Protein Kinase C β Inhibition Attenuates the Progression of Experimental Diabetic Nephropathy in the Presence of Continued Hypertension

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In addition to hyperglycemia, hypertension and the renin-angiotensin system have been consistently implicated in the pathogenesis of diabetic nephropathy. Each of these pathogenetic factors may induce changes in cellular function by a common intracellular signaling pathway, the activation of protein kinase C (PKC) β. The present study thus sought to determine the in vivo effect of PKC β inhibition in experimental diabetic nephropathy in the setting of continued hyperglycemia, hypertension, and activation of the RAS. Studies were conducted in the (mRen-2)27 rat, a rodent that is transgenic for the entire mouse renin gene (Ren-2) and develops many of the structural, functional, and molecular characteristics of human diabetic nephropathy when experimental diabetes is induced with streptozotocin (STZ). Six-week-old female Ren-2 rats received an injection of STZ or vehicle and were maintained for 6 months. Within 24 h, diabetic rats were further randomized to receive treatment with the specific PKC β inhibitor, LY333531, admixed in diet (10 mg·kg−1·d−1) or no treatment (n = 8/group). Diabetic rats developed albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis with a concomitant increase in transforming growth factor-β (TGF-β). Western blot analysis demonstrated increased PKC β in diabetic animals, localized by immunofluorescence to the glomerular mesangium. In vivo inhibition of PKC β with LY333531 led to a reduction in albuminuria, structural injury, and TGF-β expression, despite continued hypertension and hyperglycemia. Diabetes 52:512–518, 2003

Diabetic nephropathy is a leading cause of end-stage renal disease throughout much of the world (1). In addition to elevated blood glucose, hypertension and inappropriate activation of the renin-angiotensin system (RAS) have been identified as contributing to the development and progression of diabetic renal disease. Indeed, clinical studies not only have demonstrated a key role for good glycemic control in reducing the development and progression of diabetic nephropathy (2,3) but also have shown the importance of blood pressure reduction (4,5) and blockade of the RAS (6–8) in slowing the progression of renal dysfunction in both type 1 and type 2 diabetes. However, despite these advances, the incidence of end-stage renal disease as a result of diabetes continues to rise in the United States and other countries where diabetes is an important problem.

In addition to their frequent coexistence in patients with diabetic renal disease, hyperglycemia and hypertension may induce changes in cellular function by common intracellular signaling pathways (9). Indeed, several components of the diabetic milieu, implicated in the pathogenesis of diabetic nephropathy, induce activation of protein kinase C (PKC) β. These include both glucose-dependent pathways such as the formation of advanced glycation end products and hyperglycemia per se in addition to glucose-independent mechanisms such as hypertension and activation of the RAS (10–13).

Previous studies have shown that not only is PKC β the major isoform of PKC activated by hyperglycemia in the diabetic kidney (14), but also its inhibition of the β isoform reduces albuminuria (15) and mesangial expansion (16). However, whether this therapeutic strategy is also effective in the setting of coexistent hypertension and inappropriate activation of the RAS (17), as in human diabetic nephropathy, remains uncertain. Furthermore, the effects of PKC β inhibition on tubulointerstitial injury in diabetes, a major histological feature of the disease (18), also has not been reported.

The present study thus sought to examine the effects of PKC β inhibition in the diabetic (mRen-2)27 rat model of experimental diabetic nephropathy. This transgenic animal has the entire mouse renin gene (Ren-2) inserted into the genome of a Sprague-Dawley rat (19) and develops hypertension and many of the structural and functional
RESEARCH DESIGN AND METHODS

Animals. Six-week-old female, heterozygous (mRen-2)27 rats that weighed 125 ± 5 g were randomized to receive either 55 mg/kg STZ (Sigma, St. Louis, MO) dihydro 0.1 mol/l citrate buffer (pH 4.5) or citrate buffer alone (nondiabetic) by tail-vein injection after an overnight fast. Diabetic Ren-2 (n = 8 per group) were treated with the PKC β inhibitor LY333531 (Eli Lilly and Co., Indianapolis, IN) 10 mg·kg−1·d−1, milled into rat food for 6 months after STZ or vehicle. Treatment commenced within 24 h of STZ injection. All rats were housed in a stable environment (maintained at 22 ± 1°C with a 12-h light/dark cycle) and allowed free access to tap water. Each week, rats were weighed and blood glucose was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, Australia). Every 4 weeks, systolic blood pressure (SBP) was recorded in preheated conscious rats by tail-cuff plethysmography (21). Diabetic rats received a daily injection of insulin (2–4 units intraperitoneally; Ultratard, Novo Nordisk, Bagsvaerd, Denmark) to promote weight gain and to reduce mortality. 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 Bioethics Committee of the University of Melbourne.

