Chapter 2

PLASMIN, UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR AND AMILORIDE IN THE NEPHROTIC SYNDROME

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ABSTRACT

Proteinuria is a common feature of acute and chronic nephropathies and a key predictor of declining glomerular filtration rate (GFR). It is an independent factor for tubulointerstitial damage induced by direct toxicity of the filtered protein. Systemically, substantial amounts of proteinuria cause symptoms such as interstitial edema, renal salt and water retention, and hypertension. The potential causes for these disorders have been partly elucidated in models of the nephrotic syndrome (NS), in which a primary insult to the podocytes or to the glomerular basement membrane (GBM) is followed by proteinuria and functional tubular derangements. Salt retention in NS has traditionally been attributed to the hypovolemia concept, implying decreased oncotic pressure following proteinuria, loss of fluid into the interstitium, and activation of the renin–angiotensin–aldosterone (RAA) system. Meanwhile, it has been claimed that the vascular volume in NS often remains unchanged along with the absence of blood pressure and RAA system effects. It has become accepted that primary tubular salt retention may per se produce a substantial systemic volume expansion in proteinuric nephrosis. Potential sites of sodium retention along the renal tubule have mostly been attributed to changes in the proximal or distal tubule. Generally, protein overload may impair proximal tubule structure and consequent handling of proteins. Cytoskeletal changes may interfere with trafficking of transporters and channels, and the effects of inflammatory and profibrotic cytokines have also been implicated.

The diuretic amiloride plays a significant role in reducing podocyte cell motility in vitro and proteinuria in mice. Besides its diuretic properties at the distal tubule blocking the absorption of sodium and water at the $ENa^{+}C$ channel, amiloride has been recently shown

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to inhibit the synthesis of the urokinase receptor, called urokinase plasminogen activator receptor (uPAR), a molecule involved in the αvβ3 integrin activation. This integrin (also known as the vitronectin receptor) anchors the podocyte to the GBM; when activated it causes podocyte contraction and eventually contributes to the detachment of the cell from the glomerulus and its appearance in the urine (a phenomenon known as podocyturia), finally causing proteinuria. Amiloride capacity to inhibit uPAR synthesis and suPAR (the soluble circulating version of uPAR but also with glomerular permeability factor properties) secretion by T lymphocytes, macrophages and neutrophils should be taken into consideration as an adjuvant tool in the treatment of NS. Furthermore, amiloride may further decrease proteinuria by acting on the distal nephron on $ENa^{+}C$ channels, as proteinuria stimulates the activity of these channels by promoting the reabsorption of sodium and water. Tubular plasmin, already high in patients with proteinuria, would act as the mediator in sodium and water reabsorption and amiloride may inhibit its action by blocking uPAR. Thus, this would be another additional and relevant nonimmunosuppressive strategy contributing to the fall in proteinuria in NS individuals, if tolerated hemodynamically and no hyperkalemia ensues.

Keywords: Amiloride, edema, glomerulopathies, nephrosis, plasmin, proteinuria.

1. INTRODUCTION

Proteinuria is a common and relevant component of acute and chronic glomerulopathies and a key predictor of declining glomerular filtration rate (GFR). Basically, proteinuria can be caused by three different pathophysiological events: a podocytopathy, hyperfiltration situations, and/or scarring and sclerosis due to primary or secondary glomerular filtration membrane derangements. Nevertheless, and independent of the cause, proteinuria has also been viewed as an independent factor for tubulointerstitial damage induced by direct toxicity of filtered protein. [1,2] At both renal and systemic aspects, the substantial amounts of protein passing through the damaged filter to be lost in the urine are capable of causing interstitial edema, renal salt and water retention, hypertension, progressive renal function decline and is a surrogate of morbidity and mortality caused by cardiovascular events. [3-7] The potential causes for these disorders have been partly elucidated in models of the nephrotic syndrome (NS), in which a primary insult to the podocyte or to the glomerular basement membrane (GBM) is followed by proteinuria and functional tubular derangements. Salt retention in the NS has traditionally been accepted to be caused by the hypovolemia theory. This hypothesis implies that decreased oncotic pressure is due to hypoalbuminemia caused by copious proteinuria, loss of fluid into the interstitium, and activation of the renin–angiotensin–aldosterone (RAA) system. Noteworthy, vascular hyperpermeability itself has also been demonstrated as an additional culprit in edema formation and perpetuation. [8] However, it has been claimed that the vascular volume in most cases of the NS remains basically unchanged, coupled with the absence of effects on systemic blood pressure and on the RAA system. [9, 10] In this regard, it has lately become accepted that primary tubular salt retention by itself may produce a primary substantial systemic volume expansion in proteinuria. [11, 12] This hypothesis underscores the cross-talk between the glomerulus and the tubule as the first step, in which a glomerular podocyte damage translates into a proteinuric tubular fluid with interactions at the tubular cell level with subsequent fluid disbalances. Potential sites of sodium retention along the renal tubule have mostly been attributed to changes in the

