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ARTICLE *in* JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY · AUGUST 2011

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Calcium Mediates Glomerular Filtration through Calcineurin and mTORC2/Akt Signaling

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ABSTRACT

Alterations to the structure of the glomerular filtration barrier lead to effacement of podocyte foot processes, leakage of albumin, and the development of proteinuria. To better understand the signaling pathways involved in the response of the glomerular filtration barrier to injury, we studied freshly isolated rat glomeruli, which allows for the monitoring and pharmacologic manipulation of early signaling events. Administration of protamine sulfate rapidly damaged the isolated glomeruli, resulting in foot process effacement and albumin leakage. Inhibition of calcium channels and chelation of extracellular calcium reduced protamine sulfate-induced damage, suggesting that calcium signaling plays a critical role in the initial stages of glomerular injury. Calcineurin inhibitors (FK506 and cyclosporine A) and the cathepsin L inhibitor E64 all inhibited protamine sulfate-mediated barrier changes, which suggests that calcium signaling acts, in part, through calcineurin- and cathepsin L-dependent cleavage of synaptopodin, a regulator of actin dynamics. The mTOR inhibitor rapamycin also protected glomeruli, demonstrating that calcium signaling has additional calcineurin-independent components. Furthermore, activation of Akt through mTOR had a direct role on glomerular barrier integrity, and activation of calcium channels mediated this process, likely independent of phosphoinositide 3-kinase. Taken together, these results demonstrate the importance of calcium and related signaling pathways in the structure and function of the glomerular filtration barrier.

J Am Soc Nephrol 22: 1453–1461, 2011. doi: 10.1681/ASN.2010080878

The identification of genes critical to glomerular function has led to an increased understanding of the signals regulating glomerular filtration and the podocyte.[1,2] Ca^{2+} signaling through membrane-associated ion channels has recently emerged as a potential modulator of podocyte function, and several Ca^{2+}-permeable channels have been identified in podocytes.[3,4] Mutations in one of these channels, TRPC6, lead to aberrant Ca^{2+} signaling, podocyte dysfunction, and focal segmental glomerular sclerosis,[5,6] and the slit diaphragm components Nephrin and Neph1 have been shown to interact with several Ca^{2+}-permeable channels, including TRPC6.[7,8] Despite mounting evidence for the importance of Ca^{2+} signaling in glomerular function, several key questions remain. It is still unclear to what extent Ca^{2+} signaling is involved in the initiation of glomerular disease. Individuals with TRPC6 mutations generally exhibit late-onset focal segmental glomerulosclerosis,[5,6] suggesting that Ca^{2+} dysregulation acts primarily in disease progression, but *in vitro* studies indicate that Ca^{2+} signaling may also initiate earlier changes in the podocyte.[9,10]

Downstream signals activated by Ca^{2+} signaling in the podocyte remain incompletely understood. Signaling through the Ca^{2+}-activated phosphatase calcineurin (CaN) has recently emerged as a modulator of podocyte and glomerular function. In both podocytes and cardiomyocytes, TRPC6 ac-

Received August 24, 2010. Accepted April 5, 2011.

Published online ahead of print. Publication date available at www.jasn.org.

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tivates CaN, inducing nuclear factor of activated T cell (NFAT)-dependent transcription.[9,11] An NFAT-independent mechanism for calcineurin has also been identified: by dephosphorylating the actin stabilizer synaptopodin (Synpo), calcineurin renders Synpo accessible to cathepsin L (CatL)-mediated degradation, leading to cytoskeletal rearrangement and proteinuria.[12] Because CaN activity is regulated by Ca^{2+} binding, these data imply that CaN may be a key transducer of Ca^{2+}-activated signaling in podocyte injury, although the Ca^{2+} dependence of this pathway has not been formally shown.

CaN is not the sole mediator of podocyte cytoskeletal function: among other signaling pathways affecting the filtration barrier is the Mammalian Target of Rapamycin (mTOR).[13,14] mTOR is a multiprotein kinase defined by two complexes: mTORC1 and mTORC2.[15] mTORC1 is involved in nutrient sensing and redox signaling,[16] whereas mTORC2 primarily regulates the cytoskeleton.[17] mTOR signaling modulates podocyte function,[18] and rapamycin, an inhibitor of mTOR, is an immunosuppressant drug with antiproteinuric effects in several animal models of renal disease.[19,20,21] Rapamycin, initially identified as an mTORC1 inhibitor,[22] has been shown to also affect signaling through mTORC2.[23]

mTOR signaling and phosphoinositide 3-kinase (PI3K) are both linked to activation of the kinase Akt, a regulator of cytoskeletal dynamics and apoptosis.[1] mTORC2 acts as an upstream regulator of Akt in podocytes, and rapamycin blocks this interaction.[18] Interestingly, Akt activity can be modulated by Ca^{2+} in nonpodocyte cells,[24,25] raising the possibility that Ca^{2+} may also interact with Akt signaling in the podocyte.

In this study, we examined the role of Ca^{2+}-dependent molecular pathways in the initiation of foot process effacement using a modified method for assaying filtration barrier integrity in whole glomeruli. We show that protamine sulfate induces rapid foot process effacement and changes in the filtration barrier and that these early alterations require Ca^{2+} influx. In addition to establishing an early role in foot process effacement, we sought to understand the downstream consequences of Ca^{2+} signaling. We demonstrate that elevated Ca^{2+} activates both calcineurin and mTOR signaling, leading to increased albumin permeability across the glomerular filtration barrier. We further show that Akt plays a critical role in these early events and that Akt signaling is downstream of both Ca^{2+} and rapamycin. These data provide insight into the initial signaling events regulating the glomerular barrier, demonstrating the importance of tight Ca^{2+} regulation in maintaining glomerular integrity and suggesting potential avenues for therapeutic intervention in glomerular injury.

