Attenuation of Extracellular Matrix Accumulation in Diabetic Nephropathy by the Advanced Glycation End Product Cross-Link Breaker ALT-711 via a Protein Kinase C-α–Dependent Pathway

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This study investigated the role of advanced glycation end products (AGEs) in mediating protein kinase C (PKC) isoform expression in diabetic nephropathy. In vitro, vascular smooth muscle cells incubated in a high-glucose (25-mmol/l) medium demonstrated translocation and increased expression of PKC-α as compared with those from a low-glucose (5-mmol/l) environment. Coincubation with the cross-link breaker ALT-711 and, to a lesser extent, with aminoguanidine, an inhibitor of AGE formation, attenuated the increased expression and translocation of PKC-α. Streptozotocin-induced diabetic rats were randomized to no treatment, treatment with ALT-711, or treatment with aminoguanidine. Diabetes-induced increases in PKC-α as well as in the βI, βII, and ε isoforms. Treatment with ALT-711 and aminoguanidine, which both attenuate renal AGE accumulation, abrogated these increases in PKC expression. However, translocation of phosphorylated PKC-α from the cytoplasm to the membrane was reduced only by ALT-711. ALT-711 treatment attenuated expression of vascular endothelial growth factor and the extracellular matrix proteins, fibronectin and laminin, in association with reduced albuminuria. Aminoguanidine had no effect on VEGF expression, although some reduction of fibronectin and laminin was observed. These findings implicate AGEs as important stimuli for the activation of PKC, particularly PKC-α, in the diabetic kidney, which can be directly inhibited by ALT-711. Diabetes 53: 2921–2930, 2004

Hyperglycemia facilitates nonenzymatic glycation of proteins, which leads to the formation of advanced glycation end products (AGEs) (1). Experimental animals infused with AGE-rich proteins demonstrate diabetes-like complications, including increased vascular permeability, extracellular matrix deposition, and glomerulosclerosis (2). The importance of AGEs in the pathogenesis of diabetic nephropathy has been demonstrated in a number of animal studies that have shown the benefit of AGE inhibitors with different chemical structures such as aminoguanidine (3) and pyridoxamine (4). More recently, the putative AGE cross-link breaker, ALT-711 (5), has been reported to attenuate various functional and structural manifestations of diabetic microvascular disease within the kidney.

Protein kinase C (PKC) is a family of closely related enzymes that phosphorylate serine or threonine residues on various intracellular proteins and are involved in a wide range of cellular functions (6), such as basement membrane regulation and expression of growth factors (7). Increases in PKC activity have been identified in vivo in renal glomeruli from streptozotocin-induced diabetic rats (8,9) and cells cultured under high-glucose conditions (10). Several studies have also reported attenuation of experimental diabetic nephropathy with the PKC-βI inhibitor, LY333531 (11,12). More recently, studies have shown that diabetic PKC-α knockout mice are protected from albuminuria and have reduced expression of vascular endothelial growth factor (VEGF) (13). AGEs can also directly induce the expression of VEGF, whose cellular signaling actions are mediated via PKC activation (14). We (15) have previously shown that diabetes is associated with increased renal VEGF expression. Furthermore, Cha et al. (16) have also observed that downregulation of PKC can inhibit glucose-induced increases in VEGF production. In addition, PKC may be directly activated by AGEs (17), possibly via a specific AGE-receptor subtype (18). Previous studies by our own group have identified abrogation of diabetes-associated increases in PKC activation with the AGE inhibitor, aminoguanidine (8).

The aim of this study was to examine the effects of AGE-reducing therapies—namely, ALT-711, which is presumed to act via cleavage of preformed AGEs, and aminoguanidine.
guanidine, an AGE formation inhibitor—on diabetic nephropathy. This involved investigation of PKC-α phosphorylation in the context of renal functional and structural parameters, including the PKC-dependent cytokine VEGF and the extracellular matrix proteins fibronectin and laminin. Once we identified that ALT-711 was associated with reduced phosphorylated PKC-α in the diabetic kidney, our second aim was to determine if these effects were attributable to a direct effect of ALT-711 on the translocation and expression of PKC-α. This phenomenon was tested in a well-established in vitro system.

