Mammalian Target of Rapamycin Pathway Blockade Slows Progression of Diabetic Kidney Disease in Rats

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Recent data suggest that the phosphatidylinositol 3-kinase (PI3-K)/Akt/mammalian target of rapamycin (mTOR) pathway is important in diabetic nephropathy. The effect of mTOR blockade by sirolimus (SRL) in diabetic kidney disease in rats was investigated. Diabetes was induced by streptozotocin in male Sprague-Dawley rats. Sixteen weeks later, diabetic animals were divided into the following groups: diabetes (D; \( n = 8 \)), diabetes + SRL at 1 mg/kg per d, SRL trough level 2.3 ± 0.25 ng/ml (D+SRL; \( n = 7 \)); and diabetes + normoglycemia maintained by insulin implants (D+NG; \( n = 5 \)). There was an age-matched nondiabetic group (ND; \( n = 6 \)). All animals were followed for 4 wk. The D group showed glomerular hypertrophy (mean glomerular volume 5.0 ± 0.4 in D versus 3.3 ± 0.2 \( \mu m^3 \) in ND; \( P < 0.05 \)) without renal hyperplasia (calculated by reverse transcription–PCR of proliferative cell nuclear antigen) and albuminuria (29 ± 4 in D versus 1.4 ± 1.5 mg/24 h in ND; \( P < 0.05 \)). Both D+NG and D+SRL groups had a significant reduction of albuminuria, although glomerular hypertrophy was still present. SRL treatment did not modify the number of infiltrating renal ED1+ cells. Diabetic animals had greater expression of p-Akt and mTOR, unlike ND rats. NG and SRL treatment reduced p-Akt and normalized mTOR. It is interesting that D+SRL was associated with a significant reduction of renal TGF-β1 and glomerular connective tissue growth factor. SRL treatment reduced glomerular α-smooth muscle actin overexpression and reduced significantly the mesangial matrix accumulation that is characteristic of diabetic nephropathy. In conclusion, mTOR blockade by low-dose SRL has a beneficial effect in diabetic kidney disease, suggesting that the mTOR pathway has an important pathogenic role in diabetic nephropathy.


Diabetic nephropathy is the main cause of chronic renal failure and ESRD requiring dialysis in developed countries (1). Hyperglycemia generates a cascade of events in the kidney, which finally results in mesangial extracellular matrix (ECM) accumulation (2). Mesangial cell activation, characterized by an induction of α-smooth muscle actin (α-SMA) expression and matrix overproduction, is considered a major feature of diabetic nephropathy (3). Histologically, diabetic nephropathy is characterized by an early thickening of tubular and glomerular basement membranes, as a result of an excessive accumulation of ECM, leading to progressive scarring and fibrosis of the kidney (4–6).

Utimura et al. (7) reported the beneficial effect of the immunosuppressant mycophenolate mofetil (MMF) in preventing the progression of diabetic nephropathy. The authors suggested that these intriguing results are related to glomerular anti-inflammatory and antiproliferative effects of MMF. Sirolimus (SRL), or rapamycin, is a potent new immunosuppressant mycophenolate mofetil (MMF) in preventing the progression of diabetic nephropathy. The effect of mTOR blockade by sirolimus (SRL) in diabetic kidney disease in rats was investigated. Diabetes was induced by streptozotocin in male Sprague-Dawley rats. Sixteen weeks later, diabetic animals were divided into the following groups: diabetes (D; \( n = 8 \)), diabetes + SRL at 1 mg/kg per d, SRL trough level 2.3 ± 0.25 ng/ml (D+SRL; \( n = 7 \)); and diabetes + normoglycemia maintained by insulin implants (D+NG; \( n = 5 \)). There was an age-matched nondiabetic group (ND; \( n = 6 \)). All animals were followed for 4 wk. The D group showed glomerular hypertrophy (mean glomerular volume 5.0 ± 0.4 in D versus 3.3 ± 0.2 \( \mu m^3 \) in ND; \( P < 0.05 \)) without renal hyperplasia (calculated by reverse transcription–PCR of proliferative cell nuclear antigen) and albuminuria (29 ± 4 in D versus 1.4 ± 1.5 mg/24 h in ND; \( P < 0.05 \)). Both D+NG and D+SRL groups had a significant reduction of albuminuria, although glomerular hypertrophy was still present. SRL treatment did not modify the number of infiltrating renal ED1+ cells. Diabetic animals had greater expression of p-Akt and mTOR, unlike ND rats. NG and SRL treatment reduced p-Akt and normalized mTOR. It is interesting that D+SRL was associated with a significant reduction of renal TGF-β1 and glomerular connective tissue growth factor. SRL treatment reduced glomerular α-smooth muscle actin overexpression and reduced significantly the mesangial matrix accumulation that is characteristic of diabetic nephropathy. In conclusion, mTOR blockade by low-dose SRL has a beneficial effect in diabetic kidney disease, suggesting that the mTOR pathway has an important pathogenic role in diabetic nephropathy.
In addition, Wang et al. (18) showed that SRL, at concentrations between 10 and 1000 nM, inhibits mesangial proliferation derived from PDGF. Lock et al. (19) demonstrated that low-dose SRL (0.1 to 0.001 ng/ml) has no proapoptotic effect but inhibits mesangial cell proliferation and collagen IV production. Zhang et al. (20) confirmed that SRL blocked laminin synthesis that was induced by high glucose in mesangial cells.