RESULTS

Protamine Sulfate Induces Glomerular Permeability to Albumin and Podocyte Foot Process Effacement

We used isolated, protamine sulfate (PS)-treated glomeruli as an *ex vivo* model of glomerular injury. PS induces rapid podocyte foot process effacement in perfused kidneys and in isolated glomeruli.[26,27] We confirmed the presence of intact foot processes in untreated isolated glomeruli (Figure 1A, arrows), whereas foot process effacement was evident after 40 minutes of PS treatment (Figure 1B, arrows).

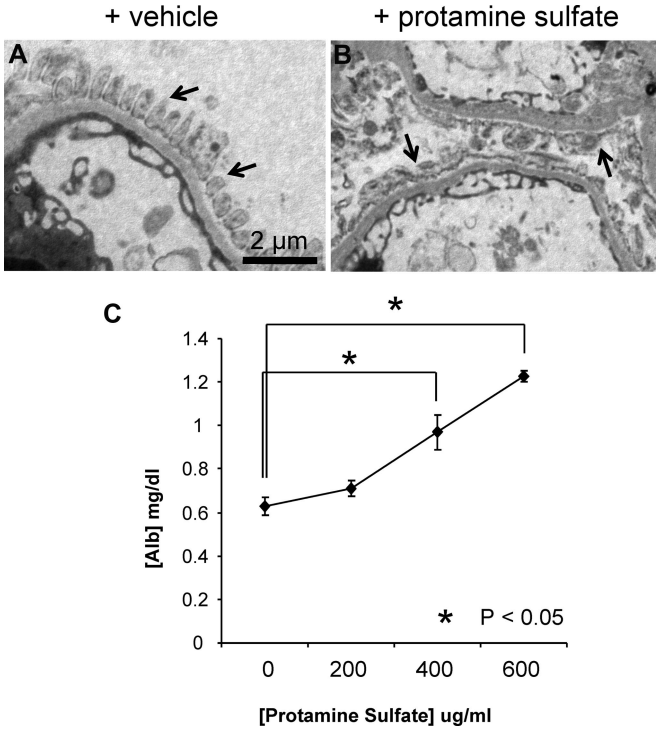

Figure 1. PS treatment causes podocyte foot process effacement and glomerular albumin leakage. (A) Electron micrograph of untreated glomeruli show intact podocyte foot processes with well delineated slit diaphragms dispersed along the glomerular basement membrane (arrows). (B) Forty-minute PS treatment leads to flattening and fusion of the foot processes (arrows). (C) PS treatment causes a statistically significant ($P < 0.05$), dose-dependent release of albumin, reflecting functional changes in the glomerular barrier.

Savin *et al.*[26] demonstrated that *ex vivo* glomeruli release albumin when the surrounding oncotic gradient is reduced and that PS treatment stimulates this release, reflecting the increased albumin permeability of the injured glomerular barrier. We modified this method, using a rat albumin-specific ELISA (Supplemental Figure 1A) rather than glomerular swelling to measure albumin release. Briefly, glomeruli are isolated and pretreated with PS before reducing the oncotic gradient. Consistent with PS-induced damage to the glomerular barrier, PS-treated glomeruli increase their albumin release in a dose-dependent manner (Figure 1C). These changes are detectable after as little as 45 minutes of PS treatment (Supplemental Figure 1B) and are reproducible in separate replicate experiments (Supplemental Figure 1C). These results demonstrate that PS-induced foot process effacement leads to changes in the glomerular barrier, providing a quantitative *ex vivo* assay of glomerular permeability.

