Aminoguanidine ameliorates changes in the IGF system in experimental diabetic nephropathy

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Abstract

Introduction. Nephropathy occurs in up to 30% of diabetic patients and is a significant cause of morbidity and mortality. Hyperfiltration and microalbuminuria are hallmarks of early nephropathy, with the subsequent development of macroalbuminuria, hypertension, and progressive renal impairment [1]. Nephropathy is characterized by renal enlargement and a number of ultrastructural abnormalities, including basement membrane thickening and mesangial expansion [1]. Although hyperglycaemia is a prerequisite for the development of diabetic nephropathy, the mechanisms underlying its development are not completely understood. Increased formation of advanced glycation end-products (AGEs), which result from the non-enzymatic reaction of glucose with biologically relevant molecules such as structural proteins, has been implicated in the development of diabetic complications including nephropathy [2]. Indeed, treatment of diabetic rats with aminoguanidine, an inhibitor of AGE formation, significantly reduces albuminuria and mesangial expansion in streptozotocin-(STZ)-diabetic rats [3]. AGE formation may directly result in the development of structural changes in tissues by chemical means, including formation of cross-links between structural proteins that may alter properties such as susceptibility to breakdown. However, there is also in vitro evidence that AGEs may also stimulate the synthesis of growth factors, including TGF-β and IGF-I [4], which have been implicated in the development of diabetic complications [5–7]. Considerable evidence links dysregulation of the IGF system and the development of experimental diabetic nephropathy. Kidney IGF-I levels are elevated during the first few days of STZ diabetes, immediately preceding the rapid phase of renal growth [8,9]. Diabetes in prepubertal rats [9], growth-hormone-deficient rats [10], and rats treated with a somatostatin analogue [11] is associated with decreased early kidney growth. In each of these models the kidney IGF-I response is attenuated or absent. Longer-term studies of diabetic growth hormone-deficient rats and rats treated with a somatostatin analogue have found

Background. Formation of advanced glycation end-products (AGEs) has been implicated in the development of diabetic complications. As well as causing changes in structural proteins, AGEs may also alter gene expression of growth factors in vitro. The insulin-like growth factor (IGF) system, including IGF-I and modulatory IGF binding proteins (IGFBPs), is dysregulated during the development of diabetic nephropathy.

Methods. Quantitative in situ hybridization histochemistry and immunohistochemistry were used to determine the effects of aminoguanidine, an inhibitor of AGE formation, on gene expression of IGF-I and IGFBPs in kidneys of long-term (8 months duration) streptozotocin-diabetic rats. Results. Diabetes was associated with increased renal expression of IGFBP-1 mRNA (diabetes 824 ± 236 vs control 264 ± 76 arbitrary units, \( P < 0.01 \)) and decreased expression of mRNAs for IGF-I (diabetes 39 ± 7 vs control 185 ± 23 arbitrary units, \( P < 0.001 \)) and IGFBP-4 (diabetes 139 ± 25 vs control 383 ± 54 arbitrary units, \( P < 0.001 \)). Aminoguanidine treatment inhibited the effects of diabetes on renal expression of mRNA for IGF-I, IGFBP-1 and IGFBP-4. The changes in IGF-I and IGFBP-1 mRNA levels were reflected in altered peptide levels. In diabetic kidneys, IGFBP-5 mRNA levels were slightly decreased to 75% of control levels (\( P < 0.01 \)); aminoguanidine had no effect on IGFBP-5 mRNA levels.

Conclusions. These results suggest that amelioration of changes in the renal IGF system by aminoguanidine may contribute to the renoprotective effects of the latter, which have been previously shown to inhibit structural and functional aspects of diabetic nephropathy in the rat.

Keywords: advanced glycation; binding protein; diabetic nephropathy; immunohistochemistry; in situ hybridization; insulin-like growth factor
decreased albuminuria and structural changes compared with 'IGF-I-replete' control diabetic rats [12,13]. Following the initial rise in kidney IGF-I levels in diabetic rats, renal IGF-I levels normalize or decrease below control levels (reviewed in [6]), indicating either that the predominant role of the IGF system is in the development of the early changes of diabetes or that more sustained changes in the other components of the IGF system are responsible for continuing effects.

