The PI3K/Akt/mTOR pathway is activated in murine lupus nephritis and downregulated by rapamycin

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Abstract

Background. The mammalian target of rapamycin (mTOR) inhibitor, rapamycin, has been shown to inhibit the progression of murine lupus nephritis by virtue of its potent immunosuppressive properties. The phosphoinositide-3-kinase (PI3K)/Akt pathway is a major upstream activator of mTOR and has been implicated in the propagation of cancer and autoimmunity. However, the activation status of the PI3K/Akt/mTOR pathway in lupus nephritis has not been studied so far.

Methods. In NZBW/F1 female mice, we examined the glomerular expression of Akt and mTOR by immunofluorescence and western blot. We also searched for specific phosphorylations of these kinases known to ensue during activation of the PI3K/Akt/mTOR pathway. In parallel, we examined the therapeutic role of rapamycin either before or after the development of overt lupus nephritis.

Results. We found that in untreated mice, as opposed to healthy controls, Akt and mTOR were over-expressed and phosphorylated at key activating residues. Rapamycin prolonged survival, maintained normal renal function, normalized proteinuria, restored nephrin and podocin levels, reduced anti-dsDNA titres, ameliorated histological lesions, and reduced Akt and mTOR glomerular expression activation.

Conclusions. These results suggest that: (i) the PI3K/Akt/mTOR pathway is upregulated in murine lupus nephritis, thus justifying treatment with rapamycin; (ii) rapamycin not only blocks mTOR but also negatively regulates the PI3K/Akt/mTOR pathway; and (iii) rapamycin is an effect-
ive treatment of murine lupus nephritis. Examination of the PI3K/Akt/mTOR pathway may offer new insights into the pathogenesis of lupus nephritis in humans and may lead to more individualized and less toxic treatment.

Keywords: Akt; lupus nephritis; mTOR; rapamycin

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by increased presence of autoreactive CD4+ memory cells which induce polyclonal B-cell activation and expansion, leading to hyper-gammaglobulinaemia and production of anti-nuclear and anti-DNA antibodies. Approximately half of the patients develop glomerulonephritis (GN) due to immuno-complexes deposited in the kidney, subsequent activation of complement cascade, and infiltration with T cells and macrophages [1–3].

One of the best characterized models of murine lupus is the New Zealand Black White (NZBW)/F1 hybrid which develops overt SLE with features reminiscent of those observed in human SLE, such as high anti-dsDNA titres and a high incidence of GN [4]. These mice begin to develop GN at the age of 5 months, and they die of renal failure by the age of 10 months.

In some recent studies [5–9], the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, improved the clinical course of lupus nephritis (LN) in NZBW/F1 mice. Rapamycin has also been shown to prolong survival and to reduce inflammatory changes in several organs, including the kidneys, in the MRL/lpr model of murine SLE [10]. Moreover, rapamycin reduced disease activity in nine patients with SLE who had been treated unsuccessfully with other immunosuppressive medications [11].

Conventionally, it has been considered that rapamycin acts by inhibiting IL-2-mediated signal transduction pathways, thus preventing cell cycle progression from the G1 to the S phase in T cells and several other cell lines [12]. Since transcription of IL-2 is downregulated in SLE [13], the beneficial role of rapamycin must be exerted via pathways different from mere IL-2 inhibition.

The phosphoinositol-3-kinase (PI3K)/Akt pathway is a major upstream activator of mTOR and has been implicated in the propagation of cancer and autoimmunity [14–16]. The PI3K/Akt/mTOR pathway has been found to be upregulated in lupus B cells [2–4,17] and T cells [18]. In a recent study, ~50% of the genes curated as lupus disease genes, from both humans and rodents, could be linked to the mTOR pathway [9].

Activation of the pathway begins with the binding of certain ligands such as growth factors, hormones, cytokines and mitogens to their respective receptors, resulting in recruitment of PI3K and subsequent phosphorylation of Akt at Thr308 (Thr308pAkt) [19–21]. Full activation of Akt requires a second phosphorylation at Ser473 (Ser473pAkt), which leads to a 5-fold increase in the activity of Akt [22]. Although the Ser473 phosphorylation can be mediated by several kinases [23–27] (collectively referred to as PDK2s), recent evidence suggests that the most possible candidate for the role of PDK2 is mTOR in the so-called mTORC2 complex [22].