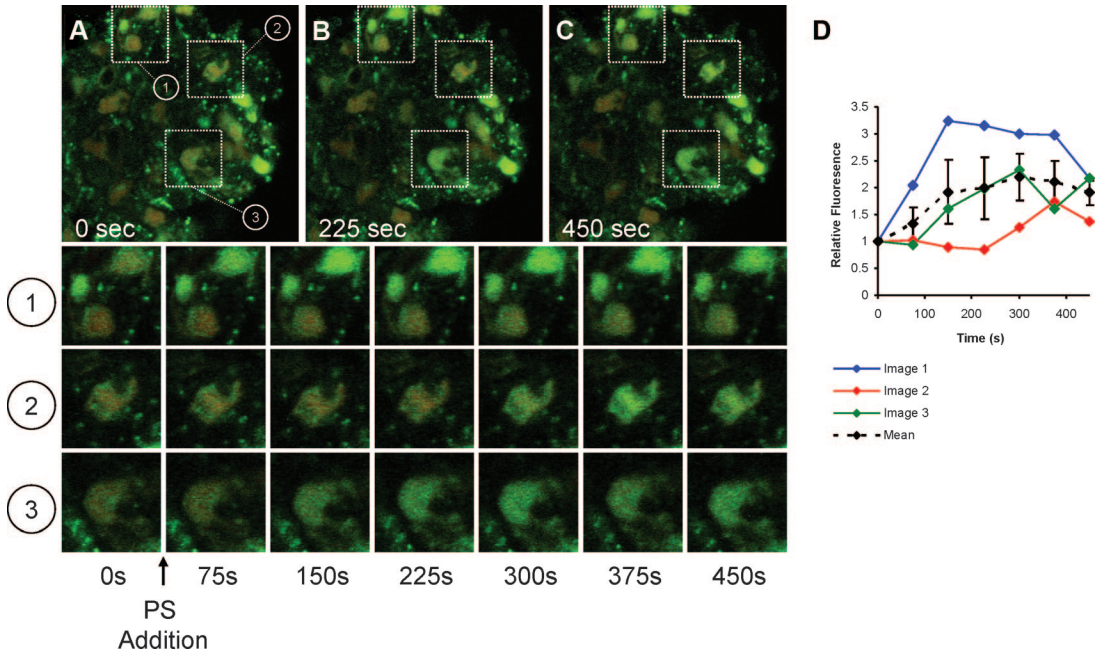

Figure 2. PS treatment induces Ca^{2+} transients in isolated glomeruli. (A through C) A representative isolated glomerulus imaged by confocal microscopy. Glomeruli were imaged for at least 2 minutes before addition of protamine sulfate (PS), during which time no change in fluorescence was observed (A). The images in B and C were taken at 75-second intervals after PS addition. Images in rows 1, 2, and 3 are magnifications of the areas marked in A, depicting changes in fluorescence over shorter periods of time. Cells in row 1 are on the outer aspect of the glomerulus and show increased fluorescence upon PS addition, indicating a slow, sustained increase in cellular Ca^{2+} levels. Cells in rows 2 and 3 are more internal to the glomerulus. These cells show a similar response, although the onset of changes in Ca^{2+} is delayed relative to the cells in row 1. (D) Image quantitation of Fluo-4 fluorescence from cells in rows 1, 2, and 3. Mean fluorescence is indicated by the dotted black line.

Treatment with PS Increases Intracellular Calcium Concentration in Isolated Glomeruli

Ca^{2+}-mediated signaling is a potential regulator of glomerular function,[5,6,12,28] and PS increases intracellular Ca^{2+} in cultured podocytes.[29] Figure 2A shows a representative glomerulus before the addition of PS. Three areas are highlighted, with enlarged images of these areas shown as rows 1, 2, and 3. Increased Fluo-4 fluorescence is evident upon PS addition at 20 seconds (Figure 2, B and C), reflecting increased intracellular Ca^{2+} upon treatment. Note that the time between PS addition and increased Fluo-4 fluorescence varies from cell to cell (Figure 2D). This may be a consequence of the heterogeneous cell population in the isolated glomerulus, cell-to-cell variations in the amount of Fluo-4 loading, or the diffusion rate of PS after addition. Nevertheless, these data indicate that, as in isolated podocytes, PS induces changes in intracellular Ca^{2+} in *ex vivo* glomeruli.

Calcium Signaling Is Required for PS-mediated Changes in the Glomerular Barrier

To determine whether changes in intracellular Ca^{2+} regulate the glomerular barrier, we manipulated Ca^{2+} signaling during PS-mediated glomerular damage. Pretreatment with Gd^{3+}, a general blocker of ion channels, or SKF96365, a selective inhibitor of receptor-mediated Ca^{2+} entry,[30] prevents PS-induced albumin release (Figure 3, A and B). Additionally, PS does not induce albumin release from glomeruli in the presence of the Ca^{2+} chelator EGTA (Figure 3C). Taken together, these experiments suggest that receptor-operated Ca^{2+} channels are at least partially required for PS-induced changes in glomerular permeability.

CaN and Synpo are Critical Early Regulators of Glomerular Barrier Function

Faul *et al.*[12] recently showed that the Ca^{2+}-dependent phosphatase CaN regulates podocyte function by dephosphorylating Synpo, a regulator of actin dynamics. CaN-dependent dephosphorylation renders Synpo accessible to CatL-mediated degradation. These data suggest that the Ca^{2+}-dependent changes we observe in *ex vivo* glomeruli may be mediated by the CaN/Synpo axis. To test this hypothesis, we examined the effect of PS on Synpo cleavage (Figure 4A). After PS treatment, we detect decreased expression of the full-length (110 kD) form of Synpo, and this effect is blocked by pretreatment with the CatL inhibitor E64. These results indicate that PS sulfate treatment rapidly induces CatL-dependent Synpo cleavage in isolated glomeruli.

Next, we examined whether blocking Synpo cleavage, either directly or through CaN inhibition, prevents early changes in albumin permeability. Pretreatment of glomeruli with the CaN inhibitors FK506 or cyclosporine A (Figure 4, B and C) or with the CatL inhibitor E64 (Figure 4D) reduces albumin release in PS-treated glomeruli. These data confirm the hypothesis that CaN activity and Synpo cleavage are essential regulators of the renal filter, further dem-

