Protein Kinase Cβ Inhibition Attenuates Osteopontin Expression, Macrophage Recruitment, and Tubulointerstitial Injury in Advanced Experimental Diabetic Nephropathy

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Tubulointerstitial macrophage accumulation is an important marker of prognosis that correlates closely with declining renal function in a range of human and experimental diseases, including diabetic nephropathy. These inflammatory cells are rich in the profibrotic growth factor TGF-β such that their presence in areas of injury is frequently associated with tissue fibrosis. The migration of macrophages occurs in response to the site-specific production of chemokines, with osteopontin closely associated with their trafficking into the tubulointerstitium of the kidney. Although cell culture studies indicate that protein kinase C (PKC) mediates the expression of osteopontin, its role in the *in vivo* setting is unknown. Accordingly, Ren-2 control and diabetic rats that were treated with or without the specific PKC-β isoform inhibitor ruboxistaurin (10 mg/kg per d) were examined. After 12 wk, diabetic rats showed increases in osteopontin expression in tubular epithelial cells of the cortex in association with macrophage infiltration, interstitial fibrosis, and activity of TGF-β as indicated by the expression of its receptor activated protein phospho-Smad2 ($P < 0.05$ for all parameters). Ruboxistaurin treatment significantly attenuated these parameters ($P < 0.05$) in diabetic rats without affecting either BP or glycemic control. These findings suggest that osteopontin and macrophage accumulation may play a role in the tubulointerstitial injury in diabetic nephropathy and that inhibition of osteopontin expression may be one of the mechanisms by which inhibition of the β-isoform of PKC confers a renoprotective effect.

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Macrophage infiltration is a well-recognized feature of glomerulonephritis. More recently, these cells have also been implicated in tubulointerstitial injury and in renal diseases that have been traditionally regarded as nonimmune (1). Indeed, tubulointerstitial macrophage accumulation is an important marker of prognosis that correlates closely with declining renal function in a range of human and experimental diseases, including diabetic nephropathy (2,3).

Macrophages secrete a range of potentially injurious products that include the profibrotic growth factor TGF-β (4) such that a reduction in their numbers is associated with attenuation in renal injury (1). Blockade of factors that induce their recruitment therefore may provide a useful target in the treatment of progressive disease. The infiltration of mononuclear cells into the kidney is largely dependent on the local tissue concentration of specific chemokines that stimulate their recruitment and activation. These small glycoproteins, elaborated in response to injury, have a site-specific pattern of expression. For instance, the C-C chemokine monocyte chemoattractant protein-1 plays a major role in glomerular disease, whereas the phosphoprotein osteopontin is more closely related to tubulointerstitial pathology (5).

In vitro studies have shown that activation of protein kinase C (PKC) is a key mediator of osteopontin expression in a range of cell types and in response to a number of stressors that include high glucose (6–8). We therefore sought to determine the effects of PKC inhibition, *in vivo*, using the specific PKC-β inhibitor ruboxistaurin in a rodent model of diabetic nephropathy in which tubulointerstitial injury, macrophage infiltration, osteopontin expression, and TGF-β1 activation all are well-documented features (9,10).

Materials and Methods

Animals

Forty 6-wk-old female heterozygous Ren-2 rats that weighed 125 ± 5 g were randomized to receive either 55 mg/kg streptozotocin (Sigma, St. Louis, MO) diluted in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone (nondiabetic) by tail-vein injection after an overnight fast. Rats were randomized further to receive treatment with the PKC-β inhibitor ruboxistaurin (LY333531; Eli Lilly and Co., Indianapolis, IN) in rat food at a dose of 10 mg/kg per day or no treatment for 6 mo. Untreated rats received nondrug control food *ad libitum*, and all animals had free access to tap water throughout the experiment. All rats were housed in a stable environment (maintained at 22 ± 1°C with a 12-h light/dark cycle). Each week, rats were weighed and blood glucose was determined (AMES glucometer; Bayer Diagnostics, Melbourne, Australia), and rat hemoglobin A_{1c} was assessed by HPLC at the end of the study. Diabetic rats received a daily injection of insulin (2 to 4 units intraperitoneally; Humulin NPH Isophane; Eli Lilly and Co.) to promote weight

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Table 1. Animal characteristics in control and diabetic rats that were treated with ruboxistaurin[a]

