renal tubules. Aminoguanidine attenuated not only the overexpression of TGF-β1 and PDGF-B but also reduced type IV collagen deposition in diabetic rats (P < 0.05). TGF-β1 and PDGF mRNA within glomeruli were also similarly increased with diabetes and attenuated with aminoguanidine. The observed beneficial effects of aminoguanidine on the tubulointerstitial in experimental diabetes suggest that AGE-mediated expression of profibrotic cytokines may contribute to tubulointerstitial injury and the pathogenesis of diabetic nephropathy.

The magnitude of tubulointerstitial injury is an important prognostic marker of renal outcome in many forms of renal diseases (1). In human diabetic nephropathy, the extent of interstitial fibrosis is strongly associated with mesangial expansion, falling GFR, and increasing proteinuria (2). In experimental diabetic nephropathy, investigation has focused almost exclusively on the glomerulus and particularly on the mesangial cell, although tubulointerstitial disease also develops in the streptozotocin model (3). Accumulation of extracellular matrix (ECM), first recognized as thickening of capillary basement membranes, is a characteristic pathologic feature of diabetes (4) and is present in the tubulointerstitium as well as the glomerulus (5). Intensive investigation into the pathogenesis of both experimental and human renal disease has consistently implicated the locally active growth factors, transforming growth factor-β (TGF)-β and platelet-derived growth factor (PDGF) (6,7). Although these two growth factors have differing effects on cell proliferation (8), both TGF-β and PDGF share an ability to augment ECM synthesis in glomerular (9) and tubular epithelial cells (10).

Chronic hyperglycemia, a necessary prerequisite for the development of diabetic nephropathy, leads to the formation of long-lived nonenzymatically glycated proteins that are referred to as advanced-glycation end products (AGE) (11). As a consequence of increased substrate (glucose) availability, AGE accumulate at an accelerated rate in patients with diabetes, where they have been postulated to play a major role in the pathogenesis of the microvascular complications of diabetes (12). In vitro studies have shown that AGE induce increased ECM production via both TGF-β and PDGF-dependent mechanisms (13,14). However, it remains to be determined whether this AGE-growth factor interaction mediates the development of renal injury in diabetes in vivo.

This study sought to determine the effects of inhibiting AGE formation on matrix accumulation and the expression of TGF-β and PDGF within the diabetic kidney with a particular focus on the tubulointerstitium.
Materials and Methods

**Animals**

Thirty-six male Sprague-Dawley rats aged 13 wk were randomized into control and diabetic groups. Diabetes was induced in 24 rats by the intravenous administration of streptozotocin 50 mg/kg body wt. Diabetic animals received 4 units of long-acting insulin daily (Ultra-Lente, Novo-Nordisk, Bagsvaerd, Denmark) to maintain body weight and prevent ketoacidosis. Diabetic rats (n = 12) were further randomized to receive the aminoguanidine hydrogen carbonate 1 g/l in drinking water (Fluka Chemica AG, Buchs, Switzerland). The remaining 12 rats were injected with citrate buffer and served as control. Systolic BP was measured in all animals at 4-wkly intervals by tail cuff plethysmography. Seven days before they were killed at 24 wk, the animals were housed in metabolic cages for 24 h to obtain urine for the measurement of albumin by RIA (15).

At 24 wk, the rats were anesthetized with sodium pentobarbital (Nembutal, Bomac, Asquith, Australia). Both renal arteries were ligated, and the kidneys were then excised, decapsulated, and weighed. The kidneys were then bisected sagittally. The anterior half-kidney was immersion fixed in 10% neutral buffered formalin for in situ hybridization studies, and the posterior half was immersed in Methyl Carnoy’s fixative for immunohistochemical studies.

Blood, obtained at decapitation, was collected in lithium heparin tubes, placed on ice, spun immediately, separated and stored at −20°C. Hemoglobin A1c at death was measured by HPLC (16). All aspects of the experiment were approved by the Animal Ethics Committee of the Austin and Repatriation Medical Center.