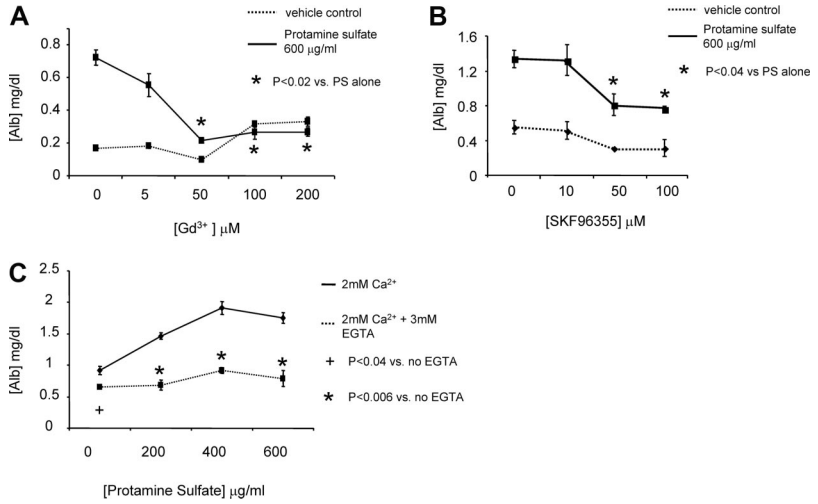

Figure 3. PS-mediated damage requires Ca^{2+} influx through membrane-associated ion channels. (A) PS treatment in the absence of Gd^{3+} increases albumin release in the glomerular permeability assay. Pretreatment with Gd^{3+} blocks the effects of PS in a dose-dependent manner, with complete inhibition at ≥50 μM Gd^{3+} ($P < 0.02$). (B) Albumin release from isolated glomeruli is significantly reduced by addition of ≥50 μM SKF96365 ($P < 0.04$). (C) The presence of 3 mM EGTA slightly reduces albumin release in the absence of PS treatment ($P < 0.04$). As increasing concentrations of PS are added, Ca^{2+} chelation by EGTA completely blocks albumin release in the assay ($P < 0.006$).

onstrating that both proteins are regulators of the initial steps in glomerular barrier function.

mTORC2 But Not mTORC1 Is Activated by PS–Induced Glomerular Injury

Although CaN signaling is clearly important, it is likely not the sole mediator of injury, as inhibition of other pathways also affects glomerular function.[31] We therefore examined additional signaling pathways in the glomerulus. Rapamycin, an inhibitor of mTOR signaling, regulates glomerular function both *in vitro* and *in vivo*[18–20] and exhibits renoprotective effects in many animal models of renal damage.[20,32,33] Concordantly, pretreatment of isolated glomeruli with rapamycin dose-dependently ameliorates the effects of PS (Figure 5A), indicating that mTOR signaling is necessary for PS injury.

To further elucidate the role of mTOR, we examined phosphorylation of downstream components of the mTORC1 and mTORC2 complexes. Phosphorylation of the mTORC1 targets 4E-BP1 and p70S6K is attenuated, but not abolished, by rapamycin pretreatment, both in the absence and in the presence of PS (Figure 5B). This is surprising given the high concentration of rapamycin and suggests that isolated glomeruli may be relatively rapamycin insensitive over the short time frame of this assay. Nevertheless, PS treatment does not appear to induce 4E-BP1 or p70S6K phosphorylation, suggesting that PS-induced changes to the filtration barrier do not require mTORC1 activation (Figure 5B).

Because rapamycin can also inhibit mTORC2, we examined phosphorylation of the mTORC2 targets PKCα and the Ser473 residue of Akt.[23] Like 4E-BP1 and p70S6K, PKCα phosphorylation is unchanged by PS treatment (Figure 5B). Akt phosphorylation at Ser473, however, is activated by PS (Figure 5B), suggesting that PS activates components of mTORC2 signaling. This and the fact that mTORC1 markers 4E-BP1 and p70S6K are not induced by PS suggests that mTORC2 is the primary mTOR pathway mediating these changes. In contrast to 4E-BP1 and p70S6K, rapamycin dramatically inhibits Akt Ser473 phosphorylation, suggesting that the mechanism of rapamycin on PS-induced albumin release may be through mTORC2/Akt inhibition.

Akt Activation Is Linked to Glomerular Damage Independent of PI3K

Akt is a serine/threonine kinase that has been linked to changes in the cytoskeleton in podocytes and other cell types.[14,34] Akt can be activated through phosphorylation at Serine 473 by mTORC2 (PDK2) or phosphorylation at Threonine 308 by phosphoinositide 3-kinase (PI3K)-mediated PDK1 activation.[14,35,36] We show that PS treatment of glomeruli dose-dependently induces Akt Ser473 phosphorylation but does not affect Thr308 phosphorylation (Figure 6).

To understand whether Akt is functionally important at the glomerular filtration barrier, we blocked Akt activity using the Akt/mTOR inhibitor curcumin.[37] Curcumin inhibits Akt Ser473 phosphorylation in PS- and vehicle-treated glomeruli (Figure 7A) and reduces albumin release without affecting untreated glomeruli (Figure 7B). We also tested the role of PI3K in activating Akt in this system. Consistent with the lack of PS-induced Akt phosphorylation at Thr308, pretreatment with the PI3K inhibitors wortmannin or LY-294002 does not reverse PS-induced glomerular damage (Figure 7, C and D), indicating that Akt activation is not PI3K dependent.

Finally, to determine whether mTORC2/Akt signaling in glomeruli is linked to Ca^{2+}, we examined the effect of receptor-operated Ca^{2+} channel blockers on Akt Ser473 phosphorylation. Pretreatment with SKF96365 reduces this phosphorylation, both in the presence and the absence of PS (Figure 8). Taken together, these data suggest that early changes in the glomerular barrier require Akt activity and that Ca^{2+} signaling activates Akt through mTORC2 in this system.

