Prevention of Albuminuria by Aminoguanidine or Ramipril in Streptozotocin-Induced Diabetic Rats Is Associated With the Normalization of Glomerular Protein Kinase C

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This study examined whether the prevention of diabetes-related albuminuria by aminoguanidine (AG) or ramipril (RAM) may be mediated by a common postglomerular basement membrane renal intracellular mechanism involving protein kinase C (PKC). The renal handling of albumin was examined over 24 weeks in control and streptozotocin (STZ)-induced diabetic rats. A radioimmunoassay (RIA) that measures intact albumin, and intravenously injected tritium-labeled rat serum albumin, was used to assess the proportion of intact albumin and albumin fragments in urine. Diabetes was induced in male Sprague-Dawley rats by the intravenous administration of STZ at a dose of 50 mg/kg. Age-matched control rats received buffer alone. Diabetes was characterized by an increase in blood glucose (>15 mmol/l), an increase in glomerular filtration rate (GFR) (4.13 ± 0.15 ml/min; control 3.54 ± 0.19 ml/min, P < 0.005), an increase in glomerular filtration rate (GFR) (4.13 ± 0.15 ml/min; control 3.54 ± 0.19 ml/min, P < 0.005), an increase in intact albumin excretion rate (expressed as geometric mean 11.64 ×/ґ 2.11 mg/24 h; control 0.74 ×/ґ 1.57 mg/24 h, P < 0.005) as measured by RIA, and an increase in glomerular PKC activity. Renal lysosomal cathepsin activity decreased in diabetic rats and this was not prevented by AG or RAM. Neither drug affected glycemic control or GFR, but RAM reduced systolic blood pressure (BP), whereas AG did not. These data indicate that urinary excretion of intact albumin and albumin-derived fragments in diabetes may be modulated independently of glycemic control (AG and RAM) and systemic BP (RAM). While both drugs are known for their different mechanisms of action, the fact that both prevent diabetes-related increases in glomerular PKC activity and albuminuria supports the hypothesis that PKC plays a central role in the development of diabetic nephropathy. Diabetes 49:87–93, 2000

A n increase in albumin excretion rate (AER), which leads to microalbuminuria, is widely acknowledged as the earliest index of diabetic nephropathy (1). Therefore, understanding the mechanism of microalbuminuria and the action of drugs that ameliorate albuminuria is essential to advances in treatment. The exact mechanisms underlying albuminuria, however, are still to be determined as we have recently demonstrated, that under physiological conditions, albumin undergoes fragmentation to small peptides during renal passage. The albumin-derived fragments are not detectable by immunochemical assays which only detect intact albumin (2,3). For this reason, it is necessary to reevaluate the mechanism of renal albumin handling in untreated diabetes and during intervention with renoprotective agents.

