Effects on protein kinase C-β inhibition on glomerular vascular endothelial growth factor expression and endothelial cells in advanced experimental diabetic nephropathy

Darren J. Kelly,1 Danielle Buck,1 Alison J. Cox,1 Yuan Zhang,1 and Richard E. Gilbert1,2

1Department of Medicine, University of Melbourne, St. Vincent’s Hospital, Fitzroy, Australia; and 2Department of Medicine, University of Toronto, St. Michael’s Hospital, Toronto, Canada

Submitted 5 October 2006; accepted in final form 16 May 2007

Kelly DJ, Buck D, Cox AJ, Zhang Y, Gilbert RE. Effects on protein kinase C-β inhibition on glomerular vascular endothelial growth factor expression and endothelial cells in advanced experimental diabetic nephropathy. Am J Physiol Renal Physiol 293: F565–F574, 2007. First published May 23, 2007; doi:10.1152/ajprenal.00397.2006.—Ruboxistaurin is an inhibitor of the β isoform of protein kinase C (PKC-β) that reduces the actions of vascular endothelial growth factor (VEGF) and attenuates the progression of diabetic retinopathy. In the glomerulus VEGF is constitutively expressed where it likely has a role in maintaining endothelial cell integrity, particularly in disease states. Given its potential use in diabetic nephropathy, we sought to determine the effects of PKC-β inhibition on VEGF and glomerular endothelial cells in experimental diabetic nephropathy. Studies were conducted in (mRen-2)27 rat, a transgenic rodent with hypertension and an enhanced renin-angiotensin system that following induction of diabetes with streptozotocin develops many of the features of diabetic nephropathy. Moreover, to mimic the clinical context, the effects of PKC-β inhibition were examined both with and without concomitant angiotensin-converting enzyme (ACE) inhibitor therapy. Diabetic Ren-2 rats were randomized to receive either vehicle, the ACE inhibitor, perindopril (0.2 mg/l in drinking water), ruboxistaurin (10 mg·kg−1·day−1, admixed in chow), or their combination and studied for 12 wk. Diabetic Ren-2 rats displayed glomerular endothelial cell loss in association with overexpression of VEGF mRNA. Both cell loss and VEGF overexpression were attenuated by the administration of either perindopril or ruboxistaurin, as single agent treatments with their combination providing additional, incremental improvements, reducing these manifestations of injury down to levels seen in non-diabetic, normotensive, nontransgenic animals. Combination therapy was also associated with additional improvements in albuminuria and glomerulosclerosis.

glomerular endothelial cells; glomerulosclerosis; albuminuria

RUBOXISTAURIN MESYATE. A SPECIFIC and selective inhibitor of PKC-β isoforms, has been shown to delay moderate visual loss in patients with diabetic retinopathy (12) and to reduce albuminuria in patients with diabetic nephropathy (28). Its beneficial effects in diabetic eye disease are thought to relate, at least in part, to its ability to reduce the actions of vascular endothelial growth factor (VEGF) (30), a pro-angiogenic and permeability-enhancing cytokine that is overexpressed in both human (1) and experimental diabetic retinopathy (10). However, to date, studies in the kidney have focused mostly on the role of the profibrotic growth factor, transforming growth factor-β, and its effects on extracellular matrix expansion (19, 20), rather than on VEGF.

In addition to extracellular matrix expansion, the integrity of the glomerular capillary network is also recognized as a key determinant of renal pathology in both the experimental and human settings (23–25). The development of this network is critically dependent on VEGF, a growth factor that not only stimulates endothelial cell proliferation and permeability but may also have a role in the maintenance of endothelial cell integrity in the mature animal (6, 29). Accordingly, we sought to examine the effects of PKC-β inhibition on glomerular endothelial cell and VEGF expression, as well as the morbidity and mortality markers of glomerular injury of albuminuria and glomerulosclerosis. Moreover, since human subjects with diabetic nephropathy mostly receive treatment with agents that block the renin-angiotensin system (RAS), we also sought to examine the effects of PKC-β inhibition both with and without concomitant angiotensin-converting enzyme (ACE) inhibitor therapy.