The actions of IGFBs are modulated by a family of six structurally related IGF binding proteins (IGFBPs). Under different circumstances, IGFBPs may potentiate or inhibit IGF actions [14]. Renal expression of IGFBPs is altered in STZ-diabetes [15–20]. Major changes include increased IGFBP-1 levels and decreased IGFBP-4 levels. The aim of this study was to investigate whether aminoguanidine, which inhibits the development of diabetic nephropathy, also normalizes changes in expression of IGF-I and IGFBPs 1–6.

Subjects and methods

Animals

Male Sprague–Dawley rats weighing 200–250 g were made diabetic by intravenous injection of streptozotocin (55 mg/kg in citrate buffer, pH4.5). Control rats were injected with citrate buffer alone. Control and diabetic rats were randomized to receive either aminoguanidine (AG, 1 g/kg in drinking water for the entire 8 months of the study) or no treatment. Diabetic rats did not receive insulin therapy. Urine of diabetic rats was regularly tested for ketones (Ketostix, Bayer, Mulgrave, Australia) and these were not detected. After 8 months, blood was taken from the tail vein for glucose measurement and animals were killed by intravenous ketamine. Kidneys were removed, weighed, and fixed in 10% neutral buffered formalin. This protocol was approved by the Austin and Repatriation Medical Centre Animal Welfare Committee.

In-situ hybridization histochemistry

In-situ hybridization histochemistry was performed as previously described [21]. Briefly, 35S-labelled complementary (antisense) or non-complementary (sense) RNA probes were synthesized for rat IGFBP-1–6 and IGF-I. The cDNAs for IGFBP, p1–2, were kindly provided by Dr S. Shimasaki (Whittier Institute, La Jolla, CA), and for IGF-I by Dr C. T. Roberts Jr (NIH, Bethesda, MD). Probes were adjusted to an average length of 150 bases by alkaline hydrolysis. The average specific activity of the RNA probes generated was 3 × 10^6 c.p.m./μg RNA.

Kidney sections (4 μm) were digested with Pronase E (Sigma, 125 mg/ml), washed in 0.1 M sodium phosphate buffer, pH 7.2, ultrapure water, dehydrated in 70% ethanol, and air-dried. The 35S-labelled RNA probes (5 × 10^5 c.p.m./25 ml hybridization buffer) were added to a hybridization buffer consisting of 300 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM Na2HPO4, pH 6.8, 5 mM EDTA, pH 8.0, 1 × Denhardt’s solution, 50 mg/ml yeast RNA, 50% deionized formamide, and 10% (w/v) dextran sulphate. The hybridization buffer containing labelled probe was preheated to 80°C (5 min), following which sections were incubated with probes in humidified (50% formamide) chambers for 14–16 h at 60°C. Sections were washed in 2 × standard saline citrate (2 × SSC: 0.3 M NaCl, 0.33 M Na2HPO4–2H2O) containing 50% formamide at 55°C for 1.5 h. Sections were then washed and treated with 150 μg/ml RNAase A for 1 h at 37°C followed by a wash in 2 × SSC at 55°C for 45 min. After final dehydration through graded ethanol, slides were air-dried and autoradiographed with Kodak XAR (Eastman Kodak, New York) film for 1–3 days at room temperature. Some slides were dipped in photographic emulsion (Amersham, UK), stored with desiccant at 4°C for 14–21 days, developed in Kodak D19, fixed in Ilford Hypam, and stained with haematoxylin for evaluation.

Quantitation of in-situ hybridization histochemistry [22]

Autoradiographs were quantitated using the MCID (Imaging Research, Ontario, Canada) computerized image analysis system. Regions of interest were traced out, and optical densities within those regions were quantified and calibrated by reference to 14C-radioactive standards (Amersham, UK) which were simultaneously exposed with slides to X-ray film. Results were corrected for non-specific labelling by subtracting the signal obtained using sense probes from that obtained using antisense probes.

Immunohistochemistry

Standard techniques were employed using rabbit anti-mouse IGBPBP-1 and rabbit anti-human IGF-I polyclonal antisera (GroPep, Adelaide, Australia), both of which cross-react with rat antigens, at dilutions of 1:200 and 1:100 respectively. The Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and the peroxidase method of labelling were used according to the manufacturer’s instructions. Negative control slides were incubated with normal goat serum instead of primary antibody.

