Molecular changes in proximal tubule function in diabetes mellitus

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Summary

1. Diabetic kidney disease is initially associated with hypertension and increased urinary albumin excretion. The hypertension is mediated by enhanced volume expansion due to enhanced salt and water retention by the kidney. The increased urinary albumin is not only due to increased glomerular leak but to a decrease in albumin reabsorption by the proximal tubule. The precise molecular mechanisms underlying these two phenomena and whether there is any link between the increase Na+ retention and proteinuria remain unresolved.

2. There is significant evidence to suggest that increased Na+ retention by the proximal tubule Na+-H+ exchange isoform 3 (NHE3) can play a role in some forms of hypertension. Increased NHE3 activity in models of diabetes mellitus, may explain in part the enhanced salt retention observed in patients with diabetic kidney disease.

3. NHE3 also plays a role in receptor mediated albumin uptake in the proximal tubule. The uptake of albumin requires the assembly of a macromolecular complex that is thought to include the megalin/cubulin receptor, NHE3, the vacuolar type H+-ATPase (v-H+-ATPase), the Cl− channel, CIC-5 and interactions with the actin cytoskeleton. NHE3 seems to exist in two functionally distinct membrane domains, one involved with Na+ reabsorption and the other involved in albumin uptake.

4. This review focuses on the evidence derived from in vivo studies as well as complementary studies in cell culture models for a dual role of NHE3 in Na+ retention and albumin uptake. We suggest a possible mechanism by which disruption of the proximal tubule albumin uptake mechanism in diabetes mellitus may lead to both increased Na+ retention and proteinuria.

Diabetes mellitus, hypertension and albuminuria

Diabetic nephropathy is the most prevalent cause of chronic renal failure and end-stage renal disease in the Western world and can account for up to 40% of the patients requiring renal replacement therapy. The onset of renal failure in patients with diabetes mellitus is associated with hypertension and increased urinary albumin excretion. Although mesangial expansion, glomerular hypertrophy and thickening of the glomerular basement membrane leading to hyperfiltration and microalbuminuria are hallmarks of diabetic nephropathy, it is the degree of interstitial fibrosis that more closely correlates with the decline in glomerular filtration rate. The tubulointerstitial represents a dynamic environment that maintains the structural and functional homeostasis within the kidney and it is the dysregulation of this highly integrated system that may lead to many of the complications associated with diabetic kidney disease.

The hypertension usually observed in patients with diabetic nephropathy is well recognised to be mediated by volume expansion due to enhanced salt and water retention by the kidney. This suggests a dysregulation of the normal mechanisms to maintain volume homeostasis occurs in the "diabetic milieu" long before a functional decline in renal function develops. Microalbuminuria is well recognised as being associated with primary glomerular pathology. However, there is now clear evidence that the renal tubule has a critical role in the reabsorption of filtered albumin and in the development of albuminuria. As microalbuminuria and volume-mediated hypertension occur in patients with diabetes mellitus, this may suggest a more direct relationship between albumin handling and Na+ reabsorption. This review will focus on the possible compartmentalised roles of NHE3 in Na+ reabsorption and albumin uptake in the proximal tubule and how the trafficking of NHE3 between the two functional compartments may provide a link to explain the coexistence of hypertension and albuminuria in diabetic nephropathy.

Under normal conditions, the kidneys filter approximately 180 litres of blood and reabsorb approximately 1.7 kg of NaCl per day. The proximal tubule facilitates ‘bulk’ reabsorption of Na+, responsible for 50-75% of tubular Na+ reabsorption. At the brush border membrane of proximal tubules approximately 0.7 moles of sodium are reabsorbed per hour. Thus relatively small changes in the capacity of the proximal tubule to reabsorb Na+ and water in response to elevations in plasma glucose or cytokine levels may result in dramatic changes in Na+ retention and volume expansion.

