Role of oxidative stress in primary sodium retention and edema formation in nephrotic syndrome

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Résumé

Le syndrome néphrotique (SN) résulte d'une altération glomérulaire, responsable d'une excrétion urinaire anormale de protéines plasmatiques induisant une hypoalbuminémie. Le SN est toujours associé à une rétention rénale de Na + qui conduit à la génération d'ascite et/ou d'œdèmes. La pathogénie de la rétention de Na + et de la constitution d'œdèmes n'est pas entièrement élucidée. Dans notre étude, nous avons évalué le rôle possible des espèces réactives de l'oxygène (ROS) dans cette pathogénie. Notre étude dans le modèle de rat aminonucléoside puromycine (PAN) de SN fournit des éléments de preuve d'un rôle critique des ROS dans les troubles hydro-électrolytiques associés au SN. Dans le rein, l'endocytose de l'albumine anormalement filtrée dans le néphron distal induit un stress oxydatif qui est responsable de l'augmentation de la Na, K-ATPase. Dans le péritoine, le SN est associé à une augmentation marquée de la perméabilité à l'eau et à une diminution du coefficient de réflexion des protéines de la barrière péritonéale. Ces modifications, déclenchées par le stress oxydatif et l'activation subséquente de NF-κB, sont responsables d'environ deux tiers du volume de l'ascite. Enfin, nous avons confirmé que le stress oxydatif participe à la sécrétion de l'aldostérone et est nécessaire à l'apparition de l'hyperaldostéronémie observée chez les rats néphrotiques PAN.

Abstract.

Nephrotic Syndrome (NS) is a nonspecific kidney disorder defined by abnormal urinary excretion of plasma proteins and hypoalbuminemia. NS is always associated with a renal retention of Na^+ leading to the generation of ascites and/or edema. The pathogenesis of Na^+ retention and edema is not fully elucidated. In our studies we evaluated the possible role of reactive oxygen species (ROS) in this pathogenesis.

Our studies in the puromycin aminonucleoside (PAN) rat model of NS provided pieces of evidence for a critical role of ROS in the hydro-electrolytic disorders associated with NS. In the kidney, endocytosis of abnormally filtered albumin in the distal nephron induces an oxidative stress which is responsible for the up-regulation of Na,K-ATPase. In the peritoneum, NS is associated with a marked increase in water permeability and a decrease in the reflection coefficient to proteins of the peritoneal barrier. These changes, which are triggered by oxidative stress and subsequent activation of NF-κB, account for approximately two-third of the volume of ascites. Finally, we confirmed that oxidative stress participates in the angiotensin-stimulated secretion of aldosterone and is required for the hyperaldosteronemia observed in PAN-nephrotic rats.

Introduction

1. Nephrotic syndrome

1.1. Definition, signs and symptoms

Nephrotic syndrome (NS) in humans is a nonspecific kidney disorder defined by abnormal urinary excretion of plasma proteins (>50 mg/kg/day) and hypoalbuminemia (<30 g/l). Diagnosis is made by determination of urine protein/creatinine ratio in a random urine sample or measurement of urinary protein content in a 24-h urine collection [1]. Causes of NS are diagnosed on the basis of history, physical examination, serologic testing, and renal biopsy. Congenital and infantile NS are those that manifest during the first year of life.

In addition to proteinuria and hypoalbuminemia, NS is commonly associated with two additional symptoms: edema and hyperlipidemia. Edema results from excess fluid accumulation in the interstitium. It takes several forms ranging from puffiness around the eye, pitting edema in the extremities, ascites, and severe form of general edema called anasarca. It is classically thought to stem from hypoalbuminemia, vascular fluid leakage towards the interstium, hypovolemia and renal sodium retention. Hyperlipidemia, stems from overproduction of lipoproteins in the liver, secondarily to stimulation of protein synthesis in the liver to compensate for proteinuria, and from decreased lipid catabolism due to the urinary loss of lipoprotein lipase, the main enzyme involved in lipid catabolism [1].

1.2. Epidemiology

Nephrotic syndrome can affect any age depending on its etiology, but in different ways in children and adults. The most frequent form of glomerulopathy in children is minimal change disease (66% of cases), followed by focal and segmental glomerulosclerosis (8%) and mesangiocapillary glomerulonephritis (6%). In adults the most common disease is mesangiocapillary glomerulonephritis (30-40%), followed by focal and segmental glomerulosclerosis (15-25%) and minimal change disease (20%). There are also differences in prevalence between genders, the disease being more common in men than women by a ratio of 2 to 1. The incidence of nephrotic syndrome varies worldwide depending on its primary cause; according to the American Society of Nephrology, diabetic nephropathy is responsible for at least 50 cases per million populations. In children, nephrotic syndrome may occur at a rate of 20 cases per million children [2] .

1.3. Complications of NS

The most common complications of nephrotic syndrome are bacterial infection and thromboembolism. Patients with NS display increased susceptibility to infections due to the presence of edema fluid (which acts as a breeding ground for infections) and urinary loss of plasma immunoglobulins. Peritonitis is the most common infection, followed by lung, skin and urinary infections.

Increased prevalence of thromboembolism is thought to result from increased platelet aggregation, increased fibrinogen concentration, decreased antithrombin III concentrations, increased blood viscosity and decreased blood flow. Venous thrombosis is most common, especially in the renal vein, the deep vessels of the extremities, and the pulmonary arteries (which carry deoxygenated blood from heart to lungs). In patients with refractory nephrosis, low doses of anticoagulants are sometimes used.

In addition, nephrotic syndrome is associated with increased risk of renal failure. The disease responsible NS can damage the glomeruli and alter their ability to clear the blood. Renal failure can either be gradual, leading to chronic renal failure (CRF), or sudden in a form of acute renal failure (ARF) as a result of hypovolemia and decreased blood supply to the kidneys.

1.4. Etiology

Nephrotic syndrome can be caused by diseases that affect only the kidneys, such as membranous nephropathy or focal segmental glomerulosclerosis (FSGS). Diseases that affect only the kidneys are called primary causes of nephrotic syndrome. In addition, nephrotic syndrome can also be caused by systemic diseases, which are diseases that affect many parts of the body, such as diabetes or lupus. Systemic diseases that affect the kidneys are called secondary causes of nephrotic syndrome. More than 50 percent of nephrotic syndrome cases in adults have secondary causes.

The primary forms of the disease, which are usually described according to their histology, correspond to either genetic alterations of proteins involved in the glomerular filtration barrier or to the idiopathic nephrotic syndrome which dstems from an unidentified circulating factor which functionally alters the glomerular barrier. The most common form of primary NS is children is called minimal change disease (MCD) owing to the fact that kidneys

appear normal at optic microscopy level, without glomerular morphologic alterations, and lesions are only visible by electron microscopy. Another important form is focal segmental glomerumosclerosis (FSGS), the most common cause of nephrotic syndrome in adults [3], which is characterized by the presence of tissue scarring in the glomeruli.

Secondary causes of NS usually have the same histological patterns as primary causes, but sometimes they exhibit some differences which suggest a secondary cause, and they are usually described by the underlying disease. The most common causes of secondary NS are: diabetic nephropathy, systemic lupus disease, sarcoidosis, syphilis, HIV and hepatitis B.

For proper treatment of NS it is essential to define the underlying cause as there are numerous causes that can lead to NS or NS range of proteinuria.

1.5. Pathogenesis and consequences

The hallmark of the disease is an increased permeability of the glomerular filtration barrier which leads to high quantities of proteins passing from the blood into the urine. The cause of NS is damage to the glomeruli, which leads to alterations in their capacity to filter the substances transported in the blood.

Although controversy exists regarding the sieving of albumin across the glomerular barrier, it is usually accepted that in healthy individuals, less than 0.1% of plasma albumin crosses the glomerular filtration barrier [4]. In experimental animal studies, it has been proposed that there is ongoing passage of several grams of albumin per day into the urine, with equivalent tubular uptake of albumin, the result being that daily renal excretion of albumin is < 80 mg [5].

The glomerular filtration barrier consists of three successive layers (Figure 1): the capillary endothelium which displays fenestrations allowing the passage of solutes and water but retaining blood cells, the basement membrane which is a negatively charged structure exerting repulsive force on negative proteins, and the vascular sheet of the Bowman's capsule made of highly differentiated podocytes, the interdigatations of which constitute the filtration diaphragm.

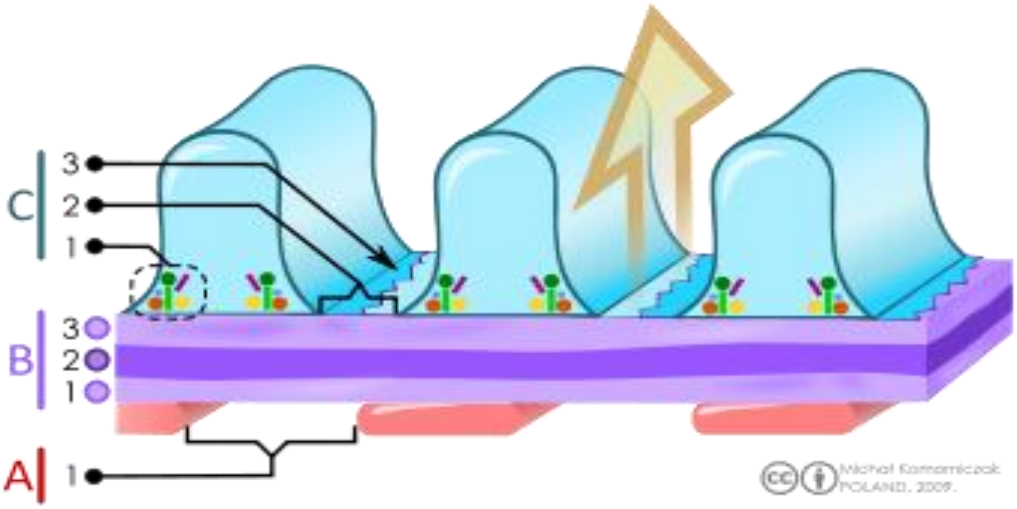


Figure (1). Filtration barrier (blood-urine) in the kidney.
A. The endothelial cells of the glomerulus; 1. Pore (fenestra).
B. Glomerular basement membrane: 1. lamina rara interna 2. Lamina densa 3. Lamina rara externa
C. Podocytes: 1. enzymatic and structural protein 2. Filtration slit 3. Diaphragm
M•Komorniczak, polish wikipedist.

In the glomerular filtration barrier, the podocytes (visceral epithelial cells) are key cell in the selective filtering action of the glomerular capillary wall, they wrap around the capillaries of the glomerulus (Figure 2). Adjacent podocytes interdigitate to cover the glomerular basement membrane which is in contact with glomerular capillaries, but they leave small gaps (the filtration slits). These filtration slits are covered by slit diaphragms (as shown in figure 2).

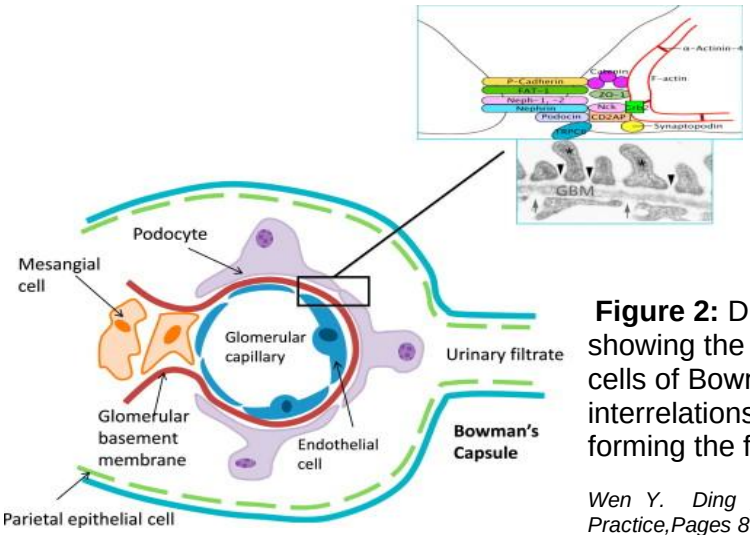


Figure 2: Diagram of a glomerular capillary, showing the three cell types, as well as the parietal cells of Bowman's capsule, and their interrelationship in supporting the glomerulus and forming the filtration barrier. (Modified)

Wen Y. Ding , Moin A. SaleemKidney Research and Clinical Practice,Pages 87 - 93Volume 31, Issue 2, 2012.

The slit diaphragms are composed of cell surface proteins like nephrin, p-cadherin and podocalyxin, which make sure that large molecules such as albumin and gamma globulins stay in the bloodstream.

Structural and/or functional changes of any of these structures may be the cause of proteinuria. One or more of these targets may be involved in each type of nephrotic syndrome. The importance of the podocytes and the filtration slits is evidenced by genetic diseases. Thus, in congenital nephrotic syndrome of the Finnish type, the gene encoding nephrin, a protein of the filtration slit, is mutated, leading to nephrotic syndrome in infancy. Similarly, podocin, a protein of the podocytes, may be abnormal in a number of children with steroid-resistant focal glomerulosclerosis. In addition, alternation in the glomerular basement membrane (GBM) may alter the glomerular barrier and results in proteinuria and nephrotic syndrome, as seen in mutations in the COL4A3, COL4A4, or COL4A5 genes that encode the type IV collagen α3, α4, and α5 chains, respectively, and cause Alport's syndrome [6, 7].

