Flow stimulated endocytosis in the proximal tubule

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Purpose of review
The proximal tubule plays a critical role in the reabsorption of ions, solutes and low molecular weight proteins from the glomerular filtrate. Although the proximal tubule has long been known to acutely modulate ion reabsorption in response to changes in flow rates of the glomerular filtrate, it has only recently been discovered that proximal tubule cells can similarly adjust endocytic capacity in response to flow. This review synthesizes our current understanding of mechanosensitive regulation of endocytic capacity in proximal tubule epithelia and highlights areas of opportunity for future investigations.

Recent findings
Recent studies have reported that the endocytic capacity of proximal tubule cells is dramatically increased upon exposure to flow and the accompanying fluid shear stress. Modulation of this pathway is dependent on increases in intracellular calcium initiated by bending of the primary cilium, and also requires purinergic receptor activation that is mediated by release of extracellular ATP. This article summarizes what is currently known about the signaling cascade that transduces changes in flow into alterations in endocytosis. We discuss the implications of this newly described regulatory pathway with respect to our understanding of protein retrieval by the kidney under normal conditions, and in diseases that present with low molecular weight proteinuria.

Summary
Primary cilia act as mechanotransducers that modulate apical endocytic capacity in proximal tubule cells in response to changes in fluid shear stress.

Keywords
calcium, fluid shear stress, mechanosensation, primary cilium, purinergic receptor

INTRODUCTION

The proximal tubules of the kidney are responsible for recovering over two-thirds of the water from the glomerular filtrate, as well as a significant fraction of the filtered Na^+, K^+, Cl^-, HCO_3^-, phosphate, and glucose. Despite sizable daily variations in glomerular filtration rate (GFR), the kidneys maintain consistent fractional fluid and solute reabsorption efficiency. Studies conducted over the past decade have shown that ion reabsorption in the proximal tubule is modulated in response to changes in GFR to preserve glomerulotubular balance. The flow of the glomerular filtrate through the proximal tubule manifests as fluid shear stress (FSS) on the apical surface of the cells lining the renal tubule and as radial stretch on the wall of the renal tubule. Apical microvilli on proximal tubule cells have been suggested to function as mechanosensors that transduce changes in FSS to regulate ion transporter trafficking and activity [1–4]. For example, changes in GFR and the accompanying FSS and stretch lead to increases in Na^+ reabsorption mediated primarily by the insertion of active transporters into the luminal membrane [3,5–7]. Changes in actin dynamics are thought to play a role in this process; however, the exact mechanism for how this leads to altered transporter distribution has not been determined. In addition to mediating mechanosensation upon bending of the microvilli, changes in the actin cytoskeleton resulting from radial stretch may also contribute to FSS-dependent signaling [1,8].

In the distal tubule, primary cilia have emerged as the organelles that transduce changes in FSS into signals that modulate ion transport and other cellular functions [9▪▪,10,11]. Defects in ciliary biogenesis and function lead to diseases (collectively termed ciliopathies) that manifest with a subset of common phenotypes, including cystic kidneys, retinal dystrophies, cognitive impairment, and polydactyly

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(reviewed in [12]). In common with distal tubule cells, proximal tubule cells also express primary cilia, but no role for cilia in modulating proximal tubule ion transport has been described [12–14].

Another major function of the proximal tubule is to retrieve low molecular weight (LMW) proteins, vitamins, hormones, and other small metabolites from the glomerular filtrate. In the kidney, the multiligand receptors megalin and cubilin are expressed exclusively in the proximal tubule, and mediate the apical binding and internalization of these LMW proteins and other ligands into proximal tubule cells (reviewed in [15]). Defects in the receptors that mediate the uptake of these filtered ligands or saturation of the clearance pathway (e.g., in diabetes [16]), lead to LMW proteinuria. Prolonged LMW proteinuria (also referred to as tubular proteinuria because it does not involve glomerular dysfunction) causes further deterioration in kidney function and leads to renal failure [17].

