Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation

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Gooch, Jennifer L., Jeffrey L. Barnes, Sergio Garcia, and Hanna E. Abboud. Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation. Am J Physiol Renal Physiol 284: F144–F154, 2003. First published September 11, 2002; 10.1152/ajprenal.00158.2002.—Diabetic nephropathy is characterized by the rapid onset of hypertrophy and ECM expansion. Previously, we showed that calcineurin phosphatase is required for hypertrophy and ECM synthesis in cultured mesangial cells. Therefore, we examined the effect of calcineurin inhibition on renal hypertrophy and ECM accumulation in streptozotocin-induced diabetic rats. After 2 wk of diabetes, calcineurin protein was increased in whole cortex and glomeruli in conjunction with increased phosphatase activity. Daily administration of cyclosporin A blocked accumulation of both calcineurin protein and calcineurin activity. Also associated with calcineurin upregulation was nuclear localization of the calcineurin substrate NFATc1. Inhibition of calcineurin reduced whole kidney hypertrophy and abolished glomerular hypertrophy in diabetic rats. Furthermore, calcineurin inhibition substantially reduced ECM accumulation in diabetic glomeruli but not in cortical tissue, suggesting a differential effect of calcineurin inhibition in glomerular vs. extraglomerular tissue. Corresponding increases in fibronectin mRNA and transforming growth factor-β mRNA were observed in tubulointerstitium but not in glomeruli. In summary, calcineurin plays an important role in glomerular hypertrophy and ECM accumulation in diabetic nephropathy.

extracellular matrix; kidney; cyclosporin A; mesangial cells

Renal cell hypertrophy and ECM expansion are controlled by a variety of hormones, cytokines, and peptide growth factors. In diabetes, elevated blood glucose concentration and factors such as transforming growth factor-β1 (TGF-β1), angiotensin II, and insulin-like growth factor-I (IGF-I) contribute to the development and maintenance of renal hypertrophy and matrix expansion (1, 39). Our laboratory and others have shown that IGF-I mediates hypertrophy and induces ECM accumulation in cultured cells (15, 28, 30). In addition, we have shown that although IGF-I activates well-known signaling pathways such as Erk1/Erk2 MAPK and PI-3 kinase, neither of these signaling mechanisms appears to be required for hypertrophy or accumulation of ECM proteins (15). Instead, IGF-I-mediated hypertrophy and ECM synthesis requires activation of the calcium-dependent, serine/threonine phosphatase calcineurin. In addition, we found that calcineurin is also required for ECM synthesis by TGF-β (14), suggesting that calcineurin may be a common signaling mechanism for multiple stimuli that regulate hypertrophy and/or ECM accumulation in glomeruli.

Calcineurin is a serine/threonine phosphatase whose activation requires increased availability of intracellular calcium. In response to a variety of stimuli, calcineurin binds calmodulin and calcium, resulting in enhanced phosphatase activity (29). Targets of calcineurin dephosphorylation include transcription factors, such as members of the nuclear factors of activated T cell (NFAT) family, myocyte enhancer-binding factors, and GATA proteins. In particular, NFATc1 and NFATc2 are activated by IGF-I and implicated in the hypertrophic effects of calcineurin (15, 27, 31). After dephosphorylation, these factors undergo translocation from the cytoplasm to the nucleus, bind other transcription factors including AP-1 components fos and jun, and modulate transcription of target genes (4, 6). Calcineurin has been best characterized as a mediator of T cell signal transduction, and inhibition of calcineurin with the drug cyclosporin A (CsA) successfully suppresses T cell-mediated immunity.

There are indications that calcineurin is expressed in the kidney and may be important in certain aspects of renal physiology. Several investigators have reported expression of calcineurin mRNA, primarily in the medulla but also in the cortex of the kidney (5, 25). Furthermore, calcineurin protein has been detected in the medulla as well as in dissected proximal tubule epithelial cells and cortical collecting ducts (36). Calcineurin phosphatase activity corresponds to expression of calcineurin protein in renal structures (36). In addition to calcineurin expression, there is evidence that calcineurin is involved in kidney function. Aperia et al. (2) report that calcineurin is involved in the...
regulation of Na-K-ATPase activity in response to angiotensin II in cultured proximal tubule cells. Adding to potential functions of calcineurin in the kidney, data from our laboratory implicate calcineurin and NFATc1 in IGF-I signaling and mesangial cell hypertrophy in vitro (15). However, the role of calcineurin and calcineurin substrates such as NFAT in diabetic renal and specifically glomerular cell hypertrophy remains unclear.