**In Situ Hybridization**

Anti-sense riboprobes for TGF-β1 and PDGF-B were generated as described previously (17). In brief, a 985 bp cDNA probe coding for rat TGF-β1 (gift of Dr. Qian, National Institutes of Health, Bethesda, MD) was cloned into pBluescript KS− (Stratagene, LaJolla, CA) and linearized with XbaI, and an antisense riboprobe was produced by T7 RNA polymerase. The cDNA coding for rat PDGF-B (gift of Dr. D.F. Bowen-Pope, Seattle, WA) was cloned into pBluescript KS+ (Stratagene) and linearized with SnaI to produce an antisense riboprobe using T7 RNA polymerase.

Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Four-μm thick sections were cut onto slides that had been precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in P buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.5]; 1 mM EDTA [pH 8.0]; 0.5 M NaCl) and then incubated with RNase A (150 μg/ml) for 1 h at 37°C. Sections were later washed in 2× SSC for 45 min at 55°C, dehydrated in graded ethanol, air dried, and exposed to Kodak X-Omat autoradiographic film (Eastman Kodak, Rochester, NY) for 3 d. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box with desiccant at 4°C for 21 d, immersed in Kodak D19 developer (Eastman Kodak), fixed in Ilford Hypam, and stained with hematoxylin and eosin.

**Quantitative Autoradiography**

Quantitative in situ hybridization autoradiography, which permits the assessment of gene expression equivalent to Northern blot analysis (18), was used to determine the magnitude of gene expression. As the tubulointerstitial accounts for >95% of kidney volume (19), whole kidney was used as an index of tubulointerstitial gene expression with more detailed structural analysis undertaken in emulsion-dipped sections and in immunohistochemical studies. Quantitative autoradiography was performed by film densitometry, as described previously (20). In brief, film densitometry of autoradiographic images obtained by in situ hybridization was performed by computer-assisted image analysis (21) by using a Micro Computer Imaging Device (MCID, Imaging Research, St. Catherine’s, Ontario, Canada). In situ autoradiographic images were placed on a uniformly-illuminating fluorescence light box (Northern Light Precision Luminator C60, Image Research, Ontario, Canada) and captured using a video camera (Sony Video Camera Module CCD, Sony Corp., Tokyo, Japan) connected to an IBM AT computer (IBM, Armonk, NY) with a 512 × 512 pixel array imaging board with 256 gray levels. After appropriate calibration by constructing a curve of optical density versus radioactivity (22), quantitation of digitalized autoradiographic images was performed using MCID software. Data were expressed as optical density.

**Table 1. Clinical characteristics of study rats**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + Aminoguanidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>617 ± 5</td>
<td>487 ± 35b</td>
<td>421 ± 30b</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.6 ± 0.1</td>
<td>2.5 ± 0.2b</td>
<td>2.3 ± 0.1b</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>4.5 ± 0.3</td>
<td>21.1 ± 1.2b</td>
<td>20.8 ± 1.0b</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>2.5 ± 0.1</td>
<td>14.9 ± 0.9b</td>
<td>13.6 ± 0.9b</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>123 ± 3</td>
<td>135 ± 3b</td>
<td>130 ± 2b</td>
</tr>
<tr>
<td>Albuminuria (mg/d)</td>
<td>2.1 ×/÷ 1.4</td>
<td>53.8 ×/÷ 1.8b</td>
<td>15.9 ×/÷ 3.4c</td>
</tr>
</tbody>
</table>

*Data are shown as mean ± SEM except for albuminuria, where geometric means ×/÷ tolerance factors are shown.

b P < 0.01 versus control.

c P < 0.01 versus diabetic.
per cm² relative to control kidneys (Relative Optical Density, ROD) which were arbitrarily assigned a value of 1.

In addition to film densitometry, grain counting of emulsion-coated sections was also used to quantitate gene expression and to explore the regional distribution of transcripts within the kidney. Data are shown as mean ± SEM of the relative optical density (OD) of specific mRNA. *P < 0.01 diabetic versus control; †P < 0.01 aminoguanidine-treated diabetic versus untreated diabetic rats.

Regional gene expression was quantitatively measured to determine the proportion of each area occupied by autoradiographic grains as described previously (23), using computerized image analysis (Analytical Imaging Station, Imaging Research Inc., St. Catherine’s, Ontario, Canada).

All sections were uniformly cut in the mid-sagittal plane, hybridized to their respective probes in the same experiment, and analyzed in duplicate under identical conditions. All analyses were performed with the observer masked to the animal study group.

