mTOR inhibition with temsirolimus causes acute increases in glomerular permeability, but inhibits the dynamic permeability actions of puromycin aminonucleoside

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mTOR comprises a complex of large serine/threonine kinases of the phosphoinositide kinase family that functions as part of two multimeric complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (28, 29). mTOR is associated with a nonenzymatic scaffolding protein, regulatory-associated protein of mTOR (Raptor) in mTORC1, while rapamycin-insensitive companion of mTOR (Rictor) is associated with mTORC2. Rapamycin binds to FK506 binding protein of 12 kDa (FKBP12) to inhibit the function of mTORC1 (but not that of mTORC2). Activation of mTORC1 results among other things in phosphorylation of two downstream targets, the ribosomal S6 kinase (S6K) and 4EBP (eukaryotic translation initiation factor 4 E binding protein), which stimulate ribosome biogenesis and translation to increase cell mass. At large, mTOR integrates information on nutrient availability and growth factors to control protein synthesis and cell size.

Podocytes are terminally differentiated cells with a limited ability to proliferate. Activation of mTORC1 is thought to play a key “protective” role in the adaptive hypertrophic podocyte responses to various noxious challenges, such as toxic, mechanical, or metabolic insults, whereby podocytes could increase in size to compensate for losses of nearby damaged podocytes in the glomerulus. Particularly in states of podocyte stress, e.g., hyperfiltration, mTORi treatment may therefore represent a “second hit,” worsening proteinuria. In hyperglycemia there is ample evidence of increased mTOR activity, conceivably by downregulation of the upstream negative mTOR regulators hamartin (TSC1) and tuberin (TSC2), which are maintained moderately active over time by the nutrient sensor 5′ AMP-activated protein kinase (AMPK). Activation of mTORC1 in diabetes following glucose-induced inhibition theremore, paricalcitol, which is supposed to interact with the Ca$^{2+}$ entry into the cells via so-called transient receptor potential canonical 6 (TRPC6) receptor-operated Ca$^{2+}$ channels, was found to be effective (3).

Inhibitors of the mammalian target of rapamycin, mTORi, such as sirolimus and everolimus, have been tried for replacing calcineurin inhibitors (CNI) as immunosuppressants in organ (kidney) transplantation, to avoid the profibrotic and glomerular filtration rate (GFR)-deteriorating effects of the latter (8, 9, 16). The use of mTORi has, however, been hampered by their antiproliferative actions; they should not be used during the first post-transplant month, and also by frequent reports of de novo proteinuria after conversion from CNI to mTORi (28). It has also been shown that long-term inhibition of mTOR may interact with pathways of autophagic flux in podocytes (11). Whether the proteinuric actions of mTORi are due to direct effects on the GFB or due to effects on the tubular reabsorption of proteins has also been under debate (10, 15).

The glomerular filtration barrier (GFB) is a highly selective sieving barrier to macromolecules, yet being dynamic (4, 22, 38). Thus the permeability of the GFB can rapidly increase due to challenges induced by trauma (2), hyperglycemia (6), oxidative stress (44, 46), or due to systemic infusions of atrial natriuretic peptide (ANP) (7) or angiotensin II (ANG II) (3, 5). We have previously demonstrated that the rapid dynamic increases in glomerular permeability induced by systemic ANG II infusions in rats can be inhibited not only by ANG II receptor blockers (ARB) but also by scavengers of reactive oxygen species (ROS) and inhibitors of the intracellular Ca$^{2+}$ signaling cascade in podocytes and endothelial cells (3). Fur-
of AMPK seems to be able to induce podocyte loss, glomerular basement membrane thickening, mesangial expansion, and proteinuria (14, 21).