Our laboratory has previously shown that activation of the serine-threonine phosphatase calcineurin is required for IGF-I-induced hypertrophy of mesangial cells in vitro (15). Because hypertrophy, ECM expansion, and increased expression of renal IGF-I are early consequences of hyperglycemia, we were interested in the role of calcineurin in an in vivo model of diabetes. Therefore, the purpose of this study was to examine calcineurin phosphatase activity in the kidney and the effect of calcineurin inhibition on the development of renal and glomerular hypertrophy and ECM accumulation in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Materials. STZ and actin and fibronectin antibodies were obtained from Sigma, CsA from Novartis, calcineurin and collagen type IV antibodies from Chemicon, and NFATc1 antibody from Santa Cruz.

Animal protocol. Male Sprague-Dawley rats weighing between 200 and 250 g were divided into four groups of 4–6 rats/group. Group 1 was injected intravenously via the tail vein with 65 mg/kg body wt STZ in sodium citrate buffer (pH 4.0) to induce diabetes. Group 2 was similarly injected with sodium citrate buffer alone. Rats in group 3 were injected with STZ and were also given daily subcutaneous injections of CsA (5 mg/kg body wt) beginning at the time of STZ injection. Group 4 received daily subcutaneous injections of vehicle alone (10% ethanol). Blood glucose concentrations were monitored by using a LifeScan One Touch glucometer (Johnson & Johnson) 24 h later to verify hyperglycemia and periodically thereafter. All rats had unrestricted access to food and water and were maintained in accordance with Institutional Animal Care and Use Committee procedures.

To inhibit calcineurin activity, CsA was utilized. CsA is a chemical inhibitor with high specificity for calcineurin. CsA doses have been carefully examined in rats with regard to nephrotoxicity and serum concentrations (26, 33). Signs of chronic nephrotoxicity were observed at doses of CsA > 10 mg/kg body wt administered for at least 2 wk. Therefore, for our experiments, we chose a dose of 5 mg/kg body wt CsA to minimize possible nephrotoxicity.

Rats were euthanized, and both kidneys were removed and weighed. A slice of kidney cortex at the pole was embedded in paraffin or flash frozen in liquid nitrogen for preparation of sections for light microscopy and image analyses. In addition, cortical sections from two kidneys from different animals within the treatment groups were pooled for isolation of glomeruli by differential sieving as described (32), and samples of cortical tissue were frozen for biochemical analyses.

Calcineurin phosphatase assay. Calcineurin phosphatase activity in renal cortex was determined following a protocol published by Fruman et al. (13). Briefly, the calcineurin-specific substrate RII was phosphorylated in vitro with 250 U recombinant PKA, 50 mM ATP, 50 μCi [γ-32P]-ATP, 0.15 mM RII peptide, and 500 μl of 2× reaction buffer ([in mM] 40 MOPS, 4 MgCl2, 0.1 CaCl2, 0.4 EDTA, 0.8 EGTA, and 0.5 DTT, as well as 0.1 mg/ml BSA and 0.2 μg/μl recombinant calmodulin). Lysates were prepared by homogenizing cortex sections in a hypotonic lysis buffer ([in mM] 50 Tris, pH 7.5, 1 EDTA, 1 EGTA, and 0.5 DTT, and (in μg/ml) 50 PMSF, 10 leupeptin, 10 aprotinin) followed by four cycles of freeze-thawing in liquid nitrogen and a 30°C water bath. Calcineurin activity in each sample was determined by incubating equal parts lystate, 3× reaction buffer ([in mM] 40 Tris, pH 7.5, 6 MgCl2, 0.1 CaCl2, and 0.5 DTT, and (in mM) 500 okadaic acid and 100 calmodulin, as well as 0.1 mg/ml BSA and 0.1 M NaCl) and labeled RII peptide at 30°C for 10 min. The reaction was stopped by addition of 0.1 M KPO4 in 5% TCA. To determine the amount of phosphate released by calcineurin in each sample, reactions were then added to PolyPrep columns (Bio-Rad, Hercules, CA) containing AG-50× Dowex ion exchange resin (Bio-Rad) prepared as described (13). Finally, 5 ml scintillation fluid were added to the flow-through from each column, and the released phosphate was measured in a scintillation counter.

