Rapamycin induced ultrastructural and molecular alterations in glomerular podocytes in healthy mice

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Abstract

Background. In the normal kidney, rapamycin is considered to be non-nephrotoxic. In the present study, we investigated whether rapamycin is indeed non-nephrotoxic by examining the ultrastructural and molecular alterations of podocytes in healthy mice.

Methods. Balb/c mice were given three different intraperitoneal doses of rapamycin for 1 week (dose model)—low-dose group: 1 mg/kg/day, intermediate-dose (ID) group: 1.5 mg/kg/day and high-dose (HD) group: 3 mg/kg/day; four mice in each group. An ID of rapamycin was also given for three different periods (time model): 1, 4 and 8 weeks; four mice were in each group. Mice treated with dimethyl sulphoxide served as controls. Body weight was measured weekly. Renal function was assessed by serum creatinine at the time of sacrifice. For estimation of albuminuria, 24-h urine collections were performed before treatment and weekly thereafter. Glomerular content of nephrin, podocin, Akt and Ser473-phospho-Akt was estimated by western blot and immunofluorescence. Nephrin and podocin messenger RNA (mRNA) were measured by real-time polymerase chain reaction. Mean podocyte foot process width (FPW) was measured by electron microscopy.

Results. Urine albumin levels increased in the HD and 4-week groups. Renal function was modestly deteriorated in the HD group. The mean FPW increased in a dose-dependent manner at Week 1, further deteriorated at Week 4 and finally improved at Week 8. Nephrin and podocin mRNA levels showed a significant decrease at Week 1 and were restored at Week 4 and 8. Nephrin and podocin protein levels were restored at Week 4 and recovered at Week 8. Ser473-phospho-Akt significantly increased in all rapamycin-treated groups.

Conclusions. Rapamycin induced significant ultrastructural and molecular alterations in podocytes in association with albuminuria. These alterations happened early during treatment and they tended to improve over an 8-week treatment period.

Keywords: Akt; nephrin; podocin; podocytes; rapamycin

Introduction

Several animal studies have shown that rapamycin is not nephrotoxic in the normal kidney in terms of renal function, haemodynamics and histopathology [1–6]. Di Joseph et al. studied the functional and histopathological effects of rapamycin on mouse kidneys after administration of very high doses (HDs) of rapamycin [up to 100 mg/kg, intraperitoneally (i.p.) for 4 or 7 days] in C3H/HeJ and Balb/cJ mice. They noticed an increase in blood urea nitrogen and some histological alterations in the tubules and concluded that rapamycin’s effects on kidney function were minimal at doses 50 times higher than its therapeutic dose established in the mouse [2]. Subsequently, it was shown that rapamycin exerts nephrotoxicity in some particular rat strains or other animal species [3–6]. An oral dose of 2 mg/kg/day for 2 weeks was associated with a 25% reduction in creatinine clearance in the Sprague–Dawley rat and in the spontaneously hypertensive rat but not in the Lewis strain [3]. In spontaneously hypertensive rats, rapamycin did not impair kidney function when administered at therapeutic doses for 2 weeks, but it accelerated the naturally occurring renal lesions when given at HDs [7]. In Wistar–Munich rats, a single infusion of sirolimus (5 mg/kg) reduced single-nephron glomerular filtration rate (GFR) by 26% [8], whereas in pigs, an intravenous infusion of sirolimus (0.4 INS> mg/kg) resulted in a mild increase in GFR [9]. In rabbits, administration of rapamycin for up to 60 days did not affect renal function but did cause mild tubular atrophy and fibrosis [5]. Moreover, rapamycin was shown to exert diverse effects in renal function in Sprague–Dawley rats, depending on the route and duration of administration without causing specific morphological alterations [10]. It is noteworthy that none of the aforementioned studies in normal kidneys has looked more closely into the podocytes ultrastructural and functional alterations after rapamycin administration. Since then, a considerable amount of data from human studies has accumulated, showing that rapamycin can indeed cause toxicity in the transplanted kidney manifested either as proteinuria [11–18] and/or as
deterioration of renal function [16, 19]. De novo proteinuria and focal segmental glomerulosclerosis develop in 5–30% of transplant patients receiving rapamycin [20, 21]. Moreover, various experimental models in diseased kidneys have shown either favourable [22–35] or untoward [36–38] results, which are often associated with the dosage and/or the timing of rapamycin administration [34, 35, 37–39]. In some recent studies, rapamycin appeared to decrease the expression of podocyte-specific proteins such as nephrin and podocin [40] and to exert its toxic effects by inhibiting the phosphorylation of Akt kinase at Ser473 [15, 41]. Rapamycin inhibits the action of a kinase known as the mammalian target of rapamycin (mTOR), mTOR, when part of the multiprotein complex mTOR complex-1, is considered a downstream effector of Akt. Moreover, mTOR in a separate protein complex known as mTOR complex-2 (mTORC2) has recently been shown to act as an upstream activator of Akt by phosphorylating Akt at Ser473 [42]. Although mTORC2 is considered insensitive to rapamycin inhibition, recent evidence suggests that prolonged treatment with rapamycin affects mTORC2 assembly and action towards Akt [43]. This action has been proposed as a possible mechanism underlying the appearance of proteinuria in some transplant patients [15, 41] as well as for the decreased nephrin and podocin expression in podocyte cultures [40]. On the other hand, rapamycin has been shown to induce Ser473 phosphorylation of Akt by inhibiting S6 kinase [44]. These two opposed effects of rapamycin on Akt phosphorylation have not been examined in the glomerular level so far. The increased incidence of de novo proteinuria and the conflicting results regarding the therapeutic role of rapamycin in various human and animal studies prompted us to examine its effects on podocyte ultrastructure, on the expression of the slit diaphragm proteins nephrin and podocin and on the activation of Akt kinase in glomeruli.