Recent studies have demonstrated that albumin does not undergo significant charge restriction by the glomerular capillary wall (GCW) or the glomerular basement membrane (4–8). In accord with these findings, estimates of glomerular sieving of albumin during inhibition of renal tubular uptake of protein demonstrate that albumin flux across the GCW is high (5). Studies have shown that postfiltration processing of albumin may involve two distinct intracellular pathways in the rat kidney. The major pathway is a high capacity pathway that processes ~1,800 µg/min per kidney of albumin and returns it to the blood intact (9); this pathway is specific for filtered albumin and has been termed the retrieval pathway, but its exact anatomical location has not been determined. It is likely to involve transcellular transport across glomerular or tubular epithelial cells. The small amount of albumin that escapes the retrieval pathway and remains in the filtrate is degraded during renal passage, most likely by endocytosis and lysosomal degradation (2,3,5,10). The albumin fragments are subsequently excreted in the urine, and this pathway has been termed the degradation pathway. In control rats, ~90% of the albumin excreted is in a heavily degraded form (<10 kDa). Degradation products are found in the urine but not in the blood.
The mechanisms whereby microalbuminuria occurs in diabetes remain to be elucidated. The formation of advanced glycation end products (AGEs) (11), activation of the local tissue renin-angiotensin system (12), and the direct pathogenetic effects of glucose have been implicated. Interestingly, these processes may be integrated by a final common pathway involving activation of protein kinase C (PKC) (13,14) and its possible influence on microtubule formation and intracellular trafficking (15), particularly in relation to intracellular albumin processing as described above. Several studies have shown that hyperglycemia increases de novo synthesis of diacylglycerol (DAG), resulting in the sustained activation of PKC in vascular tissues in diabetic animals, including glomeruli (16–20) and cultured cells exposed to high concentrations of glucose (18,21,22). Koya and King (23) have reported preferential activation of PKC-α, -β1, and -δ isoforms in glomeruli isolated from diabetic rats. The same was determined with blue dextran T2000 (Pharmacia), and 3-glucose tolerance factor. Normally distributed variants of intact albumin (25) and an intravenous injection of tritium-labeled rat serum albumin ([3H]-RSA) to assess the proportion of intact and fragmented albumin in urine, as determined by size exclusion chromatography. We also investigated the possibility that renal lysosomal enzyme activity, as measured by cathepsin B and L activity, is related to the fragmentation of albumin. Total PKC activity was quantified by an in situ assay in glomeruli isolated from control and STZ-diabetic rats treated with either AG or RAM for 8 or 24 weeks after the induction of diabetes.

RESEARCH DESIGN AND METHODS

Fasting male Sprague-Dawley rats (Animal Resource Centre, Perth, Australia) aged between 6 and 7 weeks and weighing between 200 and 250 g were randomized to receive an intravenous injection of STZ (Sigma, St. Louis, MO) at a dose of 50–55 mg/kg or sodium citrate buffer (pH 7.4) alone (control). The animals were then randomized to receive one of the following three drug regimens: 1) no treatment; 2) AG (Fluka Chemica, Buchs, Switzerland) at a dose of 1 g/l for diabetic rats and 3 g/l for control rats in drinking water; or 3) RAM (Hoechst, Frankfurt, Germany) at a dose of 3 mg/l for diabetic rats and 9 mg/l for control rats in drinking water for 24 or 32 weeks. These doses were adjusted for water intake and were chosen because they ameliorate albuminuria (25–27). Only diabetic animals with a blood glucose >15 mmol/l were included in the study. Diabetic animals were given 2 U of long-acting insulin (Ultratente, Novo Nordisk, Bagsvaerd, Denmark) daily to maintain body weight and to prevent ketoacidosis without normalizing hyperglycemia. All animals had free access to rat food and water.

The following parameters were measured at 2, 4, 8, 16, and 24 weeks during the study: body weight, blood glucose by glucometer (Accutrend, Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), systolic BP by tail-cuff plethysmography in conscious warmed rats (28), GHb by high-performance liquid chromatography (CLC330 GHb Analyzer; Primus, Kansas City, MO) (29), and glomerular filtration rate (GFR) by a single-injection isotopic technique ([125I-Pertechnetate (PTEC)] developed in our laboratory (30). In addition, urine was collected from animals placed in individual metabolic cages for 24 h for measurement of intact AER by an in-house RIA as described previously (31). The interassay coefficient of variation was 7% at a concentration of 180 ng/ml (n = 10), and the detection limit was 32 ng/ml.