To assess the potential role of mTOR blockade on diabetic nephropathy in vivo, we performed a study in STZ-induced diabetic rats with diabetic kidney disease. We treated the animals with low-dose SRL and observed that it slowed the progression of diabetic nephropathy by reducing renal p-Akt, glomerular α-SMA–positive cells, and ECM accumulation. These effects were produced without modification of the number of infiltrating macrophages.

Materials and Methods

Animals

The experiments conducted complied with current legislation on animal experiments in the European Union and were approved by our Institution’s Ethics Committee for Investigation with Animals. Young (6 to 7 wk old) male Sprague-Dawley rats (190 to 210 g body wt) were purchased from Harlam Iberica (Iberica, Spain) and housed under controlled environmental conditions (temperature 22°C, 12-h darkness period). Animals were given free access to water and standard laboratory diet.

Induction of Diabetes and Insulin Administration

Diabetes was induced by intravenous injection of STZ (Sigma, St. Louis, MO) at 60 mg/kg body wt in 0.01 M citrate buffer (pH 4.5) after 12 h of food deprivation. Three days after STZ administration and twice a week thereafter, the rats were weighed, and tail-vein blood glucose was determined by Glucocard (Menarini, Barcelona, Spain). Insulin (Insulatard NPH, NovoNordisk (Bagsvaerd, Denmark); 1 to 5 U/d, subcutaneously) was initiated 7 d after administration of STZ to maintain blood glucose between 350 and 500 mg/dl and to avoid ketosis.

Study Groups and Renal Function

Diabetes was induced in 20 rats. Sixteen weeks after diabetes induction, animals were divided into three groups, as follows: diabetic animals that continued with blood glucose between 350 and 500 mg/dl (D; n = 8); diabetic animals that continued with blood glucose between 350 and 500 mg/dl with administration of SRL (D+SRL; 1 mg/kg per d; n = 7) by daily gavage; and diabetic animals in which normoglycemia was induced by two subcutaneous implants of insulin (Linplant, Lin Shin Canada, Inc, Scarborough, ON, Canada; D+NG; n = 5). We included six age-matched nondiabetic rats as controls (ND). Rats were placed in metabolic cages for collection of 24-h urine specimens on day 0 (before therapeutic intervention) and on day 30. Serum (sCr; mg/dl) and urine creatinine levels were determined on an autoanalyzer (Beckman Instruments, Palo Alto, CA). Urinary albumin excretion was determined by an immunoturbidimetric method in a Nefelometer II (Dade Behring, Barcelona, Spain). After a follow-up of 30 d, rats were killed and tissue samples were processed and stored as needed.

Choosing the SRL Dose

SRL at 1 mg/kg per d dose was established after previous experiments with 3 mg/kg per d. Five diabetic rats were treated at a high SRL dose. These animals died early (1 wk) as a result of intestinal ulcers and hemorrhage, with SRL levels after 1 wk of treatment at 13 ± 1 ng/ml. Therefore, 1 mg/kg per d SRL was the dose chosen for the experiments. This low dose was well tolerated without associated mortality. SRL trough levels in blood after 1 wk of treatment at 1 mg/kg per d were 2.3 ± 0.25 ng/ml (range 1.4 to 2.9 ng/ml; EIA, Imx Analyzer, Abbott Lab, Abbott, IL).