Elevations in GFR increase the amount of filtered megalin and cubilin ligands that pass through the proximal tubule. A long-held assumption is that proximal tubule cells maintain a sufficiently high constitutive endocytic capacity to efficiently retrieve these proteins even at high GFR. However, until recently, whether proximal tubule cells modulate endocytosis in response to changes in flow had not been directly tested. This review summarizes recent findings that implicate cilia-mediated mechanotransduction in regulating apical endocytosis in proximal tubule epithelia and discusses new challenges in the field that emerge from this finding.

THE APICAL ENDOCYTIC PATHWAY

The epithelial cells that line the proximal tubule are specialized to internalize and recycle large amounts of apical membrane in order to effectively reabsorb megalin/cubilin ligands that enter the tubule lumen. Despite the critical role of apical endocytosis in proximal tubule function, surprisingly little is known about how this pathway is organized or regulated. Our current understanding of the apical endocytic pathway in proximal tubule cells comes primarily from electron microscopy studies examining the uptake of fluid phase and membrane markers [18–22]. Proximal tubule cells have highly irregular invaginations at the base of microvilli at the apical surface in proximal tubule cells compared with other polarized epithelia [18,20,21,23]. Fluid and membrane tracers are internalized into unevenly shaped clathrin-coated pits that form at the base of brush border microvilli. After budding and uncoating, these endocytic vesicles fuse with a dense network of subapical tubules [18]. 'Fast recycling' (i.e., rapid return of internalized receptors to the apical cell surface without transit through recycling endosomes) may occur from these sites [22]. Within 1–15 min after internalization, both membrane and fluid phase markers can be visualized in large spherical compartments termed apical vacuoles [18–20]. Tubules that arise from these apical vacuoles are thought to carry recycling proteins back to the apical surface [19,20], whereas dissociated ligands are delivered to lysosomes for degradation or transcytosed to the basolateral membrane [24].

This model for apical endocytosis in proximal tubule cells differs significantly from the pathway described in kidney cells derived from other tubule segments and best characterized in Madin–Darby canine kidney (MDCK) cells. In MDCK cells, apically internalized proteins enter Rab5-positive apical early endosomes, and membrane proteins recycle to the surface via Rab8 positive common endosomes and Rab11 positive apical recycling endosomes [25]. How the apical tubules and vacuolar compartments in proximal tubule cells correspond to the compartments described in MDCK and other polarized kidney cells is not known, although we recently found that the vacuolar structures in primary cultures of proximal tubule cells are Rab11a positive [26]. Presumably, apical endocytic compartments in proximal tubule cells have specialized adaptations that enable them to uniquely handle the large flux of internalized membrane through this pathway. Moreover, as described below, it appears that this pathway can be acutely upregulated in response to increased endocytic demand.

FLUID SHEAR STRESS MODULATES APICAL ENDOCYTOSIS IN PROXIMAL TUBULE CELLS

Two recent studies have observed that endocytosis in proximal tubule cells is modulated by FSS.

Opossum kidney cells incubated under a FSS of 1 dyne/cm^2 internalized 2–3-fold more fluorescent albumin compared with cells incubated under static conditions [27,28]. This shear stress in the human proximal tubule corresponds to a flow rate of roughly 63 nl/min (and a GFR of roughly 115 ml/min per 1.73 m^2) [29,30]. Similar results were obtained with the porcine proximal tubule cell model LLC-PK1 [28], and more recently with immortalized human and mouse proximal tubule cell lines (Raghavan and Weisz, unpublished). Additionally, exposure to FSS also increased uptake of the fluid phase marker rhodamine dextran, suggesting that FSS stimulates a generic increase in apical endocytic capacity rather than a selective increase in the uptake of megalin/cubilin ligands [28]. Consistent with the idea that this response is specific for proximal tubule cells, no effect of FSS on apical endocytosis was observed in MDCK type II cells, which have hybrid characteristics of proximal and distal tubule cells [28,31].