DISCUSSION

Disease and genetic animal models have provided substantial insight into the long-term consequences of altered signaling in the glomerulus, particularly in the podocyte, whereas early

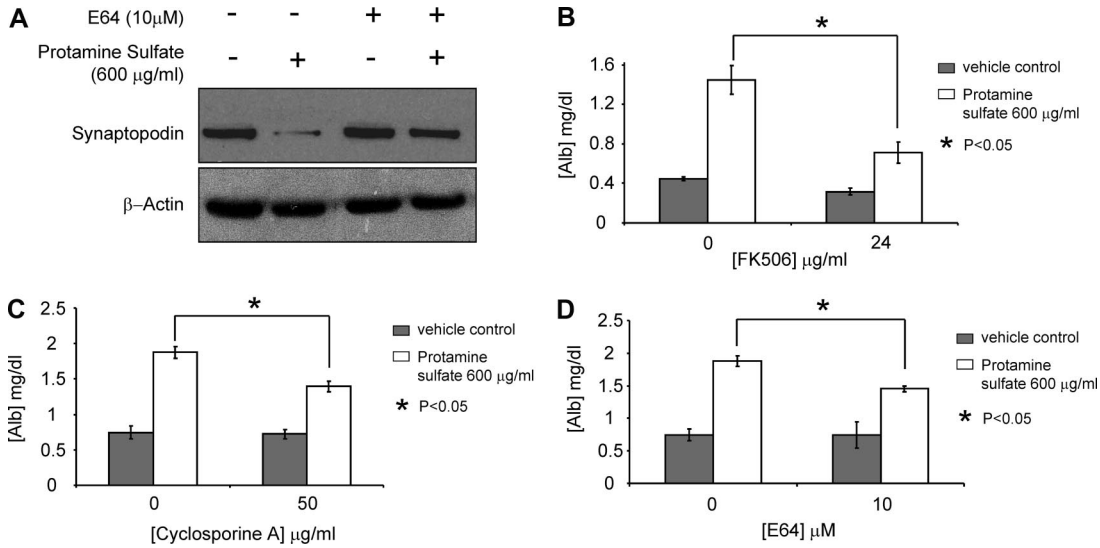

Figure 4. Protamine sulfate activates signaling through the calcineurin pathway. (A) Western blots show treatment with 600 μg/ml PS reduces the amount of full-length (110 kD) Synpo present in glomerular lysates. Pretreatment with 10 μM E64 prevents this reduction in full-length Synpo. (B and C) Pretreatment with 24 μg/ml FK506 (B) or 50 μg/ml cyclosporine A (C) reduces PS-mediated glomerular injury. (D) Treatment with 10 μM CatL inhibitor E64 partially blocks albumin release upon PS treatment.

injury responses have been identified using cultured podocytes. These two approaches are complemented by studying cell signaling in freshly isolated glomeruli, where the local environment of the glomerular barrier is relatively well preserved, but signaling is easily manipulated with pharmacologic inhibitors.

Using a method modified from Savin *et al.*,[26] we show that treatment of isolated glomeruli with PS induces podocyte foot process effacement and that effacement correlates with increased glomerular albumin permeability. These data are consistent with the effacement observed upon perfusion of intact kidneys with PS, suggesting that freshly isolated glomeruli respond to injury in a physiologically relevant manner. Thus, the measurement of albumin release provides a direct, simple, and quantitative method to assess immediate effects of PS on the glomerular barrier.

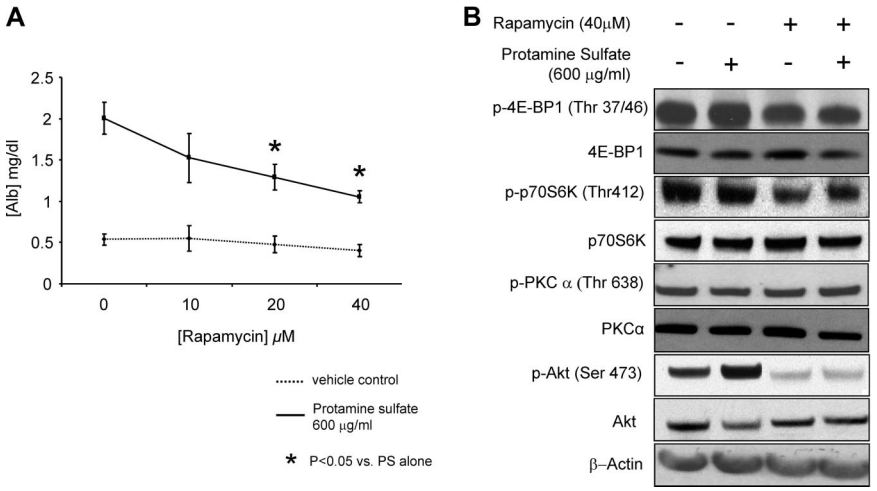

Figure 5. Rapamycin reduces glomerular damage by PS. (A) Glomerular permeability assay showing that PS damage (600 μg/ml) is reduced in a dose-dependent fashion by the addition of rapamycin. (B) Western blots of PS-treated glomeruli show that phosphorylation of the mTORC1 targets 4E-BP1 (Thr37/46) and p70S6K (Thr412) is unaffected by PS and is reduced by treatment with rapamycin, both in the absence and in the presence of PS. Phosphorylation of the mTORC2 target PKCα (Thr638) is also unchanged by PS or rapamycin. Akt phosphorylation at Ser473, however, is markedly increased by PS addition. Rapamycin reduces the basal level of phospho-Akt Ser473 and inhibits activation by PS.