Albuminuria. Rats were individually housed in metabolic cages every 2 months and habituated for 2–3 h, and measurements of urinary albumin excretion were obtained over 24 h. Animals continued to have free access to tap water and standard laboratory diet 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°C for subsequent analysis of albumin. Albuminuria was determined by a double-antibody radioimmunoassay, as previously performed in our laboratories (22).

Tissue preparation. Rats were anesthetized (Nembutal 60 mg/kg body wt – C for subsequent analysis of albumin. Albuminuria was determined by a double-antibody radioimmunoassay, as previously performed in our laboratories (22).

Histopathology. Changes in kidney structure were assessed in a masked light microscopic evaluation.

Immunofluorescence. Six-micron frozen sections were postfixed in 4% paraformaldehyde for 20 min and incubated for 20 min with normal goat serum (NGS) diluted 1:10 with 0.1 mol/l PBS at pH 7.4. Sections were then incubated for 18 h at 4°C with specific mouse anti-rat monoclonal PKC β antibody (1:250; Zymed, San Francisco, CA). Sections incubated with FITC-labeled goat anti-mouse IgG (DaKopatts, Glostrup, Denmark) diluted 1:200 with PBS for 1 h at room temperature. Sections were rinsed with PBS (2 × 5 min), rinsed in tap water for 5 min, and mounted for microscope viewing.

Immunohistochemistry. Three-micron sections were placed into histosol, hydrated in graded ethanol, and immersed in tap water before being incubated for 60 min with NGS diluted 1:10 with 0.1 mol/l PBS at pH 7.4. Sections were then incubated for 18 h at 4°C with specific primary to transforming growth factor-β (TGF-β) (1:250, Zymed). 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 incubated with FITC-labeled goat anti-rabbit IgG (DaKopatts, Glostrup, Denmark) diluted 1:200 with PBS. Sections were then incubated for 18 h at 4°C with specific primary to transforming growth factor-β (TGF-β) (1:250, Zymed). 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 incubated with a 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% diamino benzidine and 0.05% hydrogen peroxide (Pierce, Rockford, IL) in PBS at pH 7.6 for 1–3 min, rinsed in tap water for 5 min, counterstained in Mayer’s hematoxlin, differentiated in Scott’s tap water, dehydrated, cleared, and mounted in Depex (20).

Quantiﬁcation of matrix deposition and immunohistochemistry. The accumulation of matrix within the tubulointerstitium was assessed on Masson’s trichrome-stained sections using computer-assisted image analysis as, previously reported (25,26). 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, then loaded onto a Pentium III IBM computer. An area of blue on a trichrome-stained section or brown on TGF-β-stained sections was selected for its color range, and the proportional area of tissue with this range of color was then quantified. Calculation of the proportional area was then determined using image analysis (AIS, Analytical Imaging Station Version 6.0, ON, Canada) for quantification of histological sections.

Western blot analysis. Protein concentration of whole kidney samples was determined by the Bradford assay, using BSA as a standard. Samples containing 10 µg of protein were diluted to 30 µl in loading buffer, denatured for 5 min at 95°C, and separated by electrophoresis in 12.5% SDS-PAGE gels. After electroblotting onto Hybrid transfer membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), gel loading and transfer efficiency were assessed by staining the blot with 0.1% Ponceau’s Solution (Sigma Chemical Co.). Blots were blocked overnight at 4°C in 5% wt/vol nonfat dry milk before incubation with the primary antibody for 60 min at room temperature. Anti-PKC β antibody (dilution 1:1750; Zymed) was used to demonstrate the 81-kDa protein band. After the blot was incubated with a horseradish peroxidase–conjugated anti-mouse secondary antibody (dilution 1:1000; Amersham) for 60 min at room temperature, antibody binding was visualized by enhanced chemiluminescence detection reagents. The bands of the resulting autoradiographs were compared for optical density using ImageQuaNT software (Version 4.2a, Build 13, Amersham). Relative quantities were compared normalized to control values, arbitrarily assigned as 100%.