Proteinuria may concern only albumin or, in case of greater injury, all plasma proteins. Proteinuria is referred as selective when albumin accounts for > 85% of urine proteins. Because albumin has a net negative charge, it has been proposed that loss of glomerular basement membrane negative charges might be responsible for selective proteinuria. Nonselective proteinuria, would not involve changes in glomerular net charge but rather a generalized defect in permeability. This construct does not permit clear-cut separation of

causes of proteinuria, except in minimal-change nephropathy, in which proteinuria is selective.

2. Nephrotic edema and ascites

Whatever its etiology, NS is always associated with a renal retention of sodium (Na^+) leading to extracellular volume expansion and to the generation of ascites and/or edema. The edema formation and/or ascites in nephrotic syndrome is a serious issue that constraisn the functional activity of patients and make their lives difficult. It ranges from small puffiness and pitting edema to a serious ascites, pleural and cardiac effusion and, in a severe form of edema called anasarca, edema is generalized with a widespread swelling of the skin.

Classically, nephrotic edema was thought to be secondary to hypoalbuminemia, which decreases the gradient of oncotic pressure across capillary wall and increases fluid leakage from vascular towards interstitial compartment. In turn, the resulting hypovolemia stimulates the renin/angiotensin/aldosterone system (RAAS) and promotes renal sodium retention which allows refilling of the vascular compartment. During the past decade, many experimental and clinical observations have ruled out this underfill theory, and it is now admitted that 1) **renal sodium retention is a primary event independent of capillary leakage and activation of the RAAS** and 2) **capillary leakage stems not only from decreased oncotic gradient but also from intrinsic changes in the properties of the capillary walls**. However, neither the molecular basis of these renal and capillary alterations nor their linkage to proteinuria and/or hypoalbuminemia is fully elucidated. The purpose of our study was to determine the etiology of primary sodium retention and the nature and etiology of the changes in the peritoneal capillaries.

2.1. Sodium retention

2.1.1. Brief description of the mechanisms of sodium handling by the renal tubule

Sodium (Na^+) is the predominant cation in the extracellular fluid (ECF), the volume of ECF is directly proportional to the content of Na^+ in the body. The body must maintain ECF volume within acceptable limits to maintain tissue perfusion. As demonstrated by Guyton[8], kidney has a major role in controlling arterial blood pressure by maintaining sodium

homeostasis. In humans, more than 25 moles of Na^+ in 180 litter of fluid daily are delivered into the glomerular filtrate, most of which (>99%) is reabsorbed while the urine flows along the renal tubule. Tubular sodium reabsorption is a two-step mechanism including active pumping of intracellular sodium across the basolateral membrane by the Na,K-ATPase (except in distal tubule intercalated cells) and passive entry of sodium across the apical membrane. The successive segments of the nephron differ by the mechanism of apical sodium entry.

About 60 to 65% (figure 3) of this sodium load is reabsorbed along the proximal tubules (PCT). In PCT 80% of apical sodium entry is electroneutral and coupled with proton secretion by the exchanger Na^+/H^+ (NHE3). The loop of Henle (LOH) is responsible for the reabsorption of about 25% of the sodium load, mainly in the thick ascending limb (TAL). In TAL, the apical entrance of Na^+ is mediated by the electroneutral cotransporter $Na^+/K^+/2Cl$ (NKCC2) fot ~85% and by the exchanger NHE3 (the remaining 15%. Coupled with electrogenic recycling of potassium across the apical membrane and electrogenic exit of chloride across the basoalteral membrane, the transcellular reabsorption of sodium generates a lumen-positive transepithelial voltage which drives paracellular reabsorption of sodium. It is estimated that along the TAL two-third of sodium is reabsorbed via the transcellular pathway and the remaining third by the paracellular patway.

The distal part of the nephron is responsible for the fine tuning of sodium homeostasis. The distal convoluted tubule (DCT) reabsorbs 5 to 7% of the filtered load of sodium. In human DCT, apical uptake of Na^+ is mediated by the Na^+-Cl^- cotransporter (NCC); this reabsorption is electroneutral and sensitive to thiazides. In mouse DCT, it is mediated by NCC in the proximal part and by both NCC and the epithelial sodium channel (ENaC) in the distal part.

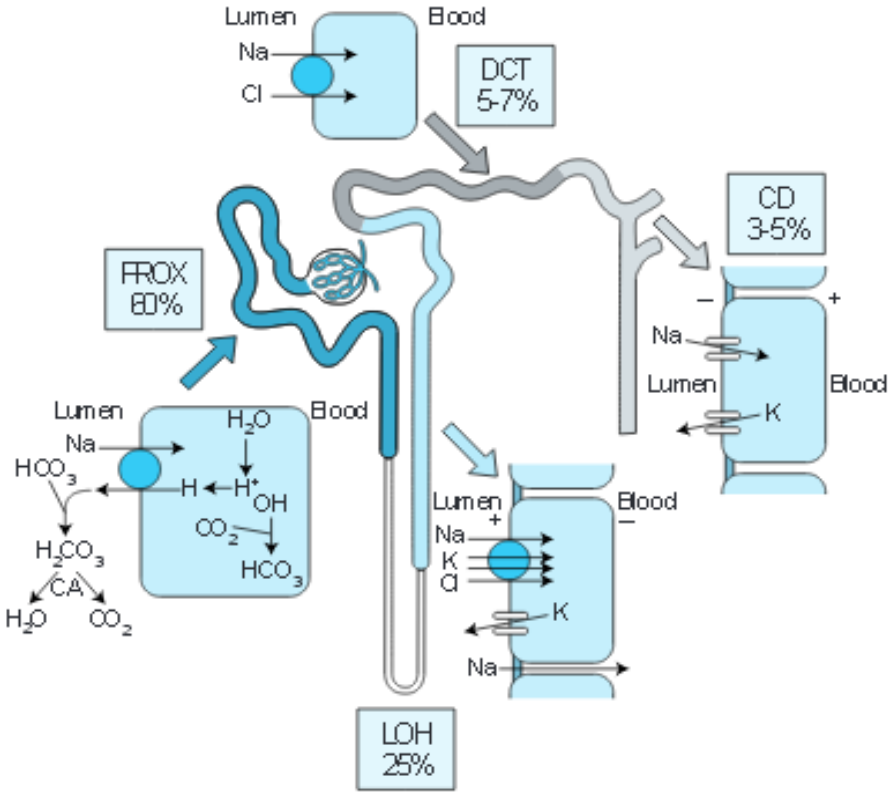


Figure 3: Sodium reabsorption along the mammalian nephron.
Modified: Disorder of water, Electrolytes, and Acid-Base. David H. Ellison

The connecting tubule (CNT) and cortical collecting duct (CCD) are the last sites of homeostatic control of sodium reabsorption which reabsorb 0 to 5% of the filtered sodium. Conversely to other nephron segments, CNT and CCD are made of several cell types: principal and intercalated cells. Classically, sodium was thought to be exclusively reabsorbed by principal cells. In these cells, apical sodium entry is mediated by amiloride-sensitive ENaC, which depolarizes the apical membrane and energizes potassium secretion via ROMK channels. This sodium reabsorption is under the positive control of aldosterone. More recently, it was demonstrated that, under some circumstances associated with high plasma mineralocorticoid levels, type B intercalated cells also contribute to 30-50% of sodium reabsorption in the CCD. In these cells, basolateral exit of sodium is mediated by a Na^+-HCO_3^- cotransporter, itself energized by the gradient of HCO_3^- generated by the activity of basolateral H-ATPase. Apical entry of sodium proceeds via the coupling of the pendrine Cl^-/ HCO_3^- exchanger with a Cl^-/ Na^+-HCO_3^- exchanger (figure 4). This transport is electroneutral and sensitive to thiazide [9].

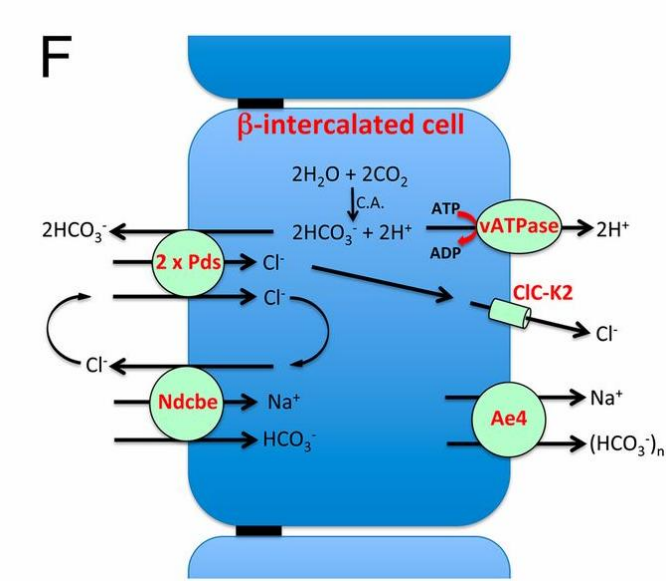


Figure 4: Schematic description of electroneutral NaCl absorption energized by the H^+ V-ATPase. Two cycles of pendrin coupled with one cycle of Ndcbe result in the net uptake of one Na^+, one Cl^-, and two HCO_3^- ions, whereas one Cl^- ion is recycled across the apical membrane. Then Cl^- ion exits the cell through a basolateral chloride channel, while Na^+ and bicarbonate exit the cell at the basolateral membrane via Ae4. All these transporters are indirectly energized by the H^+ V-ATPase.

Modified, Régine Chambrey et al. PNAS 2013;110:7928-7933

2.1.2. Site of sodium retention

During the past decades, many studies aimed at determining the site of sodium retention using the puromycin aminonucleoside (PAN) model of nephrotic rats. It is induced by a single injection of PAN: proteinuria and sodium retention develop within less than one week. PAN also induces a NS in humans but mice are resistant to PAN. In a model of unilateral PAN nephrosis, early *in vivo* micropuncture experiments demonstrated that sodium retention originates beyond the distal convoluted tubule accessible to micropuncture [10]. Later, using *in vitro* microperfusion and measurements of Na,K-ATPase activity in isolated segments of nephron, our laboratory identified the connecting tubule and cortical collecting duct (CCD) as the main sodium retaining nephron segments in bilateral PAN nephrosis [11, 12]. In addition, in children with idiopathic nephrotic syndrome, amiloride treatment restores the sodium balance to a normal levels [13], suggesting that connecting tubule and cortical collecting ducts, the two nephron segments endowed with amiloride-sensitive sodium channels (ENaC), are also the main sites of sodium retention in the human disease. In addition, it has been shown that nephrotic animals and patients display resistance of their inner medullary collecting ducts to atrial natriuretic peptide [14]. This prevents any compensation of increased sodium reabsorption in connecting tubule and cortical collecting duct.

2.1.3. Cellular mechanism and possible etiology of Na^+ retention

Our laboratory also showed that sodium retention in PAN nephrotic rats is associated with increased abundance and activity of Na,K-ATPase and of ENaC at the basolateral and apical sides of CCD principal cells respectively [15]. The site of sodium retention and its targets in the CCD resemble those of aldosterone in the kidney. Moreover, PAN nephrotic rats, like some patients with NS, show high plasma aldosterone level [11, 13]. Altogether, these findings are consistent with the underfill theory of nephrotic sodium retention and a role of the RAAS. However, clamping plasma aldosterone at normal level in PAN nephrotic rats prevents neither the retention of sodium nor the generation of ascites [15, 16]. Interestingly, in this aldosterone-clamped model of PAN nephrosis (AC-PAN), sodium retention remains sensitive to amiloride although functional ENaC is no longer detectable [15, 16], suggesting the existence of an alternate pathway for apical sodium uptake. The role of the RAAS in nephrotic sodium retention is further ruled out by the fact that in unilateral PAN nephrosis, sodium retention occurs exclusively in the PAN-injected kidney, excluding the involvement of a systemic factor.

Alternately, it has been proposed that sodium retention during nephrosis is triggered by a factor abnormally present in the luminal fluid that reaches the distal nephron of nephrotic rats and patients secondarily to the alterations of the glomerular filtration barrier. Thus, some groups proposed plasmin to be this local factor, based on the fact that 1) plasmin is detected in the urine of nephrotic patients and PAN nephrotic rats and 2) plasmin activates ENaC through proteolysis in cultured cells [17]. However, we consider it unlikely that primary activation of ENaC by plasmin may be solely responsible for sodium retention in nephrotic syndrome because 1) in the CCD of nephrotic rats there is not only an increased activity of ENaC but also an increase in its abundance at the apical membrane, 2) plasmin-induced activation of ENaC cannot account for the observed over expression of Na,K-ATPase, and 3) studies in AC-PAN rats have shown that sodium retention can occur without activation of ENaC.