proximal or distal tubule. Generally, protein overload may impair proximal tubule structure and consequent handling of luminal proteins. Cytoskeletal changes may interfere with trafficking of transporters and channels, and the effects of inflammatory and profibrotic cytokines have also been implicated. [1, 2, 13-15] At the distal nephron, epithelial Na^+ channels (ENa^+C) are normally implicated in the absorption of sodium and water. These channels appear to be upregulated in the NS primarily due to both proteinuria itself and to the increased tubular concentration of plasminogen and plasmin, also augmented in the NS due to the upregulation of urokinase plasminogen activator receptor (uPAR). [9, 10, 16-18]

Finally, amiloride- a potassium-sparing diuretic- may play a significant potential role in the treatment of the NS at different levels. Amiloride acts at the proximal tubule inhibiting the Na^+/H^+ and Na^+/Ca^{++} antiporters [19] and at the distal tubule it interferes with water and salt retention at the above-mentioned ENa^+C. Moreover, amiloride has recently been shown to inhibit the synthesis of the urokinase receptor uPAR. [16, 20-22] This molecule converts plasminogen to plasmin at the cell surface of distal tubular cells, which subsequently upregulates ENa^+C activity, resulting in salt and water retention and edema formation. [16, 23] In addition, amiloride appears to reduce podocyte cell motility in vitro and proteinuria in mice by inhibiting a molecule involved in podocyte contraction, the β3 moiety of the αvβ3 integrin. [24] This integrin (also known as part of the vitronectin receptor-complex, together with uPAR) is one of the molecules involved in the anchoring of the podocyte to the GBM. When this integrin is activated, it would cause podocyte contraction and motility. [25] This cellular volume decrease may at final stages contribute to the critical detachment of the cell from the glomerulus and its appearance in the urine (a phenomenon known as podocyturia). The denuded glomerular filtration membrane would render the pathway for chronic proteinuria unhindered. Amiloride would therefore interfere negatively on β3-composed integrins actions and also inhibiting the synthesis of uPAR. [24] These actions position amiloride -at least at the theoretical level- as a relevant drug to be employed in the management and treatment of edema and proteinuria in the NS. Thus, this would be another additional and relevant non-immunosuppressive strategy contributing to the decrease in proteinuria in nephrotic individuals. [25, 26]

2. EDEMA FORMATION IN THE NEPHROTIC SYNDROME

Edema is the characteristic clinical presentation of the NS. The mechanisms of edema formation in the NS have long been a subject of investigation and are continually debated. The ‘underfill’ hypothesis states that a decrement in oncotic pressure due to hypoalbuminemia leads to excess filtration of fluid from the intravascular space to the interstitial space (reduced intravascular-to-interstitial albumin gradient), causing hypovolemia, renal hypoperfusion, activation of the RAA system, and secondary renal sodium retention. However, at its pure expression, this situation is applied only to some cases of minimal change disease (MCD) and some cases of extreme hypoalbuminemia.