Western blotting. Cortical cortex or isolated glomeruli were resuspended in hypotonic lysis buffer ([in mM] 50 Tris, pH 7.5, 1 EDTA, 1 EGTA, and 0.5 DTT, and (in μg/ml) 50 PMSF, 10 leupeptin, and 10 aprotinin), and then protein lysates were collected by four rounds of freeze-thawing in liquid nitrogen and a 30°C water bath followed by centrifugation at 14,000 g for 30 min at 4°C. Twenty-five micrograms of protein were analyzed by SDS-PAGE. After transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk-TBST (20 mM Tris·HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and then immunoblotted with 1:2,000 dilutions of anti-fibronectin or actin antibodies and 1:1,000 dilutions of anti-collagen type IV or calcineurin antibodies. Horseradish peroxidase-conjugated secondary antibodies were added at 1:2,000, and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Determination of glomerular surface area. Light microscopy of hematoxylin- and eosin-stained sections from the different treatment groups was used for morphometric studies. The surface area (μm2) of a minimum of 50 glomerular sections from each animal was determined by using Image-Pro Plus software.

Immunohistochemistry. Paraffin-embedded tissue sections (5-μm thick) were prepared by dewaxing and unmasking. After incubation with specific primary and biotin-conjugated secondary antibodies, calcineurin and NFATc1 were identified by immunoperoxidase ABC staining following the manufacturer’s instructions (Vector Laboratories). Coverslips were mounted with Crystal Mount (Biomeca, Foster City, CA), and sections were viewed by brightfield microscopy.

In situ hybridization. Synthesis of riboprobe, tissue preparation, in situ hybridization, and autoradiography were identical to methods previously described (3). Briefly, fragments of fibronectin and TGF-β cDNAs cloned into pGEM vector were used for generation of 35S-labeled riboprobe to detect cellular localization of mRNA in sections of renal tissue. All experiments were performed simultaneously with the sense riboprobe as a negative control. Semiquantitative analyses were made by using Image-Pro Plus software. The relative intensity of signal from a minimum of 10 glomeruli from 3 animals/group was determined. Also, relative intensity of a representative interstitial field of each animal was determined.

Renal function. Two weeks after induction of diabetes, animals were placed overnight in metabolic cages, with unrestricted access to food and water. Urine was collected, urine flow rate was calculated (μl/min), and nitrogen and creatinine levels were determined. At the time of death,
whole blood was collected and serum blood urea nitrogen and serum creatinine levels were measured. Renal function was determined as the average of nitrogen clearance and creatinine clearance.

Statistics. As indicated, two-way ANOVA or Student’s t-test was used to determine statistical significance for experiments with multiple or single variables, respectively. For all Student’s t-test analyses, a paired t-test was used and a result was considered significant if \( P < 0.05 \). For all analyses using two-way ANOVA, treatment vs. time was used and a result was considered significant if \( P < 0.05 \).

RESULTS

Regulation of calcineurin in the diabetic kidney. To study the role of calcineurin in diabetic renal hypertrophy, type 1 diabetes was induced in male Sprague-Dawley rats by a single intravenous injection of STZ (65 mg/kg body wt). Control and diabetic rats were treated with CsA (daily subcutaneous injection of 5 mg/kg body wt) to inhibit calcineurin activity or vehicle alone. Glucose levels, weight, kidney mass, and kidney/body mass ratio after 14 days of diabetes are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
<th>CsA</th>
<th>CsA + STZ</th>
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<tr>
<td>Glucose, mg/dl</td>
<td>69.3 ± 1.9</td>
<td>466.9 ± 26.4*</td>
<td>69.5 ± 3.7</td>
<td>384.4 ± 34.6**</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>287.7 ± 4.2</td>
<td>222.2 ± 9.2*</td>
<td>288.3 ± 4.4*</td>
<td>245.3 ± 5.3**</td>
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<tr>
<td>Kidney mass, g</td>
<td>1.99 ± 0.06</td>
<td>2.31 ± 0.16</td>
<td>1.91 ± 0.09</td>
<td>2.13 ± 0.07</td>
</tr>
<tr>
<td>Kidney/body mass</td>
<td>0.661 ± 0.03</td>
<td>1.046 ± 0.06*</td>
<td>0.663 ± 0.02</td>
<td>0.868 ± 0.02**</td>
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Values are means ± SE, \( n = 4–6/group \). STZ, streptozotocin; CsA, cyclosporin A. *\( P < 0.05 \) or higher degree of significance compared with control; **\( P < 0.05 \) or higher degree of significance compared with STZ alone.