2.1.4. Our hypothesis for the etiology of Na^+ retention in NS

The most abundant factor abnormally present in the urine of nephrotic patients and animals is albumin. In a healthy individual, less than 0.1% of plasma albumin may cross the glomerular filtration barrier [4]. Thereafter, reabsorption via the megalin/cubulin-dependent

endocytotic pathway in proximal tubules [18, 19] delivers a protein-free luminal fluid to the distal parts of renal tubules [20]. In nephrotic patients, the proximal tubule endocytotic absorption pathway is saturated and the distal part of the nephron is exposed to luminal albumin.

It is well established now that albuminuria has a link in the progression of Chronic Kidney Disease (CKD) [21, 22]. Albumin endocytosis in proximal convoluted tubules (PCT) induces toxicity [23] and triggers an oxidative stress which, in turn, activates different signalling pathways including NF-κB and MAP kinases [24]. Moreover, *in vitro* treatment of proximal tubular cells with albumin induces oxidative stress (OS) and produces numerous reactive oxygen species (ROS) [24-26].

It has been shown recently that apical albumin is also internalized by the collecting duct cells of PAN nephrotic rats and by $mCCD_{c11}$, a mouse CCD principal cell line[27]. In CCD from PAN rats, albumin endocytosis activates the MAP kinase $ERK_{1,2}$ ([28], and in mCCD cells, it activates the canonical NF-κB pathway leading to expression of pro-inflamatory cytokines [27]. Whether or not albumin endocytosis also triggers OS and ROS production in collecting ducts during NS is not documented, but several published or preliminary results suggest that it does. Thus, our laboratory previously reported or observed that albumin endocytosis in CCDs from PAN nephrotic rats is responsible for activation of ERK and is associated with activation of NF-kB and induction of osteopontin expression (unpublished observation), three targets of ROS [29]. In addition, phospho-ERK is known to increase Na,K-ATPase activity in both CCD and collecting duct cells [30] and may thereby initiate sodium retention.

Several studies showed that ROS are an important modulator of nephron ion transport; Oxidative stress has been shown to regulate BP and sodium handling in various animal models. For example, in the proximal tubules increased oxidative stress influences a number of physiologic processes including renal sodium handling and regulation of Na+,K+-ATPase activity [31, 32] . In the medullar thick ascending limb of the loop of Henle, both exogenous and endogenous superoxides stimulate sodium absorption and enhancement of Na-K-2Cl cotransporter [33, 34]. Moreover, a group of researchers found that ROS production through NADPH oxidase system has a stimulatory effect on ENaC activity in $mpkCCD_{c14}$ cell line[35]

Based on these data, we hypothesized that luminal endocytosis of albumin in CNT/CCD induces OS and ROS production which in turn increases sodium reabsorption during nephrotic syndrome.

2.2. Edema and ascites formation

Edema and ascites result from an imbalance between capillary filtration and lymphatic drainage. Except for lympho-edema which stem from alteration of lymphatic drainage, all forms of edema result from increased capillary filtration. They are associated with either hypoalbuminemia, as in NS, or increased venous pressure (e.g. gestational edema) or increased capillary permeability (e.g. Quincke's edema).

Fluid leaking from the capillaries accumulates in the interstitium or, in case of ascites, in the peritoneal cavity. Because the compliance of these compartments is almost infinite, very large volumes of edema or ascites can accumulate without altering much the interstitium hydraulic pressure, which would oppose to the capillary leakage.

2.2.1. Peritoneum anatomy and structure

The peritoneum is a serous membrane that covers most of the intra-abdominal organs. It supports the intra-abdominal organs, provides a small space for them to move, and serves as a conduit for their blood vessels, lymph vessels and nerves. It composed of two major layers: 1) the parietal peritoneum, which is the outer layer and is attached to the abdominal and pelvic walls, and 2) the visceral peritoneum, which is the inner layer and is wrapped around the internal organs. Ultimately these two layers form a continuous sheet.

The virtual space between these two layers constitutes the peritoneal cavity which is normally filled with a small amount of serous fluid, allowing the two layers to freely slide against each other. The peritoneal cavity is the space where ascites accumulates during NS. Ascites is an iso-osmotic extracellular medium mostly devoid of proteins. Because of its important vasculatization and high filtration capacity, the peritoneum is clinically used for dialysis. During peritoneal dialysis, a glucose solution is introduced into the peritoneal cavity,

left there for a specific amount of time to absorb waste products through the mechanism of diffusion, and thereafter aspirated.

2.2.2. The peritoneal filtration barrier

The peritoneal filtration barrier is a complex structure made up of three layers: the capillary endothelium, the interstitium and the mesothelium that lines the inner surface of the peritoneum [36]. The mesothelium is a unistratified squamous epithelium with a basal membrane in contact with the submesothelial interstitium composed of collagen, fibroblasts, and adipocytes and crossed by a rich network of blood and lymph vessels.

Filtration across the peritoneum requires the crossing of five successive resistance barriers: the vascular endothelium of capillaries and post-capillary venules of the peritoneum [37], the endothelium basement membrane, the interstitial matrix, the mesothelium basement membrane and the mesothelium. Transfer across the endothelium and mesothelium proceeds through both transcellular and paracellular pathways. Four simultaneously occurring mechanisms were described for the transport of fluid and solute across the peritoneum: diffusion, convection, osmosis, and fluid absorption.

In the context of peritoneal dialysis, this complex filtration structure has been modelled according to the so-called three pore model (TPM) [38] and reviewed by Olivier Devuyst and Bengot Rippe as shown (figure 5). According to this model, the continuous endothelium lining of the peritoneum capillaries are considered as a single membrane and can be functionally described in term of TPM: The large pores (less than ,5% of all pores) allow the passage of molecules with large molecular weight like proteins and contribute to 5-8% of peritoneal ultrafiltration coeffient (L_PS), the small pores (99% of total pores) allow the passage of solutes and water and contribute to around 90% of L_PS , and finally the ultra-small pores which account only for 2% of L_PS.

In the capillary walls of the peritoneal membrane, the small pores represent the major pathway for water and solute transport and they are represented by the space in between individual endothelial cells which called inter-endothelial cleft as shown in figure 4. The radius of these clefts is around 40 A°, whereas the radius of large pores is around 250 A°[39]. The ultra-small, water-only pores of radius around 2,5 A° present in the endothelial cell membrane and account for the transcellular pathway of transport across the peritoneal membrane[40].

More recent works in the modelling of peritoneal transport have been taken beyond the simple TPM. The simplest extension of the regular TPM model is the so-called fiber matrix model or the serial three-pore model, in which includes the interstitial space as a serial transport resistance barrier acting in parallel with the capillary wall[41]. More complicated models included not only the interstitium but also the distributed complexity nature of the peritoneal capillaries in the modeling[42].

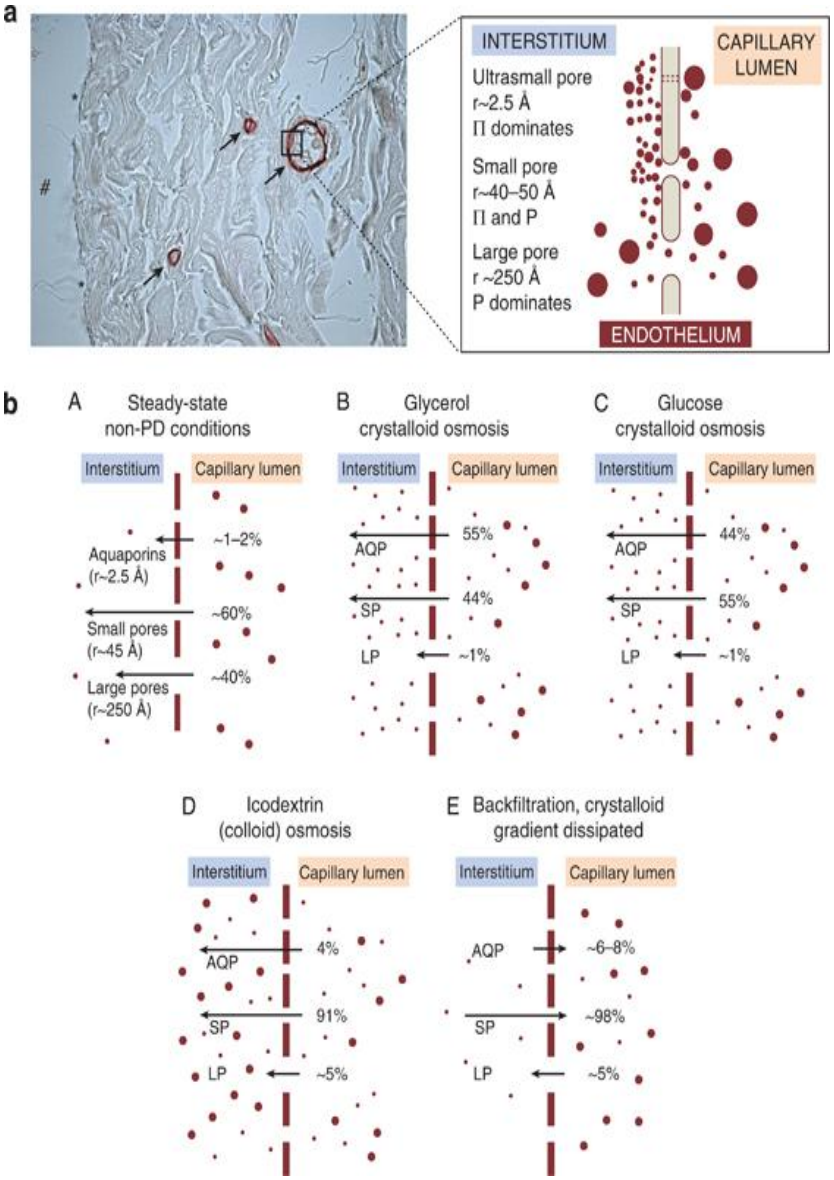


Figure 5: Structure of the peritoneal membrane and the three-pore model (TPM). a) Cross-section of the human parietal peritoneum stained for the water channel aquaporin-1 (AQP1), showing different pores in the endothelium and their sizes. b) Transcapillary UF in the TPM using different osmotic solutions in which (A) shows the Fractional fluid flows across the peritoneum under normal conditions with no dialysis. (B) With glycerol. (C) With glucose. (D) In a conventional icodextrin PD solution. (E) Reabsorption of fluid across the small pores occurs when the crystalloid (glucose) osmotic gradient has totally dissipated (usually after 4 h). (Modified).

Olivier Devuyst and Bengt Rippe Kidney Int. Mini review, 85(4):750-8 2014, April.

2.2.3. AQP1 in the peritoneum

AQP1, a 28-kD protein is a water-specific membrane channel, impermeable to urea and glycerol. Its physiological function has been characterized thoroughly in the renal proximal tubules. AQP1 is the first identified water channel, and it distributes in a variety of tissues, including the epithelium of proximal tubules in the kidney, many parts in the eye, lung alveolus, hepatic bile duct, spleen, cardiac endocardium, peritoneum, salivary glands, and erythrocyte membrane [43, 44]. There is accumulating evidence that water channel proteins, aquaporins (AQPs), have a fundamental role in water transport in many forms of tissues [45, 46].

The identification of AQP1 had a major relevance for better understanding the mechanism of water transport and ultrafiltration in the peritoneum. The atomic model of AQP1,derived from studies based on electron and X-ray crystallography, disclosed key features accounting for the high permeation rate and strict selectivity for water transport[47] and several lines of evidence have demonstrated that AQP1 is the ultra-small, water specific pore predicted by TPM.

In human peritoneum, messenger RNA level expression of AQPs revealed that the amount of mRNA level of AQP1 was much greater than AQP3, which was much greater than AQP4 [48]. AQP1 is distributed in the endothelium lining of the peritoneal capillaries and postcapillary venules [49], which is the most important barrier to solute transport during peritoneal dialysis [50]. In addition, immunohistochemistry of rat peritoneal tissues showed the expression of AQP1 in mesothelial cells, venular endothelial cells, and capillary endothelial cells [51] and shown in (figure 6).

Inhibition of AQPs with mercury induced a 66 % inhibition of peritoneal water flow [52] Similarly, peritoneal water flow is reduced by 58% in AQP1 knockout mouse [53].. All these data from literature provide solid evidence that AQP1 plays a major role in water transport across the peritoneal barrier.