**RESEARCH DESIGN AND METHODS**

Experimental diabetes was induced in male SD rats (200–250 g) by intravenous injection of streptozotocin in citrate buffer (50 mg/kg) after rats had been fasted overnight. Plasma glucose levels were measured 1 week after the induction of diabetes; animals with levels >15 mmol/l were included in the study. Control and diabetic animals were randomized into six treatment groups (n = 10 for each group): 1) no treatment (control and diabetic groups); 2) treatment with the cross-link breaker ALT-711 (4,5-dimethylimidazole-2-phenylethyl-thiazolium chloride; Alteon, Ramsey, NJ) as an intervention, 10 mg · kg⁻¹ · day⁻¹, gavaged during weeks 16–32 (CALT and DALT for control and diabetic aminoguanidine groups, respectively); and 3) treatment with aminoguanidine, an inhibitor of AGE formation, 1 g/l in drinking water during weeks 0–32 (CAG and DAG for control and diabetic aminoguanidine groups, respectively). Diabetic animals were given 2 units of ultralente insulin (Ultratard HM; Novo Nordisk, Bagsvaerd, Denmark) daily to prevent ketoacidosis, promote weight gain, and improve survival. Body weight, mean systolic blood pressure (by tail cuff plethysmography) (19), the glomerular filtration rate (using 99Tc-DTPA) (20), the albumin excretion rate (AER; measured by radioimmunoassay) (21), and 1% Triton X-100, in the absence of phosphatase inhibitors. Homogenates were centrifuged at 13,000 rpm for 1 h at 4°C and supernatant protein content was measured using the BCA protein assay (Perbio Science, Cheshire, U.K.).

For Western blotting, 10 μg of each sample were separated by 10% SDS-PAGE under nonreducing conditions. Using a secondary transfer tank (Bio-Rad, Hercules, CA), proteins were transferred to polyvinylidene fluoride membrane (Hybond P; Amersham, Buckinghamshire, U.K.). Nonspecific binding sites were blocked overnight with 5% (wt/vol) nonfat milk powder in Tris-buffered saline (TBS), then incubated for 1 h with the primary antibodies rabbit anti-PKC-α and -PKC-ε (1:15,000; Sigma, St Louis, MO), rabbit anti-PKC-β1 and -PKC-βII (1:15,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-PKC-α (1:500 Upstate Biotechnology, Lake Placid, NY), anti-β-tubulin (1:200; Santa Cruz), and the secondary antibody biotinylated goat anti-rabbit IgG (1:15,000; Dako, Carpentry, CA). Bound antibodies were amplified using a streptavidin–horseradish peroxidase conjugate (1:15,000; Dako) and detected by reaction with an enhanced chemiluminescence kit (Amersham, Piscataway, NJ). The band intensity was quantified using a microcomputer imaging device and expressed as (density × area) – background. Individual samples (n = 6 per group for a total of 36 samples) for each antibody were analyzed after correcting for β-tubulin. Representative samples from each group are shown in Fig. 1.