METHODS

Animals. Eight-week-old female, homozygous (mRen-2)27 rats (St. Vincent’s Hospital Animal House, Melbourne, Australia) weighing 170 ± 20 g were assigned to receive either 55 mg/kg of streptozotocin (STZ; Sigma, St. Louis, MO) diluted in 0.1 M citrate buffer, pH 4.5, or citrate buffer alone (nondiabetic) by tail vein injection following an overnight fast. Diabetic rats were then randomized into four groups, receiving either treatment with the selective PKC-β inhibitor ruboxistaurin mesylate 10 mg·kg−1·day−1 (LY333531, Eli Lilly, Indianapolis, IN) in rat chow, the ACE inhibitor perindopril 0.2 mg·kg−1·day−1 in drinking water (Technologie Servier Laboratories, Paris, France), combination therapy (ruboxistaurin 10 mg·kg−1·day−1 and perindopril 0.2 mg·kg−1·day−1), or no treatment for 12 wk. Treatment commenced within 24 h of STZ or citrate buffer injection. Untreated rats received nondrug control chow (Certified Rodent Diet no. 5002, LabDiet) and untreated drinking water ad libitum. A group of 10 nontransgenic Sprague-Dawley (SD) rats served as nondiabetic, normotensive controls receiving nondrug control chow. All animals were housed in a stable environment maintained at 22 ± 1°C with a 12:12-h light-dark cycle commencing at 6 AM.

Each week, rats were weighed and their blood glucose levels were measured (Accu-check Advantage II Blood Glucose Monitor, Roche Diagnostics) and only STZ-treated animals with blood glucose >15 mmol/l were considered diabetic. Every 4 wk, systolic blood pressure (SBP) was determined in preheated conscious rats via tail-cuff ple-
Table 1. Animal characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body Weight, g</th>
<th>SBP, mmHg</th>
<th>Plasma Glucose, mmol/l</th>
<th>HbA1c, %</th>
<th>Plasma Creatinine, μmol/l</th>
<th>AER, mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic Ren-2 + vehicle</td>
<td>8</td>
<td>289±15</td>
<td>204±14</td>
<td>5.78±0.51</td>
<td>3.16±0.24</td>
<td>55±4</td>
<td>14×1±1.2</td>
</tr>
<tr>
<td>Diabetic Ren-2 + vehicle</td>
<td>8</td>
<td>271±5.0*</td>
<td>208±10</td>
<td>25.7±1.94†</td>
<td>10.7±0.35†</td>
<td>70±4*</td>
<td>81×1±1.3†</td>
</tr>
<tr>
<td>Diabetic Ren-2 + ruboxistaurin</td>
<td>8</td>
<td>258±4.0*</td>
<td>218±7</td>
<td>28.5±0.84†</td>
<td>11.2±0.34†</td>
<td>60±2*</td>
<td>37×1±1.2‡</td>
</tr>
<tr>
<td>Diabetic Ren-2 + perindopril</td>
<td>8</td>
<td>265±4.0*</td>
<td>147±8†</td>
<td>28.8±0.88†</td>
<td>10.5±0.44†</td>
<td>66±4*</td>
<td>7×1±1.4‡</td>
</tr>
<tr>
<td>Diabetic Ren-2 + perindopril + ruboxistaurin</td>
<td>10</td>
<td>255±4.0*</td>
<td>145±5†</td>
<td>27.6±1.15†</td>
<td>11.0±0.34†</td>
<td>65±2*</td>
<td>2×1±1.2§</td>
</tr>
<tr>
<td>Nondiabetic SD + vehicle</td>
<td>8</td>
<td>560±16†‡§</td>
<td>137±3†</td>
<td>5.50±0.43</td>
<td>3.62±0.21</td>
<td>49±1</td>
<td>3×1±1†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE except for albumin excretion rate (AER) which is expressed as geometric mean ×/± tolerance factor. *P < 0.05 and †P < 0.01 vs. nondiabetic Ren-2. ‡P < 0.05 vs. untreated diabetic Ren-2. §P < 0.05 vs. monotherapy-treated diabetic Ren-2. SBP, systolic blood pressure; HbA1c, hemoglobin A1c; SD, Sprague-Dawley rats.