	Control	Control + Ruboxistaurin	Diabetic	Diabetic + Ruboxistaurin
Body wt (g)	555 ± 21	521 ± 16	486 ± 12[b]	481 ± 12[b]
Kidney wt (g)	2.31 ± 0.12	2.29 ± 0.15	2.92 ± 0.09[b]	2.73 ± 0.13[b]
Plasma glucose (mM)	5.7 ± 0.5	5.2 ± 0.2	24.7 ± 2.4[c]	26.9 ± 1.1[c]
Hemoglobin A_{1c} (%)	3.4 ± 0.2	3.4 ± 0.2	10.5 ± 0.6[b]	10.4 ± 0.5[b]
Plasma creatinine (mM)	0.054 ± 0.005	0.051 ± 0.002	0.070 ± 0.004[b]	0.060 ± 0.003
Albuminuria (mg/d)	0.15 x/÷ 0.10	0.11 x/÷ 0.10	4.32 x/÷ 0.27[b]	1.10 x/÷ 0.11[b,d]
Systolic BP (mmHg)	204 ± 18	202 ± 12	221 ± 21	217 ± 16

[a]Data are expressed as mean ± SEM. Albuminuria is expressed as geometric means ÷ tolerance factors.
[b]$P < 0.05$ *versus* control. [c]$P < 0.01$ *versus* control. [d]$P < 0.05$ *versus* untreated diabetic rats.

gain and reduce mortality. Before rats were killed, they were housed in individual metabolic cages to collect a 24-h urine specimen. A 5-ml aliquot of collected urine was stored at −70°C for subsequent analysis of albumin by RIA. All experimental procedures were performed in accordance with 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 Bioethics Committee of the University of Melbourne.

Tissue Preparation

Rats were anesthetized (Nembutal 60 mg/kg body wt intraperitoneally; Boehringer-Ingelheim, North Ryde, New South Wales, Australia), the left kidney was clamped, ligated, removed, and stored at −80°C for subsequent molecular biologic analyses. The abdominal aorta then was cannulated with an 18-G needle. Perfusion-exsanguination commenced at 180 to 220 mmHg via the abdominal aorta with 0.1 M PBS (pH 7.4; 20 to 50 ml) to remove circulating blood, and the inferior vena cava adjacent to the renal vein was severed simultaneously, allowing free flow of the perfusate. After clearance of circulating blood, buffered formalin was perfused for an additional 5 min (100 to 200 ml of fixative) to fix the tissues. Kidneys were removed from the animal, decapsulated, and sliced transversely. Kidneys were postfixed in the same fixative for 2 h, routinely processed, embedded in paraffin, and sectioned.

Histochemistry and Immunohistochemistry

A picrosirius red stain was performed on kidney sections to identify matrix deposition within the interstitium as described previously (11). In brief, 5-μm sections were deparaffinized, rehydrated, and then stained with 0.1% Sirius red (Polysciences, Inc., Warrington, PA) in saturated picric acid (picrosirius red) for 1 h, differentiated in 0.01% HCl for 30 s, and rapidly dehydrated and mounted in Depex (BDH Chemicals, Kilsyth, Victoria, Australia).

Osteopontin expression was localized using a specific polyclonal antibody (10). Macrophages were identified by the monoclonal rat macrophage marker (ED-1; Serotec, Raleigh, NC) (4). For TGF-β, we examined expression with a polyclonal antibody (R & D Systems, Minneapolis, MN) and assessed its activity by quantifying TGF-β's downstream signaling, as evidenced by the abundance of nuclear phosphorylated Smad2 (Cell Signaling Technology, Boston, MA) (12), in comparison with total Smad2 (Cell Signaling Technology). For immunohistochemistry, 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 diluted 1:10 with 0.1 M PBS at pH 7.4. Sections then were incubated for 18 h at 4°C with specific primary polyclonal antisera. Sections that were incubated with 1:10 normal goat serum 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, rinsed with PBS (2 × 5 min), and incubated with biotinylated goat anti-rabbit IgG (Dakopatts, Glostrup, Denmark) diluted 1:200 or goat anti-mouse IgG diluted 1:200 (Dakopatts) 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. After rinsing with PBS (2 × 5 min), sections were incubated with 0.05% diaminobenzidine 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. All studies were performed with the observer masked to the study group to which the animal had been assigned.