A growing body of evidence implicates Ca^{2+} signaling in the regulation of podocyte function and the glomerular barrier *in vivo*.[7,38] PS treatment induces changes in intracellular Ca^{2+} in cultured podocytes.[29] Here we show that PS induces similar changes in intracellular Ca^{2+} in isolated glomeruli, correlating with foot process effacement. We cannot determine whether these Ca^{2+} changes occur exclusively in podocytes due to the inability to label live cells with identity markers. Nevertheless, these data suggest that Ca^{2+}-dependent signaling plays a role in short-term regulation of the filtration barrier.

To further examine regulation of the glomerular barrier by Ca^{2+}, we tested the effects of receptor-operated Ca^{2+} channel inhibition on PS-induced glomerular damage. Blocking Ca^{2+} channels or chelating extracellular Ca^{2+} prevents increased albumin permeability, further supporting the hypothesis that Ca^{2+} influx across the cell membrane regulates barrier integrity and may be a primary injury response.

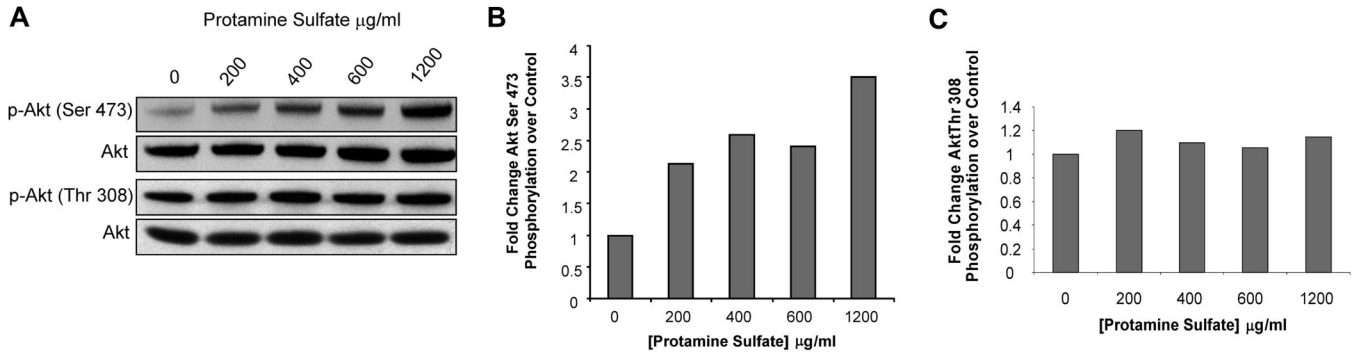

Figure 6. Akt Ser473 signaling is activated by PS in a dose-dependent manner after 40 minutes of PS treatment, whereas levels of the total protein remain stable (Western blot, panel A). Akt phosphorylation at Thr308 is unaffected by PS treatment (A). Quantitation of Akt phosphorylation (normalized to total Akt) in representative Western blots is shown in B and C.

To elucidate the consequences of Ca^{2+} influx, we examined several signaling pathways with proposed roles in glomerular injury. CaN signaling has been shown to play a role in glomerular disease, both through NFAT-mediated transcription[9] and by regulating Synpo cleavage by CatL.[12] We show that PS induces Synpo degradation, and this effect is attenuated by CatL inhibition. Inhibition of CaN or CatL also reduces PS-induced albumin release, reflecting *in vivo* experiments.[12] Importantly, because of the short timescale (40 minutes), our experiments indicate that CatL/CaN-activated Synpo degradation is an early mediator of foot process effacement.

Recent studies demonstrate that mTOR signaling regulates podocyte cytoskeletal dynamics and function,[17,18,33] but the upstream signals that activate mTOR in this context are not well understood. Using isolated glomeruli, we investigated whether mTOR plays a role in early barrier maintenance. The mTOR antagonist rapamycin blocks albumin release, indicating that this pathway is actively involved in early foot process effacement. This effect is independent of mTORC1, as phosphorylation of the mTORC1 targets, p70S6K and 4E-BP1, are unaffected by PS. We note, however, that rapamycin has only modest effects on p70S6K and 4E-BP1 phosphorylation, suggesting that mTOR in our experimental system may be relatively rapamycin insensitive. This may simply be a consequence of the rapid timescale of our studies. However, we also note that to block PS-induced changes in our system, significantly higher concentrations of rapamycin are required than are used in the clinic. In a recent review, Huber *et al.*[21] note that although rapamycin often induces proteinuria in human patients, numerous studies have shown that rapamycin prevents proteinuria in rodent models. Our ob-