The ‘overfill’ hypothesis, on the other hand, states that in the NS there is a cause of primary renal sodium retention, leading to edema, suggesting that an abnormal primary tubular alteration in the handling of water and sodium leads to edema. Proteinuria itself can also upregulate the absorption of fluid at the distal nephron. [16] Interestingly, it has been

shown in young analbuminemic Nagase rats that there is no difference in transcapillary colloid osmotic pressure, with preservation of the oncotic pressure gradient due to parallel decreases in serum and interstitial oncotic pressures. [27] This phenomenon has also been demonstrated in humans with the NS [28-30]. It has been proposed that two safeguard factors may protect against edema formation in the presence of hypoalbuminemia. [31] An increase in the passage of fluid from the intravascular space into the interstitial space could dilute the interstitial protein concentration and its oncotic pressure. In addition, an increase in fluid delivery to the interstitial space will produce an increase in lymphatic flow that will 'washout' interstitial proteins and finally delivering the protein load back to the systemic circulation. Both safeguard mechanisms could serve to maintain the plasma to interstitial protein ratio close to normal and thus defend against edema formation. Edema would appear when the interstitial protein concentration became virtually inexistent and no gradient forces could be generated due to the lack of oncotic pressure and the secondary intravascular protein decline. Finally, in the analbuminemic Nagase rats, plasma proteins such as transferrin, several globulins and fibrinogen are synthesized to compensate for the lack of plasmatic albumin, in an attempt to maintain oncotic pressure and to serve as transport molecules, replacing some of the main functions of albumin. It is not known to what extent these proteins are excluded from the interstitial space, but lipoproteins and fibrinogen, for example, which are increased in nephrotic subjects, will probably not traverse the capillary endothelium. This factor may also contribute to the preservation of the oncotic pressure and acting against the generation of edema in the NS. [27]

Finally, capillary permeability is severely altered in most nephrotic patients with primary glomerulonephritis. Capillary hyperpermeability plays a role in the pathophysiology of nephrotic edema associated with primary glomerular disease. It has been postulated that this widespread abnormality in capillary permeability could be related to the release of vascular permeability factors and other cytokines by immune cells, as histamine, endotoxins, anaphylatoxins, catecholamines, estrogens, progesterone, insulin and cytokines as vascular endothelial growth factor, Interleukin-1, Interleukin-2, tumor necrosis factor-alpha and vascular permeability factor. [32-35] This capillary permeability appears to be reverted after successful treatment of the NS. [8] In conclusion, most edematous patients with the NS may have a normal to expanded intravascular volume, whereas a minority of patients presents a depleted intravascular volume, as seen in some stages of MCD nephropathy. [36]

3. PLASMIN, UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR AND THE NEPHROTIC SYNDROME

Many primary glomerulopathies can cause NS. MCD, focal and segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), and membranoproliferative glomerulonephritis (MPGN) are among the most frequently encountered in clinical practice. It has been previously mentioned that a tubular derangement is the main culprit for the salt and water retention that occurs in most of the entities that present with NS, leading to edema formation and, eventually or concomitantly, to hypertension and renal insufficiency. In this respect, a key system to take into consideration is the uPA/uPAR binomium. Briefly, the urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) are very