Endocytosis from the apical surface of differentiated kidney cells occurs exclusively at the base of microvilli via a clathrin-dependent, dynamin-dependent, and actin-dependent pathway [32–37]. Addition of chlorpromazine (which disrupts clathrin assembly) or the dynamin inhibitor Dyngo-4a [38] to opossum kidney cells inhibited both basal and FSS-stimulated endocytosis, suggesting that the same internalization mechanism operates under both conditions [28]. Moreover, the onset of the FSS-stimulated response was rapid and statistically different from control by 15–30 min of exposure to FSS [28]. Similarly, within 15 min of removing FSS, the endocytic rate returned to the constitutive level observed in cells maintained under static conditions [28]. The delay in onset and rapid reversibility of the FSS-stimulated endocytic response suggest that this is a downstream event in a tightly regulated signaling cascade. The precise mechanism underlying this reversible cascade remains to be determined (see below).

Importantly, the endocytic response to FSS is tuned within the normal physiological range of flow across the proximal tubule. GFR ranges between 60 and 120 mL/min per 1.73 m^2 in healthy individuals, but can reach more than 140 mL/min per 1.73 m^2 in diabetic patients [39]. Our study showed that the endocytic response trended upwards starting at low FSS (>0.2 dyne/cm^2) and reaches statistical significance compared with control cells (maintained under static conditions) between 0.7 dyne/cm^2 (equivalent to a GFR of ~60 mL/min per 1.73 m^2) and 1 dyne/cm^2 [28]. Interestingly, exposure to higher FSS (1.5 dyne/cm^2, equivalent to a GFR of ~150 mL/min per 1.73 m^2) did not further enhance uptake of albumin [28]. This suggests that proximal tubule cells tune their endocytic capacity within the normal range, and that saturation of this response at the higher FSS encountered in hyperfiltering disease states may contribute to tubular proteinuria.

PRIMARY CILIA, PURINERGIC SIGNALING AND Ca^{2+} RELEASE FROM ENDOPLASMIC RETICULUM STORES MEDIATE THE ENDOCYTIC RESPONSE TO FLUID SHEAR STRESS

In distal tubule cells, bending of primary cilia in response to FSS leads to an acute and transient increase in intracellular calcium $[Ca^{2+}]_i$ [11,40]. Similarly, FSS triggered a rapid increase in $[Ca^{2+}]_i$ in polarized opossum kidney cells that was not observed when Ca^{2+} was omitted from the perfusion buffer, or when cells were deciliated by treatment with ammonium sulfate [28].

The FSS-stimulated, cilium-dependent increase in $[Ca^{2+}]_i$ in collecting duct cells is mediated by Ca^{2+}-stimulated Ca^{2+} release from endoplasmic reticulum (ER) reserves [11]. Consistent with this, FSS had no effect on $[Ca^{2+}]_i$ in opossum kidney cells treated with the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor tBuBHQ (to deplete ER reserves of Ca^{2+}) or ryanodine (to deplete ryanodine-sensitive stores of ER Ca^{2+}) [28]. Ciliary bending in distal tubule cells is also known to cause extracellular release of ATP that can trigger P2X and P2Y purinergic receptors and cause a further increase in $[Ca^{2+}]_i$ [9,41]. Consistent with this possibility, inclusion of the ATP hydrolyzing enzyme apyrase in the perfusion medium attenuated the FSS-stimulated increase in $[Ca^{2+}]_i$. Similarly, treatment with suramin, a pan-inhibitor of purinergic receptors, blocked this response. Conversely, addition of ATP or ryanodine to cells incubated under static conditions was sufficient to trigger enhanced endocytosis of albumin [28]. Together, these results suggest an important role for ciliary mechanosensitive Ca^{2+} channels, extracellular ATP, purinergic signaling, and ER stores of Ca^{2+} in regulating the endocytic response to FSS.