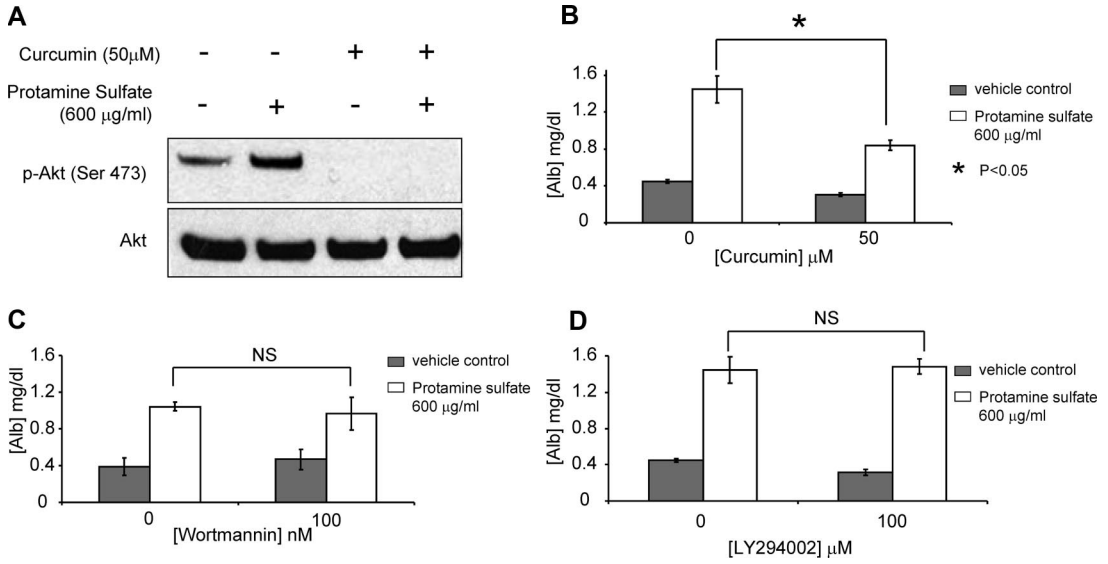

Figure 7. Akt inhibition reduces glomerular damage by protamine sulfate through a PI3K-independent mechanism. (A) Curcumin treatment reduces levels of phosphorylated Akt both in the absence and presence of PS. Total Akt protein was unchanged by either PS or curcumin. (B) Preincubation with 50 μM curcumin partially blocks the effects of PS in the permeability assay. (C and D) Pretreatment of glomeruli with the PI3K inhibitors wortmannin (C) or LY294002 (D) had no effect on PS-induced injury. NS, data points are not significantly different.

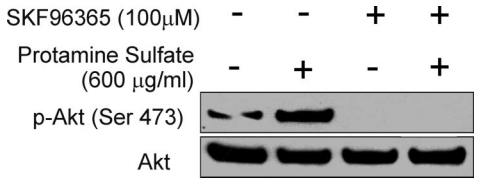

Figure 8. Inhibition of Ca^{2+} signaling blocks Akt activation. PS treatment induces Akt phosphorylation in the absence of the receptor-operated channel blocker SKF96365. Addition of 100 μM SKF96365 completely inhibits Akt phosphorylation, both in the absence and presence of PS.

servation of altered rapamycin sensitivity in isolated rat glomeruli may be consistent with this species-dependent heterogeneity, which likely reflects the complexity of the mTOR pathway.

Although several mTORC1 substrates are not affected by PS treatment, we show that PS-treatment induces Akt phosphorylation at Ser473, which lies downstream of mTORC2, but not at Thr308, which lies downstream of PI3K. Although the mTORC2 complex was initially thought to be rapamycin insensitive, recent evidence indicates that prolonged rapamycin treatment can block mTORC2 signaling.[23,39] We find that Akt phosphorylation at Ser473 is significantly inhibited by rapamycin, consistent with the hypothesis that rapamycin's effect in the albumin release assay reflects mTORC2 activation. The short-term inhibition of mTORC2 in our experimental system may be a consequence of the higher concentrations of rapamycin used.

Akt activity is required for glomerular barrier changes, as we show that the Akt inhibitor curcumin also reduces albumin release. Akt phosphorylation at Ser473 has profound effects on the cytoskeleton,[23,35] a role consistent with the rapid cytoskeletal rearrangements during foot process effacement. Because Akt is also a well characterized inhibitor of apoptosis, these data further raise the possibility that Akt coordinates several podocyte survival responses under injurious conditions. Thus, reformation of a functional filtration barrier after effacement may, in part, be due to the actions of Akt increasing both their mechanical plasticity and cell survival.

Surprisingly, our results suggest that early changes in the glomerular barrier may be independent of PI3K, as the PI3K inhibitors wortmannin and LY-294002 do not prevent albumin release and PS treatment does not cause Akt phosphorylation at Threonine 308, an indicator of PI3K activity through PDK1.[40] This, however, does not rule out a role for PI3K later in glomerular injury, because mTOR and Akt participate in several feedback loops that can affect PI3K.[35]

We demonstrate that early events in glomerular permeability involve Ca^{2+}-dependent signals, likely through receptor-operated channel activation, subsequently activating multiple downstream signaling pathways, including CaN and mTORC2. Taken together, these observations show the importance of tight cellular Ca^{2+} regulation and related signaling pathways in glomerular filtration dynamics. In Figure 9, we propose a model for regulation of podocyte foot process effacement by receptor-operated calcium channel signaling. PS initiates the activation of receptor-operated calcium channels, in turn activating both the calcineurin and mTORC2 pathways. Calcineurin dephosphorylates synaptopodin, thereby making it available for degradation by CatL and changing podocyte morphology, whereas mTORC2 induces Akt activity thereby affecting the cytoskeleton. Although we cannot conclude from our data whether activation of either pathway on its own is sufficient to induce glomerular injury, inhibition of either pathway alone reduces damage, suggesting that both arms are necessary for injury.

Understanding the molecular control points of Ca^{2+}-dependent signaling in the glomerulus will likely offer further insights into glomerular disease pathogenesis and novel therapeutic targets for the treatment of glomerulopathies.