Quantification of Histologic Parameters

The accumulation of matrix and the extent of histochemical staining and immunostaining was quantified using computer-assisted image analysis, as previously reported (13,14). Briefly, five random nonoverlapping fields from six rats per group were captured and digitized using a BX50 microscope attached to a Fujix HC5000 digital camera. Digital images then were loaded onto a Pentium III IBM computer. An area of red on a picrosirius-stained section (for matrix) or brown on immunostained sections (osteopontin, TGF-β, phospho-Smad2, and total Smad2) was selected for its color ranges. For matrix, osteopontin, TGF-β, and total Smad2, the proportional areas stained red or brown, respectively, then were determined using image analysis (AIS; Analytical Imaging Station Version 6.0, St. Catharines, ON, Canada). For phospho-Smad2, the number of positive nuclei (brown) was also quantified using image analysis software and expressed per mm^2. Macrophage number was assessed by counting the number of ED-1–labeled cells in six sections per animal from each group ($n = 6$ per group), as described previously (10).

Quantitative Real-Time Reverse Transcription–PCR

RNA extraction and cDNA synthesis were performed as described previously (15). Rat osteopontin gene expression was measured and quantified using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence-specific primers were designed to span exon–exon boundaries using the Primer Express software v1.5 (Applied Biosystems). Primers were obtained from Sigma-Aldrich, and fluorescence probes were obtained from Applied Biosystems with forward primer TGGTTTGCCTTTGCCTGTTC, reverse primer TCTCCTCTGAGCTGCCAAACTC, and probe CCTTGCCTCCTGTCTCCCGGTGA. Real-time quantitative reverse

transcription–PCR was performed using the Taqman system, as described previously (16), using a commercial, predeveloped (18S) kit (Applied Biosystems) to control for differences in loading. Experiments were performed in duplicate for each sample, and no template controls were added to ensure that amplification was not due to contamination of other components within the PCR mixture. Data analysis was performed using Applied Biosystems Comparative C_T method.

Statistical Analyses

Data are expressed as means ± SEM unless otherwise stated. Statistical significance was determined by a two-way ANOVA with a Fisher *post hoc* comparison. Analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA) on an Apple Macintosh G4 computer (Apple Computer, Inc., Cupertino, CA). $P < 0.05$ was regarded as statistically significant.

Results

Animal Characteristics

Rats that had received streptozotocin all were diabetic with plasma glucose and hemoglobin A_{1c} elevated to a similar extent in treated and untreated diabetic rat groups (Table 1). All rats had elevated BP that was not affected by treatment with ruboxistaurin. In comparison with control animals, diabetic rats had reduced body weight and increased kidney weight that was unaffected by treatment with ruboxistaurin. Plasma creatinine was increased in untreated diabetic rats but not in rats that had received ruboxistaurin. When compared with control animals, albuminuria was elevated in diabetic rats but was significantly attenuated in ruboxistaurin-treated rats.

Osteopontin and Macrophages

Tissue expression of osteopontin mRNA increased approximately 2.2-fold in untreated diabetic rats and was reduced to control levels by treatment with ruboxistaurin (Figure 1). Immunohistochemistry showed increased immunodetectable osteopontin with intense labeling in cortical tubules of diabetic rats that was substantially attenuated in diabetic animals that had received ruboxistaurin (Figures 1 and 2). Whereas only occasional macrophages were identified in control rats, diabetic rats showed an abundance of macrophages in the tubulointerstitium of diabetic rats with a significant reduction in diabetic rats that had received ruboxistaurin (Figure 3).

Tubulointerstitial Matrix, TGF-β, and Smad2

Examination of picrosirius red–stained sections revealed increased collagenous matrix deposition diabetic Ren-2 rats when compared with control rats ($P < 0.01$; Figure 4). Ruboxistaurin treatment reduced interstitial collagen deposition to levels that approached those of control rats (Figure 4). Minimal nuclear phospho-Smad2 was detected in control tissues. In contrast, kidneys from diabetic Ren-2 rats showed abundant nuclear immunolabeling that was substantially reduced by ruboxistaurin treatment (Figure 5). Similarly, TGF-β immunostaining was increased in the tubules of diabetic rats and significantly attenuated by ruboxistaurin treatment (Figure 6). In contrast, total Smad2 was detected in the cytosol of renal epithelial cells and was unchanged by either diabetes or ruboxistaurin treatment (data not shown).