**Vascular endothelial growth factor.** After extraction, Western blotting

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**TABLE 1**

Functional and structural data from all groups

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>CAG (0–32 weeks)</th>
<th>CALT (16–32 weeks)</th>
<th>Diabetic group</th>
<th>DAG (0–32 weeks)</th>
<th>DALT (16–32 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma glucose (mmol/l)</strong></td>
<td>6.3 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>28.4 ± 15*</td>
<td>30.0 ± 1*</td>
<td>26.9 ± 1*</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>4.5 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>15.2 ± 0.8*</td>
<td>14.7 ± 0.9*</td>
<td>15.9 ± 0.7*</td>
</tr>
<tr>
<td><strong>AER (mg/24 h)</strong></td>
<td>1.6 (1.5–1.7)</td>
<td>0.6 (0.5–1.5)</td>
<td>0.9 (0.6–1.6)</td>
<td>7.9 (4.8–21.6)*</td>
<td>1.6 (1.0–3.1)†</td>
<td>8.5 (5.7–14.5)*</td>
</tr>
<tr>
<td><strong>Mean systolic blood pressure (mmHg)</strong></td>
<td>115 ± 5</td>
<td>116 ± 7</td>
<td>114 ± 6</td>
<td>161 ± 4*</td>
<td>142 ± 6*</td>
<td>145 ± 6*</td>
</tr>
<tr>
<td><strong>Glomerular filtration rate (ml · min⁻¹ · g kidney wt⁻¹)</strong></td>
<td>2.2 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>2.6 ± 0.1#</td>
<td>2.8 ± 0.2#</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td><strong>Kidney-to-body weight ratio (×10⁻³)</strong></td>
<td>2.9 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>5.7 ± 0.2*</td>
<td>5.2 ± 0.4*</td>
<td>5.0 ± 0.2*</td>
</tr>
<tr>
<td><strong>Renal AGEs (% area)</strong></td>
<td>7.7 ± 0.4</td>
<td>7.3 ± 1.6</td>
<td>8.1 ± 0.9</td>
<td>14.9 ± 0.6*</td>
<td>8.3 ± 0.8†</td>
<td>7.2 ± 0.7†</td>
</tr>
</tbody>
</table>

Data are means ± SE or median (interquartile range). Data for renal AGEs represent morphometric computer-aided analysis of renal cortical AGE (CML) immunostaining. *P < 0.001 vs control group; †P < 0.001 vs diabetic group; ‡P < 0.001 for DAG vs. DALT; §P < 0.001 vs. CALT; ¶P < 0.01 vs. diabetic group; ||P < 0.05 vs. DAG; ††P < 0.05 vs. control group; †††P < 0.05 vs. diabetic group.

**FIG. 1.** Representative immunoblots stained for PKC-α (80 kDa; A), phosphorylated PKC-α (82 kDa; B), PKC-β1 (80 kDa; C), PKC-βII (80 kDa; D), PKC-ε (90 kDa; E), and β-tubulin (F) in kidneys at 32 weeks.
TABLE 2

<table>
<thead>
<tr>
<th>PKC isofroms</th>
<th>Control group (0–32 weeks)</th>
<th>CAG (0–32 weeks)</th>
<th>CALT (16–32 weeks)</th>
<th>Diabetic group (16–32 weeks)</th>
<th>DAG (0–32 weeks)</th>
<th>DALT (16–32 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.8 ± 0.2*</td>
<td>1.5 ± 0.02†</td>
<td>1.4 ± 0.02†</td>
</tr>
<tr>
<td>Phosphorylated PKC-α</td>
<td>1.0 ± 0.1</td>
<td>2.1 ± 0.1*</td>
<td>0.7 ± 0.1</td>
<td>1.8 ± 0.3*</td>
<td>2.8 ± 0.5*</td>
<td>1.0 ± 0.3†</td>
</tr>
<tr>
<td>PKC-β</td>
<td>1.0 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.7 ± 0.1*</td>
<td>1.1 ± 0.02‡</td>
<td>1.0 ± 1.1‡</td>
</tr>
<tr>
<td>PKC-βII</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.1$</td>
<td>0.7 ± 0.1$</td>
<td>1.3 ± 0.002$</td>
<td>1.0 ± 0.1†</td>
<td>0.9 ± 0.1†</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.04</td>
<td>1.7 ± 0.06*</td>
<td>1.1 ± 0.04‡</td>
<td>1.3 ± 0.1‡</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE and are expressed as relative optical density. *P < 0.01, †P < 0.05 vs. control group; ‡P < 0.01 vs. diabetic group.