CONCISE METHODS

Experimental Animals and Glomerular Isolation

Sprague-Dawley male rats weighing 200 through 300 g from Charles River Laboratories (Wilmington, Massachusetts) were used for glomerular isolation. Animals were euthanized by CO_2, and the kidneys were removed and placed in glomerular isolation buffer (115 mM NaCl, 1.2 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 5.5 mM glucose, pH 7.2) supplemented with 5% BSA. Glomeruli were isolated by pressing minced kidneys through a 100-mesh (149 μm) sieve, followed by passage through and collection from atop a 150-mesh (105 μm) and a 200-mesh (74 μm) sieve, Glomeruli were main-

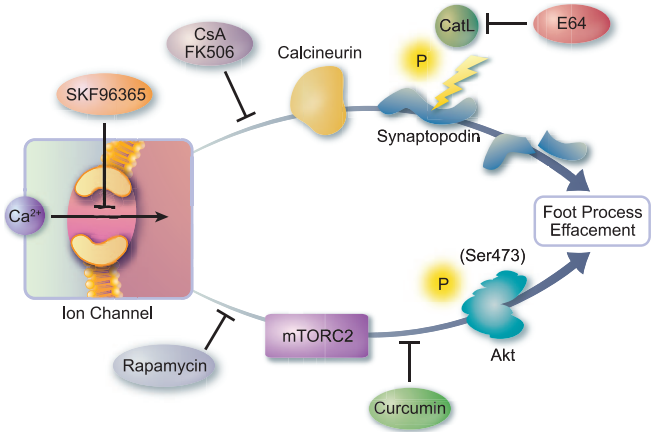

Figure 9. Proposed mechanism of podocyte foot process effacement by receptor-operated calcium channel signaling. PS initiates the activation of receptor-operated calcium channels. Calcium signaling can in turn activate both the calcineurin and mTORC2 pathways. Calcineurin dephosphorylates Synpo, thereby making it available for degradation by CatL and changing podocyte morphology. Calcium also activates mTORC2, in turn inducing Akt activity and affecting the cytoskeleton. Inhibition of either pathway is sufficient to block PS-induced damage to isolated glomeruli.

tained in the 5% BSA glomerular isolation buffer and washed three times. These preparations were free of tubules and Bowman's capsules.

Glomerular Permeability Assay

The glomerular permeability assay was performed with a modification of previously described methods,[26] using the Nephrat albumin ELISA plate (Exocell Inc., Philadelphia, Pennsylvania) to assay albumin release as a measure of glomerular damage. Glomeruli in 5% BSA glomerular isolation buffer were pretreated with either Gd^{3+}, SKF96365, FK506, cyclosporine A, or rapamycin as a 100× stock in DMSO for 15 minutes before PS treatment. PS treatments were performed in the ELISA plate, with 3000 glomeruli per well in a volume of 45 μl, and each condition was assayed in triplicate. Five microliters of PS was added for a final concentration of 600 μg/ml, and the plate was incubated for 40 minutes at 37°C. The oncotic gradient was changed from 5 to 1% BSA by the addition of 160 μl BSA-free glomerular isolation buffer and incubated for 10 minutes. The ELISA assay was then completed according to the manufacturer's instructions. Additional experiments for assay validation and reproducibility can be found in the Supplemental Data section (Supplemental Figure 1).

Electron Microscopy

Isolated glomeruli were treated with PS and after 40 minutes immediately were placed in 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, Pennsylvania). Glomeruli were postfixed in 1% osmium tetroxide and dehydrated in ethanol and propylene oxide. The tissues were embedded in Epon araldite resin and sectioned on a Leica Ultracut. Electron microscopy images were captured on a JEOL JEM-1400 electron microscope.

Fluorescence Glomerular Calcium Imaging

Glomeruli were maintained in a BSA-free glomerular isolation buffer and added to a loading solution (0.02% Pluronic F-127, 20 μM Fluo-4AM, 20 μM SNARF; Invitrogen, Carlsbad, California) for 20 minutes. The glomeruli were washed twice in a 2 mM probenecid/PBS solution. Imaging was performed using a Zeiss LSM510-META laser scanning confocal microscope and a 40× 1.2 NA water immersion objective. As a negative control, glomeruli were imaged for at least 2 minutes before addition of PS to ensure that no change in fluo-4 fluorescence was occurring. Fluorescence image quantitation was performed using ImageQuant software (IQ Solutions).

Western Blot Analysis

Glomeruli were homogenized in RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 0.1% SDS, 1% protease inhibitor cocktail [Roche] supplemented with 1 mM Na_3VO_4 and 1 mM NaF as phosphatase inhibitors), separated on a denaturing 4 to 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Primary antibodies were anti-rabbit phospho-PKCα/β (Thr638/641)/ phospho-4E-BP1 (Thr37/46)/phospho-Akt (Ser473)/ phospho-Akt (Thr308) phospho-p70S6K (Thr412) (Cell Signaling Technologies, Danvers, Massachusetts), with corresponding nonphospho primary antibodies from the same vendor. All blots were blocked in 5% BSA/TBS/0.1% Tween-20 and were visualized with SuperSignal West Pico reagent (Pierce, Rockford, Illinois). Western blot quantitation was performed using ImageQuant software (IQ Solutions).

Statistical Analyses

Comparisons between groups were performed using the *t* test. Values are reported as mean ± SEM. Statistical significance is recognized at $P < 0.05$.

DISCLOSURES

None.

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