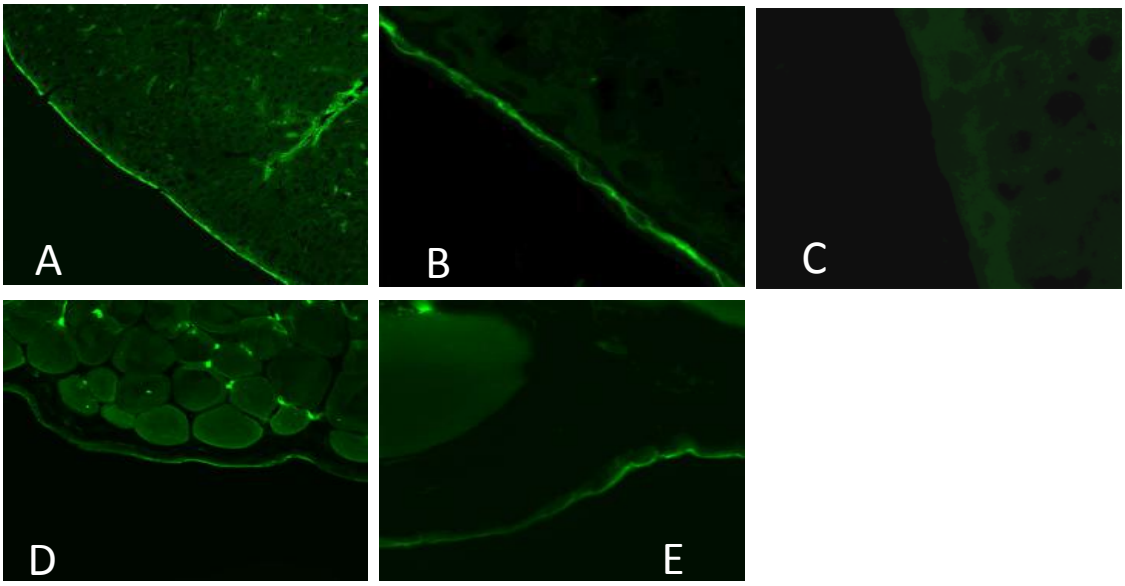


Figure 6 — Immunohistochemical localization of aquaporin-1 (AQP1) in rat peritoneum [(A) to (E)], omentum. Clear staining was observed in both the visceral peritoneum attached to the liver [(A) (200×) and (B) (1000×)] and the parietal peritoneum attached to the abdominal wall [(D) (200×) and (E) (1000×)], suggesting AQP1 was expressed in thenmesothelial cells. By contrast, staining was negative in the visceral peritoneum without incubation with an anti-AQP1 antibody [(C) (1000×)]. (Modified)
Ota et all, Peritoneal Dialysis International, Vol. 22 pages. 307–315. 2002.

2.2.4. Our hypothesis regarding edema formation in NS

Edema formation in NS results from the asymmetry of the extracellular volume expansion resulting from renal sodium retention: the vascular volume is not, or only slightly, modified whereas water and solutes accumulate in the interstitium. As a matter of fact, capillary filtration capacity is increased almost 2-fold in nephrotic patients [54].

According to Starling's law, water flux across a capillary (J_v) is governed by several parameters:

$$J_v = L_p \times S \times [(P_c - P_i) - \sigma \times (\Pi_c - \Pi_i)]$$

where L_p is the hydraulic conductivity of capillaries, S the exchange surface, P_c and P_i the hydrostatic pressure of the capillary and interstitium respectively, σ the reflection coefficient of proteins across the capillary wall, and Π_c and Π_i the oncotic pressure in the capillaries and interstitium respectively. This is a macroscopic modelling of water flux that considers a single pathway with a mean L_p and σ. With regard to the three-pore model, one should calculate the total water flux as the sum of the fluxes through the three pathways, each one being

characterized by specific L_p and σ; e.g. σ would equal zero and one for the large and ultra-small pores respectively.

According to the underfill theory, increased peritoneal filtration stems from the decreased Π_c brought about by hypoalbuminemia. However, it is known now that the transcapillary gradient of oncotic pressure (Π_c-Π_i) is almost unchanged in NS. It has been shown that the transcapillary gradient of oncotic pressure is unchanged in analbuminemic rats, because of a parallel decrease in plasma and interstitium oncotic pressure [55]. Accordingly, there is no edema or ascites formation in analbuminemic rats and patients [55]. .Moreover, diuretic treatments allow the withdrawal of significant amounts of edema without significant change in the transcapillary gradient of oncotic pressure [56]. Thus, decrease in plasma oncotic pressure in animal models as well as in nephrotic patients does not significantly alter the transcapillary gradient of oncotic pressure and is neither a determinant parameter in the genesis of edema, nor a resistance factor to edema withdrawal.

The transcapillary gradient of hydrostatic pressure (P_c-P_i) also is unchanged in NS [54] because soft tissues display an almost infinite compliance.

The 2-fold increase in capillary filtration in nephrotic patients [54] stems mainly from increased capillary hydraulic conductivity and decreased reflexion coefficient for proteins. In other words, it does not result from an increase in the driving force for water filtration but from intrinsic changes of the filtration barrier. However, neither the molecular mechanism responsible for these intrinsic changes of the filtration barrier nor their etiology is known presently.

ROS are known to induce endothelial barrier dysfunction [57] that may lead to tissue edema in the context of inflammation or ischemia [58]. TNFα, which may be involved in increased capillary hydraulic conductivity during nephrotic syndrome ( ) is a known target of NF-κB and may therefore be induced through production of ROS. **We hypothesize that nephrotic syndrome is associated with endothelial and peritoneal oxidative stress which is responsible for increased capillary permeability and edema and ascites formation via activation of NFκB pathway.**

Thus, our hypothesis stipulates that a same cellular event, i.e. oxidative stress and ROS production, is responsible for the renal and endothelial alterations leading to edema formation and ascites accumulation

2. Reactive species and oxidative stress

Most stable molecular species have the electrons in their outer orbital arranged in pairs. A molecule with one or more unpaired electrons in its outer orbital is called a free radical. This unpaired electron makes the specie very unstable and able to react with other molecules to pair this electron and thereby generate a more stable specie [59]. The term Reactive Oxygen species (ROS), often used in the literature as a collective term that includes not only oxygen-centred radicals, but also some non-radicals derivatives of oxygen (table 1). A similar term, is Reactive Nitrogen Species (RNS) is also becoming widely used nowadays (table 2).

Table (1): Reactive Oxygen Species (ROS)

Radicals	Non-radicals
Superoxide: O_2^-	Hydrogen peroxide: H_2O_2
Hydroxyl: OH^-	Hypochlorus acid: HOCL
Peroxyl: RO_2^-	Hypobrromus acid: HOBr
Alkoxyl: RO^-	Ozone: O_3
Hydroperoxyl: HO_2^-	Singlet oxygen: Δg

Table (2): Reactive Nitogen Species (RNS)

Radicals	Non-radicals
Nitric oxide: NO^-	Nitrogen dioxide: NO_2
Nitrous acid: HNO_2	Nitrosyl cation: NO
	Nitrosyl anion: $NO^- NO^-$
	Dinitrogen tetroxide: N_2O_4
	Dinitrogen trioxide : N_2O_3
	Peroxynitrite: $ONOO^-$
	Peroxinitrous acid: ONOOH
	Alkylperoxynitrites: ROONO

(Source: *Oxidative stress and human disease, Taibur Rahman, Ismail Hosen, M. M. Towhidul Islam, Hossain Uddin Shekhar, Advances in Bioscience and Biotechnology Vol.3 No.7A(2012), Article ID:25130,23 pages*)

ROS are chemically active molecules containing oxygen that are continuously produced *in vivo* in all cells: they are by-products of naturally occurring metabolism of O_2 and play a critical role in cellular metabolism, signalling and homeostasis [60]. ROS is a broad term that includes all oxy-radicals such as singlet and doublet oxy-radicals and nonradicals like hydrogen peroxide (H_2O_2). The different ROS display a wide variety of activity, half-life, mode of production and abundance. Common examples of oxygen free radicals include the hydroxyl radical (OH), superoxide anion (O_2-), and hydrogen peroxide (H_2O_2). The hydroxyl radical, is the most potent oxidant known, and accordingly shows an extremely short half-life. Superoxide O_2- is the proximal ROS generated by mitochondria as a result of oxygen reduction by various enzymes in the Krebs cycle and respiratory chain; it acts as a weak oxidizing agent, and it is likely to be more important as a source of OH and H_2O_2.

It was originally thought that ROS are only produced by phagocytic cells as part of their host cell defense mechanisms. Recent work has demonstrated that ROS have a role in cell signalling role in many biological systems, including; apoptosis; gene expression or suppression; and the activation of cell signaling cascades [61].

3.1. Sources of ROS

Intracellular ROS are generated through various mechanisms, as shown in Figure 7, depending on the cell and tissue types. The major sources of ROS production are the mitochondria, the cell membrane through NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) system, the perixosome and the endoplasmic reticulum [62].

Mitochondria are the main source of energy production (ATP) in cells. ATP production in the mitochondria is coupled to proton transport across the inner mitochondrial membrane by an electron transport chain in which electrons are passed through a series of proteins via oxidation-reduction reactions. The last acceptor of electron in this chain is an oxygen molecule which gives rise to superoxide anion ($O_2^{\cdot}-$).

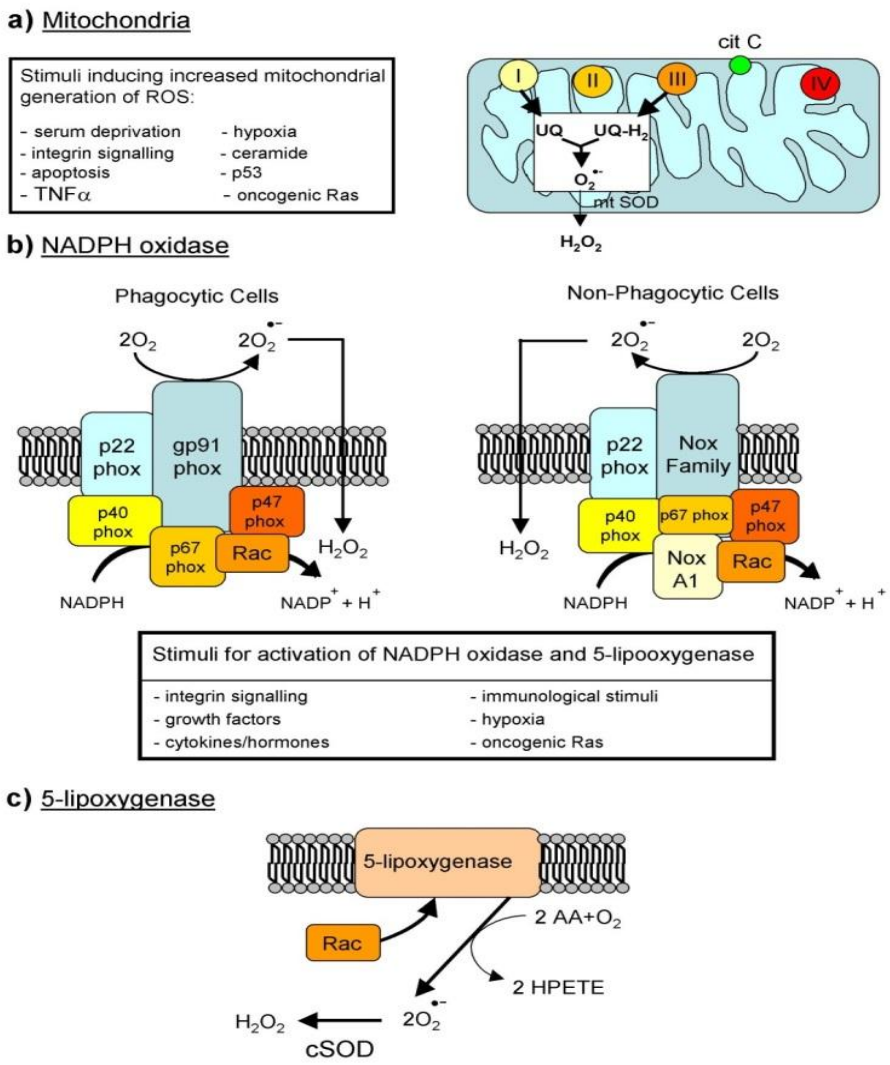


Figure 7: Major sources of ROS production in living cells. This figure summarises the major cellular cities of ROS production, in which it shows the stimuli that induce and activate the corresponding cite to produce ROS. a) Mitochondria. b) NADPH oxidase system. And c) Lipoxygenese. (Modified).

Erica Novo and Marizio Parola Fibrogenesis Tissue Repair. October ,2008.

Mitochondrial $O_2 \cdot -$ generation can then lead to the production of ROS such as hydrogen peroxide (H_2O_2), which can, in the presence of ferrous iron form a highly reactive hydroxyl radical. When mitochondrial enzymatic and non-enzymatic antioxidant systems are overwhelmed by these ROS, oxidative damage and cell death can occur. Mitochondria use approximately the 85–90% of total oxygen, thus representing the major site of oxygen consumption as well as a primary and continuous source of cellular ROS.

3.2. Oxidative stress and ROS measurements

As a result of their unstability and high reactivity, ROS either donate or accept electrons from more stable molecules (lipids, proteins, nucleic acids and

carbohydrates are possible targets), which results in a radical chain reaction with the formation of new radicals and a state of oxidative stress in the tissues.