RESULTS

Functional parameters: experimental diabetic nephropathy. Indexes of glycemic control were significantly increased in diabetic animals as compared with controls (P < 0.001 vs. control group) (Table 1). No effect of either treatment was detected for plasma glucose or HbA1c.

Urinary albumin excretion was elevated by week 16 in the diabetic and DALT groups (P < 0.001) (Table 1). By week 16, the aminoguanidine-treated diabetic animals had less albuminuria than the other diabetic animals (diabetic and DALT, 16–32 weeks), similar to that seen in the control group. By week 32, both diabetic treatment groups (DAG and DALT) had significantly lower AER as compared with the untreated diabetic group (P < 0.001 vs. diabetic group). Although the AER in the DALT group was higher than that in the DAG group, this difference was not statistically significant (P = 0.585). The glomerular filtration rate was elevated in the diabetic group when compared with controls (P < 0.05 vs. control group). No differences were observed in the GFR among untreated and treated diabetic rats (Table 1). Diabetes was associated with elevated mean systolic blood pressure, which was modestly attenuated in the DAG and DALT groups (P < 0.01 vs. diabetic group) (Table 1). Kidney–to–body weight ratios were increased in all diabetic groups compared with the control groups (P < 0.001 vs. control group) (Table 1). There was a modest but significant attenuation of kidney–to–body weight ratios in the DAG and DALT groups (P < 0.01 vs. diabetic group).

Accumulation of renal advanced glycation end products. Immunohistochemistry revealed a diabetes-induced increase in the renal accumulation of AGEs (P < 0.001 diabetic vs. control group) (Table 1). Localization was evident in all components of the kidney, including glomeruli, the tubulointerstitium, and vasculature. Treatment with aminoguanidine or ALT-711 abrogated renal AGE accumulation (P < 0.001 for DAG and DALT vs. diabetic group) (Table 1).

PKC isoform Western immunoblotting. Western immunoblotting demonstrated significant increases in diabetic as compared with control kidneys for PKC-α (80%), PKC-βI (74.5%), and PKC-ε (70.4%) (P < 0.01) (Fig. 1A, C, and E; Table 2) and to a lesser extent PKC-βII (29.3%; P < 0.05) (Fig. 1D, Table 2). Increased expression of all four PKC isoforms proceeded as for PKC isoforms with the following exceptions: 35 μg of sample protein were separated on 10% SDS-PAGE, and the primary antibody was mouse anti-VEGF (1:1,000; NeoMarkers, Fremont, CA).

Immunohistochemistry. Methyl carnosine- and formalin-fixed paraffin sections of kidney were dewaxed and rehydrated. Immunohistochemistry was performed as previously described (5). Slides for fibronectin were micro-waved in a pressure cooker for 6 min in 0.01 mol/l citrate buffer (pH 6.0) and slides for laminin were pretreated with proteinase K (Dako) for 2 min. Frozen kidney sections for PKC staining were air dried, fixed in cold acetone for 10 min, and washed in TBS. The primary antibodies used were polyclonal rabbit anti-human PKC-α (1:125), PKC-βI (1:100, 1:50 frozen samples), PKC-βII (1:100), PKC-ε (1:100; Santa Cruz Biotechnology), and phospho-PKC-α (1:100) (Upstate Biotechnology). Rabbit anti-mouse VEGF (1:200; a kind donation of Dr. Steve Stackler) (15), rabbit anti-human fibronectin (1:1,000; Dako), mouse anti-α-actin and desmin (Boehringer, Mannheim, Germany) (24).

Probes, Leiden, the Netherlands) in 1% BSA/PBS was applied for 60 min. The sections were washed three times in PBS, after which the secondary antibody, Alexa-488–conjugated anti-mouse IgG (1:200; Molecular Probes, Leiden, the Netherlands) in 1% BSA/PBS was applied for 60 min. The cells were washed and mounted with Aquamount (Polyscience, Niles, IL). A Nikon Diaphot microscope and a BioRad MRC 1024 confocal imaging system (BioRad, Freiburg, Germany) with an argon/krypton laser was used to examine at least 30 cells from at least two independent experiments under each experimental condition. Identical settings for the power of light source, confocal aperture, gain, and black level were used for each set of experiments.