Akt exerts its effects (anti-apoptosis, transcription factor regulation, cytoskeleton regulation and cell proliferation) by phosphorylating a plethora of downstream effectors including mTOR at Ser2448 (Ser2448pTOR), in the mTORC1 complex [14,28–31]. There is a growing body of evidence [3,15–17,32,33] indicating that activation of the PI3K/Akt/mTOR pathway is implicated in the pathogenesis of SLE. However, at the glomerular level, a direct activation of the pathway has not been demonstrated so far.

In the present study, we investigated the glomerular expression activation of the PI3K/Akt/mTOR pathway in proteinuric mice with full-blown LN as well as non-proteinuric and rapamycin-treated NZBW/F1 female mice. We also tested some recently published data from in vitro experiments dealing with the nephrotoxicity of rapamycin, in particular the possible effect of rapamycin on the slit diaphragm proteins nephrin and podocin.

Materials and methods

Animals

Female NZBW/F1 mice were obtained from Foundation of Research and Technology, Crete, Greece. The experiments were carried out in accordance with the current legislation on animal experiments in the European Union and approved by our institution’s Safety and Ethics Committee for Animal Research. Mice had free access to water and a standard laboratory diet, and were housed in a room with constant temperature (25°C) and a 12-h dark/light cycle.

Study design and follow-up

Thirty-two mice were divided into four groups: (i) Preventive group (PG), in which mice (n = 6) were treated with intraperitoneal (i.p.) rapamycin (purchased from LC Laboratories, MA, USA) at a dose of 1 mg/kg every other day (e.o.d.), before the development of pathological proteinuria (<4 mg/day) starting approximately at age Month 4; (ii) Therapeutic group (TG), in which mice (n = 6) were given i.p. rapamycin, 1 mg/kg e.o.d. 1 week after the development of severe proteinuria (>20 mg/day verified twice) starting approximately at age Month 5–6; (iii) Healthy control group (HC), in which mice (n = 5) were sacrificed after the development of proteinuria (approximately at 4 months); and (iv) an additional group of 15 untreated mice (UG) served as the lupus control group. As many mice in the UG died early, six of them were sacrificed when they appeared severely ill (age Month 6–7), in order to obtain kidney tissue for further studies. Treatment and follow-up lasted up to the age of 8 months. For 24-h urine collections, mice were placed in metabolic cages with free access to water, starting from the age of 3 months and weekly thereafter. Body weight was determined weekly, and rapamycin dose was adjusted accordingly. The treatment scheme was chosen based on previous experiments with different rapamycin doses and administration intervals aiming at whole blood trough levels between 5 and 10 ng/mL. At the end of the follow-up, surviving animals were sacrificed, and kidneys were processed for histological studies, western blot and real-time polymerase chain reaction (RT–PCR). Blood was obtained through cardiac puncture at the time of sacrifice.

Proteinuria was detected with Coomassie blue staining of urine samples in SDS gel electrophoresis. Urinary protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Serum creatinine levels were determined by an auto-analyser. Anti-dsDNA antibodies were measured using a commercially available ELISA kit (Alpha Diagnostic International, TX, USA). Rapamycin levels were determined by EIA in whole blood (Bmx analyser, Abbott, USA).
**Table 1.** Real-time RT–PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td>Nphs1</td>
<td>Forward</td>
<td>5'-GCGAGGCCACTTCGGAAA-3'</td>
<td>80 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CAGTTCCTCCACGGAAC-3'</td>
<td></td>
</tr>
<tr>
<td>Nphs2</td>
<td>Forward</td>
<td>5'-GTCGCAAAATGTCTTCTC-3'</td>
<td>117 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGCAACTTTCATCTTCTG-3'</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>5'-AAATTGCCTGGCTTGAGTCTGA-3'</td>
<td>84 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATGCCTTCTACACCTCT-3'</td>
<td></td>
</tr>
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Nphs1, nephrin; Nphs2, podocin; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

**Western blot analysis (WB)**

Kidney cortex tissue obtained at sacrifice was homogenized in RIPA-buffer containing protease inhibitors and stored in –80°C until analysis.