ROS are continuously detoxified by various cellular mechanisms. When an imbalance occurs between ROS production and detoxification, an oxidative stress status manifests which can somehow intensify the pathophysiological mechanisms in several diseases. Thus, during periods of stress, inflammation or infection, ROS level increases dramatically [60] and this may alter the cellular metabolism and cause significant damage to the cell structures. So, in a state of oxidative stress when the production of ROS is massive or the antioxidant defenses are deficient, damage may occur in a variety of tissues.

Unfortunately, direct *in vivo* quantification of ROS is difficult because of their short half-life; consequently, the evaluation of free radical production and the balance between pro-oxidant and anti-oxidant is based on the evaluation of markers of oxidation reactions, often in *in vitro* experiments. IOxidative stress can be assessed by measurement of oxidative damage reaction products, for example, lipid peroxidation, DNA oxidation, and protein oxidation. Another approach is based on the measurement of depletion of major antioxidants enzymes and substrate in the cells, such as ~x-tocopherol, vitamin C, and thiol groups.

3.3. NADPH oxidase

NADPH oxidase (NOX) is a membrane-bound enzyme initially found in the plasma membrane and in the membranes of phagosomes used by neutophils to kill micoorganisms via the generation of superoxide anion. NADPH oxidase was later fonud in most - if not all- mammalian cell types. NADPH oxidases constitute a family of multisubunit enzyme complexes. In most mammals, seven isoforms of NADPH oxidase have been described, each of them consisting of a core catalytic subunit (the so-called NADPH oxidase (NOX) or the dual oxidase (DUOX)) and up to five regulatory subunits (p22phox, p47phox, p40phox, p67phox, Rac1&2) (figure 8). Rodents are missing the NOX5 subunit.

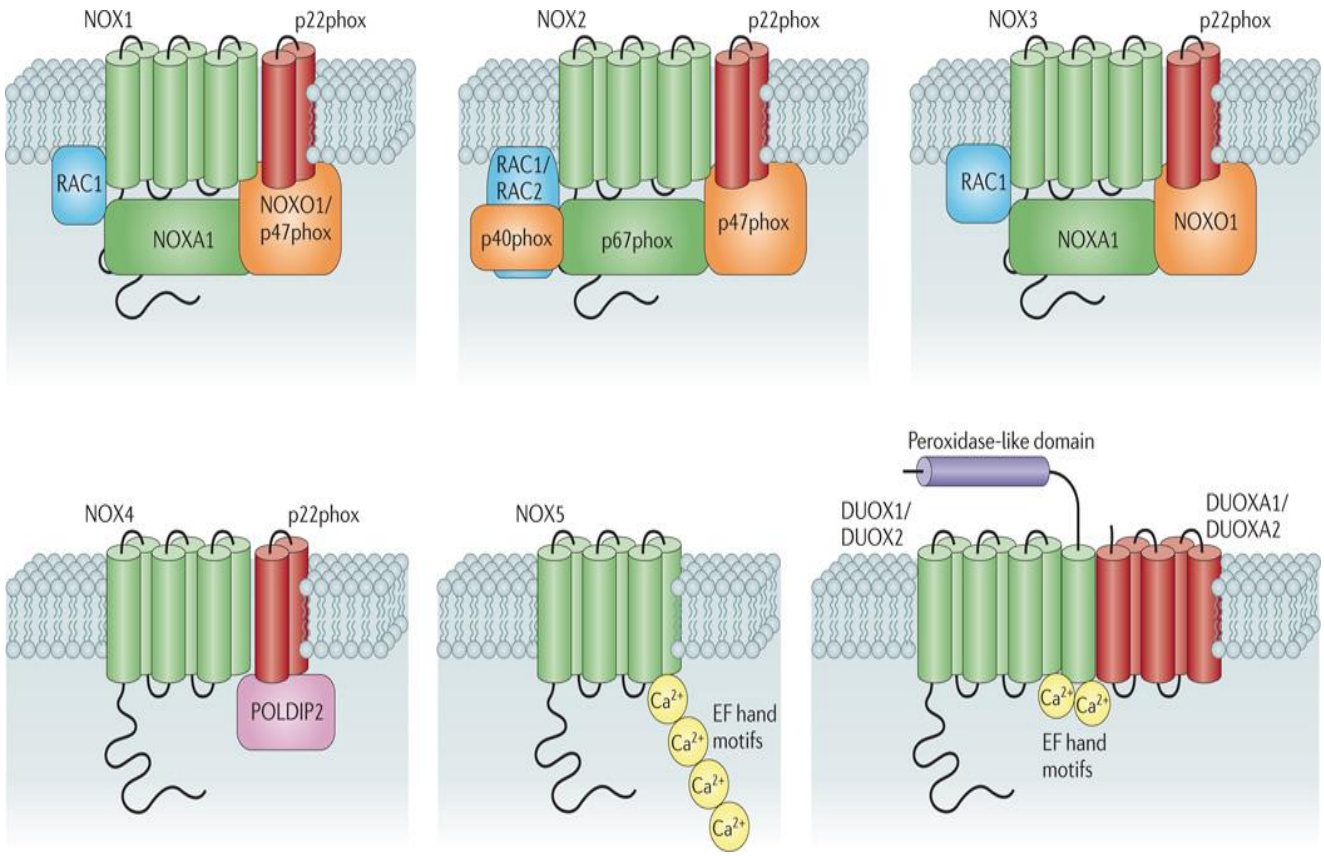


Figure (8) Subunit composition of the seven mammalian NADPH oxidase isoforms. The catalytic core subunits of the enzymes (NADPH oxidase 1 (NOX1)–NOX5, dual oxidase 1 (DUOX1) and DUOX2) are shown in green; NOX and DUOX maturation and stabilization partners (p22phox, DUOX activator 1 (DUOXA1) and DUOXA2) are shown in red; cytosolic organizers (p40phox, NOX organizer 1 (NOXO1) and p47phox) are shown in orange; cytosolic activators (p67phox and NOX activator 1 (NOXA1)) are shown in green; and small GTPases (RAC1 and RAC2) are shown in blue. Also shown (in pink) is polymerase δ-interacting protein 2 (POLDIP2), which is thought to regulate NOX4 activity and link production of reactive oxygen species by this isoform with cytoskeletal organization.
Modified: Grant R. Drummond, Stavros Selemidis, Kathy K. Griendling & Christopher G. Sobey
Nature Reviews Drug Discovery 10, 453-471 (June 2011)

With the exception of NOX4 which is constitutively active, NADPH oxidase activity is controlled by a complex regulatory system including the G-protein Rac [63].

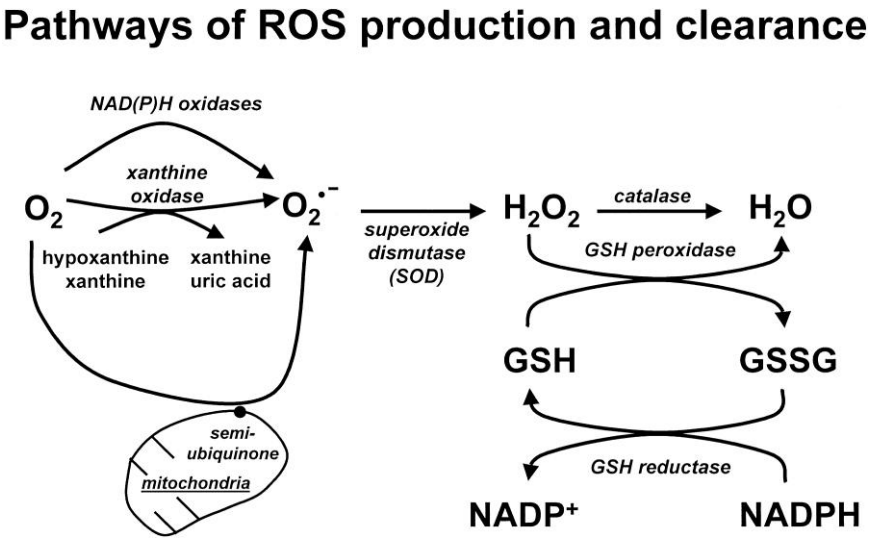
The active enzyme transfers electrons along an electron transport chain from NADPH to the final acceptor oxygen to produce superoxide (O_2^-). During this reaction, electrons are captured within the cytosol and superoxide is released at the other side of the membrane, either into the extracellular compartment or into organelles lumen. Conversely to other NADPH oxidases, NOX4 does not release superoxide but hydrogen peroxide (H_2O_2), normally produced from superoxide by superoxide dismutase [64]. NOX4 is highly expressed in kidney, particularly in the tubular system [65].

While several enzymes produce ROS, NADPH oxidase is the most significant one [61]. Overproduction of O_2^- and its downstream catabolism initiate an oxidative stress status which can inactivate critical metabolic enzymes.

3.4. Cellular mechanisms of defence against ROS

In normal healthy conditions, cells detoxify these oxygen radicals by reducing them to oxygen as shown in Figure 9 below. In particular, superoxide dismutases (SOD) are ubiquitous enzymes that catalyze the dismutation of O_2^- into O_2 and hydrogen peroxide (H_2O_2). Three forms of SOD are present in humans, SOD1 presents in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. Catalase, which is abundant in the peroxisomes located next to mitochondria, converts the resulting H_2O_2 into water and oxygen.

Another important mechanism for preventing the accumulation of ROS is to transfer the energy of the reactive peroxides to a small sulfur-containing protein (glutathione) through glutathione peroxidase (GPx). GPxs have several isozymes which encoded by different genes and vary in cellular location. So far 8 isoforms have been identified in humans (GPx1-8)[66]. Of these isoforms, GPx1 is the most abundant form and found in the cytoplasm of almost all mamalians, in which hydrogen perioxide is the preffered substrate.



Figur 9: Pathways of reactive oxygen species (ROS) production and clearance.GSH, glutathione; GSSG, glutathione disulfide.
Modified, Wulf Dröge. Physiol Rev, 82:47-95, 2002.

Oxidative stress occurs when there is excessive free radicals production, low anti-oxidant defence, or both.

3.5. ROS in nephrotic syndrome

ROS production and OS are suspected to be important and thought to be involved in the development of various diseases in humans including cancer [67], cardiovascular diseases [68, 69] and kidney disease [70]. Several lines of evidence indicate that chronic kidney disease (CKD) represents a pro-oxidant state including: 1) increased levels of oxidative markers in the plasma of patients [71, 72], and 2) decrease and defect in the antioxidant defence mechanisms in patients, which leads to a defect in the clearance of excessive ROS production [73].

In the past years, many studies explored the role of ROS in the development of kidney diseases, but several aspects remain unknown. For instance, the relative importance of each different type of ROS, the source of ROS and the exact link between this oxidative stress and the pathogenesis of the disease remain poorly defined.

In nephrotic syndrome, ROS seems to play a role in the etiology and the pathogenesis of nephrotic proteinuria. For instance, ROS was described as a possible mediator of glomelular injury that leads to proteinuria [74]. In addition, it was observed that O_2^- mediates an oxidative injury of the glomerular basement membrane and increases the glomerular permeability [75, 76]. Moreover, nephrotic patients show evidence of oxidative stress and impaired antioxidant defense in the acute phase of the disease [77]

The exact source of ROS and how it contributes to the pathogenesis of sodium retention and edema formation in NS are not well established. However, NS is associated with decreased serum albumin, and albumin is the main circulatory antioxidant [78, 79] due to its multiple ligand-binding capacities and its scavenging properties for free radicals [80]. The hypothesis of the antioxidant role of albumin is supported by the central role of oxidative stress in some pathology such as liver failure and sepsis [78, 81].

3.6. NF-κB and peritoneum

Nuclear factor-kappa B (NF-κB) is a transcription factor known to have pro-inflammatory, mitogenic and anti-apoptotic functions in many cell types [82], and it is thereby involved in several diseases. The NF-κB pathway is modulated in a cell-specific manner and interacts with other transcription pathways, providing a complex variety of cellular responses to its activation [83]. In mammals, there are five members of the transcription factor NF-κB family: RelA (p65), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively. NF-κB transcription factors bind as dimers to κB sites in promoters and enhancers of a variety of genes and induce or repress transcription[84]. NF-κB dimers are bound to inhibitory IκB proteins, which sequester NF-κB complexes in the cytoplasm. To stimulate NF-κB pathway, the degradation of IκB proteins is initiated through phosphorylation by the IκB kinase (IKK) complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO).

There are two main NF-κB-activating pathways in cells (figure 8). The canonical pathway (classical) is activated by most physiological NF-κB stimuli; for example, signals from cytokine receptors, such as the tumor necrosis factor receptor (TNFR) and interleukin 1 (IL-1) receptor (IL-1R), antigen receptors and pattern-recognition receptors, including Toll-like receptor 4 (TLR4). This pathway is dependent on IKKβ and leads mainly to the phosphorylation of IκBα and nuclear translocation mainly of p65-containing heterodimers.

In contrast, the non-canonical pathway depends on IKKα-mediated phosphorylation of p100 associated with RelB and leads to partial processing of p100 and the generation of p52-RelB complexes. Non-canonical signalling is induced by specific members of the TNF cytokine family, such as BAFF, CD40 ligand, and lymphotoxin-β2 [83].