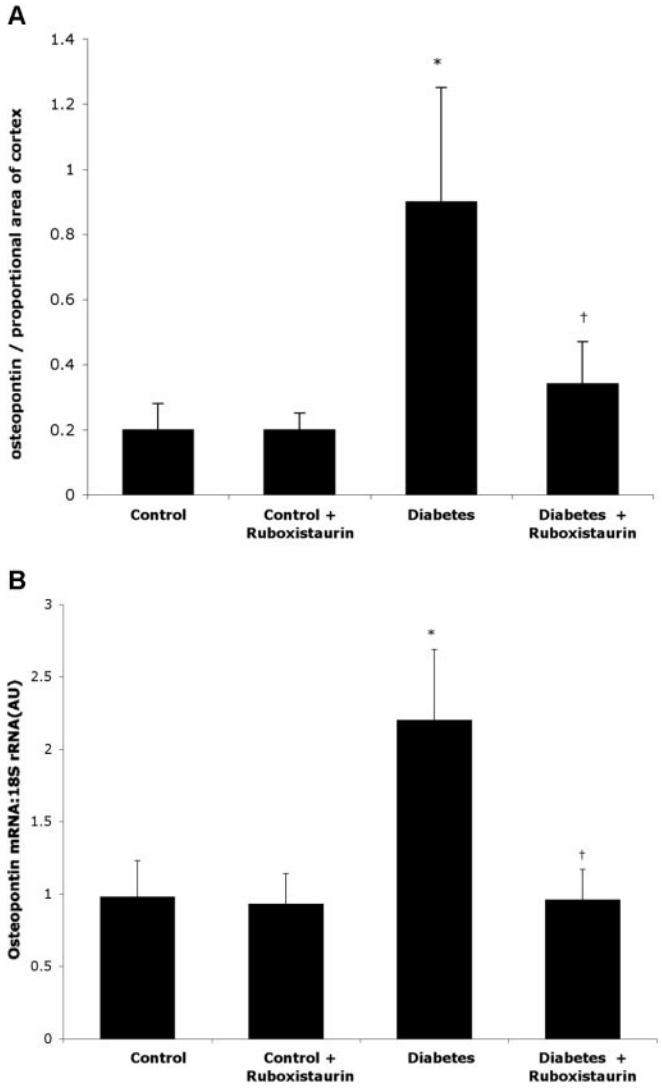

Figure 1. Quantification of protein (A) and osteopontin gene expression (B) in control and diabetic rats that were treated with and without ruboxistaurin. Data are expressed as the ratio of osteopontin mRNA:18S rRNA in arbitrary units (AU), relative to controls assigned a value of 1. Data are expressed as mean ± SEM. $^*P < 0.001$ *versus* controls; $^\dagger P < 0.05$ *versus* untreated diabetic rats.

Discussion

PKC is a ubiquitously expressed large family of serine-threonine kinases that transduce a wide range of cell-signaling processes by substrate-specific phosphorylation (17,18). Of the 12 identified PKC isoenzymes, a preferential increase in the β isoform has been described in experimental diabetes (19,20). Traditional approaches to PKC inhibition have been marred by toxicity and lack of specificity. However, more recently, a range of less toxic and more specific approaches to PKC inhibition (21), including specific β-isoform inhibitors such as ruboxistaurin (LY 333531), that have been used in preclinical and clinical studies of diabetic retinopathy (22), neuropathy (23), and vascular dysfunction (24) have been developed. In diabetic nephropathy, ruboxistaurin is currently undergoing clinical eval-