Histologic Studies

Three to 4-μm-thick tissue sections were placed in 4% formaldehyde for paraffin embedding and subsequent staining with periodic acid-Schiff (PAS) and periodic acid-silver methenamine. Masson’s trichrome staining was used to demonstrate collagen deposition. All samples were evaluated by a pathologist who was blind to the group assignment. Mesangial expansion was evaluated in periodic acid-silver methenamine– and Masson’s trichrome–stained sections as 0 (absent), 1 (mild), 2 (moderate), 3 (severe), and 4 (severe plus glomerular sclerosis). The mean glomerular volume (MGV; 10^6 μm^3) was evaluated in PAS sections according to the Weibel and Gomez formula (21), as described elsewhere (22). Interstitial fibrosis was estimated in PAS-stained sections on the 0 to 4 scale, as described (23). For evaluation of mesangial matrix area, 30 glomeruli that were cut at the vascular pole were randomly selected from each animal, and the extent of extracellular mesangial matrix was identified by the periodic acid-methenamine-positive area in the mesangium by using a soft imaging system (ANALYSIS; Münster, Germany). The mesangial matrix index represented the ratio of mesangial matrix area divided by the tuft area, as described (24).

Electron Microscopy

Electron microscopy was performed by retrieving tissue from paraffin blocks followed by deparaffinization and postfixation in 3% glutaraldehyde and 1% osmium tetroxide. Sections were stained with lead citrate and uranyl acetate and examined on a JEOL 1010 transmission electron microscope with a Bioscan digital imaging system (Gatan, Pleasanton, CA) and soft imaging system (ANALYSIS). The thickness of the basal membrane was measured by a validated simplified method (25).

Immunohistochemical Analyses

Primary and secondary antibodies were used as described previously by our group (23). α-SMA staining was evaluated as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). Connective tissue growth factor (CTGF)-positive glomerular cells per glomerular tuft were quantified in 10 glomeruli per sample. and the index of glomerular cells stained as 0 (absent), 1 (mild), 2 (moderate), 3 (severe), and 4 (severe plus glomerular sclerosis). The mean glomerular volume (MGV; 10^6 μm^3) was evaluated in PAS sections according to the Weibel and Gomez formula (21), as described elsewhere (22). Interstitial fibrosis was estimated in PAS-stained sections on the 0 to 4 scale, as described (23). For evaluation of mesangial matrix area, 30 glomeruli that were cut at the vascular pole were randomly selected from each animal, and the extent of extracellular mesangial matrix was identified by the periodic acid-methenamine-positive area in the mesangium by using a soft imaging system (ANALYSIS; Münster, Germany). The mesangial matrix index represented the ratio of mesangial matrix area divided by the tuft area, as described (24).

Determination of Rat Kidney Hepatocyte Growth Factor

Kidneys were homogenized in the hepatocyte growth factor (HGF) extraction buffer, as described previously (26). Total protein concentration was measured by the Bradford protein assay (Bio-Rad, Hercules, CA). HGF was determined with a specific commercially available ELISA kit (Rat HGF-EIA; Institute of Immunology, Tokyo, Japan). This rat HGF antibody does not cross-react with human HGF (27). Renal HGF concentration was expressed in ng/mg protein.

Western Blot Analysis of Akt

Renal tissue was sonicated in a lysis buffer that contained 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and protease inhibitor cocktail. Protein lysates (50 μg) were separated on 7% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane blocked in nonfat dry milk in Tris-buffered saline (pH 7.4)
that contained 0.1% Tween 20 (TBST) for 1 h, and incubated with phospho-Akt kinase antibody (Cell Signaling Technologies, Beverly, MA; 1:500) at 4°C overnight. Then immunoblots were washed with Tris-buffered saline (pH 7.4) that contained 0.1% Tween 20 and were incubated for 1 h with the horseradish peroxidase–conjugated antiamouse secondary antibody (1:20,000; Dako, Glostrup, Denmark). Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI). Band intensity was measured with a scanning densitometer (model GS-800; Bio-Rad). β-Actin was used as an internal loading control.