Na+-H+ Exchanger Isoform 3 and Na+ Retention

The luminal reabsorption of Na+ in the proximal tubule is achieved primarily by the secondary active transport of the Na+-H+ exchanger isoform 3 (NHE3) mediated by the Na+ gradient generated by the basolateral Na+-K+-ATPase. There are now several lines of evidence to suggest that changes in the activity of NHE3 may be linked to hypertension. Importantly, a recent study in hypertensive patients found that proximal tubule Na+ reabsorption was an independent determinant of the blood pressure in volume-dependent hypertension. Similarly, a reduction in NHE3 activity has been reported in acute hypertension implicating a role for NHE3 in pressure natriuresis.
Several studies in spontaneously hypertensive rats (SHR), a commonly used model for human essential hypertension, are consistent with a role for NHE3 in the genesis of volume expansion. In the tubules of normal rats, Na\textsuperscript+−H\textsuperscript+ exchange activity was inhibited by parathyroid hormone (PTH) and dopamine but stimulated by angiotensin II (AngII) and norepinephrine. Tubules obtained from SHR tubules, however, were not responsive to PTH or dopamine and the levels of stimulation by AngII and norepinephrine were significantly reduced\textsuperscript{13}. These imbalances could contribute to the development and maintenance of hypertension in this model\textsuperscript{13}. NHE3 activity was also found to be elevated in a further study in SHR rats, with v-H\textsuperscript+−ATPase also implicated in the regulation of Na\textsuperscript+ transport in the proximal tubule\textsuperscript{14}. Consistent with the above studies, proximal tubule cells freshly isolated from SHR demonstrate a 3-fold increase in NHE3 activity with a 50% increase in NHE3 protein\textsuperscript{15}. Furthermore, in SHR there appears to be defective coupling of the dopamine receptor to adenyl cyclase, resulting in an alleviation of the cAMP mediated inhibition of NHE3, with subsequent elevation in Na\textsuperscript+ retention\textsuperscript{16}. In a more recent study, it was found that proximal tubules of 5 week old SHR had greater levels of NHE3 and v-H\textsuperscript+−ATPase activity compared to age matched normotensive Donryu rats. These findings led the authors to conclude that enhanced proximal tubule fluid reabsorption is likely to contribute to the development of high blood pressure in young SHR\textsuperscript{17}. Immunofluorescence studies revealed that there was a significant level of distribution of NHE3 in the proximal tubules in both SHR and Goldblatt hypertensive rats providing evidence for the dynamic role of NHE3 in states known to alter proximal tubular Na\textsuperscript+ reabsorption\textsuperscript{12}. Further conclusive evidence for the role of NHE3 in control of blood pressure was demonstrated using NHE3 knockout transgenic mice. Microperfusion studies revealed that fluid and HCO\textsubscript{3}\textsuperscript{−} reabsorption were reduced by ~60-70\%, demonstrating that NHE3 is the major apical transporter mediating Na\textsuperscript+ and HCO\textsubscript{3}\textsuperscript{−} reabsorption in the proximal tubule. These changes were associated with small but significant decreases in blood pH and HCO\textsubscript{3}\textsuperscript{−} \textsuperscript{18}. Importantly, the systolic and mean arterial blood pressures in these mice were significantly reduced. These data therefore support the view that the major renal Na\textsuperscript+ transporters, including NHE3, play a central role in long-term control of arterial blood pressure\textsuperscript{19,20}. NHE3 in diabetes mellitus As discussed above, diabetes mellitus is associated with renal NaCl retention and expanded extracellular fluid volume, characterised by systemic suppression of the renin-angiotensin system. Volume expansion is largely responsible for hypertension in diabetes mellitus and may contribute to the altered haemodynamics responsible for diabetic nephropathy. Diabetes mellitus is associated with chronic or intermittently high plasma glucose levels, which are implicated in a number of adverse effects on the kidney. There is evidence to suggest that increased Na\textsuperscript+ flux with glucose via SGLT-1 transporters may contribute to increased Na\textsuperscript+ reabsorption by the kidney\textsuperscript{21}. However, when the significant role that NHE3 plays in Na\textsuperscript+ and fluid reabsorption in the proximal tubule is taken into account, hyperglycaemia-induced increases in the activity of NHE3 potentially also contribute to increased Na\textsuperscript+ retention and related volume expansion.