**Immunohistochemistry**

Type IV collagen content was immunohistochemically assessed by using a polyclonal goat anti-bovine/anti-human type IV collagen antibody (Southern Biotechnology, Birmingham, AL). AGE immunohistochemistry was assessed by using a polyclonal rabbit anti-AGE-KLH antibody as previously reported (24). Four-μm sections of kidney were rehydrated and treated with 1% H₂O₂/methanol and then incubated in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, PA) for 20 min at room temperature. Sections were then incubated with either type IV collagen antibody for 60 min at room temperature or AGE antibody overnight at 4°C, washed in PBS, and incubated with biotinylated either rabbit anti-goat Ig or goat anti-rabbit Ig (DAKO, Carpinteria, CA) and then by avidin-biotin complex (ABC, Vector Stain, Burlingame, CA). Peroxidase conjugates were subsequently localized by using diaminobenzidine tetrahydrochloride as a chromogen. Negative controls included omitting the primary antibody.

**Figure 1.** Quantitation of TGF-β1 (top panel) and PDGF-B (bottom panel) gene expression by film densitometry in control, diabetic, and aminoguanidine-treated diabetic rat kidneys. Data are shown as mean ± SEM of the relative optical density (OD) of specific mRNA. *P < 0.01 diabetic versus control; †P < 0.01 aminoguanidine-treated diabetic versus untreated diabetic rats.

**Figure 2.** In situ hybridization autoradiographs for TGF-β1 (top row) and PDGF-B (bottom row) in kidneys of control, diabetic, and aminoguanidine-treated diabetic rats. Magnification, ×8.
or replacing it with normal rabbit IgG at an equivalent protein concentration. Tissues treated in this manner showed no positive staining. Sections were then counterstained with hematoxylin. The magnitude of collagen IV immunostaining was quantified by using computer-assisted image analysis as described previously (25). In brief, for each tissue section, images from three nonoverlapping, randomly selected fields were examined by light microscopy (Olympus BX-50, Olympus Optical, Tokyo, Japan) and digitized by using a high-resolution camera (Fujix HC-2000, Fujifilm, Tokyo, Japan). All images were obtained by using a 20× objective lens. Digitized images were then captured on a Pentium III personal computer that was equipped with an in-built graphic board and opened by using analytical software (AIS, Analytical Imaging Software, Ontario, CA). An area of brown on an immunoperoxidase-stained section was selected for its color range, and the proportional area of tissue with this range of color was then quantified such that the magnitude of immunolabeling was expressed as the proportional area of the tubulointerstitium, which stained brown.

**Statistical Analyses**

Because of a positively skewed distribution, albuminuria was logarithmically transformed before statistical analysis and expressed as the geometric mean \( \times 10^{\pm} \) tolerance factor. Other results are expressed as mean ± SEM unless stated otherwise. All other data comparing treatment groups were analyzed by ANOVA with correction for multiple comparisons by using the Fisher’s Least Significant Difference test. Analyses were performed by using the Statview SE+ Graphics package (Abacus Concepts, Calabasas, CA) on an Apple Macintosh G3 computer (Apple Computer Inc, Cupertino, CA). Statistical significance was defined as \( P < 0.05 \).

**Results**

**Biological Parameters**

Rats that had received streptozotocin were all diabetic (blood glucose >15 mmol/l), with no difference in HbA1c between animals treated with aminoguanidine and those that were not (Table 1). Diabetes was associated with increased kidney weight and reduced body mass when compared with control animals. These effects on body weight and kidney weight were not influenced by treatment with aminoguanidine (Table 1). Aminoguanidine treatment was accompanied by a reduction in albuminuria compared with untreated diabetic rats (Table 1).

**In Situ Hybridization Autoradiography**

**In situ** hybridization autoradiographic film densitometry demonstrated a three-fold increase in TGF-β1 and a five-fold increase in PDGF-B mRNA in diabetic compared with control animals (Figures 1 and 2). Overexpression of both transcripts was reduced with aminoguanidine treatment to levels similar to that of control animals.

**In Situ Hybridization Microscopy**

Light microscopic examination of emulsion-dipped tissue labeled by **in situ** hybridization was used to examine gene expression within the tubulointerstitium. When compared with control animals, increased TGF-β1 and abundant PDGF-B mRNA were noted in tubular epithelial cells, particularly those of the proximal tubule in diabetic rats (Figures 3, 4, and 5). The increased expression was reduced in diabetic rats treated with aminoguanidine (Figures 3, 4, and 5). A similar increase in TGF-β1 and PDGF mRNA was observed within glomeruli with diabetes (Figure 6). The increased expression was also reduced with aminoguanidine treatment (Figure 6).