Antibodies used in WB included: guinea pig nephrin pAb (1:500) (Progen, Germany), rabbit podocin pAb (1:500) (Abcam, UK), rabbit Akt pAb (1:500), rabbit Ser473pAkt mAb (1:500), rabbit Thr308pAkt mAb (1:500) (Cell Signaling, USA) and anti-actin mAb (1:3000) (Sigma, EU). Protein bands were normalized to actin expression using the ImageJ densitometry analysis system (http://rsb.info.nih.gov/ij/).

**Real-time quantitative RT–PCR**

Total RNA was extracted from isolated kidney cortex using the acid guanidium-thiocyanate-phenol-chloroform single-step procedure with Trizol Reagent (Life Technologies; UK). One microgram of total RNA was reverse transcribed (Superscript II; Gibco) and amplified by RT–PCR. iTaq SYBR Green Supermix (Bio-Rad) was used for the reactions. Primer sequences are listed in Table 1. Products were normalized according to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression, and analysis was performed using the 2–ΔΔCt method. Measurements were performed using the ABI Prism 7000 (Applied Biosystems, USA). All samples were tested in duplicates.

**Microscopic studies**

For photon microscopy (PM), kidney tissue was fixed in neutral formalin 10%. Multiple sections were stained with haematoxylin–eosin and PAS staining and were examined by a renal pathologist blinded to group assignment.

For electron microscopy (EM), kidney tissue was fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and embedded in Epon812. Ultra thin sections (40–50 nm) were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (JEM 100C; JEOL, Japan). Microphotographs were obtained using an ES500W Enlargehen Camera and were analysed by the DigitalMicrograph software (Gatan, Germany) for measurement of the podocyte foot processes width (FPW) as described in previous studies [34].

For immunofluorescence studies (IF), kidney tissue was embedded in OCT compound (Sakura, USA), snap-frozen in liquid nitrogen and stored in –80°C until examination. Five-micrometre-thick cryosections were fixed in 4% paraformaldehyde and incubated with Triton X-100 (Sigma, EU). In order to avoid unspecific binding, sections were blocked with normal goat serum 5% and goat affinity-purified IgG Fab fragments (Jackson Immunoresearch, EU) versus mouse IgG(H+L) in a concentration of 0.1 mg/mL for 90 min. The primary antibody was applied overnight, while negative controls were obtained by omitting the primary antibody. Dilution for nephrin was 1:250, for Thr308pAkt was 1:800, for mTOR and Ser2448pTOR was 1:50 (both from Cell Signaling), for CD31 was 1:10 (rat CD31 pAb BD Pharmigen, NJ, USA), and for all other antibodies, it was 1:100. Appropriate purified goat secondary antibodies were applied (Alexa Fluor 488 and 555, Molecular Probes, Invitrogen, CA, USA). At least 20 glomeruli were examined per mouse. The intensity of the fluorescence was scored on a scale of 0–3+, where 0 = absent, 1+ = mild, 2+ = moderate and 3+ = strong staining.

**Statistical analysis**

For statistical analyses, the SPSS/PC 17 statistical package was used. Continuous variables are expressed as mean ± SD. One-sample Kolmogorov–Smirnov test was used for evaluation of normality of data. Analysis of variance (ANOVA) with post hoc Dunnett’s correction was performed to compare continuous variables. The Mann–Whitney U-test was used for non-parametric comparisons. Overall survival was analysed by the Kaplan–Meier method. Differences were considered significant for a P < 0.05 (two-tailed).

**Results**

**Akt phosphorylation at Thr308 is highly increased in lupus nephritis**

The phosphorylation of Akt at Thr308 was evaluated as a surrogate marker of PI3K activation. In the HC and treated groups of mice, there was a low expression level of Thr308pAkt by WB (Figure 1). In the same groups of protein levels were significantly lower in the untreated group of mice, while total Akt, 308pAkt and 473pAKT were significantly higher in the same group.*P < 0.001 for all proteins, between untreated group and all other groups.