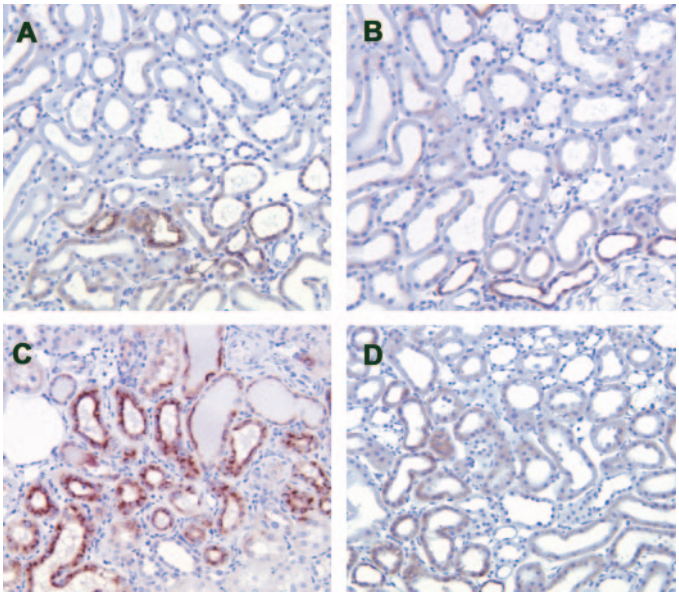

Figure 2. Osteopontin immunohistochemistry in the kidney of transgenic Ren-2 rats. Representative photomicrographs of osteopontin. Occasional immunolabeling of tubular epithelial cells is present in nondiabetic rats that were treated with either control diet (A) or ruboxistaurin (B). In contrast, widespread and intense expression of osteopontin that was attenuated by ruboxistaurin treatment (D) was noted in the tubules of diabetic rats (C). Magnification, ×340.

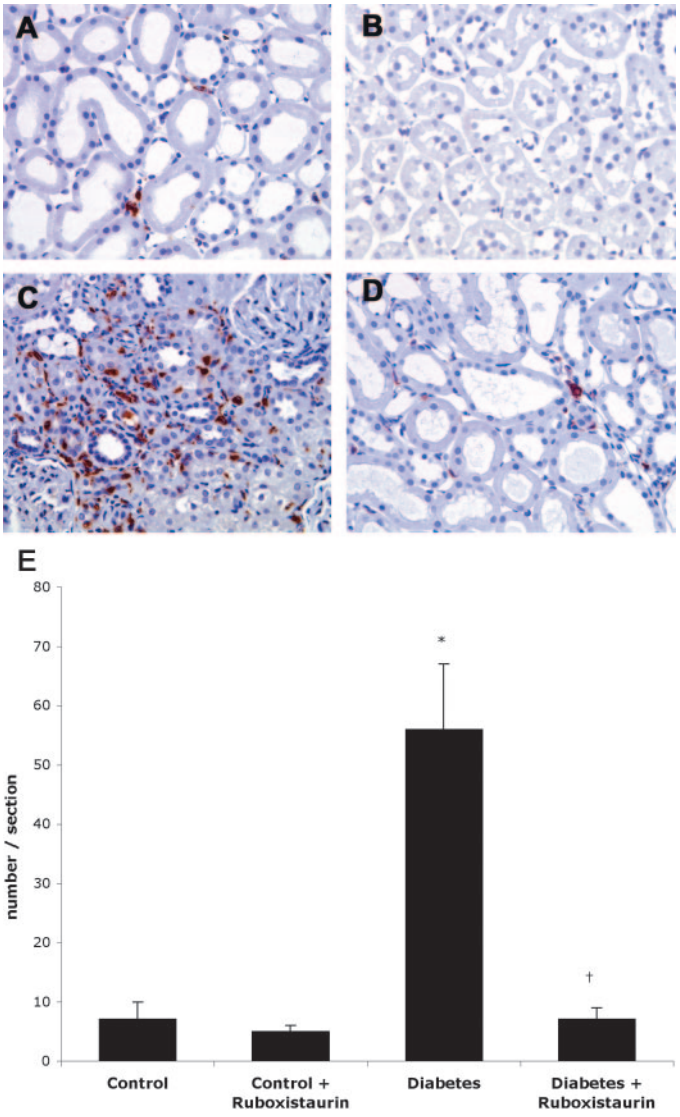

Figure 3. Macrophage infiltration in the kidney of transgenic Ren-2 rats. Representative photomicrographs (A through D) of ED-1–labeled macrophages within the tubulointerstitium of nondiabetic rats that were treated with either control diet (A) or ruboxistaurin (B). In contrast, numerous macrophages were present in the interstitium of untreated diabetic rats (C) but not in diabetic rats that received ruboxistaurin (D). Quantitative analysis of macrophage number (E), expressed as the number of ED-1–labeled cells per section. Data are expressed as mean ± SEM. $^{*}P < 0.001$ *versus* controls; $^{\dagger}P < 0.01$ *versus* untreated diabetic rats. Magnification, ×560.