similar serine proteases with the same physiological action, the activation of plasminogen. One of the receptors that converts plasminogen to plasmin, uPAR, is an extensively N-glycosylated membrane receptor tethered to the cellular plasma membrane. uPAR orchestrates a wide variety of cellular processes, including extracellular proteolysis, cell migration, adhesion, signaling, and proliferation, both under physiologic and pathologic conditions. On the plasma membrane, uPAR acts as the high-affinity binding site for uPA, promoting plasmin generation at the cell surface. The activation of proteolytic cascades after uPA–uPAR interaction is widely believed to be responsible for the biologic activity of uPAR. This interaction is blocked by amiloride, but tPA action is not modified by amiloride. [37] As reviewed by Sarra-Ferraris and Sidenius, additional studies have documented the existence of a variety of biologic activities induced solely by overexpression of uPAR or by the binding catalytically inactive uPA derivatives. These effects, referred to as the "non-proteolytic" functions of uPAR, rely on direct and functional interaction with other proteins on the plasma membrane or in the pericellular environment. The absence of a cytoplasmic tail makes uPAR unable to directly contact signaling molecules in the cytoplasm, and the signaling activity of uPAR has therefore been ascribed to interactions with a growing number of signaling receptors. It has indeed been shown that uPAR regulates signaling downstream of tyrosine kinase receptors, integrins, and G protein-coupled receptors. In addition, non-proteolytic uPAR functions include the direct effect played by uPAR on cell adhesion through its specific interaction with the extracellular matrix protein vitronectin. The importance of the uPAR-vitronectin interaction in the modulation of cell adhesion is becoming more and more compelling because it seems to be intimately connected to most of uPAR's non-proteolytic functions. uPAR modulates pericellular proteolysis by regulating the activity of the plasminogen system. [38] Interestingly, there is enough evidence to hypothesize that the inflammatory response that proteinuria causes in the renal medullary interstitium of nephrotic subjects could be enhanced by uPAR, plasmin and vitronectin. But there are other links where uPAR and the NS encounter. uPAR appears to play a critical role at least in two different ways. The first one is participating in the activation of distal tubular $ENa^{+}C$ via conversion of plasminogen to plasmin. [16,23] In addition, this phenomenon may also occur in plasma and on the podocyte surface. [16,23] The second mechanism is probably more controversial but provocative: Urokinase receptors, located on cell surface of certain cells, are bound to the cell by a glycosylphosphatidylinositol (GPI) molecule, which can be split by immunological factors. Once detached from the cell, uPAR is converted into a soluble circulating molecule, suPAR. [25] In a recent publication, Wei et al. provide ample evidence that elevated suPAR levels may play a role as a circulating factor with permeability properties on the glomerular membrane, leading to podocyte contraction and proteinuria. [23] Once described in primary FSGS, suPAR has thereafter been shown to be non-specific to this entity, having been encountered in other glomerulopathies with or without NS, and even in subjects without kidney disease. [39-43] Interestingly, it has been recently shown that both suPAR and interleukin-2 levels are elevated in patients with FSGS and are associated with the response to treatment. According to a previous study by Al-Atrash et al., interleukin-2 upregulated both uPA and uPAR in natural killer cells. An induction of uPAR mRNA and a reciprocal downregulation of uPAR mRNA-binding proteins were observed after interleukin-2 stimulation. [44] Furthermore, Nykjaer et al. demonstrated that interleukin-2 increased uPAR presentation on 20–50% of the T-cell population. [45] Recently, Shishido et al. reported that recurrent FSGS after kidney transplantation, which might be considered to be caused by

circulating factors such as suPAR, was successfully treated with immunosuppressive treatments with methylprednisolone and cyclosporine, which could suppress IL-2 levels. [46] Therefore, there is a possibility that elevated interleukin-2 might increase suPAR in patients with primary FSGS and the increased levels of suPAR could be reversed by blocking interleukin-2. [47]

Moreover, Zhang et al. showed that the calcineurin–nuclear factor of activated T cells (NFAT) axis stimulates uPAR expression [48], while Ranjan et al. demonstrated that NFAT also binds to interleukin-2 gene promoter and increases interleukin-2 synthesis and secretion. [49] Thus, the mechanism of interleukin-2 and suPAR elevation and their interaction in primary FSGS both need further investigations, but may represent another component of the inflammatory milieu in which the NS expresses its constituents and complications. As a circulating permeability factor, suPAR is regulated by several cytokines and chemokines. [25] In the pathogenesis of FSGS, Huang et al found that elevated urinary suPAR could activate β3 integrin on cultured human podocytes in vitro, suggesting that suPAR may have a direct role in mediating podocyte injury and disease development. [50] With respect to other glomerulopathies causing NS, the involvement of plasmin and uPAR in the edema generation have also been shown, as will be discussed below. However, the role suPAR may play as a generator of proteinuria is under intense debate. In this regard, elevated plasmin levels frequently occurring in the NS scene can interact with either circulating suPAR and/or cell surface attached uPAR to undertake its actions. [25] Presumably, by systemic circulation interactions, it could affect vascular sodium handling through ENa^+C activation. By glomerular filtration, it could induce podocyte contraction and proteinuria. Finally, by tubular luminal interactions, it could induce water and sodium reabsorption and edema generation stimulating ENa^+C.