**Immunohistochemistry**

In the tubulointerstitium, diabetes was also associated with an approximate ten-fold increase in immunostainable type IV collagen compared with control rat kidneys (proportional immunostained area: 0.37 ± 0.12% versus 3.3 ± 0.7%, mean ± SEM, control versus diabetic; \( P < 0.01 \)). Increased type IV collagen was noted in the interstitium of diabetic rats (Figure 7). Aminoguanidine treatment attenuated type IV collagen deposition in diabetic animals (proportional immunostained area: 1.7 ± 0.5%; \( P < 0.05 \) versus diabetic) (Figure 7). A similar pattern was observed in the glomeruli of diabetic rats, with increased collagen IV with diabetes and attenuated with aminoguanidine treatment. An increase in AGE staining was observed in glomeruli and the tubulointerstitium of diabetic rats, which were reduced by aminoguanidine (Figure 8).

**Negative Controls**

Sections hybridized with sense riboprobe or normal rabbit IgG showed no positive labeling (Figure 9).

**Discussion**

Evidence has accumulated over the last decade implicating the formation of AGE as a major factor in the pathogenesis of diabetic nephropathy (26). This study demonstrates that blockade of AGE formation by treatment with aminoguanidine was associated not only with amelioration in the overexpression of the fibrogenic growth factors TGF-β and PDGF but also with a decrease in the accumulation of the ECM protein type IV collagen in the diabetic kidney. These **in vivo** findings implicate AGE-dependent mechanisms in the pathogenesis of growth factor mediated matrix accumulation in both the tubulointerstitium and glomerulus in experimental diabetes.

AGEs accumulate in diabetic tissues at an accelerated rate as a consequence of increased glucose concentration in extracellular fluids. The administration of AGE to nondiabetic animals leads to glomerulosclerosis and albuminuria (27), suggesting that AGE alone may be sufficient to induce renal injury in diabetes. Previous **in vivo** studies have shown that inhibition of advanced glycation with aminoguanidine has renoprotective effects on a variety of structural and functional parameters in diabetes (28,29). However, the mechanisms underlying these effects had not been investigated. In the present study, overexpression of the prosclerotic growth factors TGF-β1 and PDGF-B was noted in the kidneys from diabetic rats in association with increased type IV collagen accumulation. Aminoguanidine treatment not only ameliorated the overexpression of these growth factors but also attenuated type IV collagen deposition. These findings were observed particularly within the tubulointerstitium but also in glomeruli.

Although the glomerulus and, in particular, the mesangium have been the focus of intense investigation in diabetes, recent
studies in experimental animals have shown that the proximal tubule is also a major site of growth factor and matrix expression in the diabetic kidney (30). Moreover, tubulointerstitial injury, a major histopathologic feature of diabetic nephropathy is also an important predictor of renal dysfunction in diabetes (31). In this study, the renal tubules, particularly their proximal segments, were a major site of growth-factor expression con-

Figure 3. In situ hybridization of renal cortical tubules labeled with anti-sense riboprobes to TGF-β1. Control (A), diabetic (B), and aminoguanidine-treated diabetic rats (C). Counterstained with hematoxylin and eosin. Magnification, ×320.

Figure 4. In situ hybridization of renal cortical tubules labeled with anti-sense riboprobes to PDGF-B. Control (A), diabetic (B), and aminoguanidine-treated diabetic rats (C). Counterstained with hematoxylin and eosin. Magnification, ×570.
consistent with this part of the nephron being a site of preferential clearance of glycated proteins (32). Furthermore, the tubular basement membrane, like other long-lived proteins, undergoes advanced glycation in diabetes (33). In this study, we confirmed increased AGE deposition in both the tubulointerstitium and glomerulus in experimental diabetes along with attenuation by aminoguanidine. Whether, Amadori products, intermediates in the formation of AGE that have also been implicated in the pathogenesis of diabetic nephropathy (34), are taken up by the proximal tubular epithelium or influence tubulointerstitial injury has not been delineated.