Fig. 1. In situ hybridization autoradiographs for vascular endothelial growth factor (VEGF) mRNA in nondiabetic Ren-2 (A), diabetic Ren-2 (B), diabetic Ren-2 treated with ruboxistaurin (C), perindopril (D), their combination (E), and nondiabetic, nontransgenic rats (F). Autoradiographs are presented as pseudocolorized images (green, low expression; yellow, moderate expression; red, high expression). Magnification ×8.
thymsography (4) using a noninvasive blood pressure (NIBP) controller and Powerlab (AD Instruments, New South Wales, Australia). Hemoglobin A1c (HbA1c) was measured by HPLC at the end of the study. Diabetic rats received a daily injection of insulin (2–4 U ip; Humulin NPH, Eli Lilly) to reduce mortality and to promote weight gain. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Research Ethics Committee of St. Vincent’s Hospital.

**Tissue preparation.** Rats were anesthetized (60 mg/kg body wt ip Nembutal, Boehringer-Ingelheim) and the kidneys were excised, decapsulated, sliced transversely, and fixed in neutral buffered formalin and paraffin-embedded for subsequent light microscopic evaluation.

**In situ hybridization.** In situ hybridization was performed using a cDNA encoding VEGF-A, as previously reported (10). In situ hybridization was then performed on 4-μm paraffin sections using [33P]-labeled antisense riboprobe, as previously reported (10). In brief, tissue sections were dewaxed in histol, hydrated through graded ethanol, and immersed in distilled water. Sections were then washed in 0.1 M PBS, pH 7.4, and hybridized with [33P]-labeled antisense- and sense-specific probes (5 × 10^6 cpm/25 μl hybridization buffer) which were added to hybridization buffer (300 mM NaCl, 10 mM Tris·HCl, pH 7.5, 10 mM Na2HPO4, pH 6.8, 5.5 mM EDTA, pH 8.0, 1 × Denhardt’s solution, 0.8 mg yeast RNA/ml, 50% deionized formamide, and 10% dextran sulphate), heated to 85°C, and 25 μl were added to the sections. Coverslips were placed on the sections and the slides were incubated in a humidified chamber (50% formamide) at 60°C for 14 to 16 h. Slides were then washed in 2× SSC (0.3 M NaCl, 0.33 M Na2HPO4·2H2O) containing 50% formamide at 50°C to remove the coverslips. The slides were again washed with 2× SSC, 50% formamide for a further 1 h at 55°C. Sections were then rinsed three times in RNase buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.5 M NaCl) at 37°C and treated with 150 μg RNase A/ml in RNase buffer for a further 1 h at 37°C and then washed with 2× SSC at 55°C for 45 min. Finally, sections were dehydrated through graded ethanol, air dried, and exposed to Kodak Biomax MR Autoradiography film for 4 days at room temperature. Slides were coated with Ilford K5 emulsion (Ilford, 1:1 with distilled water), stored with desiccant at room temperature for 21 days, developed in Ilford phenisol, fixed in Ilford Hypad, and stained with hematoxylin and eosin (H & E).

**Quantitative autoradiography.** Quantitative in situ hybridization by autoradiographic film densitometry which permits the assessment of gene expression equivalent to Northern blot analysis was used to determine the magnitude of gene expression, as previously reported by our group (11). In brief, film densitometry of autoradiographic images was performed by computer-assisted image analysis as previously described (22) using a micro computer imaging device (MCID; Imaging Research, St. Catherine’s, Ontario, Canada). With this method quantification of transcript is based on the changes in X-ray film density that follows exposure to the radioactive emissions of radiolabeled VEGF mRNA. In situ autoradiographic images were placed on a uniformly illuminating fluorescent light box (Northern Light Precision Luminator model C60, Image Research, Ontario, Canada) and captured using a video camera (Sony Video Camera Module CCD) connected to an IBM AT computer with a 512 × 512 pixel array imaging board with 256 gray levels. In view of the focal nature of rat glomerular VEGF mRNA, the outline of 20 glomeruli/section was defined by interactive tracing for each kidney section, as previously described (5). Following appropriate calibration by constructing a curve of optical density vs. radioactivity, quantification of digitalized autoradiographic images was performed using MCID software. Data were expressed as optical density per centimeter squared relative to control kidneys [relative optical density (ROD)].