uation (25) on the background of several preclinical studies showing attenuation of glomerular pathology in diabetes (17,26). However, the tubulointerstitium comprises approximately 90% of kidney volume and also undergoes major pathologic changes in diabetes (27). Indeed, changes in the tubulointerstitium develop commensurate with glomerular pathology and, like them, also correlate closely with declining renal function (27). Such changes include interstitial fibrosis, macrophage infiltration, and tubular atrophy, all of which were present in the diabetic Ren-2 rat and shown in this study to be ameliorated by PKC-β inhibition with ruboxistaurin.

In the past two decades, numerous studies have established a pivotal role for macrophages in the progression of renal injury, using a range of techniques that include transgenic animals, irradiation, antimacrophage sera, and cytokine blockade (3). Although well documented in glomerular disease, the relationship between tubulointerstitial macrophage number and progressive renal dysfunction seems even more striking (28–30). These inflammatory cells may mediate tubulointerstitial injury by a number of mechanisms that include the production of reactive oxygen species and the secretion of proteolytic enzymes, vasoactive hormones, and growth factors. Of the last, macrophages have been identified as a major source of the profibrotic growth factor TGF-β1 (4), a key factor in the pathogenesis of diabetic nephropathy and other forms of progressive renal disease (31). However, whereas macrophage accumulation is a consistent feature of diabetic kidney disease in humans (32), most studies in rodent models of experimental diabetic nephropathy have been confined to their examination in the glomerulus (33). In contrast, the diabetic Ren-2 rat, as demonstrated in this study, develops substantial tubulointerstitial macrophage infiltration akin to humans.

A number of chemokines have been shown to stimulate macrophage recruitment in the injured kidney with a site-specific pattern of expression. For instance, whereas monocyte chemoattractant protein-1 has a prominent role in mononuclear cell trafficking to glomeruli, osteopontin predominates in me-