**Quantification of Renal TGF-β1, Proliferating Cell Nuclear Antigen, HGF, CTGF and mTOR by Real-Time PCR**

RNA extraction and reverse transcription were performed as described previously (23). Tissue cDNA for TGF-β1, proliferating cell nuclear antigen (PCNA), HGF, CTGF, and mTOR was amplified and quantified by real-time PCR (ABI Prism 7700; Applied Biosystems, Madrid, Spain), using the predeveloped TaqMan assay reagents method and the comparative threshold cycle method (Applied Biosystems) that were valid for our pairs of amplicons (rat TGF-β1/18S, mTOR/18S, CTGF/18S, cmet/18S, and HGF/18S), because their amplifying efficiencies were similar (data not shown). For the PCR reaction, 2 µl of each cDNA sample was mixed with 2× TaqMan Universal PCR Master Mix and 20× target primers and probe in a total reaction volume of 25 µl. For rat HGF-PCR, 2 µl of each cDNA sample was mixed with primers and probe in a total reaction volume of 25 µl to reach a final concentration of 900 nmol/L for both forward and reverse primers and 200 nmol/L for the probe. Amplification followed the universal amplification program proposed by Applied Biosystems and described previously (23). Values of normal kidneys were pooled and used as the reference value. Results were expressed as “many fold of the unknown sample” with respect to the reference value (sham group).

**Statistical Analyses**

All data are presented as mean ± SEM. A t test or ANOVA for parametric values and Mann-Whitney U test or Kruskall-Wallis test for nonparametric values were used to compare group means. All P values were two-tailed, and P < 0.05 was considered statistically significant.

**Results**

**Functional Parameters in Diabetic Kidney Disease after SRL Treatment**

Baseline and postintervention functional parameters are shown in Table 1. At baseline, all diabetic groups showed more hyperglycemia and polyuria and lower body weight than the ND group. Treatment with SRL did not modify hyperglycemia or polyuria and aggravated the body weight decline that was induced by diabetes. Continuous treatment with insulin (D+NG) induced normoglycemia, reduced diuresis, and restored body weight gain. Also, at baseline, all diabetic animals had high creatinine clearance and albuminuria. Both SRL treatment (D+SRL) and normoglycemia (D+NG) reduced creatinine clearance and albuminuria down to the level observed in ND animals.

**Effect of SRL on Glomerular Hypertrophy and Cellular Proliferation Induced by Diabetes: Modification of Renal Growth Factors TGF-β1, HGF, and CTGF**

These results are summarized in Table 2. Diabetic glomerulosclerosis (D) was characterized by glomerular hypertrophy without hyperplasia, as assessed by kidney weight, MGV, and PCNA. PCNA amounts did not reach statistical significance, although it tended to be higher in D than in ND kidneys (P = 0.08). Neither SRL (D+SRL) nor NG (D+NG) modified renal...

**Table 1. Physiological and renal functional parameters in diabetic animals: Effect of SRL and continuous insulin treatment**

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>D+NG</th>
<th>D+SRL</th>
<th>ND</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td><strong>Basal (before treatment)</strong></td>
<td></td>
<td></td>
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<tr>
<td>body weight (g)</td>
<td>342 ± 17</td>
<td>345 ± 5</td>
<td>351 ± 13</td>
<td>453 ± 66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
<tr>
<td>diuresis (ml)</td>
<td>76 ± 14</td>
<td>52 ± 19</td>
<td>79 ± 13</td>
<td>15 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td>glycemia (mg/dl)</td>
<td>493 ± 21</td>
<td>438 ± 35</td>
<td>401 ± 40</td>
<td>60 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>albuminuria (mg/24 h)</td>
<td>25.6 ± 6.9</td>
<td>20.5 ± 9.2</td>
<td>25.7 ± 7.2</td>
<td>0.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>creatinine clearance (ml/min per 100 g body wt)</td>
<td>397 ± 13</td>
<td>389 ± 36</td>
<td>409 ± 24</td>
<td>311 ± 24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>After 30 d of follow-up</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>body weight (g)</td>
<td>370 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>399 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>329 ± 11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>473 ± 69&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.03</td>
</tr>
<tr>
<td>diuresis (ml)</td>
<td>75 ± 11</td>
<td>14 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>94 ± 10</td>
<td>17 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>glycemia (mg/dl)</td>
<td>500 ± 21</td>
<td>97 ± 35</td>
<td>510 ± 40</td>
<td>60 ± 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>albuminuria (mg/24 h)</td>
<td>29 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.4 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4 ± 1.5</td>
<td>0.001</td>
</tr>
<tr>
<td>creatinine clearance (ml/min per 100 g body wt)</td>
<td>387 ± 17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>332 ± 12</td>
<td>330 ± 13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>309 ± 19</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are means ± SEM. D, diabetes; NG, normoglycemia; SRL, sirolimus; D+SRL, diabetes + SRL at 1 mg/kg per d; SRL trough level 2.3 ± 0.25 ng/ml; D+NG, maintained by insulin implants; ND, nondiabetic control.