Statistics. Data are expressed as mean ± SE unless otherwise stated. Statistical significance was determined by a two-way ANOVA with a Fisher’s post hoc comparison. Albumin excretion was analyzed using log-transformed data and presented as geometric means ×/± tolerance factors. Analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA).
RESULTS
Renal functional and biochemical studies. In comparison with control animals, diabetic rats had reduced body weight, which was unaffected by treatment ($P < 0.01$). All rats, both diabetic and nondiabetic, were hypertensive with elevated SBP that was not altered by LY333531 treatment ($P < 0.01$; Table 1). Plasma glucose was elevated to a similar extent in treated and untreated diabetic rat groups ($P < 0.01$; Table 1). Diabetes was associated with an increase in urinary albumin excretion when compared with controls. Treatment with LY333531 reduced albumin excretion in diabetic rats (Table 1).

Renal structure. Glomerular injury was a prominent feature of diabetic rats, with evidence of both diffuse and nodular glomerulosclerosis (Fig. 1). These changes were significantly attenuated by treatment with LY333531 (Figs. 1 and 2). In addition to these glomerular changes, tubulointerstitial pathology was present in diabetic rats (Figs. 2 and 3). These changes were also substantially reduced in diabetic rats treated with LY333531 (Figs. 2 and 3).

PKC β Western blotting and immunofluorescence. Western blot analysis demonstrated increased PKC β in the kidneys of diabetic compared with control animals. This increased expression of PKC β in diabetic animals was significantly reduced by treatment with LY333531 (Fig. 4). The cell-specific localization of PKC β was examined using immunofluorescence microscopy. These studies demonstrated that PKC β was expressed in the glomeruli of control rats, in a pattern consistent with its presence in mesangial cells (Fig. 5). Immunostaining for

FIG. 1. Representative PAS-stained sections from control diabetic and diabetic LY333531-treated Ren-2 rats. In control rats (A), there is only minimal glomerulosclerosis, whereas diabetes is associated with a dramatic increase in glomerulosclerosis (B). Treatment of diabetic rats with the PKC β inhibitor LY333531 (C) was associated with a reduction in the number and extent of glomerulosclerosis. Magnification ×350.

FIG. 2. Glomerulosclerosis expressed as glomerulosclerotic index (top) and tubulointerstitial fibrosis expressed as percentage area occupied by extracellular matrix (blue) on trichrome-stained sections (bottom) in control, diabetic, and diabetic + LY333531–treated Ren-2 rats. *$P < 0.01$ diabetic vs. control; †$P < 0.01$ diabetic + LY333531 vs. untreated diabetic.
PKCβ was increased in glomeruli of diabetic rats, although its pattern of distribution was unchanged. Treatment of diabetic rats with LY333531 was associated with a reduction in the overexpression of PKCβ when compared with untreated diabetic animals (Fig. 5). No immunostaining of PKCβ was detected in the tubulointerstitium in either control or diabetic rats.

**TGF-β immunohistochemistry.** Minimal immunostainable TGF-β was present in the kidneys of control rats (1.68 ± 0.58% proportional area). In contrast, abundant TGF-β was expressed in the kidney (15.63 ± 2.5%; P < 0.001 versus control) of diabetic rats. This overexpression was attenuated in diabetic rats treated with LY333531 (4.38 ± 0.70%, P < 0.001 versus diabetic; Fig. 6).

**DISCUSSION**

The present study demonstrates several novel findings in relation to the pathogenesis of diabetic nephropathy. First, despite the presence of continuing hyperglycemia and hypertension, PKCβ inhibition with LY333531 reduced the development of structural and functional manifestations of renal injury in this model. Second, although immunofluorescence microscopy localized PKCβ to the glomerulus, inhibition of this enzyme also attenuated injury in the tubulointerstitium. Third, diabetes was accompanied by increased immunoreactive PKCβ, and this was also reduced with LY333531.

The pathogenesis of diabetic nephropathy is complex and involves both glucose-dependent and glucose-independent pathways. In both type 1 and type 2 diabetes, the degree of hyperglycemia influences both the likelihood of developing nephropathy and the rate of its progression (2,27,28). High intracellular glucose concentrations, perse, may lead to activation of PKC (29) and in particular the β isoform, which has been shown to be activated in the...
glomeruli in experimental diabetes (15,30). However, in addition to these glucose-dependent mechanisms, other glucose-independent components of the diabetic state contribute to the development and progression of diabetic nephropathy. In particular, both experimental and clinical studies indicate that hypertension and inappropriate acti-

FIG. 5. Representative photomicrograph of PKC β immunofluorescence in control, diabetic, and diabetic + LY333531–treated Ren-2 rats. In control rats (A), PKC β immunofluorescence was detected with more intense PKC β labeling noted in glomeruli of diabetic rats (B). Treatment of diabetic rats with LY333531 was associated with a reduction in PKC β immunofluorescence to levels similar to that of control animals (C). Magnification ×350.