Materials and methods

Study design

Two-month-old Balb/c female mice were given three different i.p. doses of rapamycin for 1 week (low-dose [LD] group: 1 mg/kg/day, intermediate-dose [ID] group: 1.5 mg/kg/day and HD group: 3 mg/kg/day, four mice in each group: dose model). An ID of rapamycin (1.5 mg/kg/day) was given i.p. for three different periods (1, 4 and 8 weeks, four mice in each group; time model). Although the ID and Week 1 groups were composed of the same mice, they are presented as separate groups for the sake of the reader’s convenience. Balb/c mice injected with the rapamycin solvent [dimethyl sulfoxide (DMSO)] served as controls. They were sacrificed in groups of three at 1, 4 and 8 weeks. Body weight was measured weekly and rapamycin dose was adjusted accordingly. Twenty-four-hour urine collections were performed in metabolic cages (Techniplast, Italy) before initiation of rapamycin administration and weekly thereafter. Mice were anaesthetized with isoflurane prior to cervical dislocation. Blood was obtained through cardiac puncture at sacrifice. Serum creatinine (Cr) was measured by an auto-analyser and rapamycin blood levels were determined in whole blood (IMx Analyser, Abbott Lab, IL). Our intention was to extend the HD regimen to 4 and 8 weeks but this was not possible due to early mortality (associated with bowel haemorrhage, severe asthenia and weight loss) that precluded us from extending the administration of HDs for longer periods. For this reason, only the intermediate dosing was chosen for the time course experiment. Laboratory animals

Balb/c mice were obtained from the Foundation of Research and Technology, Crete, Greece. Rapamycin was purchased by LC Laboratories, MA (catalogue number R5000) and diluted at a concentration of 25 mg/mL in DMSO (D5879; Sigma–Aldrich) and stored in −20°C as a stock solution. Experiments were carried out in accordance to 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.

Microscopic studies

Kidney morphology was examined by photon microscopy (PM), electron microscopy (EM) and immunofluorescence (IF) as has been described previously [33]. Microscopy was performed by a renal pathologist (L.N.) who was blinded prior to assessing sections. Ten random glomeruli were examined by EM for each mouse. Microphotographs were obtained using an ESS500W Erlangshen CCD Camera and analysed by the Digital Micrograph software (Gatan GmbH, Munchen, Germany). The mean foot process width (FPW) in each loop was calculated as has been described elsewhere [45]. The antibodies used in IF are shown in Table 1. 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.