Rat serum albumin (Sigma) was labeled with tritium as previously described by the reductive methylation procedure of Tack et al. (32). The tritium-labeling reaction involves a brief exposure to formaldehyde and sodium borohydride (Du Pont, Detroit, MI). The labeled preparation was purified before use by size exclusion chromatography as described previously (10). In vivo experiments were performed by injecting 5 × 10⁸ dpm/ml ([3H]-RSA) into the tail vein of Sprague-Dawley rats that were maintained in a metabolic cage for 3 h with free access to food and water. Urine samples obtained after 3 h were analyzed by size exclusion chromatography on a column of Bio-Gel G-1000 (Pharmacia) to determine the ratio of intact albumin (Kca–0.024–0.433, where Kca is defined as (Vca–Vc)/[Vt–Vc], where Vca is the elution volume, Vt is the void volume, and Vc is the total volume of the column) to fragmented albumin (Kca–0.721–1.186) in the urine. Samples of 1 ml were loaded onto each column, and 95 fractions of 1.7 ml were eluted with phosphate-buffered saline that contained bovine serum albumin (BSA) (136.9 mmol/l NaCl, 2.68 mol/l KCl, 8.1 mmol/l NaHPO4, 1.5 mmol/l KH2PO4, 0.2% BSA, and 0.02% sodium azide, pH 7.4) at 20 ml/h at 4°C. For routine analysis, urine containing ~15,000–75,000 dpm was applied to the column. Vca was determined with blue dextran T2000 (Pharmacia), and Vt was determined with tritiated water.

Rats were killed at 8 or 24 weeks by decapitation, and their kidneys were removed, decapsulated, and immediately frozen in liquid nitrogen and stored at −80°C. Glomeruli were isolated from half of a frozen kidney by a differential sieving method that uses various sizes of nylon mesh (80–200 µm) (33). Total PKC activity was measured by a modified method originally developed by Hasseley and Johnson (34) in digitonin-permeabilized glomeruli by using an in situ PKC assay with the phosphorylation of a PKC-specific peptide that was derived from the epidermal growth factor receptor (673–683 I/VRKRTLRLRLL-NH2). This assay exhibited linearity with time up to 12 min at 30°C. A control sample of liver was included in each assay to assess reproducibility between assays. The assay was determined to be sensitive for detection of 0.6 n mole of 32P incorporated into the PKC-specific peptide substrate per minute per milligram of protein. Nonspecific binding of 32P to the filter paper was determined by performing the assay in the absence of the substrate.

Lysosomes were isolated from one kidney according to the method of Harikumar and Reyes (35). The combined activities of cathepsin B and L were determined by measuring the fluorescence generated by cleavage of the substrate Z-Phe-Arg-NMec, which releases the highly fluorescent product NHmec (36). Cathepsin B activity was determined by the fluorescence generated by cleavage of Z-Phe-Arg-NMec. The protein content of the crude lysosomal preparation was determined by the Bradford assay (37), and cathepsin activity was expressed as units per milligram of protein. For both control and diabetic lysosomal preparations, cathepsin B and L activities were characterized by >98% inhibition with E-64 (l-trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane), a specific inhibitor of cysteine proteases.

Data are expressed as mean ± SE where n represents the number of determinations. Albuminuria data were logarithmically transformed to normalize the distribution and are given as geometric mean ± SE. All data are shown as geometric mean ± SE (tolerance factor). Normally distributed variables in different groups were compared by analysis of variance with or without repeated measures by using the Statview program (Brain Power, Calabasas, CA). Comparisons among group means were performed by using Fisher’s least significant difference method. A P value of <0.05 was considered statistically significant.

RESULTS

Biochemical and hemodynamic parameters. Table 1 shows metabolic and hemodynamic parameters at week 24 of the study. Similar trends were observed throughout the
Diabetic rats exhibited relatively constant yet significantly elevated glucose and GHb levels throughout the 24-week study when compared with control rats (Table 1). Neither AG nor RAM therapy influenced glycemic control in diabetic rats at any time. Control rats treated with AG or RAM had glucose and GHb levels similar to those of untreated control rats.

The systemic BP was significantly reduced at 4 weeks in both control and diabetic rats that received RAM therapy and remained at that level throughout the study (Fig. 1). The systemic BP was not affected by AG treatment during the entire study period.