<sup>b</sup>P < 0.05, ND versus D, D+NG, and D+SRL.

<sup>c</sup>P < 0.05, basal versus after 30 d.

<sup>d</sup>P < 0.05, ND versus D and D+SRL.

<sup>e</sup>P < 0.05, D versus D+NG, D+SRL, and ND.
hypertrophy/hyperplasia in diabetic animals. After 30 d of NG or SRL treatment, MGV and mRNA PCNA remained at the same level that was observed in diabetic animals. We also evaluated the renal expression of growth factors that are involved in diabetic kidney disease, such as HGF (renoprotective), TGF-β1, and CTGF (profibrogenic). D rats had lower HGF in kidney (D versus ND; P = 0.02). It is interesting that continuous insulin (D+NG) restored renal HGF protein (D+NG versus ND; P = 0.3) but not mRNA HGF, suggesting release of HGF from nonrenal tissue. This effect was not observed in SRL-treated animals. Renal mRNA TGF-β1 was higher in D than in ND rats (P = 0.01). SRL treatment reduced its expression (D+SRL versus D; P = 0.04) more than NG did (D+NG versus D; P = 0.2) but not as much as in ND. Renal mRNA CTGF was higher in D than in the other three groups. Glomerular CTGF staining was enhanced in diabetic rats (D versus ND; P < 0.001). Both SRL and NG reduced the number of CTGF-positive glomerular cells (Figure 1).

**SRL Did Not Modify Glomerular and Interstitial Macrophages in Diabetic Kidney Disease but Did Decrease Glomerular and Interstitial α-SMA–Positive Cells**

There were very few glomerular and interstitial macrophages in both diabetic and ND rats. The number of these cells did not change after SRL treatment, although continuous insulin treatment was linked to a slight reduction in glomerular ED1+ cells (Figure 2). α-SMA expression was enhanced in diabetic kidneys (Figures 3 and 4). In diabetic glomeruli (D), some glomerular epithelial cells expressed α-SMA (Figure 3). SRL reduced glomerular α-SMA more than normoglycemia, especially in the glomerular epithelial cells. α-SMA was slightly overexpressed in the interstitium of D kidneys. Both SRL and NG reduced interstitial α-SMA staining (Figure 4).

**SRL Prevents ECM Accumulation in Diabetic Glomerulopathy**

Mesangial matrix expansion was more prominent than interstitial fibrosis at this stage of diabetic kidney disease (Figure 5).

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### Table 2. Estimation of glomerular hypertrophy and renal hyperplasia and quantification of renal growth factors in diabetic rats: Effect of NG and SRL

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>D+NG</th>
<th>D+SRL</th>
<th>ND</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney weight (g)</td>
<td>3.2 ± 0.1b</td>
<td>2.6 ± 0.9</td>
<td>2.9 ± 0.1</td>
<td>2.3 ± 0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>MGV (10⁶ μm³)</td>
<td>5.0 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>4.6 ± 0.2</td>
<td>3.2 ± 0.2c</td>
<td>0.004</td>
</tr>
<tr>
<td>PCNA (× fold ND)</td>
<td>2.0 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>HGF (ng/mg protein)</td>
<td>71 ± 5b</td>
<td>120 ± 64d</td>
<td>82 ± 14</td>
<td>102 ± 6</td>
<td>0.003</td>
</tr>
<tr>
<td>mRNA HGF (× fold ND)</td>
<td>0.57 ± 0.38</td>
<td>0.84 ± 0.24</td>
<td>0.65 ± 0.25</td>
<td>2.75 ± 0.6c</td>
<td>0.01</td>
</tr>
<tr>
<td>mRNA TGF-β1 (× fold ND)</td>
<td>4.6 ± 1.7</td>
<td>2.7 ± 0.7</td>
<td>1.5 ± 0.1e</td>
<td>0.9 ± 0.1c</td>
<td>0.01</td>
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<tr>
<td>CTGF (+ cells/glomeruli)</td>
<td>14 ± 1f</td>
<td>5 ± 1</td>
<td>9 ± 1</td>
<td>5 ± 1</td>
<td>0.001</td>
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<tr>
<td>mRNA CTGF (× fold ND)</td>
<td>3.9 ± 1.9f</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.5</td>
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*MGV, mean glomerular volume; CTGF, connective tissue growth factor; HGF, hepatocyte growth factor; PCNA, proliferating cell nuclear antigen.