Quantification of expression was completed with National Institutes of Health software. The cells were outlined manually and the mean fluorescent intensity was obtained. Data are presented as relative intensities, setting the control mean to 100.

Statistical analysis. Results are expressed as means ± SE, unless otherwise stated. Data for albuminuria were not normally distributed and therefore were analyzed after logarithmic transformation. The albuminuria results are expressed as medians and interquartile ranges. The results of the confocal experiments are expressed as means ± SE and were analyzed by Wilcoxon’s test. Analyses were performed by ANOVA and post hoc analysis using Fisher’s least significant differences test, after correcting for multiple comparisons. P < 0.05 was considered to be statistically significant.
isoforms was significantly reduced by treatment with aminoguanidine and ALT-711 (P < 0.01 DAG and DALT vs. diabetic group for PKC-βI and -ε; P < 0.05 for PKC-α and -βII) (Table 2). PKC-βII was also significantly reduced in nondiabetic rats that received treatment (P < 0.05 CAG and CALT vs. control group) (Table 2). Phosphorylated PKC-α showed increased expression in diabetic as compared with control groups (P < 0.01 vs. control group); this increase was attenuated by ALT-711 (P < 0.05 vs. diabetic group), but not by aminoguanidine. Each PKC isoform was identified as a single band, with PKC-α, -βI, and -βII being 80 kDa and PKC-ε being 90 kDa, consistent with observations from previous studies (7). Phosphorylated PKC-α was identified as a doublet at ~82 kDa.

**Localization of protein kinase C isoforms.** In control kidneys, PKC-α (Fig. 2A) was localized to proximal and distal tubular cells. The major site of immunostaining was at the apical region of the tubular cells, with minimal staining in the glomeruli. Phosphorylated PKC-α (Fig. 2E) was localized to the glomeruli and the apical region of proximal tubular cells. In the diabetic animals there was increased staining of PKC-α in the glomeruli, predominantly in the mesangial area. There was also a clear increase in cytoplasmic staining of proximal tubule cells and, to a lesser extent, distal tubules, especially in the luminal area (Fig. 2B, arrow). Phosphorylated PKC-α was increased in the glomeruli and tubules of diabetic kidneys and appeared to have translocated to the apical region of the tubular cells (Fig. 2F, arrow). PKC-α immunostaining (Fig. 2C and D) appeared to be attenuated with both treatments, whereas phosphorylated PKC-α staining (Fig. 2G and H) was decreased only by ALT-711.

PKC-βI (Fig. 3A–D) was identified in cortical and outer medullary distal tubules, although some expression in proximal tubules was identified in the diabetic group (Fig. 3B). The staining was located at the apical luminal border of these cells. The staining in the diabetic treatment groups (Fig. 3C and D) was similar to that seen in control kidneys. Glomerular PKC-β-βI immunostaining was best demonstrated in the frozen sections (Fig. 3E and F). PKC-βI was increased in the diabetic kidney in both tubules (Fig. 3B) and glomeruli (Fig. 3F) and was attenuated by both treatments (Table 2).

PKC-βII staining (not shown) was primarily seen in the distal tubules within the kidney cortex and medulla in control animals. This staining was localized to the basolateral areas of distal tubular cells. With diabetes, staining in the basolateral area of proximal tubules was also identified. After both treatments, there was some decrease in immunostaining for this PKC isofrom (Table 2). PKC-ε (not shown) was localized to occasional glomerular cells of endothelial origin. In the control groups, focal staining was observed throughout the kidney, predominantly localized to distal tubule cells. In diabetic animals, there was intense staining of distal tubular cells, which appeared to be diminished in both treatment groups (Table 2).