At the end of 24 weeks, body and kidney weights were determined (Table 1). Diabetic rats had significantly lower body weights than control rats throughout the study. Treatment of diabetic rats with AG or RAM did not influence body weight during the 24 weeks of treatment. The body weights of control rats treated with AG or RAM did not differ from the body weights of untreated control rats throughout the study. At the conclusion of the 8- or 24-week study, kidney weight in diabetic rats increased compared with that of control rats. Treatment of diabetic rats with AG or RAM significantly reduced kidney weight, whereas similar treatment for control rats did not affect kidney weight.

Diabetes was associated with a significantly elevated GFR when compared with control rats (Table 1). AG or RAM therapy did not influence GFR. Control rats treated with AG or RAM had similar GFR levels to those of untreated control rats.

Urine volumes over a 24-h period were significantly elevated in diabetic rats when compared with those of control rats throughout the study. Treatment of diabetic rats with AG or RAM, as compared with untreated diabetic rats, did not influence urine volume. Control rats treated with AG or RAM had urine volumes similar to those of untreated control rats.

### Table 1. Metabolic and hemodynamic parameters at week 24 for control and diabetic rats treated with AG or RAM

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Glucose (mmol/l)</th>
<th>GHb (%)</th>
<th>Body weight (g)</th>
<th>Kidney weight (g)</th>
<th>GFR (ml/min)</th>
<th>Urine volume (ml/24 h)</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>5</td>
<td>6.1 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>514 ± 18</td>
<td>1.46 ± 0.06</td>
<td>3.54 ± 0.19</td>
<td>12 ± 1</td>
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<tr>
<td>C + AG</td>
<td>5</td>
<td>7.7 ± 0.4</td>
<td>3.2 ± 0.1</td>
<td>550 ± 32</td>
<td>1.43 ± 0.07</td>
<td>3.93 ± 0.33</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>C + RAM</td>
<td>6</td>
<td>8.3 ± 0.4</td>
<td>3.4 ± 0.1</td>
<td>588 ± 30</td>
<td>1.59 ± 0.02</td>
<td>4.86 ± 0.19</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>29.3 ± 1.1*</td>
<td>10.6 ± 0.4*</td>
<td>403 ± 21</td>
<td>2.49 ± 0.46†</td>
<td>4.13 ± 0.15*</td>
<td>96 ± 9*</td>
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<tr>
<td>D + AG</td>
<td>7</td>
<td>20.6 ± 1.6*</td>
<td>8.5 ± 0.5*</td>
<td>385 ± 20*</td>
<td>1.67 ± 0.07</td>
<td>5.52 ± 0.19*</td>
<td>78 ± 13*</td>
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<tr>
<td>D + RAM</td>
<td>8</td>
<td>24.8 ± 1.1*</td>
<td>10.7 ± 0.5*</td>
<td>374 ± 24*</td>
<td>1.84 ± 0.09</td>
<td>4.99 ± 0.34*</td>
<td>111 ± 13*</td>
</tr>
</tbody>
</table>

Data are means ± SE. C, control rats; D, diabetic rats. *P < 0.005 vs. appropriate control; †P < 0.01 vs. control.

During the study (data not shown). Diabetic rats exhibited relatively constant yet significantly elevated glucose and GHb levels throughout the 24-week study when compared with control rats (Table 1). Neither AG nor RAM therapy influenced glycemic control in diabetic rats at any time. Control rats treated with AG or RAM had glucose and GHb levels similar to those of untreated control rats.

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**Albuminuria.** Diabetes was associated with significantly increased intact AER (11.64 ± 3.2 mg/24 h at 24 weeks), whose GFR, systolic BP, and GHb measurements remained similar to those of untreated diabetic rats. Likewise, no effect on intact AER was observed in control rats treated with either AG (0.62 ± 0.17 mg/24 h at 24 weeks) or RAM (1.03 ± 0.17 mg/24 h at 24 weeks) (Fig. 2).