4. THE PODOCYTE AND AMILORIDE

Urokinase receptors, expressed on the cell surface of various cells, are committed to the pericellular proteolysis of plasminogen, are essential for the remodeling of the extracellular matrix, and are involved in vasculogenesis and cell migration processes. [51] uPAR is linked to the cell membrane by GPI and has a molecular weight of about 45-55 kDa. [51, 52] uPAR is present in various immunologically active cells, including monocytes, macrophages and activated T cells, and also in endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes, certain tumor cell lines, podocytes and renal tubular cells. [53-57] uPAR can be activated by various molecules, such as uPA (urokinase-type plasminogen activator, or simply urokinase), plasminogen, chymotrypsin, various metalloproteinases and some elastases. [58-61] uPAR is capable of catalyzing the conversion of plasminogen to plasmin, an important molecule in fibrinolytic processes and in the activation of several matrix metalloproteinases, in the recycling and degradation of the extracellular matrix, in cell activation, migration, contraction, vasculogenesis and in vitronectin interaction and degradation. [51, 62-66] This phenomenon may occur in plasma, on the podocyte surface or in renal distal tubular cells. [16, 23] Patients with NS present elevated serum levels of plasminogen and plasmin. [67] In turn, after being filtered, urinary plasminogen is converted to plasmin by podocyte or distal renal tubular epithelial uPAR; at this distal location, plasmin

has been reported to function as a regulator of water and sodium absorption, a key event in the pathogenesis of edema in the NS, and also as a mediator in calcium tubular transport. [16, 68, 69] Cell migration across the endothelium and into tissues is a critical component in inflammation, in immune responses against infections, and in tissue repair and remodeling after injury. The uPA/uPAR system is directly involved in these mechanisms of adhesion, migration and chemotaxis. [57, 65] For example, the adhesion and migration of monocytes involves a functional interaction between cellular uPAR and matrix integrins [70] and in uPAR-dependent changes in integrin-mediated adhesion to fibrinogen, collagen and vitronectin. [23, 71, 72] It is known that uPAR is needed to activate the integrin αvβ3 in podocytes, which promotes cell motility and activation of small GTPases that control cell division. Thus, if the αvβ3 integrin was then activated, the podocyte could contract and proteinuria would be a straight forward result. [24]

A recent study has shown that podocyte uPAR expression can be reduced using amiloride. Amiloride plays a significant role in reducing podocyte cell motility in vitro and proteinuria in mice. [24] Amiloride inhibits the synthesis of uPAR and uPAR mRNA and consequently the αvβ3 integrin activation mediated by uPAR on αvβ3 integrin. Amiloride capacity to inhibit uPAR synthesis by T lymphocytes should be of particular interest in different causes of NS, because blocking their activation would inhibit αvβ3 integrin activation and the development of proteinuria with final renal dysfunction. [23-25]

5. THE TUBULE AND AMILORIDE

It is well known that although sodium handling occurs along the entire nephron, it is the distal tubule where the fine tuning of sodium adjustment takes part. The ENa^+C located in the distal tubule is one of the main pathways by which sodium and water are absorbed. These channels are positively upregulated by plasmin and by proteinuria, and inhibited by amiloride.[16, 24, 73]

Ichikawa et al. created a unilateral puromicin amoninucleoside nephrosis (PAN) model in the rat such that one kidney was nephrotic and the other functioned normally. They showed that although the nephrotic kidney was proteinuric and sodium avid, the contralateral normal kidney had no proteinuria and handled sodium normally as in control rats. This suggested that an intrarenal defect caused sodium retention. There was a significant increase in sodium reabsorption beyond the distal convoluted tubule in the nephrotic kidney. [74]