The first evidence that NHE3 was increased in the proximal tubule in diabetes was provided by Harris and coworkers in 1986\textsuperscript{22} who demonstrated increased Na\textsuperscript+−H\textsuperscript+ exchange in brush border vesicles from rats induced to diabetes with streptozocin (STZ). Microperfusion studies in our own laboratories have also clearly demonstrated in STZ rats that there is a pronounced increase in tubular Na\textsuperscript+ reabsorption\textsuperscript{23,24} and that this increase was primarily due to enhanced NHE3 activity\textsuperscript{25}. In vivo models of diabetes mellitus using STZ rats have also demonstrated altered renal handling of H\textsuperscript+ and increased HCO\textsubscript{3}\textsuperscript{−} absorption, a result attributable to increased NHE3 activity\textsuperscript{26}. In vitro analysis of intact tubules and freshly isolated proximal tubule cells from STZ rats has shown increased NHE3 protein expression and activity\textsuperscript{27}. In addition, studies from our lab and others in cultured opossum kidney (OK) cells have shown that exposure to high glucose for 48 hours results in a significant increase in both NHE3 mRNA and protein\textsuperscript{28,29}. Furthermore, there have been at least two reports in humans that show increased proximal tubular Na\textsuperscript+ reabsorption in patients with diabetes mellitus. A study in children with Type 1 diabetes found a significant increase (~20\%) in proximal tubular Na\textsuperscript+ reabsorption as determined by fractional lithium clearance\textsuperscript{30}. Similar studies in adults with Type 2 diabetes also found a ~20\% change in reabsorption rates\textsuperscript{31}. Thus, considerable evidence exists that the NHE3 mediated component of renal salt reabsorption may be at least in part responsible for the hypertension observed in patients with diabetes mellitus.

**Albumin uptake in the proximal tubule - a macromolecular complex reliant on NHE3 activity?**

It has long been recognised that the proximal tubule has a crucial role in reabsorbing any filtered albumin\textsuperscript{32}. The concentration of albumin in the glomerular filtrate in rats and dogs ranges from <1 to 50 mg/l\textsuperscript{33}. Recently, the concentration of albumin in humans has been estimated to be 3.5 mg/l\textsuperscript{33} which translates to approximately 630 mg of albumin being filtered per day by the human kidneys. However, only around 30 mg is normally excreted in the urine per day, indicating that the tubules reabsorb at least 95\% of all albumin filtered at the glomerulus. The uptake of albumin by the proximal tubule from the glomerular filtrate has been shown to occur by a highly active receptor-mediated endocytotic pathway involving the megalin/cubulin complex\textsuperscript{34} (Figure 1). The albumin is then trafficked to the lysosomes where it is broken down to its constituent amino acids\textsuperscript{34}. Importantly, the C-terminus of megalin contains numerous potential protein binding domains\textsuperscript{35}. Recently it has been demonstrated that efficient
Figure 1. Macromolecular complex involved in proximal tubule albumin endocytosis. (1) In the plasma membrane at the intravillar cleft ClC-5, v-H⁺-ATPase, NHE3 and megalin associate by C-terminal tail interactions with scaffold proteins that anchor the complex to the actin cytoskeleton. (2) When albumin binds the megalin/cubulin complex, endocytosis is initiated. (3) As the nascent endosome forms it is pinched off from the membrane by dynamin. Entry into the cytoplasm requires the dissolution of the local actin filaments. This involves the C-terminal tail of ClC-5 recruiting the actin depolymerising protein coflin to the complex. At this stage the endosome contains extracellular fluid high in Na⁺ with a neutral pH. It is thought that NHE3 may initiate endosomal acidification by electroneutral exchange of endosomal Na⁺ for cytosolic H⁺. (4) When the Na⁺ gradient is dissipated, the v-H⁺-ATPase continues the acidification and ClC-5 provides the necessary anion shunt and albumin dissociates from the megalin/cubulin complex.
binds megalin via a C-terminal tail interaction, suggesting that NHE3 may play an additional role as a molecular scaffold. Although there are no reports of proteinuria in NHE3 knockout mice, this model is characterised by severe volume depletion, a significant reduction in glomerular filtration and an associated reduction in filtered protein. Hence the specific role of NHE3 in Na+ reabsorption in this model is difficult to ascertain.