The percentage of intact albumin excreted in urine of rats intravenously injected with [3H]-RSA was determined by measuring the area under the bell-shaped curve of the chromatographic profiles. A representative elution profile of [3H]-RSA in urine collected 3 h after the intravenous administration of [3H]-RSA from a control rat at 16 weeks is shown in Fig. 3A. This profile reveals that most of the [3H]-RSA in the urine of a control rat is degraded to small fragments (intact albumin represents ~12% of total albumin products excreted). This low molecular weight material was neither free tritium, which would elute at the Vt of the column, nor free-labeled amino acid, which also elutes at the Vt (5). With increasing duration of diabetes, there was progressively more inhibition of the fragmentation of albumin (Fig. 3B) so that intact albumin represented 18.3 ± 2.5% (n = 10) of total urinary albumin products at 8 weeks, 45.5 ± 7.9% (n = 10) at 16 weeks, and 48.2 ± 10.8% at 24 weeks.
(n = 9) at 24 weeks (Fig. 3). Treatment of diabetic rats with either AG or RAM significantly reduced the percentage rise in intact albumin excretion during the entire study period (Fig. 4). The proportion of intact albumin excreted by diabetic rats treated with either drug did not differ from that of control rats (Fig. 4). Furthermore, no effect on intact albuminuria was seen in control rats treated with either drug (Fig. 4).

**PKC activity in glomeruli.** Total PKC activity was significantly increased to a similar level in glomeruli from diabetic rats at both 8 and 24 weeks compared with control rats, as shown in Fig. 5A and B, respectively. PKC activity was normalized in glomeruli from diabetic rats treated with RAM at both 8 and 24 weeks. However, PKC activity remained increased in glomeruli from diabetic rats treated with AG for 8 weeks but was normalized after 24 weeks of AG treatment (Fig. 5). Glomeruli isolated from control rats treated with AG or RAM exhibited levels of PKC activity similar to those observed in untreated control rats.

**Renal cathepsin activity in crude lysosomal extracts.** The activities of cathepsin B and cathepsin B and L in the crude renal lysosomal extracts isolated from kidneys at 24 weeks are shown in Table 2. There was a significant decrease in lysosomal activity in kidneys from diabetic rats compared with that of control rats. Lysosome activity in control rats treated with RAM was similar to untreated controls, but control rats treated with AG showed a decrease in activity that was not different from AG-treated diabetic rats. The major feature of the data, apart from diabetes-induced inhibition of lysosomal activity, is that treatment with AG did not affect lysosomal activity in diabetic rats.

**DISCUSSION**

**Drug effects and PKC activity.** Along with previous studies, we have observed in this study that both AG and RAM prevent the rise in albuminuria, which is characteristic of the STZ-diabetic rat model. The two drugs, which are known for their different mechanisms of action, have similar effects in preventing albuminuria.

AG is an inhibitor of advanced glycation and glycoxidation of proteins and has previously been shown to retard the development of albuminuria and mesangial expansion in the STZ-diabetic model (25,26). The major renal effects of ACE inhibitors, such as RAM, including the effects on the long-term development of albuminuria and glomerular ultrastructural injury, are mediated by their capacity to inhibit angiotensin II and to lower systemic BP (27). These drugs appear to be affecting pathways in the development of albuminuria which are independent of glycemic control and GFR because neither normalizes glucose, GHb levels, and GFR (Table 1). AG was also shown not to affect systolic BP, while RAM was shown to significantly lower systolic BP (Fig. 1). Moreover, urine volumes in diabetic rats treated with either drug, as compared with control rats, did not decrease.