Western blot analysis

Kidney cortex tissue was obtained at sacrifice and homogenized in Radi-Oimmuno-Precipitation Assay-buffer containing protease inhibitors. Samples were incubated at 100°C for 5 min in reducing gel loading buffer and separated on 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels. The proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 5% low-fat milk or 5% bovine serum albumin (BSA) and incubated overnight at 4°C with the primary antibody. Antibodies used in western blots are shown in Table 1. After washing with 0.05% Tween-20 in Tris-buffered saline, the appropriate secondary affinity-purified peroxidase-conjugated antibody (Cell Signalling Technology) was applied. The nitrocellulose membranes were incubated with enhanced chemiluminescence (Amersham Biosciences, UK) and exposed to autoradiography film. Nephrin, podocin, Akt, Ser473pAkt and actin protein bands were measured using the ImageJ densitometry analysis software. Nephrin and podocin levels were normalized to the levels of actin, while Ser473pAkt was normalized to total Akt levels.

Real-time quantitative polymerase chain reactions

Total RNA was extracted from isolated murine kidneys using the acid guanidinium thiocyanate–phenol–chloroform single-step procedure with TRZol Reagent (Life Technologies; Gibco, Paisley, UK). One microgram of total RNA was reverse transcribed (Superscript II; Gibco) and amplified by real-time polymerase chain reaction. iTaq SYBR Green Supermix with ROX (Bio-Rad) was used for the reactions. % sequences

Table 1. Antibodies used in WB or in IF analysis

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host species</th>
<th>Supplier</th>
<th>Application (dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-nephrin</td>
<td>Guinea-pig</td>
<td>Progen GmbH, Germany</td>
<td>IF (1:250), WB (1:500)</td>
</tr>
<tr>
<td>Anti-podocin</td>
<td>Rabbit</td>
<td>Abcam PLC, UK</td>
<td>IF (1:100), WB (1:500)</td>
</tr>
<tr>
<td>Anti-Akt</td>
<td>Rabbit</td>
<td>Cell Signalling Technology, USA</td>
<td>IF (1:100), WB (1:500)</td>
</tr>
<tr>
<td>Anti-Ser473pAkt</td>
<td>Rabbit</td>
<td>Cell Signalling Technology, USA</td>
<td>IF (1:100), WB (1:500)</td>
</tr>
<tr>
<td>Anti-actin</td>
<td>Rabbit</td>
<td>Chemicon International, USA</td>
<td>WB (1:3000)</td>
</tr>
</tbody>
</table>
are listed in Table 2. Results were normalized according to glyceraldehyde 3-phosphate dehydrogenase quantity and analysis was performed using the 2-ΔΔCt method. All samples were tested in duplicate. Measurements were performed using the ABI Prism 7000 System (Applied Biosystems, CA).

### Table 2. RT-PCR sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
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<tr>
<td>Nphs1</td>
<td>Forward</td>
<td>5'-GGGAGGCACTTCGTGAAAC-3'</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CACCTGCTCACCAGGAAC-3'</td>
<td>117</td>
</tr>
<tr>
<td>Nphs2</td>
<td>Forward</td>
<td>5'-GTGTCTAAAGCCACCTCAT-3'</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGCAAACCGGACACCTGGG-3'</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>5'-GATGCTGTTCCACACCTTCT-3'</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATGCTGTTCCACACCTTCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Change in the 24-h albumin excretion in the study groups. 1-week group, rapamycin 1.5 mg/kg for 1 week; 4-week group, rapamycin 1.5 mg/kg for 4 weeks and 8-week group, rapamycin 1.5 mg/kg for 8 weeks. *P < 0.001 versus control group. BSA concentration was 0.12 μg in 20 μL loading volume. The bars represent mean values (±SD) of the 24-h urine albumin excretion of the collections performed the day before sacrifice.

**Fig. 2.** PM examination of 3-μm thick sections stained with periodic acid-Shiff. (A) Sections from the HD group without any essential histological abnormalities, ×300, (B) sections from the control group without abnormalities, ×300.

**Urinary albumin estimation**

Urinary albumin excretion was estimated by urine electrophoresis in 7.5% SDS-polyacrilamide after staining with Coomassie blue. A known quantity of BSA served as control.

**Statistical analysis**

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 with post-hoc Dunnett’s correction for multiple comparisons was performed to compare continuous variables. The Mann–Whitney U-test was used for non-parametric comparisons. Differences were considered significant for a P-value < 0.05 (two tailed).