Next, we examined the effect of CsA on protein expression and cellular localization of calcineurin. In glomeruli, daily CsA administration decreases accumulation of calcineurin associated with diabetes (Fig. 2A). In contrast, in cortical homogenates, increased calcineurin protein associated with STZ diabetes was not inhibited by CsA treatment (Fig. 2B). By immunohistochemistry, calcineurin expression was determined to be relatively low in extraglomerular cortex and generally localized to the basolateral surface of tubules. Calcineurin appears to be expressed at a much lower level in glomeruli (Fig. 2C, a). Two weeks after induction of diabetes, calcineurin expression is markedly increased in both extraglomerular cortex and glomeruli, in agreement with calcineurin expression determined by immunoblotting in Fig. 1, A and B. Interestingly, increased calcineurin is detected in extraglomerular cortex of animals treated with CsA alone (Fig. 2C, c). Consistent with protein levels determined by immunoblotting, increased calcineurin expression associated with diabetes in glomeruli is inhibited by CsA treatment (Fig. 2C, d), whereas expression in extraglomerular cortex remains high compared with control, again in agreement with data in Fig. 2A.

Protein extracts were collected from homogenized cortical sections, and calcineurin activity was determined by an in vitro phosphatase assay (13). After 14 days of diabetes, calcineurin activity is increased in the renal cortex of diabetic animals compared with control, 539.8 ± 30.1 vs. 3,168 ± 1,214 released counts·min⁻¹·µg protein⁻¹ (\( P < 0.05 \)), respectively. The increased activity associated with diabetes was blocked by daily CsA injection, and calcineurin activity in the cortex of control, normal rats was not changed with CsA treatment −626.3 ± 69.9 and 543.0 ± 26.8 released counts·min⁻¹·µg protein⁻¹, respectively.

NFATs are one group of proteins that are targets of calcineurin dephosphorylation. Our laboratory’s previ-
ous work showed that CsA treatment of cultured mesangial cells inhibited IGF-I-mediated hypertrophy and ECM accumulation as well as NFATc1 nuclear localization, suggesting that NFATc1 is involved in glomerular hypertrophy and/or ECM regulation (15). Figures 1 and 2 and our in vitro phosphatase assay demonstrate that calcineurin activity is increased in the diabetic kidney. Therefore, we examined NFATc1 localization in the renal cortex. Immunoperoxidase staining in control and STZ-, CsA-, and CsA + STZ-treated animals demonstrated a dramatic increase in NFATc1 protein expression in the renal cortex of diabetic animals (Fig. 3). In addition, although NFATc1 appears to be localized to the basolateral surface of tubular cells in control animals (Fig. 3, A and E), NFATc1 can be found in the nucleus in tubules of diabetic rat kidneys, consistent with activation of calcineurin phosphatase (compare with Fig. 3, B and F). Also consistent with inhibition of calcineurin activity, NFATc1 expression and localization are normalized in diabetic animals treated with CsA.

Inhibition of renal hypertrophy by CsA. Our data show that calcineurin is activated in the diabetic kidney and that calcineurin phosphatase activity (and
corresponding upregulation of calcineurin protein and NFATc1 nuclear localization) can be inhibited by daily administration of CsA. In vitro, calcineurin is involved in hypertrophy and ECM accumulation (15); therefore, we examined the effect of calcineurin inhibition in vivo on hypertrophy and ECM accumulation in the diabetic kidney. Whole kidney hypertrophy (total kidney wt/body mass) was assessed at time points up to 14 days after STZ-induced diabetes. Figure 4 shows that as early as 3 days, there is a statistically significant increase in kidney/body mass ratio in the STZ-treated animals. Over the course of the experiment, there is a significant decrease in whole kidney hypertrophy in CsA-treated diabetic animals (CsA + STZ) compared with diabetic animals (STZ alone) ($P < 0.01$, two-way ANOVA). In addition, at both 10 and 14 days, CsA treatment significantly reduced whole kidney hypertrophy ($P < 0.05$, Student’s *t*-test). There is no difference in kidney/body mass ratio between control and CsA-treated control animals. As summarized in Table 1, the effect of CsA treatment on STZ-induced whole kidney hypertrophy is seen in a trend toward a decrease in total kidney weight as well as an improvement in weight loss at 14 days. The result is a significant decrease in the kidney/body mass ratio due to calcineurin inhibition with CsA.