**Expression of vascular endothelial growth factor protein.** Western immunoblotting demonstrated a diabetes-associated increase in VEGF, with a single band present at ~45 kDa (P < 0.001 diabetic vs. control group) (Fig. 4A). This increase in VEGF protein expression was ameliorated by ALT-711 (P < 0.001 DALT vs. diabetic group), but no effect was identified with aminoguanidine (P = 0.79 DAG vs. diabetic group; P < 0.001 DALT vs. DAG). There was a trend to reduced VEGF accumulation in CALT as compared with the control group, but this did not reach statistical significance (P = 0.052). VEGF was immunohistochemically detected in the glomeruli of control groups, including the treated groups. VEGF quantitation showed a similar trend to the immunoblotting with an increase in glomerular staining with diabetes (P < 0.005 diabetic vs. control group) (Fig. 4B) that was ameliorated by ALT-711 (P < 0.01 DALT vs. diabetic group), but not aminoguanidine (P = 0.51 DAG vs. diabetic group; P < 0.001 DALT vs. DAG). In addition to glomerular staining in the diabetic groups, which was especially evident in the podocytes, there was also staining in distal tubules within the renal cortex that was not influenced by treatment (immunohistochemistry not shown).

**Fibronectin and laminin accumulation.** Immunohistochemical analysis of fibronectin identified an increase in untreated diabetic animals (P < 0.001 diabetic vs. control group) (Fig. 4C), which was reduced with aminoguanidine (P < 0.002 DAG vs. diabetic group) and ALT-711 (P < 0.001 DALT vs. diabetic group) treatment. Fibronectin protein expression was localized mainly to the mesangial matrix of glomeruli and, to a lesser extent, to the tubulointerstitium. Immunostaining for laminin was increased in the diabetic as compared with the control group (P < 0.001 diabetic vs. control group) (Fig. 4D). Similar to fibronectin, laminin protein expression was attenuated in both of the treated diabetic groups (P < 0.001 DAG and DALT vs. diabetic group) (Fig. 4D). There was also a modest but significant decrease in immunostaining for laminin in the treated nondiabetic rats (P < 0.05 CAG and CALT vs. control group). Laminin was localized primarily to glomerular and tubular basement membranes.

**Confocal microscopy.** The confocal images of VSMCs shown in Fig. 5A demonstrate the effect of 5 mmol/l (control) (top left panel) versus 25 mmol/l glucose (bottom left panel) on the intracellular distribution of PKC-α. In the control panel, PKC-α shows a coarse granular distribution in the cytosol, especially in the perinuclear region. Glucose at 25 mmol/l led to an increase in immunoreactivity in the cytosol nucleus and along the cell membranes (P < 0.0001 low vs. high glucose) (Fig. 5B). No differences were seen in cells cultured in 5 mmol/l glucose and treated with ALT-711 or AG. Treatment of cells cultured under high-glucose conditions with ALT-711 showed a significant decrease in PKC-α staining (P < 0.0001 high glucose vs. high glucose + ALT-711) (Fig. 5A and B). Coincubation with aminoguanidine in high glucose moderately attenuated this increase with PKC-α, but was less effective than ALT-711 on this phenomenon (P < 0.0001 high glucose + ALT-711 vs. high glucose + aminoguanidine) (Fig. 5A, right panel, and Fig. 5B).

FIG. 2. Representative photomicrographs of renal cortical immunostaining at 32 weeks for PKC-α in control (A), diabetic (B; arrow demonstrates cytosolic distribution), DAG (C), and DALT (D) groups, and for phosphorylated PKC-α in control (E), diabetic (F; arrow demonstrates membranous translocation), DAG (G), and DALT (H) groups. Magnification ×200.
FIG. 3. Representative photomicrographs of renal cortical immunostaining for PKC-β1 at 32 weeks in fixed sections from control (A), diabetic (B), DAG (C), and DALT (D) groups, and in frozen sections from control (E) and diabetic (F) groups. Magnification ×200.
DISCUSSION