**Results**

**Albuminuria, renal function and rapamycin levels**

During the course of the study, selective 24-h albumin excretion increased in all rapamycin-treated mice and reached the level of statistical significance in the HD and 4-week groups (both P < 0.001 versus controls; Figure 1). Serum Cr increased significantly only in the HD group (Cr: 0.34 ± 0.06 versus 0.24 ± 0.02 mg/dL in the control group, P < 0.001). The mean trough levels of rapamycin in whole blood ranged from 30 ± 8 ng/mL in the LD group to 55 ± 11 ng/mL in the ID group and 150 ± 25 ng/mL in the HD group.

**Morphologic alterations**

On PM examination, there was no evidence of any significant histopathological abnormalities either in controls or in rapamycin-treated mice (Figure 2). On EM studies, however, there was a variable degree of segmental fusion of foot processes in all rapamycin-treated groups (Figure 3). In a few glomeruli, the podocytes were enlarged, oedematous and occupied the entire Bowman’s space. Endothelial cells also showed focal and segmental lesions such as oedema, loss of fenestrae and subendothelial accumulation of electro-lucent, finally granular, material. The above changes were more frequently seen in the HD, ID and 4-week groups. The LD and 8-week groups presented only focal and segmental fusion of podocyte foot processes. In order to quantify podocyte lesions, the mean FPW was measured...
Fig. 3. EM examination. (A) Control group, normal architecture of glomerular capillary loops; (B) ID group, segmental foot process fusion and subendothelial accumulation of electro-lucent, finally granular, material (white arrow); (C) HD group, complete fusion of foot processes in a capillary loop (black arrow); (D) HD group, oedema of endothelial cells and loss of fenestræ (white arrow), oedema of podocytes and complete occupation of the Bowman’s space (black arrow); (E) 4-week group, variable degrees of foot processes fusion (black arrow) and (F) 8-week group, mild segmental fusion of foot processes.

Fig. 4. FPW in study groups. There is a significant increase of the median values of FPW, already from the first week of rapamycin treatment (dose model, left diagram). In the time model (right diagram), the highest value is noticed at Week 4, whereas at Week 8, there is partial restoration. 1-week group, rapamycin 1.5 mg/kg for 1 week; 4-week group, rapamycin 1.5 mg/kg for 4 weeks; 8-week group, rapamycin 1.5 mg/kg for 8 weeks; Median values, inter-quartile ranges and the 95% confidence intervals are illustrated. *P < 0.001 controls versus all other groups.
in overall 850 glomerular capillary loops. The mean FPW, as compared to controls (336 ± 60 nm), was significantly higher in all rapamycin-treated groups (LD: 385 ± 77 nm, ID: 420 ± 65 nm, HD: 423 ± 74, 4-week: 456 ± 145 nm, 8-week: 401 ± 91 nm, all P < 0.001; Figure 4).

Nephrin and podocin expression

Nephrin and podocin messenger RNA (mRNA) levels decreased during the first week, irrespectively of the dosage group (40–65% lower versus control, P < 0.01 for all comparisons; Figure 5). In the time model, nephrin and podocin mRNA levels restored to near normal in the 4-week group and even more so in the 8-week group (Figure 5). Regarding nephrin and podocin protein levels as measured by WB, no significant difference was observed compared with controls in the dose model. In the time model, a reduction in both protein levels was observed in the 4-week group (statistically significant only for podocin), while in the 8-week group, both protein levels were quantified at or above baseline values.

Fig. 5. RT-PCR. In the dose model, podocin and nephrin mRNA levels were both decreased irrespectively of the dosage group (40–65% lower versus controls). In the time model, both nephrin and podocin mRNA levels were lower than controls in the 1-week group and similar to controls in the 4- and 8-week groups. Bars represent mean values (±SD). *P < 0.01 versus controls.

Fig. 6. Blots of nephrin, podocin and actin in the two models (dose model to the left and time model to the right). Mean values (±SD) are shown in the diagrams below. The mean value of expression for both proteins did not change during the first week irrespectively of the administered dose. At 4 weeks, there was a reduction of both proteins as compared to controls (statistically significant only for podocin; *P = 0.007), whereas at 8 weeks, an increase of both values was observed (statistically significant only for nephrin; **P = 0.03).
(Figure 6). By IF, we were not able to show any abnormality in the distribution of nephrin and podocin or in the staining intensity scores among groups.