b P < 0.05, D versus D+NG and ND.

c P < 0.05, ND versus D, D+NG, and D+SRL.

d P < 0.05, D + NG versus D+SRL.

e P < 0.05, D + SRL versus D.

f P < 0.05, D versus D + NG, D+SRL, and ND.

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Figure 1. Glomerular connective tissue growth factor (CTGF) expression in diabetes. Effect of sirolimus (SRL) and normoglycemia (NG). Diabetic kidneys had more resident glomerular cells expressing CTGF, which was also enhanced in juxtaglomerular arterioles (D, arrow). The peripheral glomerular distribution suggests that these CTGF+ cells are mainly epithelial cells. Both NG (D+NG) and SRL (D+SRL) reduced glomerular CTGF expression at the level of age-matched nondiabetic (ND) rats. See also Table 2. Magnification, ×400.

D rats showed a significant increase of mesangial matrix expansion (D versus ND; P = 0.001) and mild interstitial fibrosis (D versus ND; P = 0.006; Figure 5A). Furthermore, the percentage of mesangial matrix area was assessed by morphometry and was higher in D than ND (P = 0.0002; Figure 5B). SRL treatment decreased ECM accumulation in diabetic kidney, achieving values of mesangial matrix expansion, percentage of mesangial matrix area, and interstitial fibrosis similar to ND. Despite continuous insulin therapy (D+NG), NG did not re-

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**Table 2.** Estimation of glomerular hypertrophy and renal hyperplasia and quantification of renal growth factors in diabetic rats: Effect of NG and SRL

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duce glomerular ECM accumulation to the same extent as SRL (Figure 5). The glomerular basement membrane (GBM) thickness was increased in diabetes. Both SRL treatment and NG reduced GBM thickness to the same level observed in ND rats (Figure 6).

**Effect of mTOR Blockade on Levels of Phosphorylated Akt and mRNA mTOR in Diabetic Kidney Disease**

In diabetic kidneys (D), there is intense p-Akt overexpression (Figure 7A). p-Akt was markedly lower in the D+SRL and D+NG than in the D group (Figure 7B). We found enhanced mRNA mTOR in diabetic kidneys. SRL treatment and NG normalized mRNA mTOR (Figure 7C).

**Discussion**

Treatment of diabetic nephropathy by immunosuppressants has emerged as a new challenge because of the findings of Sassy-Prigent et al. (28) and Utimura et al. (7). Sassy-Prigent et al. (28) showed that very early glomerular macrophage recruitment occurred in STZ-induced diabetic rats and could be related, in a secondary way, to late glomerular ECM accumulation. Utimura et al. (7) found that treatment with MMF reduced macrophage infiltration into the diabetic kidney, which was associated with a later prevention of glomerular sclerosis. Therefore, these authors hypothesized that MMF may have a therapeutic effect on diabetic nephropathy as a result of reduction of mesangial cell proliferation and/or minimization of renal inflammatory cells. Our results suggest that SRL acts differently, because it did not modify infiltration of inflamma-

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**Figure 2.** Estimation of infiltrating renal ED1+ cells. (A) There were few macrophages infiltrating both glomeruli (▵) and interstitium (■) in established diabetic nephropathy. Therefore, D and ND groups had similar numbers of ED1+ cells. Intensive insulin therapy (D+NG) was associated with a slight reduction of glomerular macrophages (*P = 0.01 versus ND), whereas SRL was not. (B) A representative image shows ED1+ cells in glomeruli and interstitium of diabetic kidney Magnification, ×400.