TGF-β has been consistently implicated as playing a pivotal role in the pathogenesis of ECM accumulation in diabetic nephropathy. This growth factor exerts its prosclerotic effects by increasing matrix synthesis, decreasing its degradation, and modulating cell-matrix interactions (35). Factors that have been implicated in mediating TGF-β overexpression in diabetes follow the activation of both glucose-dependent and glucose-independent mechanisms that accompany the diabetic state (36). In vitro studies have shown that AGE induce the expression of both TGF-β and PDGF (13,14). Pathway studies suggest a similar increase in TGF-β expression in response to AGE in a tubular cell line (37). Although the signal transduc-
Figure 7. Representative photomicrographs of renal cortex from control (A), diabetic (B), and aminoguanidine-treated diabetic rats (C) immunostained with antibody to type IV collagen. Counterstained with hematoxylin. Magnification, ×320.

Figure 8. Representative photomicrographs of renal cortex from control (A), diabetic (B), and aminoguanidine-treated diabetic rats (C) immunostained with antibody to advanced glycations endproducts (AGE). Counterstained with hematoxylin. Magnification, ×270.
tion pathways mediating these AGE-induced effects have not been fully elucidated, AGE can activate a range of intracellular second messengers, including MAP kinase, as has been observed in a renal tubular cell line (38). In addition to these in vitro effects, exogenous AGE administration to nondiabetic rodents leads to upregulation of TGF-β expression in the kidney (39) in association with tissue fibrosis and albuminuria (27). Furthermore, in the diabetic rat, recent studies that focus on vascular tissue, another site of diabetic complications, have shown that diabetes-induced structural injury and increased TGF-β expression can be ameliorated by aminoguanidine treatment (40). The present study extends these findings by demonstrating that AGE also contribute to the overexpression of TGF-β1 and accumulation of type IV collagen in the diabetic kidney, providing a mechanistic link between AGE and diabetes-associated renal injury.

In addition to TGF-β, several other growth factors, including PDGF, have been shown to be overexpressed in the diabetic kidney (41). Moreover, PDGF itself may lead to upregulation in TGF-β expression (42). Although PDGF is a potent pro-proliferative growth factor for a wide variety of cell types, its mitogenic action is curtailed in the presence of high local concentrations of TGF-β (42). However, in contrast with their differing effects on cell cycling, both PDGF and TGF-β stimulate ECM synthesis (43,44). Thus the reduction in AGE-mediated overexpression of PDGF, in addition to TGF-β, may contribute to the amelioration of matrix accumulation demonstrated in the present study with aminoguanidine treatment.

Although the principal mechanism of action of aminoguanidine is inhibition of AGE formation, it may also inhibit nitric oxide (NO) production (45,46). This action is potentially relevant to the complications of diabetes as NO is a potent vasodilator and may modulate growth factor expression. However, NO reduces expression of both TGF-β (47) and PDGF (48), such that aminoguanidine would be expected to increase expression of these growth if inhibition of NO production were its dominant mechanism of action in the present study. It is likely that the renoprotective effects of aminoguanidine are primarily via its effects as an inhibitor of AGE formation, since chemically related derivatives which do not inhibit AGE formation, yet inhibit NO synthase, such as methylguanidine and also inhibitors of NO synthase such as L-NAME do not reproduce the renoprotective effects of aminoguanidine. Furthermore, a recent study using a derivative of aminoguanidine, ALT473, which inhibits AGE formation but does not inhibit NO synthase reproduce similar renal effects as aminoguanidine (49).

In summary, aminoguanidine treatment in this study was associated with reduced renal expression of TGF-β1, PDGF-B mRNA, and type IV collagen accumulation in experimental diabetes, implicating AGE-induced activation of prosclerotic cytokines in the pathogenesis of diabetic nephropathy.

Acknowledgments

This project was supported by a program grant from the Juvenile Diabetes Foundation International. Dr. Darren Kelly is a recipient of a Juvenile Diabetes Foundation International Research Fellowship. Dr. Richard Gilbert is a recipient of a Career Development Award from the Juvenile Diabetes Foundation International.

References


Figure 9. Photomicrographs of renal cortex hybridized with sense riboprobe to TGF-β1 (A), PDGF-B (B), or normal rabbit IgG (C) showing no positive hybridization or immunolabelling. Counterstained with hematoxylin and eosin. Magnification, ×320.