![Fig. 1](https://academic.oup.com/ndt/article-abstract/26/2/498/1893724/249816832)
mice, it was shown by IF either an absence or a low level (0–1+) of expression of Thr308pAkt in the vast majority of glomeruli (Figure 2B, E and H, and Table 2). On the other hand, in the UG, the expression of Thr308pAkt was significantly increased both by WB (Figure 1) and by IF (Table 2) in the mesangium and the capillary loops (Figure 2K). Some cortical tubular segments from all study groups presented a moderate to intense brush border staining for Thr308pAkt (Figure 2B and E).

Table 2. Percentage of glomeruli with intensity scores >1+, for total Akt, Thr308pAkt, Ser473pAkt, total mTOR and Ser2448pTOR

<table>
<thead>
<tr>
<th></th>
<th>PG (%)</th>
<th>TG (%)</th>
<th>HC (%)</th>
<th>UG (%)</th>
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<tbody>
<tr>
<td>Total Akt</td>
<td>4.8</td>
<td>5.3</td>
<td>0</td>
<td>67.1*</td>
</tr>
<tr>
<td>Thr308pAkt</td>
<td>1.2</td>
<td>5.7</td>
<td>0</td>
<td>83.1*</td>
</tr>
<tr>
<td>Ser473pAkt</td>
<td>6.3</td>
<td>5.9</td>
<td>0</td>
<td>62.7*</td>
</tr>
<tr>
<td>mTOR</td>
<td>1.2</td>
<td>5.7</td>
<td>0</td>
<td>86.9*</td>
</tr>
<tr>
<td>Ser2448pTOR</td>
<td>0</td>
<td>2.6</td>
<td>0</td>
<td>77.2*</td>
</tr>
</tbody>
</table>

PG, preventive group; TG, therapeutic group; HC, healthy controls; UG, untreated group. Values represent percentages (within groups) of glomeruli with immunofluorescence intensity scores of 2+ or 3+. The Mann–Whitney U-test did not show statistically significant difference among the PG, TG and HC groups for any of the kinases.

* P < 0.001 UG versus all other groups.

Total Akt is increased in murine LN

The glomerular expression of total Akt was significantly increased both by WB (Figure 1) and by IF (Table 2) in the mesangium and the capillary loops (Figure 2K). Some cortical tubular segments from all study groups presented a moderate to intense brush border staining for Thr308pAkt (Figure 2B and E).

The Akt kinase is fully activated in murine LN

The second phosphorylation of Akt at Ser473 was used as a marker of complete Akt activation. In the HC and treated groups of mice, there was either an absence or a low level (0–1+) of glomerular expression of Ser473pAkt in the vast majority of glomeruli (Figure 2B, E and H, and Table 2). In the contrary, the untreated group of mice (J, K, L) showed intense glomerular staining for total Akt, Thr308pAkt and Ser473pAkt. Negative controls (NC) did not show any background staining (M, N, O). HC, healthy controls; PG, preventive group; TG, therapeutic group; UG, untreated group. Values represent percentages (within groups) of glomeruli with immunofluorescence intensity scores of 2+ or 3+. The Mann–Whitney U-test did not show statistically significant difference among the PG, TG and HC groups for any of the kinases.

* P < 0.001 UG versus all other groups.
majority of the glomeruli. In contrast, in the UG, the glomerular expression of Ser473pAkt was significantly (P < 0.001) increased, indicating complete activation of Akt by upstream kinases (Figures 1 and 2, and Table 2).

The mTOR kinase is upregulated in the glomeruli of mice with LN

In analogy to Akt, the glomerular expression of mTOR was increased in untreated mice as was shown by IF (Figure 3G and Table 2). The spatial distribution of mTOR was similar to that of Akt, in that both were confined mainly in the mesangium and the capillary loops (Figures 2G and 3G). On the other hand, healthy controls and treated mice showed a low level of glomerular expression of mTOR in the periphery of the capillary loops (Figure 3A, C and E, and Table 2).

The majority of the glomeruli in the UG presented moderate to intense staining for Ser2448pTOR as opposed to HC and treated mice (P < 0.001, Figure 3H), indicating downstream activation of mTOR by Akt. Some tubular segments from all study groups presented a moderate to intense cytoplasmic staining for mTOR and Ser2448pTOR (Figure 3B). We performed double-labelling studies using the endothelial marker CD31 (also known as platelet endothelial cell adhesion molecule-1, PECAM) or the podocytes marker nephrin along with Ser2448pTOR. We could demonstrate that the increased staining for Ser2448pTOR in the capillary loops of the affected glomeruli was due to increased expression by both podocytes and endothelial cells (Supplementary Figures 1 and 2; See online supplementary material for a colour version of these figures).