TOWARD A COMPREHENSIVE MODEL FOR FLOW-STIMULATED ENDOCYTOSIS

The observations above suggest a model whereby ciliary bending activates cation channels (possibly polycystin-2) that lead to a small increase in $[Ca^{2+}]_i$ that triggers extracellular ATP release to activate P2Rs and stimulates further Ca^{2+} release from ER stores, ultimately leading to enhanced endocytosis
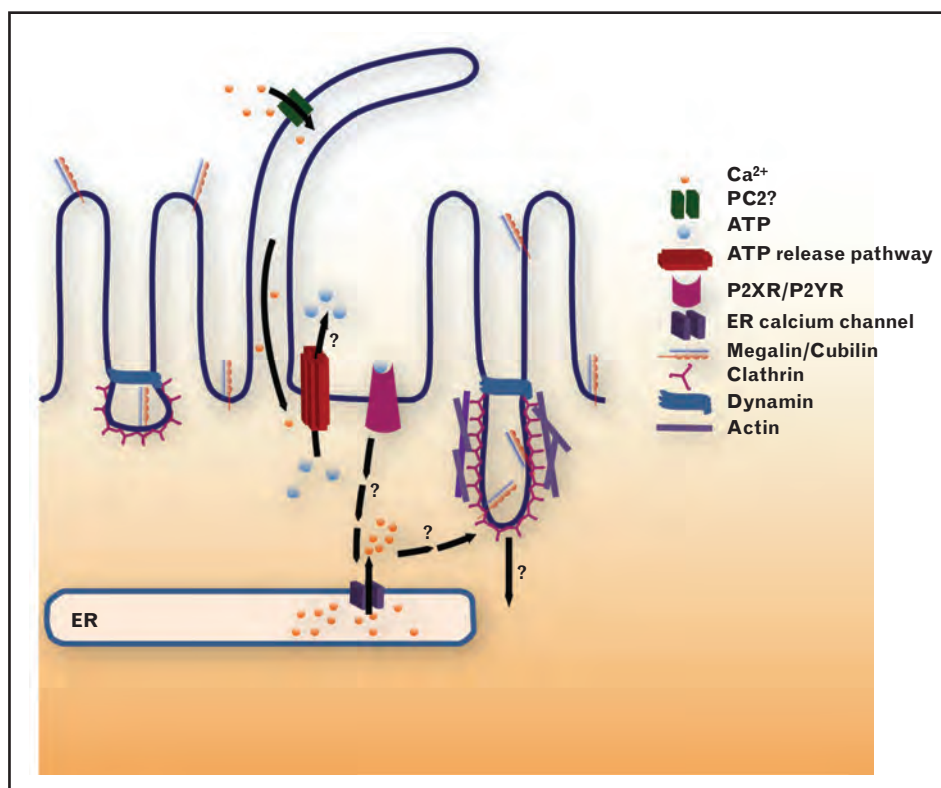
FIGURE 1. Working model for fluid shear stress-stimulated endocytosis in the proximal tubule. We hypothesize that ciliary bending activates a cation channel [possibly polycystin-2 (PC2)] that elevates ciliary and periciliary $[Ca^{2+}]_l$ to trigger luminal release of ATP via any of several pathways (see text for details). ATP activates purinergic P2X or P2Y receptors (P2XR/P2YR) that ultimately leads to Ca^{2+} release from endoplasmic reticulum stores via ryanodine and IP_3 receptor-mediated pathways. Increased $[Ca^{2+}]_i$ in turn ultimately leads to enhanced apical endocytosis. The downstream signaling cascade leading to the endocytic response remains to be determined. Endocytosis from the apical surface of polarized cells occurs at the base of microvilli via a pathway that requires clathrin and dynamin. Coated pit morphology at the apical surface of proximal tubule cells is highly irregular compared with other polarized epithelial cells consistent with the possibility that endocytosis is stimulated by an increase in individual endocytic pit size. Alternatively or in addition, the increase in endocytic capacity in response to fluid shear stress may be caused by an increase in the number of apical clathrin coated pits that form.

(Fig. 1). Needless to say, many gaps in our understanding of the steps and implications of this model remain, and are discussed further below.

Although primary cilia are required for the endocytic response to FSS, the mechanosensitive channel that initiates the response remains to be identified. An obvious candidate is the transient receptor potential (TRP) channel polycystin-2 (PC-2), which is important for flow-dependent responses in the distal tubule [11]. The related TRPV4 channel has also been implicated in mechanosensation in the distal tubule, possibly by forming a complex with PC-2 [42–45]; however, TRPV4 is not expressed in the proximal tubule [46].