In contrast to mTORC1, mTORC2 is largely rapamycin-sensitive, and phosphorylates cellular targets, such as protein kinase B (Akt), suppressor of kex2 gas1 synthetic lethality (SKG1) and PKC to control cell survival and cytoskeletal (F-actin) organization (51). Interestingly, mTORC2, but not mTORC1, seems to be activated by certain insults, such as those induced by protamine sulfate (PS). Recent data thus suggest that the rapid changes in the glomerular barrier conformation and permeability by PS require increased Akt activity and that PS induces increases in Ca$^{2+}$ signaling, which in turn activates Akt through mTORC2 (48). Since temsirolimus is primarily an mTORC1 inhibitor, it is not, however, expected to be protective in early, rapid permeability responses, such as those produced by PS. On the contrary, temsirolimus itself may adversely affect glomerular permeability, which was actually tested in this study.

Despite their reputation to induce proteinuria in human and animal models, the use of mTORi has been successful in ameliorating glomerular hypertrophy and albuminuria in the diabetic kidney (21, 30, 52) and to reduce cyst formation in animal models of polycystic kidney disease (41, 54). Given the context-dependent actions of mTORi (17, 24, 40, 47), implying that it may increase basal glomerular permeability, we first sought to assess its acute, direct actions on the glomerular permeability in rats. Second, since the effects of mTORi have been reported to confer protection of the GFB during conditions of glomerular hyperpermeability, we tested mTORi also on the acute, dynamic effects of puromycin amino-nucleoside (PAN) and ANG II, which are known modulators of glomerular permeability (5, 25, 32, 34, 47). Furthermore, since oxidative stress and induction of reactive oxygen species (ROS) is an established mechanism of PAN-induced and ANG II-induced glomerular hyperpermeability, we also studied, for comparison, the actions of the superoxide (O$_2^-$) scavenger tempol on PAN-induced glomerular permeability and also on the permeability effects of temsirolimus by itself.

To assess glomerular permeability in intact rats, we assessed the glomerular sieving coefficient ($\theta$) i.e., the primary urine-to-plasma-concentration ratios of FITC-Ficoll 70/400 (M$_r$ 70,000 and 400,000, respectively) of Stokes-Einstein radii ($a_c$) ranging from 10 to 80 Å after mTORi and PAN or ANG II infusions. Ficoll is a neutral copolymer of sucrose and epichlorohydrine, which is not significantly reabsorbed in the proximal tubules and therefore can be used as a direct probe of glomerular permeability. By contrast, albumin, for example, is normally almost fully reabsorbed by the proximal tubules. We found that mTORi per se increased baseline glomerular permeability to Ficoll$_{30,80}$A, but that it blunted the actions of PAN-induced permeability changes, underpinning the complex dual and context-dependent interactions of mTORi with the GFB.

MATERIALS AND METHODS

Animals and general surgery. Experiments were performed in 41 male Wistar rats (Møllergard, Lille Stensved, Denmark) with an average body weight of 260.8 ± 2.4 g. The rats were given water and standard chow ad libitum. The animal Ethics Committee at Lund University approved the animal experiments.

Anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt) and maintained during the experiments through repeated intra-arterial injections. The rats were placed on a heating pad to maintain body temperature at 37°C. The tail artery was cannulated (PE-50 cannula) for administration of anesthesia and for continuous monitoring of blood pressure and heart rate (HR; MP 150 system, AcqKnowledge for MAC, Biopac Systems). A tracheotomy was performed to facilitate breathing. The left carotid artery and left and right jugular veins were cannulated (PE-50) for blood sampling and infusion purposes, respectively. After an intravenous bolus dose of furosemide (0.375 mg/kg body wt, Furix, Takeda Pharma, Solna, Sweden), and after a small abdominal incision (−6–8 mm), the left ureter was cannulated (PE-10 connected to a PE-50) for urine-sampling purposes, after which the incision was closed by a small suture.

Experimental procedures. All experiments started with an initial resting period of at least 20 min following the cannulation of the left ureter. Following the resting period, a baseline measurement (time 0) of FITC-Ficoll in plasma and urine (see below) was done in all of the experiments just before the injection of mTORi.

Glomerular permeability after a 30-min preincubation period with temsirolimus. In one group of animals temsirolimus (Torsiel, Pfizer, Sollentuna, Sweden) was administered in one single dose systemically intravenously (iv; mTORi, $n = 7$, 3.8 mg/kg body wt) 30 min before the start of the experiment. Measurements of Ficoll concentrations in plasma and urine were performed sequentially before the start of the mTORi injection (baseline) and at 5, 15, and 60 min after the end of the 30-min mTORi incubation period.