Two disparate agents were used to attenuate AGE accumulation in the diabetic kidney: aminoguanidine, an inhibitor of AGE formation that has been shown to retard the development of albuminuria and structural damage in experimental diabetic nephropathy (3,21), and ALT-711, a new generation AGE cross-link breaker that has been postulated to act via cleavage of preformed AGE cross-links, thereby allowing the kidney to excrete fragmented AGEs (25). As has been seen with aminoguanidine, ALT-711 also has beneficial effects on renal structural and functional damage in diabetes (5). The present study demonstrated that ALT-711 and, to a lesser extent, aminoguanidine attenuated the increase in expression of PKC-α in the diabetic kidney. Despite continuing hyperglycemia in the diabetic animals, both treatments ameliorated diabetes-associated increases in the renal AGE carboxymethyllysine and abrogated the increase in other PKC isoforms, specifically βI, βII, and ε. Phosphorylated PKC-α was reduced only by ALT-711, a finding that correlates with the attenuation of VEGF expression. These effects were not seen with AG. Based on a positive in vivo finding linking ALT-711 treatment to changes in PKC-α, a series of in vitro studies were performed that demonstrated that ALT-711 attenuated the increase in expression and translocation of PKC-α in culture under high-glucose conditions.

PKC activation has been demonstrated in organs susceptible to diabetes-related injury, and has been implicated in the initiation and progression of the cellular dysfunction linked to complications such as diabetic nephropathy (7,10). However, in this study, increases in the expression of the phosphorylated form of PKC-α in the diabetic kidney were abrogated only by ALT-711. This finding was consistent with our cell culture studies performed under high-glucose conditions where translocation and expression of PKC-α were potentially inhibited by ALT-711. These findings suggest that ALT-711 may have a direct effect on PKC-α. Although both agents influenced PKC-α immunostaining, only ALT-711 had a significant effect on phosphorylation, even though both agents conferred renoprotection. This result is consistent with PKC-α phosphorylation not being the sole determinant of renal injury in experimental diabetes. Furthermore, the protocols for the two agents that attenuated renal AGE accumulation differed, with ALT-711 being administered after albuminuria was already established. This could also partially explain some of the differences observed on various parameters with the two drugs.
In contrast, the expression of phosphorylated PKC-α was paradoxically increased in both the control and diabetic groups receiving aminoguanidine. This, however, may have been a direct result of the inducible nitric oxide synthase inhibiting actions of aminoguanidine, as a previous study has demonstrated potent PKC-α activation by this agent in ischemic neural tissues (26). Furthermore, a separate study in experimental diabetes has demonstrated no effect of aminoguanidine on PKC activity in the context of reduced AGEs (27), thereby confirming our current results. Other studies, however, have shown that treatment with aminoguanidine attenuates increases in PKC activity after 24 weeks of diabetes in glomeruli (8), retina, and mesenteric artery (28), although specific PKC isoforms were not addressed in that study. It appears likely that some of the differences seen in that study relate to the

FIG. 5. A: Confocal photomicrographs of PKC-α translocation and expression showing the effects of treatment with ALT-711 and aminoguanidine (AG) on VSMCs incubated in low (5 mmol/l) and high (25 mmol/l) glucose. B: Semiquantitative analysis of immunofluorescence (>30 cells of two independent experiments) 15 h after exposure to low-glucose (LG) and high-glucose (HG) concentrations with and without treatment. Data are means ± SE. *P < 0.0001 vs. LG; †P < 0.0001 for HG vs. HG + ALT; ‡P < 0.0001 for HG + ALT vs. HG + AG.
lack of examination of nonglomerular sources of PKC, such as tubules, which in this and previous studies have been shown to be major sites of expression and phosphorylation of various PKC isoforms (6,7).