All sections were cut in a uniform manner in the midsagittal plane, hybridized 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.** Glomerular capillary endothelial cell density was evaluated by immunostaining with JG-12 (Bender Med Systems, Burlingame, CA), a rat endothelial cell-specific monoclonal antibody (14), monoclonal anti-rat endothelial cell antigen-1 (RECA-1) antibody (Serotec, Oxford, UK), polyclonal anti-TGF-β (R&D Systems, Minneapolis, MN), and anti-rat VEGF antibodies (R&D Systems). Three-micrometer sections were placed into histosol to remove the paraffin wax, hydrated in graded ethanol, and immersed in tap water before being incubated for 20 min with normal goat serum (NGS) diluted 1:10 with PBS at pH 7.4. Sections were then incubated for 18 h at 4°C with JG-12 or RECA-1. Sections incubated with 1:10 NGS instead of the primary antiserum served as the negative control. After thorough washing with PBS (3 × 5-min changes), the sections were flooded with a solution of 5% hydrogen peroxide (freshly prepared with PBS, 2 × 5 min) and incubated with biotinylated goat anti-mouse IgG (Dakopatts, Glostrup, Denmark) diluted 1:200 with PBS. Sections were rinsed with PBS (2 × 5 min) and incubated with an avidin-biotin peroxidase complex (Vector, Burlingame, CA) diluted 1:200 with PBS. Following rinsing with PBS (2 × 5 min), sections were incubated with 0.05% dianisobenzene and 0.05% hydrogen peroxide (Pierce, Rockford, IL) in PBS at pH 7.6 for 1 to 3 min, rinsed in tap water for 5 min, counterstained in Mayer’s hematoxylin, differentiated in Scott’s tap water, dehydrated, cleared, and mounted in Depex.

The magnitude of glomerular JG-12, RECA-1, and TGF-β immunostaining was quantified using image analysis as previously described (22). Briefly, the glomerulus, considered as the area internal to and including Bowman’s capsule, was outlined by interactive tracing, as previously described (16). Images were then captured using a BX50 Olympus microscope attached to a Fujix HC-2000 digital camera and a Pentium III IBM computer. The color range for immunolabeled cells (brown on immunoperoxidase-labeled sections) was selected and image analysis was performed using a chromogen-separating technique (22). The proportional glomerular area showing positive immunostaining was measured from three sections per rat (n = 6/group), providing in excess of 50 glomeruli/treatment group.

To quantify JG-12 immunolabeling in the tubulointerstitium, five random nonoverlapping fields of renal cortex without glomeruli from six rats per group were captured, digitized, and quantified as described above. All tissues were processed in an identical manner with the observer masked to study group identity.

**Histopathology.** Changes in glomerular structure were also assessed in a masked protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with either H & E and periodic acid Schiff’s stain (PAS). In 3-μm kidney

Table 2. Kidney gene expression of VEGF as assessed by densitometric quantification of in situ hybridization autoradiographic films

<table>
<thead>
<tr>
<th></th>
<th>VEGF mRNA, density/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic Ren-2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Diabetic Ren-2</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>Diabetic Ren-2 + perindopril</td>
<td>0.8 ± 0.2†</td>
</tr>
<tr>
<td>Diabetic Ren-2 + ruboxistaurin</td>
<td>0.8 ± 0.1†</td>
</tr>
<tr>
<td>Diabetic Ren-2 + perindopril + ruboxistaurin</td>
<td>0.6 ± 0.11†‡</td>
</tr>
<tr>
<td>Nondiabetic SD</td>
<td>0.6 ± 0.11†‡</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE for density/cm² relative to nondiabetic Ren-2 kidneys, arbitrarily assigned a value of 1. *P < 0.05 vs. diabetic Ren-2. †P < 0.05 vs. diabetic Ren-2 + perindopril or ruboxistaurin. §P < 0.05 vs. nondiabetic Ren-2.
sections stained with PAS, 150 to 200 glomeruli from rats were examined in a masked protocol. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4, as previously described (17), with grade 0, normal; grade 1, sclerotic area up to 25% (minimal); grade 2, sclerotic area 25–50% (moderate); grade 3, sclerotic area 50–75% (moderate to severe); and grade 4, sclerotic area 75–100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula

\[
\text{GSI} = \sum_{i=0}^{4} F_i (i)
\]

where \(F_i\) is the percent of glomeruli in the rat with a given score \(i\).