After activation, NF-κB transcription factor promotes the expression of over 150 target genes, the majority of which participate in the host immune response, in the regulation of the acute phase of stress response such as the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

Nephrotic syndrome is associated with a high level of TNFα [85]. Therefore, we tested the possibility of the involvement of NF-κB pathway activation in nephrotic edema, and its dependence on oxidative stress.

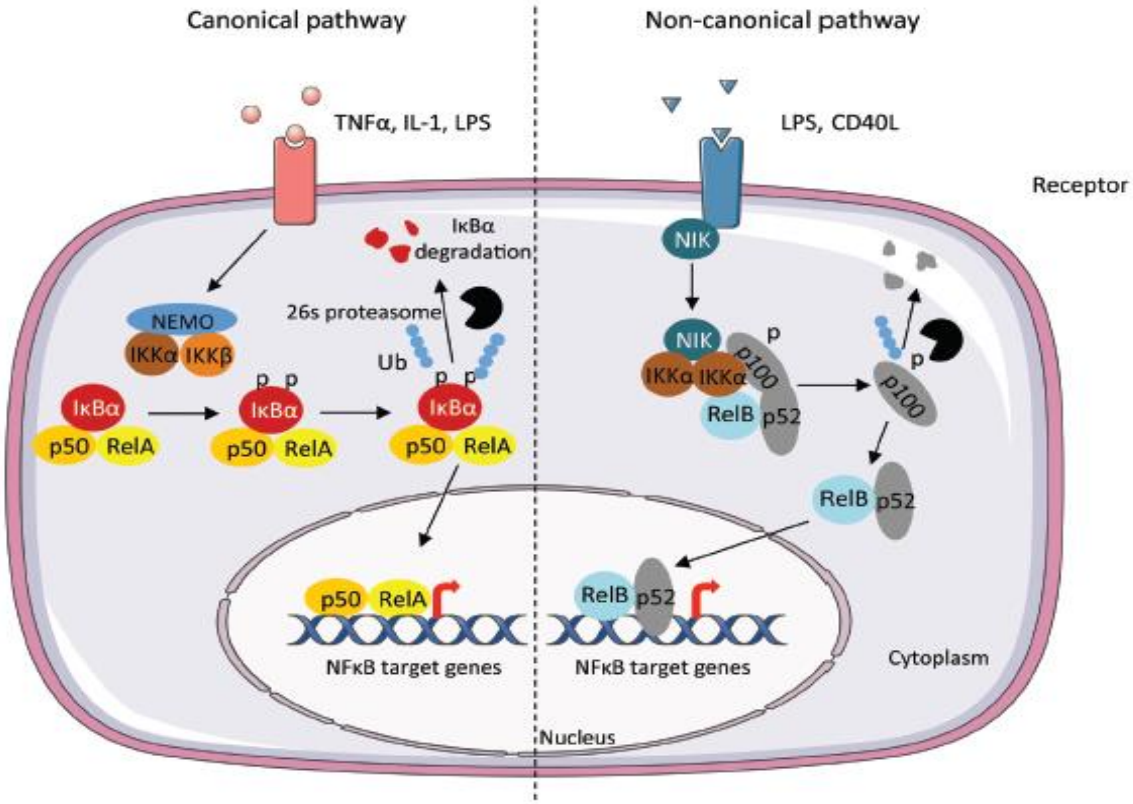


Figure 8: Scheme illustrates the canonical and non-canonical pathway leading to the activation of NF-κB. The canonical pathway is induced by cytokines such as Tumor Necrosis Factor (TNFα), Interleukin-1 (IL-1). The activation results in the phosphorylation of IκBα by the IKK complex, leading to its ubiquitylation (Ub) and subsequent degradation by the 26S proteasome. The RelA/p50 complex is free to translocate to the nucleus to activate the transcription of target genes. The non-canonical pathway results in the activation of IKKα by the NF-κB-inducing kinase (NIK) after stimulation by TNF cytokine family. The formation of the complex NIK-IKKα-p100 leads to the phosphorylation of the p100 subunit. This results in 26S proteasome dependent processing of p100 to p52, which can lead to the activation of p52-RelB that target distinct κB element and induce the transcription of target genes. (Modified)

Emilie Viennois, Fengyuan Chen, Didier Merlin . review. Transl Gastrointest Cancer, Vol 2, No 1 (January 2013)

3.7. Nrf2 Pathway

The transcription factor NF-E2 p45-related factor 2 (Nrf2) has a crucial role in maintaining the cellular redox homeostasis by regulating the gene expression of various cytoprotective proteins, including antioxidants and detoxifying enzymes. It regulates the biosynthesis and regeneration of glutathione, thioredoxin and NADPH; control the production of ROS by mitochondria and NADPH oxidase.

Nrf2 itself is controlled primarily at the level of protein stability. At basal levels, Nrf2 has a short half-life of about 20 minutes, and it is subjected to continuous ubiquitination and proteasomal degradation. There are three known ubiquitin ligase systems which contribute to the degradation of Nrf2. In which, Kelch-like ECH associated protein 1 (Keap1) is the first negative regulator of Nrf2 to be discovered [86]. Inducers that block the cycle of Keap-1 mediated degradation of Nrf2 result in the accumulation of the transcription factor, which translates after to the nucleus. Nrf2 binds to antioxidant response elements (AREs), the upstream regulatory regions of its target genes, and initiates transcription of cytoprotective proteins[87].

Nrf2 directly regulates the expression of both the catalytic and the regulatory subunits of γ-glutamyl cysteine ligase (GCL), the rate-limiting enzyme in the biosynthesis of reduced glutathione (GSH)[88]. In addition to its rule on GSH biosynthesisn, Nrf2 provides a mean to maintain the glutathione in its reduced form by the coordinate transcriptional regulation of glutathione reductase 1 (GSR1)[89]. Nrf2 also regulates the gene expression of thioredoxin (TXN), thioredoxin reductase 1 (TXNR1) [90] which are essential for reduction of oxidized protein thiols.

Cells is which Nrf2 has been disrupted showed higher levels of ROS compared to WT cells[91]. Moreover, Nrf2 is also critical for the maintenance of the mitochondrial redox homeostasis. Thus, compared to WT, the total mitochondrial NADH pool is significantly increased in Keap1-KO and dramatically decreased in Nrf2-KO cells[91].

4. Objectives

The aim of our study is to provide further insight in the role of OS and ROS in sodium retention and ascites formation in nephrotic syndrome. Specifically, we addressed the following questions:

- Does luminal endocytosis of albumin induce OS in the CCD from nephrotic rats?
- Is OS in CCD responsible for sodium retention and how does it control the activity of ENaC and Na,K-ATPase?
- Is there OS in the peritoneum of nephrotic rats
- Is peritoneal OS responsible for the formation of ascites, and how does it control the water permeability of the peritoneum filtration barrier
- What ROS-induce pathway mediates change in peritoneal permeability
- Are ROS a possible therapeutic target for the treatment of nephrotic Na^+ retention and edema?

Results

1. Pathophysiology of nephrotic ascites: Role of ROS and NF-κB

1.1. Introduction

The production of edema and ascites in the nephrotic syndrome was traditionally thought to result from the leakage of intravascular fluid towards the insterstitium brought about by the decrease in plasma oncotic pressure due to hypoalbuminemia. However, it is now proposed that expansion of the insterstitium volume is also accounted for by changes in the intrinsic properties of the vascular endothelium, including increased water permeability and decreased reflection coefficient to proteins. However, the mechanism responsible for these changes is not characterized yet. This first study aimed at characterizing changes in the intrinsic properties of the peritoneal filtration barrier and at determining their pathogenesis. We hypothesized that the changes in water permeability and reflection coefficient for protein stem from oxidative stress.

For these purposes, we developed an original dialysis method that allowed us to evaluate the permeability and reflection coefficient of the peritoneum of control and PAN nephrotic rats, and we tested the effect of a ROS scavenger, N-acetylcysteine (NAC), on these parameters. The peritoneal filtration coefficient (L_p x S) was estimated by the slope of the regression line connecting the osmotic pressure gradient (which we induce experimentally) to the water flux (measured by osmotic dilution). These determinations were done either in the absence or the presence of $HgCl_2$, an inhibitor of aquaporin, to evaluate the involvement of the transcellular and paracellular water transport pathways. We also evaluated ROS production and accumulation in the peritoneum and their role in the induction of ascites using NAC. Finally, we tried to characterize the molecular mechanism and signaling pathway by which ROS could initiate the edema in nephrotic syndrome.

First we found a two-fold increase in mannitol-induced water flux across the peritoneum of PAN rats compared to control rats, and this increase was associated with increase in the water filtration coefficient of the paracellular and transcellular pathways, and a decrease in the reflection coefficient to proteins. The increase in transcellular water flux in the peritoneum was associated with increased expression of aquaporin 1 (AQP1). Moreover, we found that the peritoneum of PAN rats displayed a significant oxidative stress, as revealed by dihydroethedium staining and

dysregulation of mRNA expression of NADPH oxidase and ROS detoxifying enzymes.

We found also that NAC treatment decreased the volume of ascites by >60%. This decrease in ascites volume was not associated with an increase in the blood albumin level or with a change in the albuminuria. In addition NAC treatment normalized to control levels the filtration coefficients of the transcellular and paracellular pathways, the reflection coefficient to proteins and the expression of AQP1.

In these experiments we also found an activation of NF-κB in the peritoneum of PAN rats attested by increased p50 nuclear labeling and mRNA expression of NF-κB target genes (RANTES and TNFα mRNAs). Activation of NF-κB was abolished in NAC-treated PAN rats. To evaluate the functional role of NF-κB, we treated the PAN nephrotic rats with the NF-κB inhibitor JSH-23. JSH-23 treatment mimicked the effects of NAC: the ascites volume decreased significantly, and the increase in water transport and the overexpression of AQP1 were blunted.

In summary, our findings show that:

- In the peritoneum of nephrotic PAN rats there is an increase in water permeability and decrease in the reflection coefficient to proteins which account for two-third of nephrotic ascites.
- Increased water permeability results from alterations of the paracellular and transcellular pathway, and the later stems from over-expression of aquaporin 1.
- Increased water permeability and over-expression of aquaporin 1 are mediated by oxidative stress-induced activation of NF-kB.
- It is concluded that changes in the intrinsic properties of the peritoneal permeability barrier are more important than changes in the driving force for water leakage out of the capillaries in the constitution of nephrotic ascites.

1.2. Article #1

Reactive oxygen species and NFκB increase peritoneal filtration and contribute to ascites formation in nephrotic syndrome

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Running headline: Increased peritoneum water transport in nephrotic syndrome
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Abstract

Clinical and experimental observations suggest that nephrotic ascites does not solely stem from hypoalbuminemia but also from changes in peritoneal permeability. We investigated the mechanisms underlying these changes in rats with puromycin aminonucleoside (PAN)-induced nephrotic syndrome. The peritoneum of PAN rats displayed an increase in the water filtration coefficient of the paracellular and transcellular pathways, and a decrease in the reflection coefficient to proteins. The transcellular water permeability increase was associated with over-expression of aquaporin 1 (AQP1). The peritoneum of PAN rats displayed oxidative stress, as attested by the accumulation of ROS and the dysregulation of mRNA expression of NADPH oxidase and ROS detoxifying enzymes. Administration of the ROS scavenger N-acetylcysteine (NAC) prevented the changes in water permeability, reflection coefficient, and AQP1 expression, and reduced the volume of ascites by 60% without changing albuminuria and hypoalbuminemia. The peritoneum of PAN rats also displayed activation of NF-κB, as attested by increased p50 nuclear labeling and expression of RANTES and TNFα mRNAs. Activation of NF-κB was blunted in NAC-treated PAN rats. Administration of NF-κB inhibitor JSH-23 mimicked the effects of NAC. It is concluded that nephrotic syndrome is associated with an increased filtration coefficient of the peritoneum, accounted in part by increased expression of AQP1, and a decreased reflection coefficient to proteins. These changes are triggered by oxidative stress and subsequent activation of NF-κB and account for over half of ascites.

Keywords: nephrotic syndrome, ascites, albuminuria, peritoneum, water permeability, aquaporin 1, reactive oxygen species, NF-κB.

Introduction

Nephrotic syndrome (NS) is a multifactorial glomerular disease defined by massive proteinuria and hypoalbuminemia. Irrespective of its etiology, NS is commonly associated with a renal retention of sodium leading to extracellular volume expansion and to the generation of ascites and/or edema (Broyer et al., 1998; Cameron, 1998). Nephrotic edema ranges from puffiness and pitting edema to massive ascites (up to 30% of body weight) with possible pleural and cardiac effusion. It is a serious symptom which constrains the functional activity of patients.

Classically, this disabling expansion of interstitial volume was thought to result from hypoalbuminemia, a decreased oncotic gradient across capillary walls and increased fluid leakage towards the interstitium. In turn, the resulting hypovolemia stimulates the renin/angiotensin/aldosterone system (RAAS) and promotes renal sodium retention (Epstein, 1952). During the past decade, experimental and clinical observations have ruled out this so-called underfill theory, and it is now admitted that the renal retention of sodium is a primary event that is independent of RAAS activation (Deschenes et al., 2003). This conclusion is in agreement with clinical observations showing that most patients with idiopathic NS display normal volemia and plasma aldosterone levels (Geers et al., 1984; Vande Walle et al., 1995; Vande Walle et al., 1996). It also agrees with the observation that in rats with unilateral NS, only the proteinuric kidney retains sodium (Ichikawa et al., 1983), ruling out the involvement of a hormonal factor.