The critical role of epithelial ion transport in tubular albumin transport is exemplified in Dent’s disease, where inactivating mutations of the Cl− channel, CIC-5, significantly inhibit tubular albumin reabsorption. In patients with Dent’s disease there are genetic abnormalities inactivating mutations of the Cl− channel in CIC-5 leading to defects in channel trafficking or channel function that in turn result in low molecular weight proteinuria as well as albuminuria due to defective proximal tubular protein reabsorption. A similar effect on tubular protein uptake is observed in CIC-5 knockout mice. It has been considered that the main role of CIC-5 was to provide an anion shunt for the positive charge translocated by the v-H+-ATPase into the endosome during acidification. Figure 1. In support of this, in the kidneys of CIC-5 knockout mice, the uptake of markers of receptor-mediated and fluid phase endocytosis is severely impaired and the acidification of the endosomes is decreased. This finding is also consistent with the fact that many channels of the CIC family are believed to be involved principally in regulating intracellular Cl− movement.

More detailed analysis, however, of the CIC-5 knockout mouse suggests that CIC-5, as well as acting as an anion shunt, plays an additional role in albumin endocytosis. If CIC-5 were acting solely as an anion shunt, it would be predicted that the nascent endosome would be able to form and that the trafficking of the endosome would only be affected at a later (early endosome) stage when significant electrogenic H+ movement occurs. This is particularly relevant when considering the role of electroneutral NHE3 exchange in initiating endosomal acidification, because this would remove the need for electrogenic transport of H+ immediately following the budding of the endosome from the membrane. In support of this, there are reports indicating that the v-H+-ATPase is not required for acidification of the early endosome.

In the brush borders of CIC-5 knockout mice exposed to the endocytic marker horseradish peroxidase, the marker was found to be trapped in a sub-plasmalemmal pre-endocytotic compartment and failed to enter the endosomal pathway. This is somewhat surprising, since if the v-H+-ATPase and hence anion shunt are not required during nascent endosome formation, it would be expected that the label would enter the early endosomal compartment. This raises the important point that the endocytotic defect may also be occurring earlier, at the formation of the nascent endosome. Further investigations in patients with Dent’s disease showing that the loss of part of the C-terminus of CIC 5 also results in mis trafficking of the v-H+-ATPase and in CIC-5 knockout mice there are significantly reduced levels of megalin/cubulin at the plasma membrane also attributed to defective trafficking. These findings strongly suggest that CIC-5 has an additional role in targeting key proteins involved in albumin uptake to the plasma membrane. Consistent with the role of CIC-5 at the plasma membrane, we have used surface biotinylation to demonstrate that CIC-5 is present at the cell surface (unpublished observations; cf).

We have recently investigated a potential mechanism by which the C-terminal tail of CIC-5 can regulate albumin uptake. We found using a yeast 2-hybrid screen and glutathione S-transferase (GST)-pulldowns that CIC-5 interacted with the ubiquitously expressed actin binding protein cofilin that is involved in actin depolymerization. We reasoned that the passage of the nascent endosome through the cortical actin web required remodelling of the actin microfilament network. By phosphorylating cofilin with LIM kinase and thereby inhibiting the remodelling of the actin web we were able to inhibit albumin uptake in OK and LLC-PK1 cells. This study demonstrates a critical role for CIC-5 via its C-terminal domain in mediating remodelling of actin microfilaments essential for albumin endocytosis. Our current hypothesis is that, although CIC-5 is expressed at the plasma membrane, the ion channel activity is redundant and that the protein plays a key role in mediating macromolecular complex assembly. This occurs via C-terminal tail scaffolding interactions with proteins directly involved in albumin endocytosis (v-H+-ATPase and megalin/cubulin) as well as accessory proteins such as cofilin to form a localised and specialised endocytic complex (Figure 1).

Taken together, these data suggest that albumin uptake by the proximal tubule requires the assembly of a macromolecular complex at the plasma membrane that involves megalin/cubulin, CIC-5, NHE3 and v-H+-ATPase. Determining the molecular composition and scaffolds associated with this complex and the precise regulatory mechanisms represents a key research focus in renal cell physiology.