Statistics

Quantitative in-situ hybridization histochemistry data were log-transformed to stabilize variance prior to analysis. Results were analysed by analysis of variance followed by Fisher’s protected least significant difference test for specific comparisons between groups. Results are expressed as mean ± SE. Two-sided P values are shown.

Results

As expected, diabetic rats had higher plasma glucose levels and urinary volumes than control rats, but aminoguanidine had no effect on severity of diabetes as measured by either parameter (Table 1). Control and diabetic rats treated with aminoguanidine weighed less than vehicle-treated rats (P = 0.003, Table 1). However, diabetes resulted in a similar degree of weight loss in vehicle- and aminoguanidine-treated rats (diabetes × treatment interaction, P = 0.23). Absolute and relative kidney weights were significantly higher in diabetic rats (P < 0.0001, Table 1) and treatment with aminoguanidine had no effect on either parameter.
Table 1. Characteristics of rat groups

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Control</th>
<th>Control + AG</th>
<th>Diabetes</th>
<th>Diabetes + AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total body weight (g)</td>
<td>633 ± 8</td>
<td>541 ± 30a</td>
<td>445 ± 11b</td>
<td>405 ± 25c</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.6 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>21.2 ± 0.3b</td>
<td>18.2 ± 2.6c</td>
</tr>
<tr>
<td>Urine volume (ml)</td>
<td>18 ± 1</td>
<td>24 ± 1</td>
<td>107 ± 16b</td>
<td>104 ± 13c</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>3.10 ± 0.09</td>
<td>2.96 ± 0.11</td>
<td>4.29 ± 0.2b</td>
<td>3.77 ± 0.15a</td>
</tr>
<tr>
<td>Kidney weight/total body weight (%)</td>
<td>0.49 ± 0.02</td>
<td>0.55 ± 0.04</td>
<td>0.97 ± 0.04b</td>
<td>0.97 ± 0.08c</td>
</tr>
</tbody>
</table>

Results are shown as mean ± SE.
aP < 0.01, bP < 0.001 vs control; cP < 0.01, vs control + AG.

Aminoguanidine ameliorates diabetes-related changes in renal IGF-I, IGFBP-1, and IGFBP-4 expression

As previously described [15–20], the major effects of diabetes on gene expression of IGF system components were increased IGFBP-1 mRNA levels and decreased IGFBP-4 and IGFBP-1 mRNA levels.

Overall, IGFBP-1 mRNA levels were increased by 212% in untreated diabetic rats (P < 0.01, Figure 1a and b), whereas this increase was abrogated by aminoguanidine treatment (control + AG vs diabetes + AG, P > 0.20). The major sites of IGFBP-1 mRNA expression were the inner stripe of the outer medulla, reflecting expression in distal tubules and thick ascending loops of Henle, and the cortex, representing cortical collecting duct expression (results not shown). IGFBP-4 mRNA levels were significantly increased by 206 and 219% in the inner stripe of the outer medulla and cortex of untreated diabetic rats respectively, whereas levels in aminoguanidine-treated diabetic rats were not significantly higher than in their non-diabetic controls (Figure 1a, c and d).

Immunohistochemical analysis showed that IGFBP-1 protein was most prominent in collecting ducts (Figure 2a). IGFBP-1 was also found in distal tubules and thick ascending limbs of the cortex and outer medulla. IGFBP-1 levels were markedly increased in all of these sites in diabetic rats (Figure 2b). Aminoguanidine attenuated the diabetes-related increase in IGFBP-1 levels (Figure 2c). Changes in IGFBP-1 mRNA and protein levels were therefore coordinate.

Steady-state cortical IGFBP-4 mRNA levels were decreased by 64% in untreated diabetic rats compared with untreated controls (P < 0.001, Figure 3). Although levels of IGFBP-4 mRNA were decreased by 36% in aminoguanidine-treated diabetic rats compared with aminoguanidine-treated controls, this difference was not statistically significant (P < 0.10, Figure 3). Levels of IGFBP-4 mRNA were significantly higher in aminoguanidine-treated than untreated diabetic rats (P < 0.001, Figure 3). Cortical IGFBP-4 mRNA predominantly reflects proximal tubular expression as confirmed by light microscopic examination of emulsion-dipped slides (results not shown).