An increase in cortical collecting duct Na^+/K^+ ATPase activity and amount has been shown in the PAN model of NS. [75-78] An inverse linear relationship between cortical collecting duct Na^+/K^+ ATPase activity and urinary sodium excretion has been noted. [76] The expression and apical targeting of ENa^+C has been shown to be increased in the cortical collecting duct in the PAN model of the NS and is aldosterone dependent. [10, 78-80] A linear correlation between plasma aldosterone and ENa^+C abundance was found in the PAN model of nephrosis [78], and treatment with amiloride prevented the sodium retention in the rat PAN model of nephrosis whereas treatment with an aldosterone receptor blocker did not. [77, 78] It is noteworthy that in adrenalectomized nephrotic rats, amiloride returned sodium excretion to normal despite the fact that ENa^+C expression was not increased. The observed increase in ENa^+C that occurs in animal models of the NS is due to aldosterone, but the

development of nephrotic edema is not dependent on aldosterone. The importance of the ENa^+C channel in the development of nephrotic edema, however, is underscored by the reversal of sodium retention by amiloride in the above animal models. Evidence suggests that the Na^+/K^+-ATPase activity is increased in the NS and appears to be important in the sodium retention seen in the NS. Regulation of ENa^+C occurs through two primary mechanisms: regulation of channel density at the apical membrane and regulation of the open channel probability. [81] The ENa^+C receptor density is regulated by both aldosterone and vasopressin. Open channel probability is regulated by proteolytic processing [65] and by anionic phospholipids present on the inner cell membrane. Noteworthy, it has been reported that both plasminogen and plasmin are present in the urine of proteinuric rats with the metabolic syndrome and heavy proteinuria but not in control rats. [82, 83] As mentioned above, Svenningsen et al. have shown that plasmin present in the urine of nephrotic rats and humans can activate ENa^+C. [16] Additionally, they showed that uPA activator present in the rat and human kidney can convert inactive plasminogen (which is filtered by the nephrotic kidney) to the active-form plasmin. In the rat PAN nephrosis model, they demonstrated that amiloride increases urine sodium excretion due to the ability of amiloride to inhibit ENa^+C and the ability of amiloride to inhibit uPA and thus reduce the amount of active plasmin present. The above mentioned proteolytic processing of ENa^+C is undertaken by nephrotic urinary serine proteases, of which plasmin appears to play a main role. [16]

The above mentioned statements suggest a dominant role of ENa^+C in sodium and water retention in the NS and provide a rationale for specific diuretic blockade of ENa^+C. These important findings provide a mechanism of an intrinsic renal defect whereby abnormally filtered proteins in nephrotic patients can cause sodium retention via ENa^+C activation. It also explains the predictable course of recovery seen in MCD patients treated with steroids. First, there is a decrement in urinary protein excretion, then a reduction in aldosterone (urine and serum), followed by natriuresis, and then diuresis. [36,84-88]

Another interesting aspect of this topic is the one that relates plasmin to calcium handling in the NS and another potential protective role for amiloride. [68] In the kidney, the fine regulation of calcium balance occurs through the activity of the epithelial calcium channel Transient Receptor Potential ion channel called TRPV5. [89] TRPV5 is mostly expressed in the distal convoluted tubule and connecting tubule of the nephron, where it constitutes the apical entry mechanism for transcellular calcium reabsorption. TRPV5 is a constitutively active ion channel that bears unique electrophysiologic characteristics, including calmodulin and calcium-dependent inactivation and high selectivity for calcium. [90, 91] The activity of TRPV5 is tightly controlled at multiple levels by an array of different factors, including parathyroid hormone and the serine protease tissue kallikrein. Both parathyroid hormone and tissue kallikrein initiate the phosphorylation of TRPV5. [92, 93] Pathologic leakage of glomerular proteins causes multiple tubulointerstitial abnormalities, such as interstitial inflammation and eventually fibrosis, but does not affect tubular structure. [94] However, tubular transport processes could be affected by direct effect of urinary protein on membrane transporters at the luminal side of the distal convoluted tubule, such as ENa^+C. [16] The observation of nephrocalcinosis in patients with nephrotic-range proteinuria, associated with impaired transcellular calcium reabsorption via TRPV5 in a mouse model, suggests that proteinuria might affect tubular calcium handling. [95-97] Patients with NS present elevated