**Albuminuria.** Rats were individually housed in metabolic cages at 12 wk, habituated for 2 to 3 h, and urine was collected over 24 h. Animals continued to have free access to tap water and standard laboratory chow during this period. After 24 h in metabolic cages, an aliquot of urine (5 ml) was collected from the 24-h urine sample and stored at \(-70^\circ\text{C}\) for subsequent analysis of albumin by radioimmunoassay, as previously performed (17).

**Statistics.** Data are expressed as means \pm SE unless otherwise stated. Statistical significance was determined by a two-way ANOVA with a Fisher’s post hoc comparison. Albuminuria was skew distributed and was analyzed following log transformation and presented as geometric means \(\pm\) tolerance factors. Analyses...
PKC-β INHIBITION IN THE DIABETIC NEPHROPATHY

### Graph M

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td><img src="image" alt="Non-diabetic" /></td>
</tr>
<tr>
<td>Diabetic</td>
<td><img src="image" alt="Diabetic" /></td>
</tr>
<tr>
<td>Diabetic + ruboxistaurin</td>
<td><img src="image" alt="Diabetic + ruboxistaurin" /></td>
</tr>
<tr>
<td>Diabetic + perindopril</td>
<td><img src="image" alt="Diabetic + perindopril" /></td>
</tr>
<tr>
<td>Diabetic + perindopril + ruboxistaurin</td>
<td><img src="image" alt="Diabetic + perindopril + ruboxistaurin" /></td>
</tr>
<tr>
<td>Non-diabetic Sprague-Dawley</td>
<td><img src="image" alt="Non-diabetic Sprague-Dawley" /></td>
</tr>
</tbody>
</table>

### Graph N

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Ren-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td><img src="image" alt="Non-diabetic" /></td>
</tr>
<tr>
<td>Diabetic</td>
<td><img src="image" alt="Diabetic" /></td>
</tr>
<tr>
<td>Diabetic + ruboxistaurin</td>
<td><img src="image" alt="Diabetic + ruboxistaurin" /></td>
</tr>
<tr>
<td>Diabetic + perindopril</td>
<td><img src="image" alt="Diabetic + perindopril" /></td>
</tr>
<tr>
<td>Diabetic + perindopril + ruboxistaurin</td>
<td><img src="image" alt="Diabetic + perindopril + ruboxistaurin" /></td>
</tr>
<tr>
<td>Non-diabetic Sprague-Dawley</td>
<td><img src="image" alt="Non-diabetic Sprague-Dawley" /></td>
</tr>
</tbody>
</table>

* * *
were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA) on an Apple Macintosh G4 computer (Apple Computer, Cupertino, CA). A P value < 0.05 was regarded as statistically significant.

RESULTS

Animal characteristics. In comparison with Ren-2 control animals, diabetic rats had reduced body weight that was unaffected by any of the assigned treatments (P < 0.01). Untreated diabetic and nondiabetic rats were hypertensive with elevated SBP. Perindopril treatment reduced SBP while ruboxistaurin treatment did not affect SBP when administered either as single agent treatment or in combination with perindopril (Table 1). Plasma glucose and HbA1c were elevated to a similar extent in all diabetic rat groups, irrespective of assigned treatment (Table 1). SD control rats were normotensive and normoglycemic (Table 1).

VEGF expression. In situ hybridization autoradiography revealed punctate cortical expression of VEGF mRNA consis-

![Fig. 4. Representative photomicrographs of cortical tubulointerstitium labeled with JG-12 showing endothelial cell immunostaining from nondiabetic Ren-2 (A), diabetic Ren-2 (B), diabetic Ren-2 treated with perindopril (C), ruboxistaurin (D), their combination (E), and nondiabetic, nontransgenic Sprague-Dawley (SD) rats (F). Original magnification ×350. G: quantitation of tubulointerstitial endothelial cell density as the proportional area immunostained with JG-12 antibody. Data are shown as means ± SE. *P < 0.05 vs. nondiabetic Ren-2 rats. †P < 0.05 vs. untreated diabetic Ren-2 rats.](http://ajprenal.physiology.org/)