**Albumin uptake in diabetes mellitus**

Patients with diabetes mellitus show a clear reduction in the capacity of the proximal tubule to reabsorb albumin, and this may even precede glomerular damage, demonstrating the importance of preventing tubular dysfunction early in the course of diabetes mellitus. Further evidence comes from studies in rat models of diabetes mellitus. Absolute tubular reabsorption of albumin is decreased in STZ rats and ultrastructural studies have shown a decrease in albumin uptake and a reduction in the levels of megalin in the kidneys of STZ rats. It is important to note that the presence of increased tubular protein overload leads to the development of inflammation and fibrosis via activation of the nuclear factor-κB (NF-κB) transcriptional pathway. This in turn induces the production of a number of proinflammatory stimuli such as regulated upon activation, normal T cell expressed and induced genes (RANK).
secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor beta (TGF-β). TGF-β is regarded as the key inflammatory cytokine in diabetic nephropathy. Furthermore, it has been shown that elevated levels of intrarenal Ang II may in fact mediate the autocrine production of TGFβ1. In fact, a recent study in STZ rats has shown that Ang II blockade restored tubular albumin uptake, further highlighting the renin-angiotensin system in the development of diabetic nephropathy. In the OK cell model of albumin uptake, it has been shown that TGFβ1 can regulate albumin uptake, by decreasing the binding, internalization and trafficking of the megalin/albumin complex. Given that TGFβ1 levels are elevated in the diabetic kidney, this may provide a partial explanation for the molecular basis for the reduction in albumin uptake observed in vivo. Interestingly, we have found that in OK cells, exposure to high glucose results in an increase in albumin uptake. This effect is specific for glucose and not due to an osmotic effect and may occur as a result of the increase in NHE3 activity that is known to accompany exposure to high glucose. It is likely that under these in vitro conditions, levels of autocrine TGFβ1 cannot reach the levels required to inhibit albumin uptake. Thus there appears to be a direct link between NHE3 activity and albumin uptake in OK cells. Furthermore, it has been shown in OK cells exposed to pathophysiological levels of albumin similar to those expected in diabetic nephropathy, that albumin uptake is reduced. This is due to a decrease in the number of albumin binding sites by an as yet undetermined mechanism that may involve altered rates of trafficking of megalin to or from the cell membrane. It has been shown, however, in both primary cultures of human proximal cells and OK cells, that exposure to high concentrations of albumin results in an increase in NHE3 expression and activity. A similar increase in NHE3 activity in response to increased tubular albumin has been reported in puromycin aminonucleoside nephrotic rats.

These data collectively suggest that NHE3 may exist in different functional pools, one associated with albumin uptake and the other involved in Na+ reabsorption and not involved in albumin uptake (Figure 2). The evidence for the presence of NHE3 in two different pools in the proximal tubule brush border was presented in a recent review by McDonough and Biemesderfer. One pool is located in the microvilli and the other in the intermicrovillar cleft where NHE3 co-localises with megalin. A number of studies have shown that NHE3 can shuttle between the two pools in

Figure 2. Possible alteration in NHE3 trafficking pathways in diabetes mellitus. Panel A: Under normal conditions in proximal tubule cells the majority of NHE3 exists in recycling endosomes from where it is inserted into the microvilli to reabsorb Na+. Because of its role in albumin uptake, a proportion of the NHE3 may then translocate to the intravillar cleft where it associates with megalin/cubulin. This complex is then internalised and the NHE3 either returned to the recycling endosomes or degraded in the lysosomes. Panel B: The proteinuria associated with diabetes mellitus is in part due to an inhibition of the normal albumin uptake pathway in the proximal tubule. As a result, the endocytosis of NHE3 via the megalin-associated pathway is inhibited. However, insertion from the recycling endosomal pool is not affected, resulting in an accumulation of NHE3 in the microvillar pool and increased Na+ reabsorption with proteinuria.
response to acute hypertension and other stimuli. It is this association with megalin by an as yet uncharacterised molecular interaction that may explain the apparent role that NHE3 plays in albumin uptake. Furthermore, recent studies in OK cells have shown that a fraction of NHE3 is located in lipid rafts and that this may represent a different functional microdomain within the plasma membrane.