serum plasminogen levels. [67] After leakage into the urine, plasminogen is converted into active plasmin by tubular uPA. [16, 98] Tudpor et al. demonstrated that plasmin inhibits TRPV5 activity through the activation of uPAR. [68] Urinary plasmin, by inhibiting TRPV5 activity, could potentially be involved in disturbances in renal calcium handling found in nephrotic patients and may play a role in the tubulo-toxic effects of proteinuric urine. Plasmin inhibition by amiloride could therefore contribute to further tubulointerstitial protection by the inhibition of calcium cortico-medullar deposition, besides the already mentioned podocyte relaxation via uPAR inhibition of β_3 action. 65, 68]

6. THE ENDOTHELIUM AND AMILORIDE

Dietary sodium and potassium contribute in part to the control of the blood pressure. Endothelial cells are targets for aldosterone, which activates the apically located ENa^+C. The activity of these channels is negatively correlated with the release of nitric oxide (NO) and determines endothelial function. [99] A mediating factor between channel activity and NO release is the mechanical stiffness of the cell's plasma membrane, including the submembranous actin network (the cell's 'shell'). Changes in plasma sodium and potassium, within the physiological range, regulate the viscosity of this shell and thus control the shear stress- dependent activity of the endothelial NO synthase located in the shell's 'pockets' (caveolae). High plasma sodium gelates the shell of the endothelial cell, whereas the shell is fluidized by high potassium. Accordingly, this concept envisages that communications between extracellular ions and intracellular enzymes occur at the plasma membrane barrier, whereas 90% of the total cell mass remains uninvolved in these changes. Endothelial cells are highly sensitive to extracellular sodium and potassium. This sensitivity may serve as a physiological feedback mechanism to regulate local blood flow. It may also have pathophysiological relevance when sodium/potassium homeostasis is disturbed. [99]

Endothelial NO synthase (eNOS) is located at the caveolae of the apical cell membrane and its expression and/or activity is regulated by various factors. It appears to be likely that sodium ions control eNOS activity, which is also inhibited by aldosterone, possibly indirectly through ENa^+C-mediated sodium influx. [100] Conversely, inhibition of ENa^+C-mediated sodium influx by amiloride activates eNOS. [101] An increase in the intake of salt induces the production of asymmetrical dimethyl-L-arginine, which is a competitive eNOS inhibitor, and increasing extracellular sodium downregulates eNOS expression and angiogenesis. [102,103] It is noteworthy that there exists a negative correlation between stiffness and eNOS activity, that extracellular sodium concentration strongly determines stiffness and eNOS function, and also that extracellular potassium concentration influences eNOS activity and stiffness only at low sodium concentrations. Vascular endothelial cells undergo large changes in shape and can best adjust to such alterations if the deformability (physical compliance) of the cells is high. The cortical cytoskeleton of vascular endothelial cells is highly 'dynamic' and the state of polymerization of cortical actin determines the structure and mechanical properties of this layer. [104,105] Monomeric globular actin (G-actin), which can rapidly polymerize into filamentous actin (F-actin), can cause a rapid increase in local viscosity (gelation). Alternatively, switching from F-actin to G-actin is associated with solation of the cortex,