Downloaded from http://ajprenal.physiology.org/ by 10.220.33.1 on October 21, 2017
Densitometric analysis of autoradiographic images confirmed a significant increase in VEGF expression in nondiabetic Ren-2 rats, compared with control SD animals (Table 2). Diabetes was associated with a further incremental increase in VEGF, which was attenuated by both perindopril and ruboxistaurin and to a greater extent with their combination (Fig. 1, Table 2). Immunohistochemistry showed VEGF protein localized to podocytes within the glomerulus (Fig. 2).
Microvascular changes. Intense localization of JG-12 to the endothelial cells of glomerular capillary loops was noted in all groups. When compared with control SD rats, JG-12 immunolabeling was reduced in the glomeruli of nondiabetic Ren-2 rats with further reduction evident in diabetic Ren-2 animals (Fig. 3). This decrease was attenuated by both perindopril and ruboxistaurin to levels found in nondiabetic Ren-2 rats. Furthermore, the combination of perindopril with ruboxistaurin resulted in a further incremental increase in glomerular JG-12 labeling, beyond that of single agent treatment, reaching levels similar to those of SD control rats (Fig. 3). Glomerular RECA-1 labeling was similar to that of JG-12.

In addition to the changes within the glomerulus, diabetes was also associated with a reduction in JG-12 immunolabeling within the tubulointerstitium that was incrementally improved with perindopril, ruboxistaurin, and their combination (Fig. 4).

Fig. 6. Representative periodic acid Schiff’s-stained sections from control and diabetic Ren-2 rats treated with ruboxistaurin, perindopril, or their combination. In control (A) rats only minimal glomerular thickening was noted, while diabetes (B) was associated with a dramatic glomerulosclerosis. Treatment of diabetic rats with ruboxistaurin (C) or perindopril (D) was associated with a reduction in the glomerulosclerosis. Glomeruli from combination therapy-treated rats showed only minimal sclerosis (E), similar to nondiabetic SD animals (F). Original magnification ×350. G: glomerulosclerotic index (GSI) in SD and Ren-2 rats treated with ruboxistaurin, perindopril, or their combination. Data are shown as means ± SE. *P < 0.05 vs. nondiabetic Ren-2 rats. †P < 0.05 vs. untreated diabetic Ren-2 rats. ‡P < 0.05 vs. ruboxistaurin-treated and perindopril monotherapy-treated diabetic Ren-2 rats. §P < 0.05 vs. ruboxistaurin + perindopril-treated Ren-2 diabetic rats.
TGF-β. Glomeruli from untreated diabetic rats displayed prominent immunolabeling for TGF-β when compared with nondiabetic rats, which was substantially diminished by treatment with perindopril, ruboxistaurin, and their combination (Fig. 5).

Glomerulosclerosis. Glomerular injury was a prominent feature of diabetic rats, with evidence of both diffuse and nodular glomerulosclerosis (Fig. 6A). These changes were attenuated by treatment with perindopril and ruboxistaurin (Fig. 6, A and B). When compared with single agent treatment, combination therapy with perindopril and ruboxistaurin resulted in a further reduction in the extent of glomerulosclerosis (Fig. 6, A and B). However, despite combination treatment, the glomerular structure was not normalized and the extent of sclerosis remained greater than in SD control rats.

Albuminuria. Diabetes was associated with an increase in urinary albumin excretion. Treatment with perindopril, and ruboxistaurin to a lesser extent, both reduced albuminuria in diabetic Ren-2 rats (Table 1). Combination therapy with both perindopril and ruboxistaurin resulted in a further incremental reduction in albuminuria, beyond that of single agent treatment, reaching levels similar to those of SD control rats (Table 1).

DISCUSSION

With blockade of the RAS an established therapy for diabetic nephropathy, new therapies need to show incremental efficacy. In the present report, the addition of the PKC-β inhibitor ruboxistaurin to background ACE inhibition had additive effects in attenuating glomerular injury. These effects included incremental improvements not only in traditional measurements of glomerular structure and function such as glomerulosclerosis and albuminuria but also on glomerular capillary endothelial cells, recently recognized as a key determinant in recovery from injury (24, 25).