Sodium retention in nephrotic patients is associated with edema and ascites rather than hypertension, indicating an asymmetry of expansion of extracellular compartments. This asymmetry suggests an increased fluid flux across the capillary endothelium and peritoneum, and accordingly the capillary filtration capacity was shown to be increased 2-fold in nephrotic patients (Lewis et al., 1998). According to Starling's principle, water flux is given by the product of the filtration coefficient of the barrier and the driving force (determined by the hydrostatic and oncotic pressure gradients and the reflection coefficient of the barrier to proteins) across the barrier, i.e. intrinsic properties of the barrier and extrinsic factors. As recalled above, until now fluid leakage towards the interstitial compartment was mainly attributed to a decreased oncotic gradient, although many observations militated against this conclusion. Namely, 1) the transcapillary gradient of oncotic pressure is almost unchanged in nephrotic patients (Fauchald et al., 1985; Koomans et al., 1985) 2)

during steroid-induced remission of nephrotic syndrome, natriuresis resumes and edema decreases before normalization of serum albumin levels (Oliver, 1963), and 3) diuretic treatments reduce edema without significantly changing the gradient of oncotic pressure (Koomans et al., 1985). It is therefore now admitted that the asymmetry of volume expansion in NS results from alterations of the intrinsic properties of the capillary endothelium: an increased filtration coefficient and a decreased reflection coefficient to proteins. However, neither the molecular basis of these capillary alterations nor their connection to proteinuria and/or hypoalbuminemia is fully elucidated. The first aim of this study was to characterize the molecular alterations accounting for the increased permeability coefficient of the peritoneum of nephrotic rats.

Several studies have highlighted the pathophysiological importance of reactive oxygen species (ROS) in nephrotic patients (Ece et al., 2005; Granqvist et al., 2010; Mishra et al., 2011), and ROS induce endothelial barrier dysfunction (Boueiz and Hassoun, 2009) that leads to tissue edema in the context of inflammation or ischemia (Lucas et al., 2009). The second aim was to determine the role of oxidative stress and ROS in the peritoneal dysfunctions leading to ascites formation in NS.

Material and methods

Ethics approval. All animal experimental protocols were approved by the Charles Darwin Ethic Committee (#Ce5/2012/041) and performed according to the French legislation for animal experimentation.

Animals. Male Sprague–Dawley rats (Charles Rivers, L'Abresles, France) weighing 150–170 g at the onset of the study were fed a standard laboratory chow (A04, Safe, Augy, France) with free access to water. Rats were anaesthetized by intraperitoneal injection of a mix containing Xylazine (Bayer health care, 13.2 mg/kg body wt) and ketamine (Vibrac France, 86 mg/kg body wt). NS was induced by intrajugular injection of puromycin aminonucleoside (PAN) (Sigma-Aldrich, 150mg/kg body wt). The antioxidant N-acetyl cysteine (NAC) and the inhibitor of NF-κB transcriptional activity JSH-23 (Symansis) were given by daily gavage (NAC, 0.5g/kg body wt (Fiordaliso et al., 2004); JSH-23, 3mg/kg body wt, (Kumar et al.)) starting the day before PAN injection. Control rats were treated with either NAC or JSH-23 or water using the same protocol.

Rats were sacrificed at day 6 post PAN injection, when sodium retention, proteinuria, and ascites are maximal (Deschenes and Doucet, 2000). The greater omentum was immediately dry-frozen in liquid nitrogen and stored at -80°C. Pieces of liver and abdominal muscle samples were rapidly immersed in OCT, frozen in liquid nitrogen and stored at -80°C. Blood was centrifuged and plasma was separated and stored at -20°C.

Metabolic studies. Animals were housed in individual metabolic cages with free access to food and water, starting 3 days before the onset of experimentation for acclimatization. Food and water consumption and body weight were measured daily and 24 h urine was collected. Urine creatinine and protein concentrations and plasma protein concentrations were measured in an automatic analyzer (Konelab, Thermo, France). Urinary excretion of protein was expressed as a function of creatinine excretion. The amount of ascites was measured by moistening and weighing an absorbent paper.

Peritoneal transport of fluid. After anesthesia, animals were placed on a heating table and their body temperature was monitored at 37°C. A customized short catheter was introduced into the peritoneal cavity, through the abdominal wall, and firmly attached to the abdominal muscle to avoid leakage. When present, the ascites was drained via the catheter. Four ml of saline solutions with different osmotic

pressures (see composition below), equilibrated at 37°C, were rapidly infused in the peritoneal cavity, the stomach of animals was gently massaged, and aliquots of ≈200 µl were taken at various times after infusion. The peritoneal cavity was successively infused with solutions of increasing osmolarity (from 340 to 400 mOsm/l, with 20 mOsm/l steps). Between each dwell, the peritoneum was drained, rinsed with the forthcoming solution and drained anew. To assess the role of aquaporins on water fluxes, in some animals we performed two successive series of measurements, before and after administration of the aquaporin inhibitor mercury chloride. In these experiments, we tested only three solutions of increasing osmolarity (from 340 to 380 mOsm/l, with 20 mOsm/l steps). After the first three dwells in absence of mercury chloride, 10 ml of saline containing 0.1 mM $HgCl_2$ were infused in the peritoneum and drained after 10 min. Thereafter, the peritoneum was rinsed with the 340 mOsm/l solution and the three measurements with solutions of increasing osmolarities containing 0.1 mM $HgCl_2$ were performed. Blood samples were collected before and at the end of the experiment.

The osmolarity of the solutions and plasma was determined by osmometry (Micro-osmometre digital, Roebling). The water flux (J_v in ml/min) across the peritoneum was calculated based upon the osmotic dilution of the infused solution.

$$J_v = V_i\,[(Osm_i/Osm_c) - 1]/t$$

where V_i is the volume of solution injected (in ml), Osm_i and Osm_c are the osmolarities of the injected and collected solutions, and t is the time between infusion and collection.

The infused solution contained (in mM): 120 NaCl, 25 $NaHCO_3$, 4 KCl, 1 $CaCl_2$, 1 glucose, and mannitol up to the required osmotic pressure, at pH 7.4. The osmotic gradient across the peritoneum was calculated as the difference between the osmotic pressure of the infused solution and that of the plasma. Osmolarities (Osm in mOsm/l) were converted to osmotic pressure (Π_{osmo} in mmHg) using the formula

$$\Pi_{osm} = Osm \times 10^{-3} \times R \times T$$

where R and T are the ideal gas constant and the absolute temperature, respectively. At 37°C, RT was taken as 18.583×10^3 l .mmHg/mol.

Plasma protein concentration was determined on an automated analyzer (Konelab, Thermo, France) and the oncotic pressure was calculated according to the Landis-Pappenheimer equation for plasma proteins (Nitta et al., 1981).

Calculation of filtration coefficients and reflection coefficient to proteins. The peritoneal filtration barrier is a complex structure made up of three layers: the capillary endothelium, the interstitium, and the mesothelium that lines the entire surface of the peritoneum (Flessner, 2005). Functionally, it is modelled as a three-pore system consisting of water-specific ultra-small pores, water- and small solute-permeable small pores and water- and macromolecule-permeable large pores (Devuyst and Rippe, 2013). The ultra-small pores are accounted for by the mercury-sensitive aquaporin 1 (AQP1), which is expressed in both endothelial and mesothelial cells (Ota et al., 2002; Devuyst and Ni, 2006), and represent the only transcellular pathway for water filtration. The water permeability of the paracellular pathway is determined by the tightness of adherens and tight junctions (Bazzoni, 2006), but the molecular counterpart of small and large pores remains mostly unknown. In this study, the peritoneal barrier was considered as a simple, uniform structure with two water pathways: a $HgCl_2$-sensitive pathway accounted for by AQP1 and a $HgCl_2$-insensitive pathway corresponding to the large and small pores. According to this model and to Starling's principle, the water flux across the peritoneal barrier (J_v) can be estimated as:

where K_1 and K_2 are the filtration coefficients (the product of the hydraulic conductance L_p and the filtration surface area S) of the $HgCl_2$-sensitive and -insensitive pathways, respectively, ΔP, $\Delta\Pi_{onco}$, and $\Delta\Pi_{osmo}$, are the gradients of hydrostatic, oncotic, and osmotic pressure across the barrier, respectively, and σ_{pl} and σ_{man} are the reflection coefficients of the barrier to proteins and mannitol, respectively. Note that these two reflection coefficients characterize the large- and small-pore pathway; the reflection coefficients corresponding to the AQP1 pathway are implicitly taken as unity (water-specific pathway).

The slopes of the regression lines connecting J_v to $\Delta\Pi_{osmo}$ in the absence and presence of $HgCl_2$ (S_1 and S_2, respectively) are:

(Eq 1)

(Eq 2)

The x-axis intercepts of the regression lines (J_v = 0) determined under the same conditions (I_1 and I_2, respectively) are:

(Eq 3)

(Eq 4)

This system of four equations cannot be solved to determine the 4 unknown variables (K_1 and K_2, σ_{pl} and σ_{man}) because these 4 equations are not independent. However, if we fix the value of σ_{man}, we can then calculate the three other variables as:

$\Delta\Pi_{onco}$ was determined as described above. Because we did not find estimates of ΔP across the rat peritoneum, we used values derived from human measurements, i.e. 17.6 mmHg and 0.9 mmHg for capillary and peritoneal pressure, respectively (Lewis et al., 1998; Rusthoven et al., 2005), yielding a ΔP of 16.7 mmHg. Importantly the capillary hydrostatic pressure does not change in nephrotic patients (Lewis et al., 1998) and putative changes in peritoneal hydrostatic pressure associated with ascites have no major effect on ΔP (Rippe, 2006). σ_{man} was taken as 0.02 (Waniewski et al., 2009). K_1, K_2 and σ_{pl} were determined in each animal using the values of ΔP, $\Delta\Pi_{onco}$, and σ_{man}, and the results shown below are the means from different animals.

DHE staining. Dihydroethidium (DHE) is a dye which, upon oxidation by superoxide to ethidium, enters the cell nucleus where it binds with DNA and exhibits a red fluorescence (excitation, 488 nm; emission, 610 nm) (Mu et al., 2008). DHE was injected intravenously (5mg/Kg rat) 2 hours before sacrifice. Thereafter, pieces of liver and abdominal muscle were collected, frozen in OCT and kept at -80°C until use. Five µm cryosections were transferred to Superfrost Gold+ glass slides, rinsed twice with PBS. Slides were mounted and observed on a fluorescence microscope (×40, Zeiss observer Z1, LSM710).

Immunohistochemistry. Five µm cryosections of liver and abdominal muscle were fixed with acetone, washed 3 times with PBS, permeabilized with Triton (Triton x100, 0.1% in PBS) for 10 minutes at room temperature, washed 3 times with PBS, blocked

for 10 minutes with 2% BSA in PBS, incubated for 2 hours at 37°C with anti-aquaporin 1 (AQP1) antibody (Santa Cruz, sc-20810, 1/200) or an antibody against the p50 subunit of NF-κB (Santa Cruz, sc-114, 1/1000), rinsed and incubated for 1 h with a FITC-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA, 1/500), and observed on a fluorescence microscope (Zeiss observer Z1, LSM710, x40).

RNA extraction and RT-PCR. RNAs were extracted from the greater omentum using Tri Reagent solution, Dnase I-treated, and purified on columns according to the manufacturer's instruction (Qiagen, Hilden, Germany). Reverse transcription was performed using a first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics), according to the manufacturer's protocol. Real-time PCR was performed using a LightCycler 480 SYBR Green I Master qPCR kit (Roche Diagnostics) according to the manufacturer's protocol. Specific primers (table 1) were designed using ProbeDesign (Roche Diagnostics).

Statistical analysis*.* Results were expressed as means ± SE from several animals. Comparison between groups was performed by unpaired Student's *t* test or by variance analysis, when comparing several groups together; p values <0.05 were considered significant.

Results

Validation of the method for water flux measurements. Injection of a hyper-osmotic solution into the peritoneal cavity primarily induces a water flux from the capillaries towards the peritoneal cavity. This water flux decreases with time mainly because it dilutes the intra-peritoneal fluid and thereby reduces the driving force. It is therefore important to determine the osmotically-driven water flux under initial rate conditions. For this purpose, we evaluated the early kinetics of changes in the peritoneal fluid osmolarity following the injection of a solution with the highest osmolarity used in this study, i.e. 400 mOsm/l. Results (Figure 1A) show that the osmolarity of the collected fluid decreased linearly as a function of time up to 30 seconds after injection (R^2 = 0.9965). We therefore decided to collect the peritoneal fluid 20 sec after injection to determine the water flux (J_v) under initial-rate conditions.