which in other words refers to the submembranous multimeric actin filament meshwork disaggregating into actin monomers. [106] G-actin is known to colocalize with eNOS and to increase eNOS activity. [107] It is possible that in this system potassium is an agonist, while sodium is a functional antagonist. Sodium influx, mediated by aldosterone-activated $ENa^{+}C$, stiffens the cytoskeleton by increasing the viscosity of the submembranous layer. It is hypothesized that when sodium is in the high physiological range, filamentous actin dominates over monomeric actin. This would explain the sodium-induced increase in cell stiffness. When potassium is elevated, actin filaments disaggregate into actin monomers and endothelial cells soften. Both F-actin and G-actin are negatively charged molecules and their interaction with positively charged ions as sodium and potassium will finally depend on the local concentrations and specific affinities of the respective ions. These changes are supposed to take place at the cytosolic submembranous zone, the cell shell (caveolae). [108] The solation-gelation hypothesis, based on the different interactions between sodium and potassium with the submembranous actin network, is supported by the observation that sodium has a greater affinity to protein surfaces than potassium. [109] It is assumed that when sodium enters the cell (e.g., through $ENa^{+}C$ activation) it binds with high affinity to actin displacing potassium from the carboxylate groups within the amino-acid side chains. Thus, increasing the concentration of sodium, which has a higher affinity to actin as compared to potassium, effectively modulates its protein–protein interaction strength. [25] These small changes in sodium or potassium in the submembranous zone should control the state of actin polymerization and thus the cell stiffness and functionality. [99]

As mentioned previously, the genesis of edema in the the NS, can be mainly due to three factors: tubular retention of water and sodium stimulated by plasmin and proteinuria; vascular tone dysregulation and increased permeability secondary to capillary dilation; and hypoalbuminemia and a decreased oncotic pressure, particularly at some stages of acute MCD. In this regard, the first two causes are mainly due to ENa+C activity, which can be managed or ameliorated by amiloride.

Another interesting cellular site in the endothelial cell at which amiloride can be indirectly useful (as any antiproteinuruc agent) is at the endothelial glycocalyx. It has already been mentioned that proteinuria is a marker of cardiovascular morbidity and mortality and NS subjects belong to this clinical situation. It has been beautifully demonstrated by Salmon et al. that in Munich-Wistar-Fromter rats, a primary insult to the endothelial GBM glycocalyx translates with time to a systemic widespread loss of this layer in other organic vascular beds, such as the coronary and mesenteric vessels. This phenomenon was accompanied by local defects in water and sodium permeability. Therefore, this could be another mechanism of edema in the NS. Additionally, considering the relevant role the glycocalyx plays in the endothelium as a primary sensor for the secretion of NO, as a mechanotransductor, as a leukocyte adhesion molecule, as a stimulator for endothelial paracrine functions implicated in vascular medial smooth muscle and myofibroblasts actions, its loss could also link proteinuria to vascular dysfunction. [110-113] Loss of endothelial glycocalyx has been shown to accelerate atherosclerosis. [114] Whether amiloride has direct effects on the endothelial glycocalyx has not been assessed, but its antiproteinuric actions may also prove to be protective for the endothelium and the systemic vascular bed.

7. THE NEPHROTIC SYNDROME AND AMILORIDE. FINAL CONSIDERATIONS

7.1. Tubular Aspects

The asymmetry of volume expansion, which has been traditionally attributed to the decrease in plasma oncotic pressure, is more likely due to an alteration of the capillary hydraulic conductivity, possibly linked to functional changes at the level of intercellular junctions. The low plasma oncotic pressure does not unbalance the transcapillary oncotic gradient and cannot be considered as a determining factor in edema generation or as a resistance factor for edema resorption. Therefore, diuretics preventing renal sodium retention remain the cornerstone of the treatment of nephrotic edema. Accordingly, the association of amiloride and furosemide provides a powerful treatment allowing progressive removal of edema from nephrotic patients. [115,116] As to the dose to be employed to commit these goals, it varies between 5 and 10 mg/day, depending on the clinical picture, concomitant drugs employed, laboratory results and patient tolerance.

7.2. Glomerulo-Tubular Aspects

On the basis of the temporal association among an initial glomerular insult, the appearance of plasmin in urine and primary, renal ENa^+C-mediated sodium hyper-reabsorption, these findings provide a putative novel mechanistic link between damage to the glomerular filtration barrier, proteinuria, and stimulation of sodium reabsorption in distal tubules and collecting ducts in the NS. This aspect is a target for amiloride. Moreover, the role amiloride plays on podocyte's integrin β_3 is important to reducing cellular contraction and proteinuria. Amiloride offers itself as a low-cost, non-immunosuppressant and complementary tool acting both at the glomerular and tubular level, for the management of complications of the NS as proteinuria, edema and hypertension.

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