Consistent with findings in human diabetic nephropathy (23), we also noted a reduction in glomerular endothelial cells in the advanced disease that is characteristic of the diabetic Ren-2 rat (18). Paradoxically, this endothelial cell loss occurred in the setting of increased glomerular VEGF, a growth factor named for its potent induction of endothelial cell proliferation (7). However, while traditionally viewed as proliferative, recent studies showed that these actions are context dependent. In particular, Ferrari and colleagues (8) reported that in the setting of high levels of TGF-β, VEGF acts together with TGF-β to induce endothelial cell apoptosis. Consistent with that report, the present study found that VEGF and TGF-β were both elevated in the glomeruli of diabetic rats in the setting of endothelial cell loss. Moreover, the aforementioned interaction between VEGF and TGF-β may also contribute to the beneficial effects of VEGF blockade (9, 27) as well as TGF-β inhibition (31) in experimental diabetic nephropathy.

Angiotensin II (15) and PKC (21) are potent inducers of VEGF expression in cultured podocytes. Consistent with these in vitro findings, we noted that intervention with either a PKC-β or ACE inhibitor in the in vivo context attenuated the diabetes-associated increase in VEGF mRNA, restoring levels to those of nondiabetic Ren-2 rats. Moreover, the combination of perindopril and ruboxistaurin led to a further reduction in VEGF down to levels found in control SD rats. Similar to the findings in untreated diabetic animals, the relationship between VEGF expression and endothelial cell number in the intervention groups of this study may at first glance also seem contradictory. That is, when compared with untreated animals, those receiving drug therapy had lower levels of glomerular VEGF but a greater number of endothelial cells. We speculate that a number of mechanisms may account for this paradox. First, in addition to inducing VEGF expression in podocytes, angiotensin II and PKC also cause endothelial cell injury. Thus reducing the levels of these injurious agents with perindopril and ruboxistaurin would likely lead to endothelial cell preservation, independent of VEGF. Furthermore, ACE and PKC inhibition both led to reduced TGF-β expression thereby attenuating the proapoptotic effect of this growth factor combination, as discussed above.

As in previous studies (13, 17, 19), the present report also shows that ruboxistaurin, when used as single agent therapy, reduces albuminuria, albeit to a lesser extent than perindopril monotherapy. However, the effects of ACE inhibition reflect contributions from both blood pressure lowering as well as diminution in angiotensin II production. In contrast, ruboxistaurin’s effects were independent of any change in blood pressure. Furthermore, when used in combination with the ACE inhibitor perindopril, ruboxistaurin provided a further incremental reduction in albuminuria, a key marker and pathogenetic factor in renal disease progression (26). Indeed, animals receiving combination therapy had urinary albumin excretion levels that were similar to those of nondiabetic, normotensive, nontransgenic animals.

Histopathologically, diabetic nephropathy is characterized by glomerulosclerosis, an important predictor of declining renal function (23). In the present study, when compared with SD controls, mild glomerulosclerosis was noted in nondiabetic Ren-2 rats, consistent with the effects of hypertension and an active tissue-based RAS on glomerular structure (3). However, as previously reported, diabetes led to a further substantial increase in the extent of glomerulosclerosis that was attenuated by both single agent treatment with either perindopril or ruboxistaurin (17, 18), and as shown in the present study, a further, albeit small reduction with combination therapy was also noted. However, even this combination of agents failed to normalize the extent of glomerulosclerosis to levels seen in nondiabetic, normotensive, nontransgenic SD rats.

In summary, combination therapy with inhibitors of PKC-β and ACE has additive effects in attenuating endothelial cell loss, reducing glomerular VEGF expression, lowering albuminuria, and lessening glomerulosclerosis.

ACKNOWLEDGMENTS

The authors thank M. Pacheco, J. Court, S. Glowacka, and L. Di Rago for expert technical assistance. We also thank Sirirat Reungjui and Richard J. Johnson for assistance with immunohistochemical techniques.

GRANTS

This project was supported by a team grant from the Canadian Institutes of Health Research, Juvenile Diabetes Research Foundation, and the National Health and Medical Research Council of Australia. D. Kelly is a recipient of a Career Development Award from the Juvenile Diabetes Research Foundation.
REFERENCES