Systemic PAN infusion. PAN (P7130, Sigma-Aldrich, St. Louis, MO) was administered immediately after baseline (time 0), as an initial bolus dose of 4.4 mg, followed by a continuous iv infusion (PAN, $n = 8$, 37.5 mg/kg and 266 μg·min$^{-1}$·kg$^{-1}$). Measurements of glomerular Ficoll sieving were performed sequentially before the start (baseline) and at 5, 15, 30, 60, and 120 min of the PAN infusion.

Effects of mTOR inhibition with temsirolimus on PAN- and ANG II-induced glomerular hyperpermeability. In separate groups of animals, temsirolimus was administered iv (3.8 mg/kg body wt) 30 min before the start of the administration of either PAN ($n = 7$, 37.5 mg/kg) or ANG II ($n = 6$; bolus dose of 50 ng followed by 16 nmol·min$^{-1}$·kg$^{-1}$). PAN or ANG II infusions were thus started immediately after the end of the mTORi incubation period. Measurements of glomerular Ficoll sieving coefficients were performed sequentially at baseline (before the mTORi injection) and at 5, 15, 30, 60, and 120 min after the start of the PAN administration, and at baseline and 5 and 15 min after the start of the ANG II infusion, respectively.

Scavenging of ROS. During either PAN or ANG II infusions, or, in some experiments, following the temsirolimus injection, the ROS scavenger and SOD mimetic compound 4-hydroxy-tempol (tempol; Sigma-Aldrich, St. Louis, MO) was administered in separate experiments. Tempol was given as a continuous infusion iv (15 mg·h$^{-1}$·kg$^{-1}$)$^{-1}$, starting 5 min before the start of either PAN or ANG II infusions, or 25 min after the temsirolimus injection (3.8 mg/kg), and was continued throughout the experiments: the tempol-PAN group ($n = 7$), tempol-ANG II group ($n = 6$), and tempol-temsirolimus group ($n = 6$), respectively. Measurements of Ficoll sieving coefficients were performed sequentially at baseline and at 5, 15, 30, 60, and 120 min after the start of the PAN infusion, and at baseline and at 5 and 15 min after the start of the ANG II infusion, or 30, 35, and 45 min after the temsirolimus injection, respectively.

Sieving of FITC-Ficoll. A mixture of FITC-Ficoll-70 (10 mg/ml) and FITC-Ficoll-400 (10 mg/ml; TdB Consultancy, Uppsala, Sweden) in a 1:24 relationship was administered as a bolus dose together with FITC-inulin (10 mg/ml, TdB Consultancy). The bolus dose (FITC-Ficoll-70, 40 μg; FITC-Ficoll-400, 960 μg; FITC-inulin, 500 μg; and $^{51}$Cr-EDTA, 0.3 MBq) was followed by a constant infusion of 10 μl·kg$^{-1}$·h$^{-1}$ (FITC-Ficoll-70, 20 μg/ml; FITC-Ficoll-400, 0.48 mg/ml; FITC-inulin, 0.5 mg/ml; and $^{51}$Cr-EDTA, 0.3 MBq/ml) for at least...
20 min before sieving measurements, after which urine from the left kidney was collected for 5 min, with a midpoint (25 min) plasma sample collected. A high-performance size-exclusion chromatography (HPSEC) system (Waters, Milford, MA) was used to determine size and concentration of the Ficoll samples. Size exclusion was achieved using an Ultrahydrogel-500 column (Waters). The mobile phase was driven by a pump (Waters 1525), and fluorescence was detected with a fluorescence detector (Waters 2475) with an excitation wavelength at 492 nm and an emission wavelength at 518 nm. The samples were loaded to the system with an autosampler (Waters 717 plus), and the system was controlled by Breeze Software 3.3 (Waters). The column was calibrated with Ficoll and protein standards described in a previous paper (1).