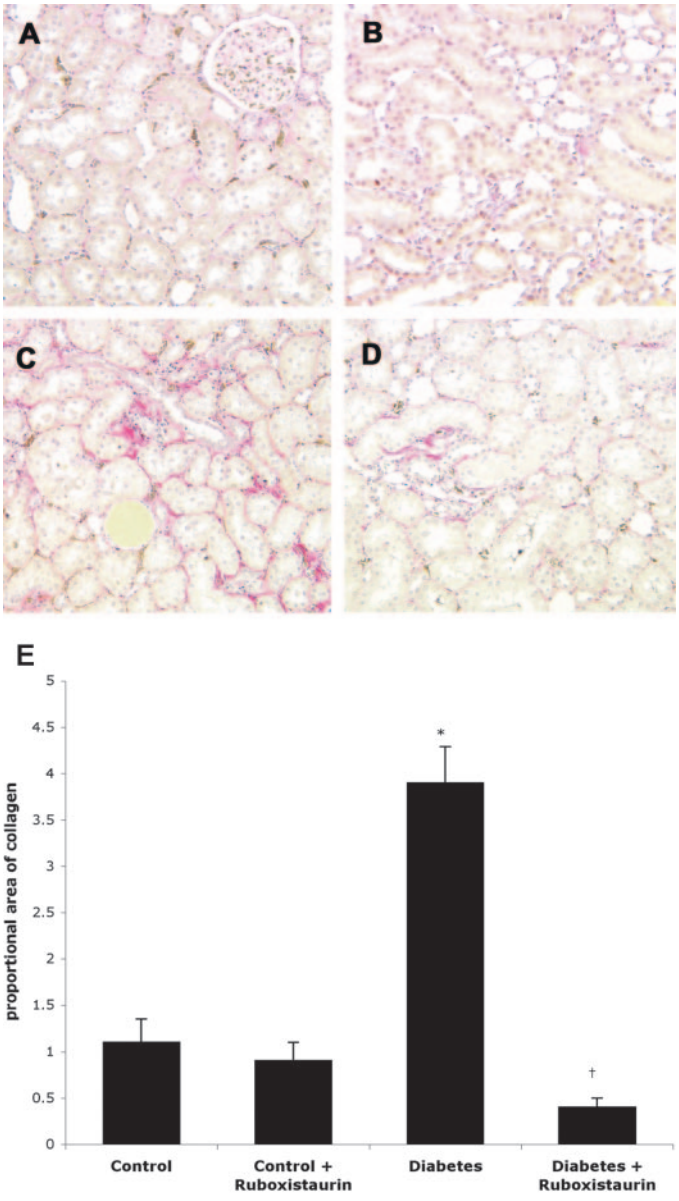

Figure 4. Picrosirius red–stained sections of kidney from transgenic Ren-2 rats. Representative photomicrographs (A through D) and quantitative analysis (E) of tubulointerstitial collagen as assessed by picrosirius red labeling. Minimal picrosirius red–labeled collagen is detected within the tubulointerstitium of nondiabetic rats that were treated with either control diet (A) or ruboxistaurin (B). In contrast, diabetes (C) was associated with increased collagen deposition that was attenuated by treatment with ruboxistaurin (D). Quantification of collagenous matrix (E) as expressed as the proportional area occupied by picrosirius red–labeled material. Data are expressed as mean ± SEM. $^{*}P < 0.001$ *versus* nondiabetic rats; $^{\dagger}P < 0.001$ *versus* untreated diabetic rats. Magnification, ×340.

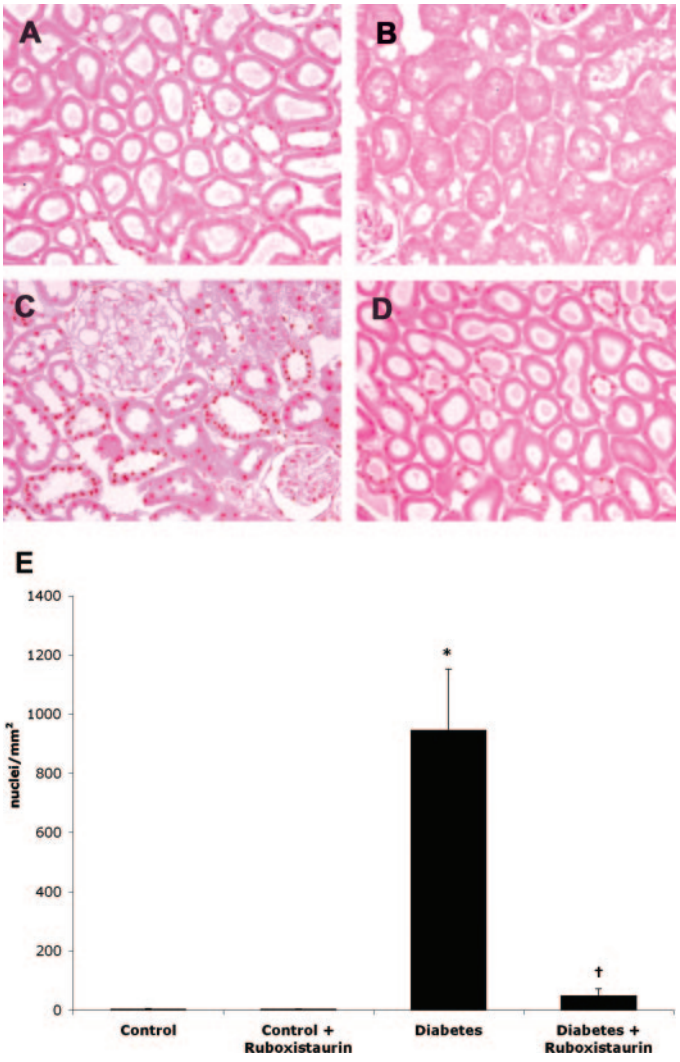

Figure 5. Immunohistochemistry for phospho-Smad 2 in the kidney of transgenic Ren-2 rats. Representative photomicrographs (A through D) and quantitative analysis (E) of nuclear phospho-Smad 2 expression. Only occasional nuclear phospho-Smad 2 immunolabeling was detected within the tubulointerstitium of nondiabetic rats that were treated with either control diet (A) or ruboxistaurin (B). In contrast, diabetes was associated with numerous tubular epithelial cells displaying positive phospho-Smad 2 immunolabeling (C) that was attenuated by treatment with ruboxistaurin (D). Quantification of phospho-Smad 2 (E), expressed as the number of labeled nuclei per mm^2. Data are expressed as mean ± SEM. $^{*}P < 0.001$ *versus* nondiabetic rats; $^{\dagger}P < 0.001$ *versus* untreated diabetic rats. Magnification, ×340.