The effect of calcineurin inhibition was also determined on diabetes-induced glomerular hypertrophy. Figure 5 shows that after 14 days of treatment, glomeruli of STZ animals are significantly larger than control or CsA-treated control animals ($P < 0.001$, Student’s *t*-test). Glomeruli of CsA + STZ animals are significantly smaller than STZ animals ($P < 0.01$, Student’s *t*-test) and are not different from glomeruli of control animals. CsA alone had no effect on the size of glomeruli of normal rats. No evidence of ischemia was observed in either group of animals treated with CsA, and normal arterioles were identified in all groups.

Next, we determined the effect of calcineurin inhibition on ECM accumulation in glomeruli and whole cortex. Isolated glomeruli from STZ-treated animals demonstrated increased accumulation of the ECM proteins fibronectin and collagen type IV compared with control animals (Fig. 6A). Inhibition of calcineurin resulted in decreased accumulation of these proteins in glomeruli of CsA + STZ animals compared with STZ animals. CsA treatment alone has no effect on expres-
of ECM proteins compared with controls. Figure 6B shows that ECM proteins are also increased with STZ treatment in whole cortex. In contrast to the finding in glomeruli, expression of fibronectin and collagen type IV is increased in cortex homogenates of animals treated with CsA alone and inhibition of calcineurin with CsA does not decrease diabetes-induced ECM accumulation.

CsA increases transcription of fibronectin in cultured proximal tubule cells (37). However, it is not clear what effect CsA has on fibronectin mRNA in glomerular cells or what role calcineurin may play in regulation of fibronectin associated with diabetes. Therefore, we examined fibronectin mRNA in both glomeruli and cortex of control and STZ-, CsA-, and CsA + STZ-treated animals by in situ hybridization. Fibronectin mRNA was upregulated by diabetes in both glomeruli and cortex (Fig. 7, b and h compared with a and g). Animals treated with CsA showed increased fibronectin mRNA in the cortex but not in glomeruli (Fig. 7, i and c, respectively). Finally, diabetes-induced fibronectin mRNA was reduced in glomeruli but not in the cortex (Fig. 7, d and j, respectively).

One mechanism for regulation of fibronectin in renal cells is upregulation of TGF-β. Therefore, in Fig. 8, we examined TGF-β mRNA expression by in situ hybridization. Similar to fibronectin regulation in diabetic animals, TGF-β mRNA was increased in both glomeruli and cortex of STZ-treated animals (Fig. 8, b and h compared with a and g). Also consistent with increased fibronectin mRNA, TGF-β was upregulated in the cortex but not glomeruli of animals treated with CsA alone. Finally, TGF-β mRNA was reduced by calcineurin inhibition in glomeruli but was further increased in the cortex of diabetic animals (Fig. 8, d vs. h).

Effect of CsA on STZ-induced diabetic rats. To verify that the dose of CsA administered daily was sufficient to achieve therapeutic and nontoxic circulating levels, serum CsA levels were measured in animals receiving only CsA and in diabetic animals receiving CsA at multiple time points from 1 to 14 days. Administration of CsA alone resulted in a mean level of 797.9 ± 98.3 ng/ml over a course of 2 wk, whereas CsA + STZ

Fig. 5. Inhibition of calcineurin blocks glomerular hypertrophy. Glomerular area was determined by microscopic examination of hematoxylin- and eosin-stained paraffin-embedded cortex sections. The areas of a minimum of 50 glomeruli/section were determined by using Image-Pro software. A: representative glomeruli from each treatment group. Arrows, normal arterioles in CsA and CsA + STZ animals. *P < 0.001 compared with control (Student’s t-test). **P < 0.01 compared with STZ (Student’s t-test). B: each bar represents the mean ± SE of glomerular area from 4–6 animals.