FIG. 6. Representative photomicrograph of TGF-β immunohistochemistry in control, diabetic, and diabetic + LY333531–treated Ren-2 rats. In control rats (A), TGF-β immunostaining was detected with more intense TGF-β labeling noted in glomeruli of diabetic rats (B). Treatment of diabetic rats with LY333531 was associated with a reduction in TGF-β immunostaining to levels similar to that of control animals (C). Magnification ×420.
vation of the RAS are likely key contributors (4,6,17). Both angiotensin II (31), the effector molecule of the RAS, and cell stretch, the in vitro counterpart of hypertension, activate PKC (10). The m(Ren-2)27 rat, used in the present studies (19), is not only hypertensive but also displays overactivity of the intrarenal RAS (20,32), both key features in the pathophysiology of progressive kidney disease in humans with diabetes. In the present study, LY333531 significantly attenuated the structural and functional manifestations of diabetic renal injury along with a reduction in the overexpression of the profibrotic growth factor TGF-β. The finding that these beneficial changes occurred despite the continued presence of hyperglycemia, hypertension, and constitutive activation of the RAS is consistent with PKC β activation as a final common pathway for these pathogenetic attributes of the diabetic milieu.

Although the glomerulus, in particular the mesangium, has largely been the focus of studies in diabetes, tubulointerstitial injury is also a major feature of diabetic nephropathy and an important predictor of both renal dysfunction (33,34) and its response to therapeutic interventions (35–37). In the present study, PKC β was localized to the mesangial region of the glomerulus but was not detected in tubular epithelium of either control or diabetic animals. However, despite the pattern of distribution, inhibition of PKC β with LY333531 attenuated tubulointerstitial as well as glomerular injury. These findings suggest that tubulointerstitial injury in diabetic nephropathy may develop as a consequence of glomerular damage. Indeed, experimental studies have indicated that multiple pathogenetic mechanisms may account for the tubulointerstitial injury that follows glomerular injury (8). These include excessive protein load to the proximal tubule leading to peritubular inflammation and fibrosis, postglomerular vascular constriction with peritubular capillary rarefaction, tubulointerstitial injury, and misdirection of filtrate into the periglomerular and peritubular space (38).

PKC is a ubiquitously expressed large family of serine-threonine kinases that transduce a wide range of cell-signaling processes by substrate-specific phosphorylation (39,39). Of the 11 identified PKC isoenzymes, a preferential increase in the β isof orm has been described in experimental diabetes and in nondiabetic renal disease in humans (9,15,40), although this has not been a universal finding (41). Although enhanced PKC activity in diabetes occurs as a consequence of glucose-induced generation of diacyl glycerol and the resultant membrane translocation (9,42), recent studies suggest that other mechanisms may also contribute. Indeed, in the present study, diabetes was associated with an increase in immunoreactive PKC β as determined by both Western blot analysis and immunofluorescence. These findings suggest that high glucose, and possibly other aspects of the diabetic milieu, not only induce activation of PKC enzymatic activity but also lead to increased PKC β protein expression. Similar changes have also been demonstrated in the in vitro setting, in which exposure of cultured mesangial cells to 48 h of high glucose resulted in a doubling of total PKC βII protein, detected by immunoblotting (43). In the present study, the diabetes-associated increase in PKC β expression was attenuated by LY333531, suggesting that PKC β activation may induce its own expression. The mechanisms underlying the possible autoinduction of PKC β in the diabetic kidney is uncertain. Recent studies suggest that the epidermal growth factor receptor may be involved in both the induction and response to PKC activation (43,44). Alternatively, the increase in PKC β may reflect a mesangial cell expansion in the setting of diabetes.

In summary, the present study demonstrates that, in a model of advanced diabetic nephropathy, inhibition of PKC β significantly attenuated the structural and functional manifestations of injury despite continued hyperglycemia and hypertension. These findings suggest the potential role for this therapeutic strategy in the treatment and prevention of diabetic kidney disease.

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