diating their recruitment to the tubulointerstitium (5), where studies using both neutralizing osteopontin antibodies and knockout mice have shown a reduction in macrophage infiltration and tubulointerstitial injury (34–36). In our study, prominent osteopontin expression was identified in the cortical tubular epithelium of diabetic but not control rats. A number of *in vitro* studies have demonstrated a key role for PKC in the stimulation of glucose-stimulated osteopontin expression (6,37). More recently, studies in immortalized rat renal proximal tubular epithelial cells, using another PKC-β inhibitor, in combination with specific antisense have shown a key role for the β isoform of PKC, in glucose-induced osteopontin expression (38). To examine the role of PKC-β in the *in vivo* setting, we

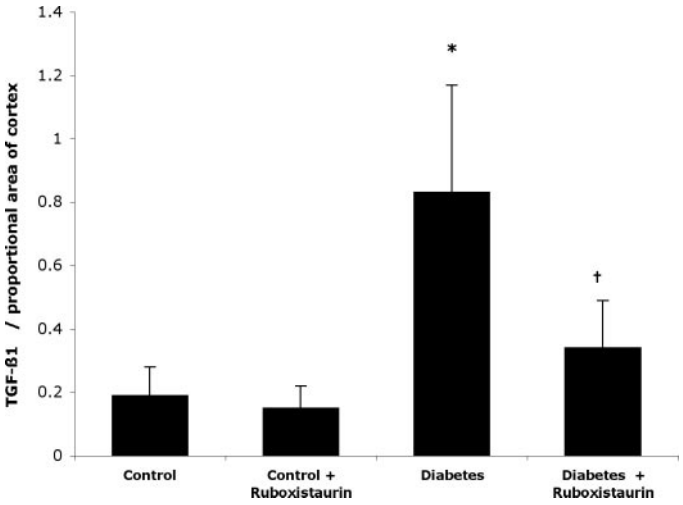

Figure 6. Quantification for TGF-β1 immunohistochemistry in the kidney of transgenic Ren-2 rats. Quantification of TGF-β1, expressed as percentage of positive stained tubules per cortex. Data are expressed as mean ± SEM. $^{*}P < 0.001$ *versus* nondiabetic rats; $^{\dagger}P < 0.001$ *versus* untreated diabetic rats.

used the specific PKC-β isoform inhibitor ruboxistaurin, showing a reduction in osteopontin expression and consequent macrophage infiltration in diabetic rats.

Both correlative and interventional studies have repeatedly shown a key role for TGF-β1 in the development of matrix accumulation at this site (39,40). TGF-β1 is synthesized as a 391–amino acid precursor molecule with little biologic activity, requiring cleavage of its N-terminal latency-associated peptide to give rise to its active form (41). In addition, its biologic effects may be modified by the presence of the proteoglycan decorin (42) and the scavenging protein α2-macroglobulin (43) such that increased TGF-β1 mRNA or protein may not accurately reflect TGF-β activity. Accordingly, in this study, in addition to assessing TGF-β protein, we examined the biologic effects of TGF-β by examining one of its specific intracellular actions, the phosphorylation of the TGF-β1 receptor-activated protein Smad2 (44,45). In contrast to control rats, prominent nuclear staining of phosphorylated Smad2 that was attenuated by treatment with ruboxistaurin was noted in diabetic rats. However, although macrophages are an important source of TGF-β1, this growth factor is also synthesized by tubular epithelial cells in the setting of diabetes (40), where its upregulation has recently been shown to be diminished by PKC-β inhibition (46).

In summary, the findings of this study suggest that osteopontin and macrophage accumulation may play a role in the tubulointerstitial injury in diabetic nephropathy and that inhibition of osteopontin expression may be one of the mechanisms by which inhibition of the β-isoform of PKC confers a renoprotective effect.

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