Diabetes was associated with increased expression of VEGF, particularly within glomeruli but also within distal tubules. This observation is consistent with those of previous studies by our group (15) and other investigators (16) who have shown that in the early phase of diabetes, VEGF expression is limited to the glomeruli, whereas at later stages of the disease, expression of this growth factor is also present in the tubules. Moreover, Cha et al. (16) have shown that PKC downregulation inhibits increases in VEGF production. VEGF expression is also increased in the plasma of type 1 diabetic patients with diabetic nephropathy (29). In a recent study, it has been suggested that VEGF expression may be regulated by PKC-ε, as diabetic PKC-ε knockout mice do not have a diabetes-associated increase in VEGF. Furthermore, the effect of PKC on VEGF expression occurs in the context of reduced albuminuria in these diabetic knockout mice (13). These findings are consistent with those of the present study in which diabetes-induced phosphorylation of PKC-α and increases in VEGF expression were selectively decreased with ALT-711. Kang and colleagues (7,30) have demonstrated increases in renal PKC-α in streptozotocin-induced diabetes and have suggested that PKC-α may mediate renal endothelial cell permeability, thereby resulting in organ damage. Such an effect may involve a cytokine such as VEGF, which was shown in this study to be upregulated in the diabetic kidney.

Both AGEs and the PKC pathway have been implicated in diabetes complications, including nephropathy (31), but the exact interaction between these two pathways has not been fully defined. Previous groups have identified links between AGEs or PKC and the expression of extracellular matrix proteins. PKC pathways have been shown to influence induction of fibronectin in various cell types, including fibroblasts (32). In addition, our group has identified a relation between AGEs and fibronectin (33). Indeed, in the present study, fibronectin was shown to be increased in the untreated diabetic animals. Laminin, a glycoprotein involved in the maintenance of structural integrity and selective permeability function of the glomerular basement membrane (34), was also found to be increased in our untreated diabetic animals. This finding is supported by those of other studies showing increased laminin expression in the cortex of diabetic animals (35). Studies by Yang et al. (36) have shown that administration of AGEs per se, even in the absence of hyperglycemia, results in the upregulation of laminin in mouse kidneys. Furthermore, it has been reported that gene and protein expression of laminin is increased in human and rat mesangial cells grown in AGE BSA (37). Interestingly, we observed that ALT-711 is more effective in reducing the accumulation of fibronectin and laminin than aminoguanidine, a result that may relate to ALT-711’s greater ability to reduce AGE accumulation. However, it could also be linked to the specific effects of ALT-711 on PKC-α expression/phosphorylation and VEGF expression. Although the effects of ALT-711 on renal fibronectin and laminin expression have not been previously reported, our findings are consistent with studies linking inhibition of PKC isoforms to reduced extracellular matrix expression in diabetes (11).

Although the present study focused on the activation PKC-α, it is evident that other PKC isoforms play a role in diabetic nephropathy. Our results showed that ALT-711 has the ability to reduce other PKC isoforms, possibly linked with the ability of VEGF to activate several isoforms, including PKC-α, -βI, -βII (38,39), and -ε (40), or the regulation of other PKC isoforms by PKC-α. Indeed, PKC-α has been shown to directly influence a number of other PKC isoforms, including PKC-θ (41) and -ε (42).

In conclusion, this study demonstrated in experimental diabetic nephropathy that the renoprotective effects of treatments that attenuate the accumulation of AGEs may, in part, occur via inhibition of PKC-α activation in the renal cortex. This finding was supported by in vitro experiments in VSMCs where ALT-711 decreased the expression and translocation of PKC-α in a high-glucose environment. Furthermore, ALT-711 ameliorated the expression of VEGF and the various extracellular matrix proteins, including fibronectin and laminin. The present study has shown that ALT-711 appears to have multiple actions relevant to conferring renoprotection, including inhibition of renal AGE accumulation and a reduction in PKC-α activation.

ACKNOWLEDGMENTS
This work was completed with support from the Juvenile Diabetes Research Foundation (JDRF) and the European Foundation for Studying Diabetes. Dr. Josephine Forbes is a JDRF postdoctoral research fellow. Color figure costs were paid for by Alteon.

The authors would like to thank Gavin Langmaid for his expert care of the animals throughout the study and Mary Ann Arnstein for her technical expertise.

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