Rapamycin prolongs survival, preserves normal renal function and reduces anti-dsDNA levels

All treated mice survived to the 8-month point when they were sacrificed. To the contrary, in UG, only two of the original nine mice (22%) survived to the 8-month point (log-rank test = 7.4; P = 0.02).

Proteinuria remained within normal limits (<4 mg/day) in the PG at all times. Complete normalization of proteinuria (from 30.6 ± 5.3 to 2.3 ± 1.4 mg/day) was noticed in the TG, 4–5 weeks after initiation of rapamycin treatment. Proteinuria in the UG was not selective (Figure 4) and exceeded 30 mg/day at the end of the study (Table 3).

Kidney mass, serum creatinine and anti-dsDNA levels were significantly lower in treated mice and HCs, as compared with the UG (Table 3). In particular, all mice in the HC and PG had normal renal function (serum creatinine ≤0.3 mg/dL). In the TG, only one mouse developed abnormal creatinine levels (0.5 mg/dL) despite restoration of proteinuria. Conversely, all mice in the UG had increased creatinine levels ranging from 0.5 to 0.9 mg/dL. With the predefined rapamycin dose used, blood trough levels lay within the preset therapeutic target levels in all treated mice.

Rapamycin prevents or alleviates histological lesions

Specimens from the UG presented mesangial and endocapillary proliferation, hyaline thrombi, subendothelial deposits and wire loops, while some glomeruli showed severe unselected proteinuria.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Immunofluorescence in kidney specimens from the healthy control group (A, B), the preventive group (C, D) and the therapeutic group of mice (E, F) showed weak peripheral glomerular staining for mTOR and Ser2448pTOR. Arrows indicate fluorescence for mTOR in some tubules. In the contrary, the untreated group of mice (G, H) showed intense glomerular staining for mTOR and Ser2448pTOR. Negative controls (NC) did not show any background staining (I, J). HC, healthy controls; PG, preventive group; TG, therapeutic group; UG, untreated group (original magnification ×400).

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Coomassie blue staining of urine samples in SDS gel electrophoresis. Samples of two mice from each group are shown. HC, healthy controls; PG, preventive group; TG, therapeutic group; UG, untreated group; BSA, bovine serum albumin. Healthy controls and treated mice did not display traceable proteinuria, while untreated mice showed severe unselected proteinuria.
Table 3. Clinical outcomes and mRNA levels of nephrin and podocin

<table>
<thead>
<tr>
<th></th>
<th>PG</th>
<th>TG</th>
<th>HC</th>
<th>UG</th>
</tr>
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<tbody>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.24 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.32 ± 0.12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.28 ± 0.04&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.58 ± 0.24</td>
</tr>
<tr>
<td>Anti dsDNA (kU/mL)</td>
<td>28.6 ± 4.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td>26 ± 3.8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>30.3 ± 10.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>44.3 ± 9.8</td>
</tr>
<tr>
<td>Urinary protein at sacrifice (mg/day)</td>
<td>2.7 ± 1.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.3 ± 1.4&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2 ± 0.9&lt;sup&gt;i&lt;/sup&gt;</td>
<td>32.2 ± 25</td>
</tr>
<tr>
<td>Spleen mass (g)</td>
<td>0.20 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.15 ± 0.04&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.17 ± 0.11&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.26 ± 0.1</td>
</tr>
<tr>
<td>Nephrin mRNA</td>
<td>3.26 ± 0.65&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.36 ± 0.41&lt;sup&gt;h&lt;/sup&gt;</td>
<td>3.85 ± 0.6&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.72 ± 0.7</td>
</tr>
<tr>
<td>Podocin mRNA</td>
<td>2.95 ± 0.8&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.42 ± 0.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3.48 ± 0.7&lt;sup&gt;g&lt;/sup&gt;</td>
<td>1.73 ± 0.3</td>
</tr>
</tbody>
</table>

All P-values are vs. UG. mRNA values are adjusted to a housekeeping gene expression (glyceraldehyde-3-phosphate dehydrogenase).