**Figure 3.** Estimation of resident glomerular α-smooth muscle actin (α-SMA)-positive cells. (A) Diabetic kidneys had marked α-SMA staining in glomeruli, which was reduced by SRL rather than NG (*P < 0.05, D versus D+SRL and ND). (B) α-SMA was constitutively expressed by smooth muscle cells of the vascular wall (black arrow, glomerular arterioles). D+NG rats showed intense α-SMA staining in glomerular epithelial cells of the Bowman capsule (red arrow). SRL treatment (D+SRL) reduced glomerular expression of α-SMA. Magnification, ×630.
tory cells. Moreover, we found that hyperglycemia was not associated with a late increase of ED1^+ cells in glomeruli and renal interstitium. Discrepancies between the two studies could be attributed to differences in the experimental model. In fact, Utimura et al. (7) performed uninephrectomy in their diabetic animals to accentuate histologic lesions. Renal mass reduction per se may produce and aggravate renal inflammatory changes (29).

Experimental and clinical data showed metabolic control as the main tool to prevent the progression of diabetic nephropathy at early stages (30). Our data showed that NG reduced hyperfiltration and microalbuminuria at the level of renal function observed in ND rats. An interesting finding was that SRL impaired weight gain in diabetic rats without affecting insulin requirements and/or diabetic control. In fact, we (31) and others (32) observed previously low body weight in SRL-treated rats. Along these lines, Um et al. (33) showed that absence of S6K1, an effector pathway of mTOR, protects against age- and diet-induced obesity in mice; this offers a potential explanation for the consistent effect of SRL on weight gain. However, whether this effect is related to SRL directly or is associated with low food consumption is not known. This metabolic consequence did not account for the beneficial action of SRL, because SRL treatment did not improve glycemic control and insulin requirements in diabetic rats. Therefore, although hyperglycemia was maintained, SRL treatment significantly improved renal function parameters in a rat model of established diabetic kidney disease.

In diabetic nephropathy, there is a two-stage mesangial growth response, with early proliferation and subsequent hypertrophy (34). Our results showed that cellular proliferation in the kidney was not enhanced several months after diabetes induction. Moreover, we found that treatment with low-dose SRL did not modify cell proliferation in diabetic kidney disease. The absence of mesangial cell proliferation several weeks after diabetes induction is consistent with previous reports (34). However, glomerular hypertrophy was significantly higher in diabetic animals, as expected. After 4 wk of treatment, neither NG nor SRL reduced glomerular hypertrophy. These results do not exclude the possibility that a more prolonged intervention would reduce glomerular hypertrophy.

Today, there is growing evidence that regulation of CDK and their specific inhibitors (10), as well as the PI3-K/Akt pathway, have an important role in the renal hypertrophy of diabetic nephropathy (14). Importantly, mTOR is involved, at least in part, in the regulation of both pathways. On the one hand, the mTOR blockade theoretically could increase CDK inhibitors (13) and aggravate diabetic nephropathy, whereas on the other hand, its effect could be beneficial because it interferes with the Akt/mTOR pathway. Indeed, in vitro studies showed that SRL inhibits mesangial cell hypertrophy (14) and ECM production (20) that is induced by hyperglycemia. Consistent with previous data (15), we found that p-Akt and mTOR increased in diabetic kidneys. It was reported previously that hyperglycemia and other stimuli such as angiotensin II activated Akt/mTOR (35). In our study, NG and especially SRL treatment attenuated Akt phosphorylation, which suggests that mTOR activation also amplifies Akt pathway activation by a positive feedback. These results were consistent with a previous report from Risterer et al. (36) in which mTOR blockade reduced Akt phosphorylation in vascular endothelial growth factor–stimulated endothelial cells. Unlike the full mortality observed in diabetic nephropathy when SRL was administered at the high dose, a low SRL dose protected the kidney. Therefore, it can be hypothesized that the SRL dose is crucial to the therapeutic effect.

Lock et al. (19) showed that low-dose SRL inhibits mesangial cell collagen IV production without increasing apoptosis.