In vehicle-treated diabetic rats, levels of IGF-I mRNA in the inner stripe of the outer medulla, representing expression in thick ascending loops of Henle (results not shown), were decreased by 79% compared with untreated control rats (P < 0.001, Figure 4). In aminoguanidine-treated diabetic rats, levels of IGF-I mRNA were decreased by only 39% compared with aminoguanidine-treated controls, a difference that did not reach statistical significance (P < 0.10, Figure 4). Levels of IGF-I mRNA were significantly higher in aminoguanidine-treated than untreated diabetic rats (P < 0.01, Figure 4).

Immunohistochemical analysis demonstrated IGF-I peptide in distal tubules and thick ascending limbs of the cortex and outer medulla (Figure 2e). Levels of IGF-I peptide were decreased in diabetic rats (Figure 2f), and aminoguanidine treatment restored peptide levels towards normal (Figure 2h). Changes in IGF-I mRNA and peptide levels were therefore coordinate.

Aminoguanidine has no effect on other IGFBPs

IGFBP-5 mRNA was predominantly expressed in the inner medulla and inner stripe of the outer medulla with lower levels of expression in the cortex (results not shown). Expression of IGFBP-5 mRNA was 25% lower in diabetic than control rats (diabetes 4069 ± 259 vs control 5459 ± 578 arbitrary units, P < 0.01) and aminoguanidine treatment had no effect on levels in diabetic or control animals. Diabetes and aminoguanidine treatment had no effect on expression of mRNA for IGFBPs-2, -3 and -6 (results not shown).

Discussion

Dysregulation of the IGF system has been implicated in the development of experimental diabetic nephropathy [6]. Renal IGF-I levels are transiently elevated in the first 24–72 h after induction of STZ-diabetes and a role for increased IGF-I activity has been postulated in the development of early diabetes-related kidney growth [8,9]. Long-term studies of diabetic growth hormone/IGF-I-deficient diabetic rats [13] and octreotide-treated rats [11], both of which have decreased urinary albumin excretion and glomerular volume, also suggest a role for the IGF system in the longer-term development of structural and functional changes of diabetic nephropathy.
In the present study, long-term STZ-diabetes resulted in changes in renal gene expression of a number of components of the IGF system. Firstly, levels of IGF-I mRNA were decreased. Although some [23,24], but not other [25], authors have found transient increases in renal IGF-I mRNA levels soon after the induction of STZ-diabetes, the observations of the present study are consistent with studies demonstrating decreased IGF-I mRNA levels in kidneys from long-term diabetic rats [15,19,26]. Second, expression of IGFBPs, which are important regulators of IGF actions [14], is altered early in STZ-diabetic kidneys with many changes persisting for up to 6 months, the longest duration previously studied [19,20]. Cortical IGFBP-1 mRNA levels were increased in STZ-diabetic kidneys in the present study, consistent with previous observations [16,19,20,27]. IGFBP-1 mRNA levels in the inner stripe of the outer medulla were also increased in diabetic rats in the present study as was observed in our previous study of short-term diabetic rats [20]. These findings contrast with the markedly decreased levels of medullary IGFBP-1 mRNA observed by Landau et al. in both short- and long-term diabetic rats [19]. These inconsistencies may relate to differences in rat strains or severity of diabetes.

The changes in IGF-I and IGFBP-1 mRNA levels were reflected at the protein level in the present study. Localization of proteins was more widespread than that of the mRNA, which is consistent with previous studies [28,29]. IGF-I and IGFBP-1 found at sites other than those of mRNA expression may reflect paracrine transport of proteins synthesized in the kidney or proteins sourced from the circulation. Cortical IGFBP-4 levels were decreased in diabetic rat kidney, which is also consistent with previous studies [19,20,27]. Unfortunately, attempts to perform IGFBP-4 immunohistochemistry with two different antisera were unsuccessful in the present study. A small decrease in IGFBP-5 mRNA was also observed in diabetic kidneys that is similar in magnitude to the decrease in cortical IGFBP-5 mRNA levels previously reported [19]. In that study, medullary IGFBP-5 levels were unchanged after 180 days of diabetes. Whereas aminoguanidine attenuated diabetes-related changes in expression of IGF-I, IGFBP-1, and IGFBP-4, expression of IGFBP-5 mRNA was not influenced. This suggests that components of the renal IGF system may respond differently to diabetes-induced AGE accumulation.