Based on the existence of different functional pools of NHE3, we postulate the following model that links increased Na\(^+\) retention and proteinuria in diabetic nephropathy (Figure 2). (i) NHE3 exists primarily in subplasmalemmal pools where it is available for insertion in to the plasma membrane in response to numerous stimuli. (ii) A significant proportion of NHE3 is recycled/removed from the membrane in conjunction with albumin, such that NHE3 opportunistically exploits the highly active albumin endocytic pathway for its recycling and that this represents a constitutive regulatory pathway for regulation of surface levels of NHE3. (iii) The NHE3 associated with megalin is not primarily involved in Na\(^+\) reabsorption. (iv) When cells are exposed to high albumin, the endocytic pathway is reduced by an as yet uncharacterised mechanism, resulting in proteinuria (reduced albumin uptake) and a reduction in the internalisation rates of NHE3. (v) This in turn may result in a shift in the normal trafficking equilbrium of NHE3 with increased surface levels of NHE3 and potentiation of Na\(^+\) retention. We are currently investigating the exact molecular mechanisms that may underlie the differences in NHE3 trafficking and albumin uptake in conditions of high glucose and high albumin.

It is also reported that exposure to high glucose results in significant alterations in the cytoskeleton in many different cell types. In terms of the kidney, studies in mesangial cells exposed to high glucose have shown pronounced rearrangements of the actin cytoskeleton that may contribute to the hyperfiltration associated with diabetes mellitus. In addition, microarray analysis of mesangial cells have demonstrated altered levels of expression of actin regulatory proteins in response to high glucose. It is also clear that both the trafficking of NHE3 and albumin uptake depend on an intact cytoskeleton. In fact, we believe that it is critical to use the inhibition of albumin uptake by actin depolymerising agents to demonstrate that proximal tubule cells in culture are taking up albumin by a receptor-mediated pathway, as all cells have the ability to take up limited amounts of albumin by pinocytotic mechanisms. In proximal tubule cells, the actin at the microvillar core and in the terminal actin web must be in a constant state of remodelling to facilitate albumin endocytosis. Thus interactions between the membrane proteins and the cytoskeleton are essential for the regulation of ion transport activity and transporter/channel trafficking, control of vesicle movement and uptake as well as assembly of signalling and macromolecular complexes at the apical membrane.

It is important to note that, cultured proximal tubule cells do not have the extensive microvillar complex and intermicrovillar clefts characteristic of their in vivo counterparts (for review see) despite retaining the core functional features of the proximal tubule, namely NHE3 dependent Na\(^+\) uptake and megalin/cubulin mediated albumin uptake. Therefore, although much can be learned from studies in OK cells about endocytic complex assembly and regulation of albumin and NHE3, care must be exercised when extrapolating these data to the situation in the intact proximal tubule. Nevertheless, experiments in the cultured cell system can yield much valuable information regarding precise molecular interactions under defined conditions. For example, studies the on the role of NHE3 uptake in OK cells have highlighted an apparently facilitative function of NHE3 in albumin uptake that may not have been as readily identified in studies in the intact proximal tubule or in NHE3 knockout mice.

**Conclusion**

There is now compelling evidence for increased proximal tubule NHE3 activity contributing to the Na\(^+\) retention that may underlie certain forms of hypertension including the hypertension often associated with diabetes mellitus. The existence of functionally different membrane domains and signalling/transporting complexes in the proximal tubule brush border may in part explain the relationship between increased Na\(^+\) retention and reduced albumin uptake observed in diabetic kidney disease. It is becoming apparent that the location of NHE3 in different membrane domains is a critical determinant of NHE3 function. In addition, albumin uptake by the proximal tubule involves a macromolecular complex at the plasma membrane that involves megalin/cubulin, CIC-5, NHE3 and v-H\(^+\)-ATPase. Determining the molecular composition and scaffolds associated with this complex and the precise regulatory mechanisms represents a key research focus in renal cell physiology. A precise understanding of how these molecular interactions are altered in disease states such as diabetes mellitus will allow novel approaches to the diagnosis and management of diabetic kidney disease.

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Proximal tubule function in diabetes mellitus


Received 30 October 2003; in revised form January 21 2004. Accepted 29 January 2004.

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