An unresolved general question of broader significance is the relationship between cilia length, Ca^{2+} signaling, and the endocytic response. Mutations in many proteins involved in ciliary biogenesis lead to cystic kidney disease, and both shorter and longer cilia can be observed under these

conditions. However, the relationship between FSS, ciliary length, and Ca^{2+} release from ER stores is unknown: is Ca^{2+} release graduated or binary when the other variables are altered? Surprisingly, there have been no studies in which the effect of different FSS on $[Ca^{2+}]_i$ in cells with varying cilia length has been systematically measured. A modeling study predicts that longer cilia are more sensitive to flow, and consistent with this idea, two reports suggest that FSS-stimulated Ca^{2+} signaling is blunted in cells with shortened cilia [47–49]. However, one of these groups also observed blunted Ca^{2+} responses in young mice, which have longer cilia than their older counterparts [48]. Thus, there may be an optimal cilia length that enables maximal signaling.

Despite the sizable body of evidence demonstrating a role for primary cilia in flow sensing, the availability of new methods to directly record and quantify the concentration of Ca^{2+} in the cilioplasm has sparked controversy surrounding

the role of this organelle in mechanosensation Studies by the Clapham group clearly demonstrate that the cilioplasm is segregated from the cytosol, and can function as a discrete Ca^{2+} signaling domain [50]. In these experiments, increases in ciliary Ca^{2+} did not lead to substantial alterations in intracellular calcium levels. Moreover, the authors found that polycystins were not especially sensitive to changes in pressure (although they did not apparently measure activity in response to changes in FSS) [51]. Taken to the extreme, these observations could argue that ciliary bending does not trigger mechanosensation. However, more recent work by Nauli and colleagues clearly demonstrates that exposure to flow causes a PC-2-mediated increase in $[Ca^{2+}]_i$ [52▪▪]. These studies also found differences in $[Ca^{2+}]_i$ responses to chemical versus mechanical stimuli, as the increase in Ca^{2+} in response to chemical stimulation of the ciliary CaV1.2 channel was restricted to the cilioplasm [52▪▪].

Another essential question to address is what maintains the endocytic response to flow after the initial and transient spike in $[Ca^{2+}]_i$? Endocytosis rates increase in proximal tubule cells within 15–30 min after exposure to FSS, and this increased rate of uptake persists long after $[Ca^{2+}]_i$ has returned to baseline levels, so long as the FSS is maintained (at least up to 3 h [28▪▪]). Moreover, once the flow is removed, endocytic rates return to normal very rapidly. This suggests that an as yet undetermined and sustained signal/secondary messenger is initiated by the increase in $[Ca^{2+}]_i$ that continues to sense the level of FSS and maintain enhanced endocytic capacity.

Our model hypothesizes that subsequent to ciliary bending, the release of ATP into the extracellular milieu is critically important for the FSS-stimulated response. Based on recent studies from other labs, ATP could also initiate signaling upstream of ciliary bending [50,53]. Moving forward, it is important to define the mechanism(s) underlying flow-mediated ATP release from proximal tubule cells. Several pathways are known to enable ATP release in the kidney and other organs [54]. First, ATP can be released by the connexin (Cx) family of gap-junction channels. Cx30 hemichannels have been demonstrated to play a role in luminal ATP release in the distal tubule [55], and several members of the connexin family are reported to be expressed in the proximal tubule, including Cx26, Cx32, Cx37, Cx40, and Cx43 [56–60]. Second, the channel-forming pannexin1 (Panx1) protein is widely expressed in the kidney, including the proximal tubule, and has been found to play a role in luminal ATP release [61]. Several chloride channels, including maxi anion channels, voltage-regulated ion channels, and tweety anion channels have also been implicated in ATP release from cells

[54]. Finally, ATP may be delivered to the extracellular milieu through constitutive vesicular release or through Ca^{2+}-triggered exocytosis, as has been demonstrated in MDCK cells [62].