The sieving coefficients (θ) of FITC-Ficoll 70/400 were determined as the fractional clearance from θ = (CFU·CIP)/(CFP·CIU), where CFU represents the Ficoll urine concentration, CIP represents the inulin concentration in plasma, and CIU the inulin concentration in urine.

GFR. GFR was measured in the left kidney during the experiment using 51Cr-EDTA. A priming dose of 51Cr-EDTA (0.3 MBq in 0.2 ml iv, Amersham Biosciences, Buckinghamshire, UK) was administered and followed by a continuous infusion (10 ml·h\(^{-1}\)·kg\(^{-1}\)) of 51Cr-EDTA (0.3 MBq/ml) throughout the experiment. Urine was collected from the left ureter repeatedly and blood samples, using microcapillaries, taken to be able to calculate GFR, approximately every 5–10 min. Radioactivity in blood and urine was measured in a gamma counter (Wizard 1480, LKP, Wallac, Turku, Finland). Hematocrit was assessed throughout the experiments to able to convert blood counter (Wizard 1480, LKP, Wallac, Turku, Finland). Hematocrit was assessed in the experiments to be able to convert blood counter (Wizard 1480, LKP, Wallac, Turku, Finland).

**Fig. 1.** The glomerular sieving coefficient (θ) for Ficoll70Å as a function of time for temsirolimus [mammalian target of rapamycin inhibitor (mTORi)]. A transient increase in θ at 15 min, i.e., 45 min after the temsirolimus injection, was evident, but θ returned to baseline within the next 50 min.

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**Fig. 2.** Ficoll θ vs. Stokes-Einstein radius (a\(_L\)) in animals infused with temsirolimus (mTORi). There was a marked increase in θ for Ficoll50-80Å, at 15 min after the temsirolimus incubation period (dotted line) (P < 0.01), but not at 5 (dashed line) and 60 min (dashed-dotted line). Dotted grey line represents the baseline glomerular sieving curve before temsirolimus infusion.

The major parameters of the two-pore model are: the small-pore radius (r\(_s\)), the large-pore radius (r\(_L\)), the unrestricted pore area over unit diffusion path-length (A\(_U\)/A\(_X\)), and the fraction of the glomerular UF-coefficient accounted for by the large pores (α\(_L\)).

**Statistical analysis.** Values are presented as means ± SE. Differences among groups were tested using nonparametric analysis of variance with the Kruskal-Wallis test and post hoc testing using the Mann-Whitney U-test. Bonferroni corrections for multiple comparisons were made when applicable. Significance levels were set at *P < 0.05 and **P < 0.01. All statistical calculations were made using IBM SPSS 20.0 for Windows (SPSS, Chicago, IL).

**Fig. 3.** Glomerular θ for Ficoll molecules of radius 70 Å as a function of time after administration of puromycin aminonucleoside (PAN; solid line and filled squares), temsirolimus (mTORi) and PAN (mTORi-PAN; dashed line and filled circles), and tempol and PAN (tempol-PAN; dotted line and filled pyramids). There was already a marked transient increase in θ at 5 min of PAN administration, and a second, more sustained increase in θ at 60 and 120 min. Tempol and temsirolimus effectively reduced the glomerular permeability increase at the latter timepoints, and additionally tempol reduced the PAN-induced changes in permeability at 5 min.
RESULTS

Effects of temsirolimus (after 30 min of preincubation) on glomerular permeability. Temsirolimus increased the glomerular permeability (θ) to Ficoll70Å at 15 min after the mTORi preincubation period, but this permeability increase was spontaneously reversed within the next 40–50 min (Fig. 1). θ for Ficoll70Å thus increased from 2.91 \times 10^{-5} \pm 1.18 \times 10^{-5} at baseline (before the temsirolimus incubation) to 2.27 \times 10^{-5} \pm 5.02 \times 10^{-5} (P < 0.01) at 15 min after the end of the preincubation period, after which the glomerular permeability returned to control (Fig. 1). Figure 2 shows the Ficoll θ vs. αe for animals treated with temsirolimus alone. An increase in θ for Ficoll50-80Å was observed at 15 min, but was not seen at 5 and 60 min. Tempol completely abrogated the permeability increment at 15 min. Thus θ for Ficoll70Å was 1.40 \times 10^{-5} \pm 3.80 \times 10^{-6} (n = 6) 30 min after the temsirolimus injection but remained unchanged at 1.95 \times 10^{-5} \pm 5.25 \times 10^{-6} (P = 0.42; n = 6) 15 min later in the tempol-temsirolimus group.