We assessed some growth factors that have a pathogenic role in diabetic glomerulopathy. We found a reduction of renal HGF and an increase in glomerular CTGF and renal TGF-β1. Both TGF-β1 and hyperglycemia induce CTGF upregulation in mesangial cells (2). In addition, it was found that angiotensin II, an important mediator of diabetic nephropathy, induces CTGF
renal expression (37). This growth factor has an important function in ECM accumulation in diabetic nephropathy (18). However, a reduction in renal HGF contributed to unsettling the equilibrium between HGF and other profibrotic growth factors such as TGF-β1 and CTGF. We recently demonstrated that human HGF gene therapy regresses glomerular sclerosis in diabetic nephropathy (38). NG restored renal HGF protein but not mRNA HGF, suggesting release of HGF from nonrenal tissues and HGF downregulation by maintained high glucose, as previously reported (39). Accordingly, SRL did not modify blood glucose and did not restore HGF. Conversely, both NG and SRL partially reduced glomerular CTGF and TGF-β1, suggesting that both high glucose and the mTOR pathway have an important role in upregulation of these growth factors.

In diabetic glomeruli, mesangial cell activation, characterized by α-SMA induction by growth factors, is regarded as a central

Figure 5. Mesangial matrix accumulation and interstitial fibrosis in diabetic kidney. Effect of SRL and NG. (A) D rats showed higher mesangial expansion (口) and interstitial fibrosis (■) than ND. SRL treatment was associated with a significant decrease in mesangial matrix expansion and interstitial fibrosis. NG did not significantly reduce the amount of sclerosis ($P < 0.05$, mesangial matrix expansion in D versus D+SRL and ND; $P < 0.05$, mesangial matrix expansion in D+SRL versus D and D+NG; $P < 0.05$, interstitial fibrosis in D versus D+SRL and ND). (B) Percentage of mesangial matrix was evaluated using the ratio of silver-methenamine–positive area to tuft area (mesangial matrix index) in 30 glomeruli of each group ($P < 0.01$, D versus D+SRL, D+NG, and ND; $P = 0.01$ D+SRL versus D+NG). (C) A representative Masson’s trichrome–stained glomerulus from each group. Magnification, ×400.
event that leads to ECM accumulation. Diabetic kidneys showed α-SMA-positive glomerular cells, including glomerular epithelial cells of the Bowman capsule, and renal interstitial cells, suggesting that there was some degree of epithelial to mesenchymal transition (EMT). In fact, EMT is emerging as a major pathway that leads to generation of the matrix-producing effector cells in diseased kidney (40). We found several factors for EMT in diabetic glomerulopathy, such as upregulation of

Figure 6. Glomerular basement membrane (GBM) thickness in diabetic kidneys. Effect of SRL and NG. (A) Diabetic animals showed an increase in GBM with respect to ND. Both SRL and NG reduced GBM thickness down to the level observed in ND (*P < 0.001, D versus D+SRL, D+NG, and ND). (B) Representative glomerular loop electron microscopy photographs from each group. Red arrows signal GBM thickness. Magnification, ×25,000.
TGF-β1 (a major stimulus of EMT) and activation of Akt, which is involved in the loss of epithelial adhesion observed at the initial stages of EMT (41). SRL treatment reduced glomerular-SMA induction and consequently ECM accumulation in diabetic kidney. D+NG rats showed some degree of glomerular and interstitial myofibroblast staining with glomerular sclerosis. These results suggest that >4 wk of NG would be required to obtain histologic benefit.

Conclusion
We observed that mTOR blockade by SRL had a beneficial effect on established diabetic kidney disease in rats, suggesting that the mTOR pathway has an important pathogenic role in diabetic nephropathy. Given our findings and the potential role of CDK inhibitors in diabetic nephropathy, further studies are needed to investigate mechanisms for mTOR triggering and mTOR effector pathways before SRL can be considered a therapeutic agent in diabetic nephropathy.

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Figure 7. Phosphorylated Akt and mRNA mammalian target of rapamycin (mTOR) in diabetic kidneys. (A) Western blot analysis of the p-Akt in two samples from each experimental group. (B) Graph of the values (p-Akt/β-actin ratio) from each experimental group (*P < 0.05, D versus D+SRL, D+NG, and ND). Data are mean ± SEM of at least three independent experiments. (C) Renal mRNA mTOR (*P < 0.03, D versus D+SRL, D+NG, and ND).