We consistently observed that the pan-purinergic receptor blocker suramin inhibits both the increase in $[Ca^{2+}]_i$ and endocytosis in response to FSS, consistent with a critical role for purinergic signaling in mediating these FSS-dependent responses. Purinergic receptors that have been variously reported to be expressed in the proximal tubule include P2X1, P2X4, P2X5, P2X6, and P2X7, and P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 [63–68]. Some discrepancies in expression of these receptors have been reported in different studies, and there may also be species-dependent differences. The purinergic receptor involved in this cascade can be identified by knocking down candidates and through pharmacological interventions.

P2X receptors typically trigger ER Ca^{2+} release via activation of ryanodine receptors, whereas P2Y receptors are commonly thought to signal via IP3 receptors. Although we observed that ryanodine added at concentrations in which it functions as an agonist of the ryanodine receptor can trigger endocytosis even in cells maintained under static conditions, we do not know whether it is required for the FSS-stimulated endocytic response. Identifying the ER Ca^{2+} release channel involved in the endocytic response to FSS is challenging, but may also provide clues as to which purinergic signaling mechanism (P2X or P2Y) is relevant.

Finally, we lack an understanding of how increases in $[Ca^{2+}]_i$ are transduced into enhanced endocytic capacity. The increase in endocytosis could be due to an increase in the number of coated pits, an increase in their average, or a combination of these factors. There is precedence for actin-dependent modulation of coated vesicle size, as recent studies have shown that actin is required for clathrin-mediated uptake of larger cargoes such as virus particles, whereas endocytosis of most cargoes in nonpolarized cells is largely actin-independent [69]. Coated pit morphology at the apical surface of proximal tubule cells is highly irregular compared with other polarized epithelial cells consistent with dynamic variation in endocytic pit capacity. Our recent unpublished results suggest that Cdc42, a small GTPase involved in regulating actin dynamics, may play a role in coupling the initial Ca^{2+} response to flow into enhanced apical endocytosis.

CONCLUSION

This review describes our current understanding of how proximal tubule cells respond endocytically to

changes in FSS. A limitation of the studies, to date, is that they have been performed only in immortalized cell culture models of proximal tubule. Clearly, more work is necessary to assess the implications of these findings with respect to kidney function. For example, how are the apparently distinct FSS-dependent signals that regulate the abundance of surface ion transporters and the modulation of endocytic capacity integrated to maintain both aspects of proximal tubule function?

A confounding variable in assessing the physiological relevance of these observations is the current controversy regarding the role and magnitude of proximal tubule endocytic uptake in maintaining efficient clearance of proteins that enter the tubule lumen. Several recent studies have concluded that the fraction of albumin that escapes the glomerular filtration barrier and enters the tubule lumen is considerably higher than previously reported using microperfusion and other approaches [70–72]. These studies further suggested that albumin is efficiently endocytosed by proximal tubule cells, and rather than being degraded, is largely transcytosed to the basolateral surface and released into the extracellular fluid for reclamation [73]. An implication of these conclusions is that defects in proximal tubule function, rather than in the glomerular barrier, account for clinical proteinuria [74]. In this model, constitutive proximal tubule endocytic capacity is predicted to be considerably higher than previously assumed, and might be expected to be less sensitive to changes in GFR.

A related issue to be addressed is whether defects in the modulation of FSS-mediated endocytosis contribute to the LMW proteinuria observed in acute, chronic, and genetic diseases. Understanding the molecular mechanism that drives the endocytic response to FSS could lead to the development of therapeutic interventions to improve kidney function, as reducing GFR by modulating hemodynamics could be effective in retarding kidney damage in these patients. One disease that may link the response to FSS with LMW proteinuria is Lowe syndrome, an X-linked disease caused by mutations in the phosphatidylinositol 5′-phosphatase OCRL1 (oculocerebrorenal syndrome of Lowe). The primary cause of mortality of patients with Lowe syndrome is renal failure as a consequence of chronic LMW proteinuria [75]. Interestingly, loss of OCRL1 has been demonstrated to alter cilia length in kidney and other cells [76–78], suggesting a possible link between OCRL1 function and the endocytic response to FSS. Whether this is the case for Lowe syndrome and other diseases that present with LMW proteinuria is an important next question to be addressed in the field.

Conflicts of interest

There are no conflicts of interest.

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