PAN rapidly increased the glomerular permeability to Ficoll50-80Å. PAN rapidly and transiently induced increases in glomerular permeability (θ for Ficoll70Å), the initial permeability increase peaking at 5 min, after which permeability was reduced, but later again increased at 60–120 min (Fig. 3). θ for Ficoll70Å thus increased from 1.52 \times 10^{-5} \pm 6.60 \times 10^{-6} at baseline to 2.23 \times 10^{-4} \pm 6.99 \times 10^{-5} after 5 min (P < 0.01). At 60 and 120 min, there was again an increase in θ for Ficoll70Å to 1.12 \times 10^{-4} \pm 2.98 \times 10^{-5} and 1.15 \times 10^{-4} \pm 6.13 \times 10^{-5} at 60 and 120 min, respectively (P < 0.01).

Effects of temsirolimus and tempol on PAN-induced glomerular hyperpermeability. Systematic administration of temsirolimus significantly reduced the late permeability increase induced by PAN at 60 and 120 min to baseline values (P < 0.01) but did not affect the initial permeability peak at 5 min. Tempol, however, effectively reduced the early increase in θ induced by PAN as well as that at 120 min of PAN administration (P < 0.05) (Fig. 3). At 120 min, both mTORi and tempol effectively reduced the sieving coefficient for Ficoll70Å in PAN-treated animals. At 120 min, θ for Ficoll70Å had thus decreased by 10.220.33.5 on April 3, 2017 http://ajprenal.physiology.org/ Downloaded from
Effects of mTORi on ANG II-induced glomerular hyper-permeability. Temsirolimus did not significantly affect the acute increase in glomerular permeability induced by ANG II (Fig. 5). For comparison, the effect on glomerular permeability of ANG II (alone) from a previous study (5) is shown (hatched line) in Fig. 5.

**Hemodynamic parameters.** There were no significant changes in GFR (Fig. 6) or mean arterial pressure (MAP) or HR (data not shown) during the course of any of these experiments, except for a significant decrease in HR, starting immediately after the start of the infusion of mTORi (data not shown) (P < 0.05).

**Two-pore parameters.** The best curve fit of θ vs. αL for Ficoll according to the two-pore model were obtained using the parameters listed in the tables below. Temsirolimus alone caused (at 15 min) an increase in the fractional ultrafiltration coefficient accounted for by large pores (αL) (P < 0.05) and the rL (P < 0.01). Baseline data and data at 60 min are shown for comparison (Table 1). PAN administration increased the rL at all time points and also fractional fluid flow through large pores (JvL/GFR) and αL significantly at 5 min (P < 0.05) (Table 2), indicating an increase in the number of large pores in the glomerular filter at this time point.

In the mTORi-PAN group (Table 3), significant increases in the rL were observed at 15, 30, and 60 min (not shown) after the start of PAN administration, while there were no significant changes in either αL or JvL/GFR or the effective pore area over unit diffusion path length (As/ΔX). The same pattern of unchanged As/ΔX and JvL/GFR and rL was seen for tempol-PAN at 120 min, while the rL was significantly increased at 15 min (Table 4). In Table 5 both rL and αL increased significantly at 15 min in the mTORi-LoAng group, consistent with previous findings of the effects of LoAng (16.2 ng/min/kg) alone in this experimental setup.

**DISCUSSION**

In the present study, we tested the effects of systemic mTORi on glomerular permeability under normal resting conditions in vivo and during acute podocyte and endothelial stress evoked by PAN or ANG II in anesthetized rats. Although mTORi in the form of temsirolimus exhibited transient effects on glomerular permeability, evidently invoked by ROS generation, it ameliorated the nonimmediate increases in glomerular permeability induced by PAN. However, mTORi had no effect on the glomerular hyperpermeability induced acutely by ANG II. Tempol, a superoxide anion scavenger, was, however, efficient in abrogating the immediate increases in permeability induced by PAN and also by temsirolimus similar to its documented effects on ANG II-induced GFB hyperpermeability.