Activated Akt kinase

The glomerular expression of Ser473pAkt was increased as compared to controls in all rapamycin-treated mice by IF (all $P < 0.001$, Figure 7), irrespectively of the dose used or the duration of administration. Similar results were obtained by WB after adjustment of Ser473pAkt to total Akt. All rapamycin-treated groups showed a statistically significant increase of pAkt/Akt ratio except for the LD group in which the difference was not significant (Supplementary Figure 1).

Discussion

With regard to kidney function, we noticed a statistically significant increase (40%) of serum creatinine in the HD group. This increase was noticed in young healthy mice on a regular sodium diet. HD of rapamycin was associated with focal ultrastructural lesions such as podocyte oedema, foot processes fusion, subendothelial apposition of electro-lucent material, loss of fenestrae and oedema of endothelial cells. The absence of essential histological lesions in tubules and interstitial tissue suggests that the deterioration of kidney function observed in the HD group might be related to the glomerular histological lesions noticed more frequently in that group. These effects were principally dose-dependent since they were increasingly noticed from LD to HD of rapamycin. Extending the HD regimen to 4 or 8 weeks would be more informative as per the changes happening in glomerular cells but it was not possible to attest due to early mortality in preliminary experiments. On the other hand, the alterations encountered in podocyte mean FPW displayed a time dependency. They were evident from the first week of treatment, deteriorated further at Week 4 and improved, but not restored, at Week 8. In accordance with the highly

Fig. 7. Glomerular expression of Ser473pAkt by IF. All rapamycin-treated mice presented increased glomerular Akt phosphorylation as compared to controls; *$P < 0.001$ for all comparisons (Mann–Whitney $U$-test). (A) Control group; (B) LD group; (C) ID group; (D) HD group; (E) 4-week group and (F) 8-week group. The graph below shows the percentage of glomeruli with intensity scores $>1+$.
increased FPW at Week 4, the lowest nephrin and podocin protein levels were noticed at the same time point. Moreover, the alterations of nephrin and podocin protein levels were preceded by parallel alterations of their respective mRNAs, indicating that rapamycin negatively regulates the transcription of these podocyte-specific mRNAs. Therefore, rapamycin may acutely and significantly reduce nephrin and podocin gene expression followed by a relevant reduction of protein levels. This initial reduction was followed by restoration of mRNA and protein levels at Week 4 and 8, respectively, indicating the potential presence of an escape from the action of rapamycin mechanism. Hence, some ‘escape’ mechanisms have already been described in vitro [46] but they are mostly related to control of translation rather than transcription. However, it is currently unknown which specific transcriptional factors control the expression of nephrin and podocin genes and if they are differentially regulated by rapamycin.

Rapamycin also induced Akt phosphorylation at Ser473, a key step for activation of this kinase. This finding may be explained in the early phase, due to inhibition of the negative feedback loop between S6 kinase and Akt [44]. However, the increased glomerular phosphorylation of Akt persisted even after prolonged treatment, indicating that the assembly and action of mTORC2 were not disrupted by rapamycin in the present in vivo model. It is interesting that despite restoration of nephrin and podocin mRNA and protein levels at Week 8, some degree of foot process widening still persisted. Whether the increased activation of Akt has a protective role or contributes, via effects on the cytoskeleton, to the persisting widening of podocyte foot processes is not known and warrants further investigation. In the everyday clinical practice, rapamycin is usually kept at much lower trough levels compared to those achieved in the present study. As such, its use can be considered much safer. However, pharmacodynamic differences between the two species make any comparisons difficult if not impossible.

The clinical relevance of our findings could be that (i) inadvertently high rapamycin levels or transient increases of its levels may pose a threat to the glomerular filtration barrier. (ii) Rapamycin-associated toxicity may be diminished with lower doses and improves over time.

In conclusion, the administration of rapamycin in healthy mice was associated with reduced kidney function when given in supratherapeutic doses and with mild alterations of podocyte architecture and function in lower doses. These alterations were seen early after treatment and tended to improve in the long term, indicating the presence of an escape from the action of rapamycin mechanism. Whether these early alterations are also seen in transplant patients is currently unknown.

**Supplementary data**

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

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**Conflict of interest statement.** None declared.

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