Fig. 6. Inhibition of calcineurin decreases accumulation of ECM in glomeruli but increases ECM accumulation in the cortex. A: total protein was collected from isolated glomeruli from control, STZ, CsA, and CsA + STZ animals. Fibronectin and collagen type IV proteins were detected by direct immunoblotting. B: total protein was collected from homogenized cortical sections from STZ, CsA, and CsA + STZ animals. Fibronectin and collagen type IV proteins were detected by direct immunoblotting. Lanes 1 and 2: control animals. Lanes 3 and 4: STZ-treated animals. Lanes 5–7: CsA-treated animals. Lanes 8–10: CsA + STZ animals.
animals had serum levels of 445.6 ± 50.1 ng/ml, a statistically significant difference (P < 0.005, two-way ANOVA). Despite the difference in mean circulating levels, both treatment groups had CsA levels considerably below what has been described to induce adverse side-effects, including nephrotoxicity (16, 26, 33). Furthermore, we did not find evidence of nephrotoxicity in any of the groups when cortical sections were examined by light microscopy.

Finally, glucose concentrations, body weight, and glomerular filtration rates (GFRs) were examined. STZ-treated animals exhibited an increase in serum glucose over 14 days, with a combined mean of 371.6 ± 15.4 mg/dl (Fig. 9A). Diabetic animals treated with CsA had a slightly lower mean glucose level of 343.3 ± 10.4, which is significantly different over time from STZ alone (P < 0.001, two-way ANOVA). In addition, there is a statistically significant difference in the glucose levels of CsA + STZ animals at day 14 compared with STZ alone (P < 0.05, Student’s t-test). There is no significant difference in the mean glucose levels between controls and control animals treated with CsA alone (69.4 ± 1.4 and 72.0 ± 1.4 mg/dl, respectively), indicating that CsA does not reduce blood glucose concentration in the absence of diabetes.

Interestingly, diabetic animals treated with CsA showed a significant improvement in weight loss over time compared with STZ animals (P < 0.001, two-way ANOVA) (Fig. 9B). The time course of improved weight loss corresponds to a similar time frame as the reduc-
tion in glucose levels in CsA + STZ-treated animals. Administration of CsA alone resulted in slower weight gain over the 2-wk period than in control animals. This is consistent with other reports that CsA administration is associated with anorexia (17, 21).

Prolonged CsA treatment is frequently associated with nephrotoxicity, particularly glomerular ischemia and impaired filtration. To assess the effect of CsA treatment on renal function, GFR was determined as the mean of nitrogen clearance and creatinine clearance per gram body weight (Fig. 9C). As expected, diabetic animals have a slightly increased GFR [P < 0.05 compared with control (Student’s t-test)]. The GFR of animals treated with CsA alone was not different from control. However, the mean GFR of CsA + STZ-treated animals is slightly higher than STZ-treated animals [P < 0.05 (Student’s t-test)].

DISCUSSION

Whole kidney, including glomerular cell, hypertrophy are early manifestations of diabetic nephropathy. Previous work in our laboratory has demonstrated that calcineurin is an important signaling mediator of mesangial cell hypertrophy and ECM accumulation in vitro (14). However, calcineurin activity has never been examined in the diabetic kidney, and the role of calcineurin in diabetic glomerular hypertrophy is unknown. In this study, we show for the first time that calcineurin phosphatase is activated in the renal cortex of diabetic rats. Furthermore, we show that increased

Fig. 8. Transforming growth factor-β (TGF-β) mRNA is upregulated in both glomeruli and cortex of STZ animals and in cortex of CsA animals. A: TGF-β mRNA was detected in glomeruli of control (a), STZ (b), CsA (c), and CsA + STZ (d) animals and in cortex of control (g), STZ (h), CsA (i), and CsA + STZ (j) animals by in situ hybridization. Hybridization with sense probe is shown as a negative control in glomeruli (e) and in cortex (f). B: semiquantitation of data shown in A. Data are representative of 10 glomeruli and 10 cortical fields from 3 animals/group. *P < 0.05, significantly different from control.
expression of calcineurin is associated with nuclear localization of a target transcription factor, NFATc1, suggesting a possible transcriptional mechanism for calcineurin action. Our work demonstrates that inhibition of calcineurin with CsA reduces whole kidney hypertrophy and completely blocks glomerular hypertrophy and ECM accumulation. These results suggest that calcineurin is a mediator of diabetic hypertrophy and ECM regulation in glomeruli in vivo.