There is now accumulating evidence that both overactivation and underactivation of the mTOR complex(es) can lead to glomerular dysfunction and proteinuria, suggesting that a balanced regulation of mTOR activity and a tight control of its effectors are crucial for maintaining a normal function of the GFB. Aside from its immunosuppressant actions in organ transplantation, a number of nonimmunological effects of mTORi on podocyte dynamics (the F-actin cytoskeleton) have been described (24, 25, 48). In podocyte cell cultures, the exposure of the cells to PAN for 2 days induced smaller cells with a “polarized” shape and reduced adhesion, reminiscent of a migratory fibroblast phenotype with diminished central stress fibers and substantial accumulation of thin (and less organized) actin fibers in the cell periphery. In such an in vitro system of cultured podocytes, mTORi (everolimus) had a protective effect with respect to PAN-induced podocyte injury, leading to a more normalized cell shape and recovery of actin stress fibers and an enhanced cell adhesion (25).

By contrast, short-term exposure of human podocytes to mTORi (sirolimus) under nonstressed conditions was found to downregulate VEGF synthesis and Akt phosphorylation.
Table 4. Pore parameters for the tempol-PAN group (n = 7)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Tempol-PAN 5 min</th>
<th>Tempol-PAN 15 min</th>
<th>Tempol-PAN 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small-pore radius (rs), Å</td>
<td>43.11 ± 0.75</td>
<td>43.79 ± 0.25</td>
<td>43.71 ± 0.46</td>
<td>42.16 ± 0.69</td>
</tr>
<tr>
<td>Large-pore radius (rl), Å</td>
<td>101.5 ± 6.44</td>
<td>122.21 ± 5.43</td>
<td>174.51 ± 10.32*</td>
<td>126.76 ± 10.23*</td>
</tr>
<tr>
<td>α × 10³</td>
<td>2.51 ± 1.36</td>
<td>1.64 ± 0.27</td>
<td>3.46 ± 0.101</td>
<td>2.21 ± 0.64</td>
</tr>
<tr>
<td>Jv/GFR × 10³</td>
<td>6.12 ± 1.48</td>
<td>4.61 ± 1.07</td>
<td>11.5 ± 3.49</td>
<td>5.95 ± 1.60</td>
</tr>
<tr>
<td>Av/ΔX, cm/g × 10⁻⁵</td>
<td>6.52 ± 0.69</td>
<td>5.81 ± 0.55</td>
<td>5.66 ± 0.25</td>
<td>6.18 ± 0.52</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE. Statistical difference between experimental groups and baseline: *P < 0.05.

Table 5. Pore parameters for the temsirolimus-ANG II group (n = 6)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Temsirolimus-LoANG 5 min</th>
<th>Temsirolimus-LoANG 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small-pore radius (rs), Å</td>
<td>43.81 ± 0.39</td>
<td>43.83 ± 0.04</td>
<td>43.71 ± 0.08</td>
</tr>
<tr>
<td>Large-pore radius (rl), Å</td>
<td>112.14 ± 10.47</td>
<td>155.06 ± 9.48*</td>
<td>187.68 ± 7.55*</td>
</tr>
<tr>
<td>α × 10³</td>
<td>2.69 ± 1.04</td>
<td>4.19 ± 0.85</td>
<td>7.82 ± 1.09*</td>
</tr>
<tr>
<td>Jv/GFR × 10³</td>
<td>8.14 ± 2.15</td>
<td>12.9 ± 2.64</td>
<td>26.7 ± 3.93*</td>
</tr>
<tr>
<td>Av/ΔX, cm/g × 10⁻⁵</td>
<td>6.80 ± 0.29</td>
<td>6.54 ± 0.63</td>
<td>7.19 ± 0.31</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE. Statistical difference between experimental groups and baseline: *P < 0.05.
proposed, by which mTORC2 is activated (at level 3), which, in turn, induces Akt activity by phosphorylation of Akt at Ser473, resulting in reorganization of the cytoskeleton (48). However, as it seems, only prolonged treatment with or high doses of sirolimus would block mTORC2 signaling. Hence it is likely that in the present acute experiments the amelioration of the effects of PAN after 60 and 120 min occurred by inhibition of mTORC1 and not by mTORC2, and that the earliest stage of mTORC1 inhibition resulted in an acute activation of Nox and cellular Ca2+ signaling, transiently increasing glomerular permeability. The subsequent actions of temsirolimus are conceivably of a different nature. Thus more long-term effects of mTORi (prolonged sirolimus treatment) have been shown to include downregulation of the diaphragm slit protein, nephrin, and also of TRPC6 Ca2+ channels, as well as the expression of the cytoskeletal adaptor protein Nck, and to reduce podocyte adhesion and proliferation (49). Furthermore, a recent study demonstrated that mTORC1 activation can directly lead to increased Nox4 levels in podocytes, and that mTORi, in contrast to its acute and transient effects, may function as a major downregulator of ROS generation in the kidney (14).