PG, preventive group; TG, therapeutic group; HC, healthy controls; UG, untreated group.

Discussion

One of the best studied downstream substrates of Akt is the serine/threonine kinase mTOR in the protein complex called mTORC1, which is sensitive to rapamycin. Akt can directly activate mTORC1 by phosphorylating the Ser2448 residue of mTOR [28–31]. Akt can also activate mTORC1 indirectly, via the tuberous sclerosis proteins complex pathway [20,35]. Subsequent activation of mTORC1 results in the phosphorylation of S6 kinase (p70S6K) as well as other kinases controlling protein synthesis, gene transcription and ribosome biogenesis [21,36].

mTOR is generally considered a downstream effector of Akt; nevertheless, mTOR in mTORC2 has recently been shown to act as an upstream activator of Akt by phosphorylating Akt at Ser473, thereby providing a level of positive feedback on the pathway [22]. Although mTORC2 is considered insensitive to rapamycin inhibition, recent evidence suggests that prolonged treatment with rapamycin affects mTORC2 assembly and action towards Akt [37]. Finally, the p70S6K can also regulate the pathway by catalysing an inhibitory phosphorylation on insulin receptor substrate (IRS) proteins preventing further activation of PI3K by growth factor receptors, setting thus a negative feedback loop in the PI3K/Akt/mTOR pathway [21].

Examination of the phosphorylation status of Akt can be a useful diagnostic and research tool, since phosphorylation of Akt at Thr308 serves as a surrogate marker of PI3K activation [38–41], while the second phosphorylation at Ser473 can be an indicator of complete Akt activation towards mTOR and other downstream substrates. Furthermore, phosphorylation of mTOR at Ser2448 offers a direct evidence for activation of mTOR by Akt [28]. At the podocyte level, a linking pathway between nephrin and PI3K/Akt has recently been reported to regulate cytoskeleton [42].

In the present study, Akt and mTOR showed a low level of expression and phosphorylation in non-proteinuric mice (HC), indicating that the PI3K/Akt/mTOR pathway displays a low expression activation level in normal glom-
Fig. 5. PAS stain. Glomeruli, tubules and interstitium in the healthy control group (A) and the preventive group (B) were within normal limits. In the therapeutic group, there were rare hyaline thrombi and mild mesangial widening (C). In untreated mice, glomeruli showed various degrees of mesangial expansion, subendothelial deposits, wire looping and segmental areas of solidification; some tubules were atrophic, dilated and filled with proteinaceous material (D, E, F). There were also areas of perivascular and interstitial inflammation (E) [original magnification: ×300 (A), ×350 (B), ×350 (C) and ×400 (D, E, F)].
Fig. 6. Electron microscopy: healthy controls (A) and mice in the preventive group (B, C) maintained normal glomerular architecture and podocyte foot process width. There was a complete absence of mesangial, subendothelial and subepithelial deposits in the preventive group (C) and scarce subepithelial deposits (arrow) in the therapeutic group (D). The least affected glomeruli in the untreated group (E) showed extended mesangial deposits (arrow) with moderate to severe mesangial expansion. More severely affected glomeruli appeared with complete occlusion of the capillary lumina with massive subendothelial and subepithelial deposits (F, H), endocapillary proliferation (arrow in G) and complete fusion of foot processes (F, G, H).
eruli. On the other hand, the tubular expression of the pathway appears upregulated in cortical segments, possibly due to the increased proliferation and metabolic activity of renal tubules.

In contrast to HC, mice with full-blown LN (UG) displayed increased glomerular expression activation of both kinases along with severe mesangial and endocapillary proliferation. The increased expression of Thr308pAkt in glomerular cells indicates activation of PI3K in LN, most likely by growth factors and/or cytokines. The increased expression of Ser473pAkt indicates that Akt is fully activated and that its downstream effectors have also to be activated. Indeed, the increased expression of Ser2448pTOR offers further evidence for activation of Akt and comprises a direct evidence for activation of mTOR. Considered together, these findings show an upregulation of the PI3K/Akt/mTOR pathway in glomerular cells in active LN. It is therefore reasonable to assume that the hyperplastic lesions seen in LN may be linked to upregulation of the PI3K/Akt/mTOR pathway, especially when considering the established role of Akt and mTOR in promoting cellular proliferation [14]. Additionally, the mice with overt LN showed reduced expression and disturbed association of nephrin and podocin in line with the appearance of severe proteinuria and the retraction of podocyte foot processes.