We show that calcineurin protein levels are increased 3 days after induction of diabetes and that calcineurin is activated in the renal cortex of diabetic rats after 2 wk of hyperglycemia. Coincident with this early time frame of disease is the increase in expression of polypeptide growth factors such as TGF-β and IGF-I in the kidney (1, 11, 12). We have shown in vitro that IGF-I induces hypertrophy and ECM accumulation in mesangial cells via calcineurin (15). Therefore, the increase in calcineurin protein and the stimulation of calcineurin activity may be due to increased availability of these, or other, peptide factors. The increased protein levels of calcineurin in the glomerulus and activation of calcineurin in the renal cortex are inhibited by treatment with CsA. Additional evidence of calcineurin activation in vivo is provided by increased expression and nuclear localization of the calcineurin substrate NFATc1 in the renal cortex of diabetic animals. NFATc1 regulation is clearly dependent on calcineurin activity, because treatment of animals with CsA abolished changes in both protein expression and nuclear localization.

Of major interest is that unlike its effects in glomeruli to decrease ECM expansion, CsA treatment alone increased both calcineurin protein levels and ECM accumulation in extraglomerular cortex. It is possible that binding of CsA/cyclophilin complexes to calcineurin inhibits normal turnover of the protein, leading to a subsequent accumulation of calcineurin in addition to inhibition of phosphatase activity. However, the mechanism of this differential effect of CsA on calcineurin expression is unclear. CsA-mediated increases in ECM proteins, however, have been described in several cell types, including cortical fibroblasts (19, 37), human endothelial and epithelial cells (9), and proximal tubule cells (37). In addition, CsA treatment has been associated with increased TGF-β expression (18, 19, 38), leading to the hypothesis that TGF-β mediates the subsequent increase in ECM proteins. Our data would support this model because CsA increased TGF-β and fibronectin mRNA levels in the tubulointerstitium. Of considerable interest is the finding that a similar event does not occur in the glomeruli. The data show no increase in fibronectin mRNA or protein and no increase in TGF-β mRNA with CsA treatment in glomeruli. Furthermore, we have previously shown that in cultured mesangial cells, there was no increase in fibronectin or collagen type IV protein expression in response to CsA treatment (15).

Also of interest is the fact that although animals in our experiments were administered daily subcutaneous injections of 5 mg/kg body wt CsA, there was a significant difference in the serum levels of CsA achieved in CsA alone and CsA + STZ animals. Diabetic animals were found to have roughly one-half the levels of CsA compared with nondiabetic animals. However, because CsA is at least partially secreted in
the urine (22), it is possible that increased clearance observed in CsA + STZ- vs. CsA alone-treated animals is responsible for decreased circulating levels. It is also possible that STZ or the diabetic state could activate metabolic pathways, leading to an increased rate of breakdown of CsA, and it could accelerate metabolism of the drug within the liver (the site of the majority of CsA metabolism). In either case, no toxicity was observed in animals treated with CsA alone, consistent with reports that nephrotoxicity of this drug within the glomeruli is not observed when it is administered even at higher concentrations (10 mg/kg body wt) for a similar time period (16, 26, 33). Next, there was a mild reduction in glucose levels in CsA + STZ-treated animals. It is possible that the mild improvement in hyperglycemia and slight increase in body weight contributed to the changes that were observed. However, the relatively small differences in glucose levels and body weight are not sufficient to explain the complete normalization of glomerular hypertrophy. This result suggests a direct role for calcineurin in hypertrophy of cells within the glomeruli. Indeed, our laboratory’s previous finding using cultured mesangial cells lends support to this interpretation (15). Furthermore, a slight decrease in hyperglycemia cannot account for the disparity in ECM regulation and TGF-β expression. Activation/deactivation of renal Na/K-ATPase: a final common pathway for regulation of natriuresis. FASEB J 8: 438–439, 1994.


The role of growth hor-

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