In this study, no permeability measurements were performed during the 30 min of preincubation with temsirolimus. For that reason, we cannot exclude that temsirolimus had altered glomerular permeability already early during the incubation period, after which glomerular permeability was again restored to baseline, to again transiently increase. Such a cyclic pattern of glomerular permeability has been observed after e.g., systemic ANP infusion (7). The rationale for having a “silent” preincubation period of 30 min before the starting of permeability measurements was to standardize the conditions for all the experiments, irrespective of the agent tested (ANG II or PAN). In all likelihood, the measured permeability changes for temsirolimus alone may thus represent a second peak of permeability increase coinciding with the assigned measurement period.

This group has previously demonstrated that a number of challenges to the GFB, such as anaphylaxis (4), acute hyperglycemia (6), systemic ANP infusions (7), or infusions of ANG II (5) or fetal Hb (46) can transiently open the GFB, conceivably by affecting the contractility of the podocytes and/or the endothelium. The exact nature of these acute permeability alterations is not known. However, podocytes may be crucial for maintaining or increasing barrier permeability. Although there is good evidence that the ultimate sieving barrier to proteins is not at the podocyte level (31), podocyte interactions with the rest of the GFB are important for its integrity. Podocytes are anchored to the GBM by integrins, and they seem to exert pressure on the GBM by their contractility, conceivably adapting the wall tension to the glomerular hydrostatic capillary pressure. Any changes in podocyte actin dynamics may thus directly affect the uphill components of the GFB, and hence, its function. Furthermore, the endothelial cells may also be involved in regulating the permeability of the GFB in a way similar to their role in peripheral capillaries, where paracellular gaps can open and close in a transient and cyclic fashion in response to various permeability challenges (35). At any rate, there is good evidence supporting cross talk between the glomerular endothelium and podocytes in the regulation of the permeability of the GFB (23).

Given the close similarity of θ for albumin and θ for Ficoll50,80A or more precisely, θ for Ficoll55A, as demonstrated in several previous studies from this group (2, 4, 44), we employed in the present study glomerular θ for high-molecular-weight Ficoll as a surrogate marker for θ of albumin. Indeed, θ values for albumin and those for Ficoll55A have been shown to be almost identical under various conditions, demonstrated also by other groups (37). Therefore, we feel confident that glomerular θ for Ficoll50,80A are good indicators of glomerular albumin permeability.

In summary, the present study demonstrates that in the acute setting mTORi, in the form of systemic temsirolimus administration, has itself acute and dynamic effects on the GFB, causing ROS-dependent, transient glomerular permeability alterations. While temsirolimus was able to blunt the permeability actions of PAN after 60 and 120 min, it exerted no protective effects on the initial (0–30 min), ROS-dependent permeability peaks induced by either PAN or ANG II. The present study amply demonstrates the dual, opposing effects of mTORi on the GFB also in an acute experimental setting in vivo.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