The inhibition of mTOR by rapamycin prevented or ameliorated the course of the disease, reduced the glomerular protein expression and the effective concentration of Akt and mTOR, and restored the expression and association of nephrin and podocin. The therapeutic effect of rapamycin clearly shows that not only is the pathway upregulated but also its inhibition is beneficial in ameliorating murine LN. It also appears that rapamycin not only blocks the downstream signal transmission but also affects the upstream components of the pathway by reducing the glomerular expression activation of Akt and mTOR. The increased Akt and TOR kinase levels in lupus glomeruli represent an interesting finding of the present study that necessitates further research. A hypothesis that could explain this phenomenon is that the chronic activation of the pathway results in altered transcriptional or translational control of both kinases leading to over-expression in lupus kidneys. Similar findings have been described in diabetic nephropathy. For example, in the study by Zdychová et al. [43], the kidney concentrations of total Akt, mTOR, Ser473pAkt and Ser2448pTOR were all increased in diabetic obese Zucker rats. In one more study, it was shown that mTOR mRNA was increased in diabetic kidneys and restored to normal after rapamycin treatment [44]. Although there is not much evidence for a direct regulation of these kinases at the transcriptional or translational level by rapamycin, there are at least four other independent studies showing a similar reduction of total Akt and/or mTOR expression after rapamycin administration [28,43,45,46].

The inhibition of Thr308 phosphorylation of Akt in treated mice was also an interesting finding. It has been shown that mTOR inhibition eliminates the negative feedback loop between the p70S6K and the IRS leading to unopposed Thr308 phosphorylation of Akt [20]. Nevertheless, we did not observed increased Thr308 phosphorylation in the treated mice in comparison with healthy controls which did not receive rapamycin. Therefore, rapamycin may decrease upstream signals by growth factors/cytokines, which are responsible for the observed Thr308 phosphorylation. A different and more provocative explanation that has been proposed and could explain the present findings is that phosphorylation of mTOR at Ser2448 by Akt may be required for the transmission of signals other than those leading to regulation of p70S6K activities [28].

Moreover, we noticed that rapamycin also impaired the glomerular expression of Ser473pAkt. This can be explained by inhibition of either mTORC2 or other kinases capable to phosphorylate Akt at Ser473 [23–27]. Inhibition of mTORC2 assembly can occur in certain cell types in vitro, after prolonged administration of rapamycin [37], and has been proposed as a possible underlying mechanism for the appearance of proteinuria in some transplant patients [47,48] or for a decrease of nephrin and podocin expression in podocyte cultures [45]. In the present study, the disruption of mTORC2 by rapamycin appears a likely scenario, but in contrast to the aforementioned studies, it did not result in proteinuria or decreased nephrin and podocin protein and mRNA levels. This discrepancy may be explained by the rapamycin blood levels achieved in the present study (5–10 ng/mL) which were much lower compared with the high concentrations of rapamycin (up to 100 ng/mL) that were used in the aforementioned studies.

We have also to notice that the anti-dsDNA levels were reduced by 40% (at levels similar to HC) in the rapamycin-treated mice in consistency with the immune-regulatory action of rapamycin. Although moderate, this reduction seems to be sufficient since immune deposits were almost completely absent after treatment.

In conclusion, this study shows that: (i) activation of the PI3K/Akt/mTOR pathway plays an important role in the pathogenesis of murine LN; (ii) at the kidney level, rapamycin not only blocks the downstream signal transduction of the activated mTOR but also negatively regulates the PI3K/Akt/mTOR pathway itself; and (iii) rapamycin monotherapy can completely prevent the development of murine LN and significantly ameliorate the already established disease.

Our findings justify further research in humans suffering from SLE. Examination of the PI3K/Akt/mTOR pathway activation may offer new insights into the pathogenesis of the disease and may lead to a more individualized and less toxic treatment of LN.

**Supplementary data**

Supplementary data is available online at http://ndt.oxfordjournals.org.

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