It is important to note that previous interpretations of PKC activity in experimental diabetic retinopathy and nephropathy have emphasized a role for PKC in modulation of blood flow (16,23). Decreases in diabetes-related renal blood flow and increases in GFR have been associated with increased PKC activity and prevented by PKC inhibition.
The present data suggest that the role of PKC may not be confined to regulation of blood flow. In this study, we were able to measure total glomerular PKC activity without partial purification of PKC from the tissue by an in situ assay using the phosphorylation of a highly PKC-specific peptide derived from the epidermal growth factor receptor. Isolation of PKC into membranous and cytosolic fractions may not reflect activities in vivo because of losses of protein or PKC activity during the isolation procedure (34). Our findings that total PKC activity is significantly elevated in glomeruli that have been isolated from diabetic rats at both 8 and 24 weeks are similar to those of other investigators who observed rats with short-term STZ-diabetes (16,20,21). The prevention of the rise in AER by RAM in diabetic rats was accompanied by the normalization of diabetes-induced increases of PKC activity in glomeruli. The rise in AER was also prevented in diabetic rats treated with AG, but the inhibition of the diabetes-induced increase in PKC activity was only observed for AG at 24 and not at 8 weeks.

Similarities in the way AG and RAM inhibit the development of albuminuria and PKC activity suggest that they act on a shared intrarenal pathway when compared with their different modes of action at a systemic level. AGE receptor interactions have been shown to include PKC activation in cultured cells (38). Angiotensin II binding to AT1 receptors in proximal tubular cells has been shown to lead to G-protein-coupled activation of phospholipase C and D, which in turn increases DAG production and PKC activity (39). The shared intrarenal pathway is likely to involve PKC through its direct actions on the intracellular processing of albumin. The effect of PKC on vesicular trafficking has been demonstrated to be a regulatory mechanism that controls endo- and exocytosis of albumin in proximal tubular cells (15). It is possible that PKC may contribute to the regulation of proteins involved in microtubular-based intracellular transport or in the cytoskeleton itself, thereby affecting the associated transcellular passage of transport vesicles. Although the requirements for microtubular structures in transcytosis may not be homogeneous (40), PKC regulation may be a direct mechanism by

<table>
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<tr>
<th>Cathepsin activity ($10^6$)</th>
<th>Cathepsin B (U/mg protein)</th>
<th>Cathepsin B + L (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.14 ± 0.24</td>
<td>222 ± 14</td>
</tr>
<tr>
<td>C + AG</td>
<td>1.81 ± 0.20†</td>
<td>126 ± 24†</td>
</tr>
<tr>
<td>C + RAM</td>
<td>3.85 ± 0.77</td>
<td>217 ± 38</td>
</tr>
<tr>
<td>D</td>
<td>1.46 ± 0.15*</td>
<td>81 ± 5.7*</td>
</tr>
<tr>
<td>D + AG</td>
<td>2.02 ± 0.26</td>
<td>123 ± 17</td>
</tr>
<tr>
<td>D + RAM</td>
<td>2.28 ± 0.38*</td>
<td>116 ± 17*</td>
</tr>
</tbody>
</table>

Data are means ± SE. C, control rats; D, diabetic rats. *P < 0.005 vs. appropriate control; †P < 0.005 vs. control.
which angiotensin II and AGEs modulate the trafficking of albumin in epithelial cells of the nephron. Renal lysosomal activity. While lysosomal activity was decreased in diabetes, which is in agreement with other studies (41,42), it was not normalized by RAM. This finding is the most striking feature of the observations on lysosomal activity, for it means that a decrease in renal lysosomal activity is not required for the prevention of albuminuria. The major quantitative influence of changes in lysosomal activity in diabetes appears to be early in the onset of albuminuria. Changes in albumin fragment excretion rate may be independent of lysosomal activity but may also reflect changes in the kinetics of the intracellular trafficking of substrate delivery to the lysosome, which is a process that may be controlled by PKC (43,44).

Concluding remarks. As in previous studies, we have observed that both AG and RAM prevent the rise in albuminuria that occurs in STZ-diabetic rats. The two drugs, which are known for their different mechanisms of action, are of interest for the prevention of albuminuria. The major quantitative influence of changes in lysosomal activity in diabetes appears to be early in the onset of albuminuria and that both drugs exert their influence on glomerular PKC activity and its role in the intracellular processing of filtered albumin.

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