The diabetic rats in the present study were not treated with insulin and had body weights ~30% lower than control rats. Although it is possible that haemodynamic and metabolic changes may have contributed to the changes in renal IGF expression in the diabetic rats, it should be noted that the rats were not ketotic or severely catabolic as ketonuria was absent and renal hypertrophy was observed. Further, since aminoguanidine ameliorated many of the diabetes-associated changes in the renal IGF system without affecting glycaemic control or changes in body weight, it is likely that these abnormalities are not merely related to haemodynamic and metabolic changes.
Aminoguanidine is an inhibitor of AGE formation. Aminoguanidine treatment substantially retards the development of albuminuria [30–33] and prevents mesangial expansion [3,31,33] in diabetic rats. Some [33,34] but not all [3,31] studies have shown that aminoguanidine also inhibits glomerular basement membrane thickening induced by diabetes. In the present study, aminoguanidine treatment significantly blunted the diabetes-related reduction in IGFBP-4 mRNA levels. Since IGFBPs may inhibit or potentiate IGF actions under different conditions [14], their role in the development of diabetic kidney disease has not been completely defined. However, IGFBP-4 is thought to be a purely inhibitory IGFBP [14], so that relatively increased levels of this IGFBP are likely to inhibit IGF actions in the kidney. Aminoguanidine abrogated the diabetes-induced increase in IGFBP-1 mRNA and protein levels. Cell-associated IGFBP-1, which may be present on proximal tubular cells of diabetic rat kidneys [16], has been implicated in potentiation of IGF

Fig. 2. Immunohistochemical analysis of IGFBP-1 and IGF-I in rat kidney. IGFBP-1 expression in collecting ducts (CD) of (A) control, (B) diabetic, (C) control + aminoguanidine and (D) diabetes + aminoguanidine rats. IGF-I expression in distal tubules (DT) of (E) control, (F) diabetic, (G) control + aminoguanidine and (H) diabetes + aminoguanidine rats (× 200).
actions [35]. Inhibition of the elevation of levels in this IGFBP by aminoguanidine may therefore also act to inhibit renal IGF actions.

The present study did not address the mechanism by which aminoguanidine affected the IGF system. It is unlikely that AGEs play a role in the changes in the IGF system that occur soon after the induction of STZ-diabetes, since they would not have had time to accumulate. AGE formation may lead to the persistence of abnormal expression of IGF system components by signalling via specific AGE receptors [36]. Aminoguanidine treatment would inhibit formation of AGE ligands and thereby result in ‘normalization’ of IGF system expression following the initial perturbation due to induction of diabetes. An alternative, indirect mechanism for the effect of aminoguanidine is that AGEs regulate synthesis of extracellular matrix components [37], which in turn may regulate expression of IGFBPs [38].

Many of the changes in the renal IGF system were most readily detected in tubules. Indeed, there has been increasing interest in the role of the tubulointerstitium in diabetic nephropathy, with declining renal function more closely correlated to tubulointerstitial rather than glomerular changes [39,40]. Renal tubules are a major site of AGE accumulation and many of the receptors involved in mediating AGE-induced tissue injury or in the clearance of AGEs are located in tubules [41,42]. Renoprotective therapies have been shown to ameliorate tubular as well as glomerular injury in experimental and human diabetic nephropathy [43,44].

AGEs have been shown to increase IGF-I expression in monocytes [36] and glomerular mesangial cells [4] via a receptor-specific mechanism. It may appear that these findings contradict the present observations that an inhibitor of AGE formation ameliorates the decrease in renal IGF-I levels in diabetic rats. However, these in-vitro experiments do not take the in-vivo milieu, whereby renal IGF-I mRNA expression is decreased in long-term diabetic rats, into account.
Aminoguanidine and renal IGF system in diabetes

In conclusion, long-term treatment of diabetic rats with aminoguanidine, which has been previously shown to inhibit the development of structural and functional aspects of experimental diabetic nephropathy [3,30–33], ameliorated diabetes-induced changes in expression of IGF-I, IGFBP-1, and IGFBP-4. Since dysregulation of the IGF system has been implicated in the development of diabetic nephropathy, these findings suggest a possible mechanism that may contribute to the protective effects of aminoguanidine.

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References


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