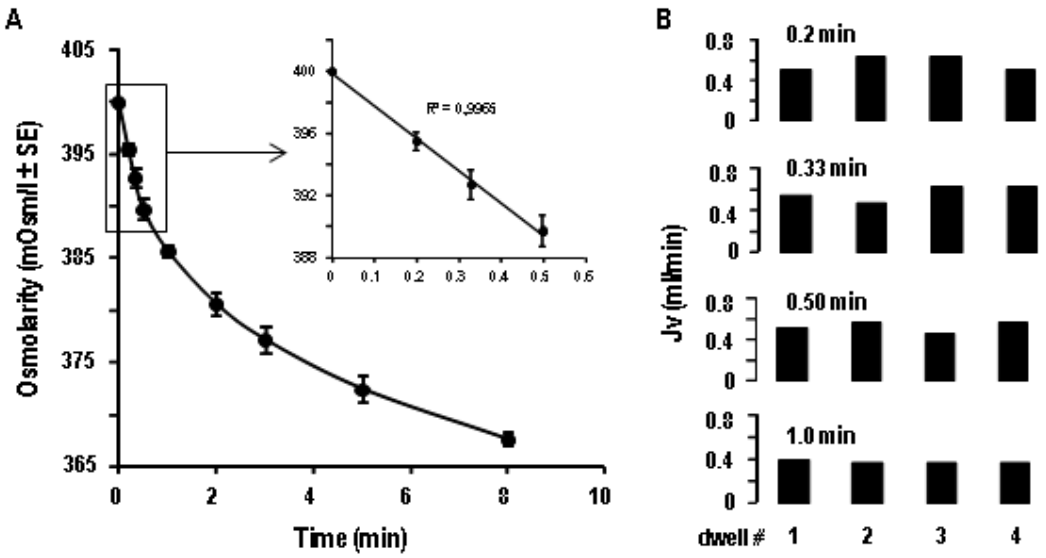

Figure 1. Kinetics of osmotically driven water flux across the peritoneum. **A.** After infusion of 10 ml of a hyper osmotic solution (400 mOsm/l) into the peritoneal cavity at time 0, 200 µl aliquots were withdrawn at various times (from 0.2 to 8 min) and their osmotic pressure was determined. Values are means from 4 experiments successively carried out on the same animal. The inset focuses on the first 30 sec of the experiment. **B.** Water flux calculated from samples collected after 0.2, 0.33, 0.5 and 1.0 min during the four successive dwells.

The technique developed to measure water fluxes exhibits two potential flaws that may lead to the underestimation of the effective osmotic gradient: 1) mannitol, used to generate the osmotic gradient, is a small solute that may leak towards the capillary compartment via the small and large pores, and 2) incomplete draining of the peritoneal fluid at the end of dwell n may dilute the fluid infused during dwell n+1. However, the impact of mannitol diffusion was limited by the short duration (20 sec) of the successive dwells. Furthermore, even when the dwell time was 8 minutes, as in the previous experiment, we found no systematic decrease in J_v throughout the four successive dwells (Figure 1B), attesting that the osmotic gradient across the peritoneal barrier did not decrease significantly after >30 min of exposure to a hyper-osmotic peritoneal fluid. The residual volume of peritoneal fluid remaining after drainage of dwell n (always less than 0.25 ml) would decrease the effective osmolarity of peritoneal fluid during dwell n+1 by approximately 1 mOsm/l. To limit this uncontrolled drift, the peritoneal cavity was rinsed with the n+1 solution between dwells n and the n+1. Under such conditions, the calculated drift in osmolarity was less than 0.05 mOsm/l and was thus neglected in the calculation of J_v.

Water flux and AQP1 expression in the peritoneum of nephrotic rats.

In both control and PAN rats, J_v increased linearly as a function of the gradient of osmotic pressure $\Delta\Pi_{osmo}$ (R^2 = 0.7944 and 0.8539 in control and PAN rats, respectively), but the regression line was much steeper in PAN rats than in controls (in µl/min/mmHg; Control: 0.26; PAN: 0.64; $p<0.001$) (Figure 2A). The increased water flux in PAN rats was associated with a 3-fold increase in the expression of the water channel AQP1 mRNA in the greater omentum (Figure 2B) and increased AQP1 immunolabeling of visceral and parietal peritoneum, mainly in the mesothelium layer (Figure 2C).

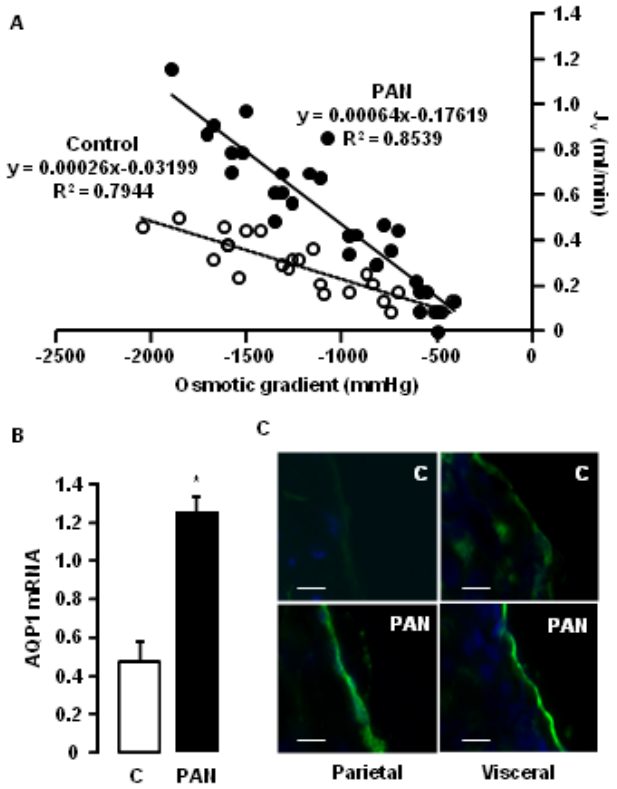

Figure 2. Water flux and AQP1 expression in the peritoneum of PAN rats. **A.** Water flux across the peritoneum as a function of the osmotic gradient. Values are individual measurements in 8 control and 9 PAN rats (3-4 conditions per animal). *, p<0.01; **, p<0.001. **B.** mRNA expression of AQP1 in the greater omentum of control and PAN rats. Values were normalized as a function of Rps23 expression and data are means ± SE from 6 control and 5 PAN rats. *, p<0.01. **C.** Immuno-labelling with anti-AQP1 antibody (green) of parietal (lining the abdominal muscle) and visceral peritoneum (lining the liver) from control or PAN rats. Scale bars: 10µm.

To determine the contribution of AQP1 over-expression to the increased water flux, we carried additional experiments in which we measured water fluxes before and after inhibition of AQP1 via intraperitoneal administration of $HgCl_2$; we then calculated the filtration coefficients through the mercury-sensitive and -insensitive pathways (K_1 and K_2, respectively) and the reflection coefficient of the filtration barrier to proteins (σ_{pl}). Assuming a reflection coefficient to mannitol of 0.02 (Waniewski et al., 2009), a mean gradient of hydraulic pressure of 16.7 mmHg (see methods), and mean gradients of oncotic pressure of 15.6 and 12.7 mmHg in control and PAN rats respectively (as calculated from measured plasma protein concentrations), results showed that 1) the $HgCl_2$-sensitive pathway accounts for less than 3% of the total filtration coefficient, 2) K_1 and K_2 increased 2.9-fold and 1.5-fold respectively in PAN rats and 3) σ_{pl} decreased by ~90% in PAN rats (Table 2). In view of this decrease in σ_{pl}, we postulated that σ_{man} might also decrease in PAN rats and recalculated the different parameters with a σ_{man} of 0.01 instead of 0.02. When comparing controls (with a σ_{man} of 0.02) with PAN rats (with a σ_{man} of 0.01), results indicate that both K_1

and K_2 increased ~3-fold in PAN rats whereas σ_{pl} did not change significantly (Table 2).

Role of ROS.

RT-qPCR analysis revealed a decreased expression of the superoxide detoxifying enzymes catalase and superoxide dismutase (although not statistically significantly for the latter) in the greater omentum from PAN rats and over-expression of NADPH oxidase subunits (Figure 3A). This suggests increased ROS levels in PAN rats. This was confirmed by histochemistry which showed weak but detectable DHE labeling in the parietal and visceral peritoneum of PAN rats but not in that of control rats (Figure 3B).

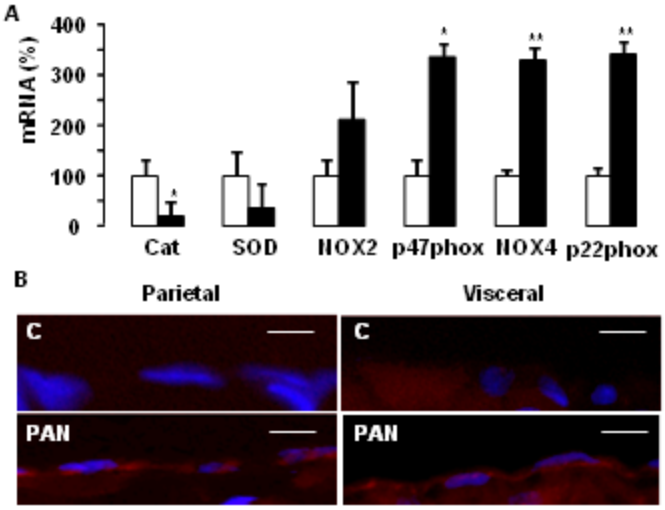

Figure 3. Oxidative stress in peritoneum of PAN rats. **A**. mRNA expression of catalase (Cat), super oxide dismutase 3 (SOD), NADPH oxidase 2 and 4 (NOX2 and NOX4), p47phox and p22phox in the greater omentum from control (open bars) and PAN rats (black bars). Values were normalized by Rps23 expression and expressed as percent of controls. Data are means ± SE from 6 control and 5 PAN rats. *, $p<0.05$; **, $p<0.01$. **B**. Dihydroethidium labeling (red) of cryosections of abdominal muscle (parietal peritoneum) and liver (visceral peritoneum) from control and PAN rats. Scale bars: 5µm.

Because ROS scavengers reduce glomerular damage and proteinuria in nephrotic animals (Matsumura et al., 2006), we tested the effect of NAC treatment on the urinary excretion of proteins in PAN rats. NAC altered neither the kinetics of

proteinuria following PAN injection nor its intensity. Accordingly, it did not alter PAN-induced hypoalbuminemia (Figures 4A & 4B) indicating that the transcapillary oncotic gradient was similar in control PAN and NAC-treated PAN rats. However, the volume of ascites was markedly reduced in NAC-treated PAN rats (Figure 4D), suggesting an effect of ROS on the peritoneal permeability.

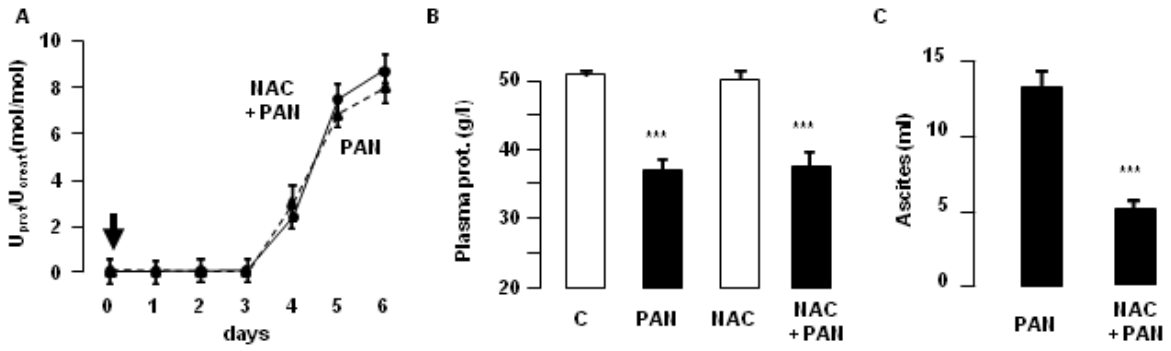

Figure 4. Effects of ROS scavenging on renal handling of proteins, proteinemia and ascites in PAN rats. **A**. Urinary excretion of proteins before (day 0) and 1-6 days after PAN injection (arrow) to control and NAC-treated rats. Protein excretion is expressed relative to creatinine excretion and data are means ± SE from 6 rats. **B**. Plasma protein concentration in the 4 groups of rats at day 6 after PAN or sham injection. Values are means ± SE from 6-8 rats. ***, $p<0.001$ as compared to the corresponding controls. **C**. Volume of ascites in control and PAN rats at day 6 post PAN injection. Values are means ± SE from 7-8 rats. **, $p<0.01$.

Accordingly, NAC treatment prevented the increase in J_v in the peritoneum of PAN rats (Figure 5A) and the over-expression of AQP1 (Figures 5B & 5C). Calculations showed that NAC treatment reversed the PAN-induced changes in K_1, K_2 and σ_{pl} (Table 2).

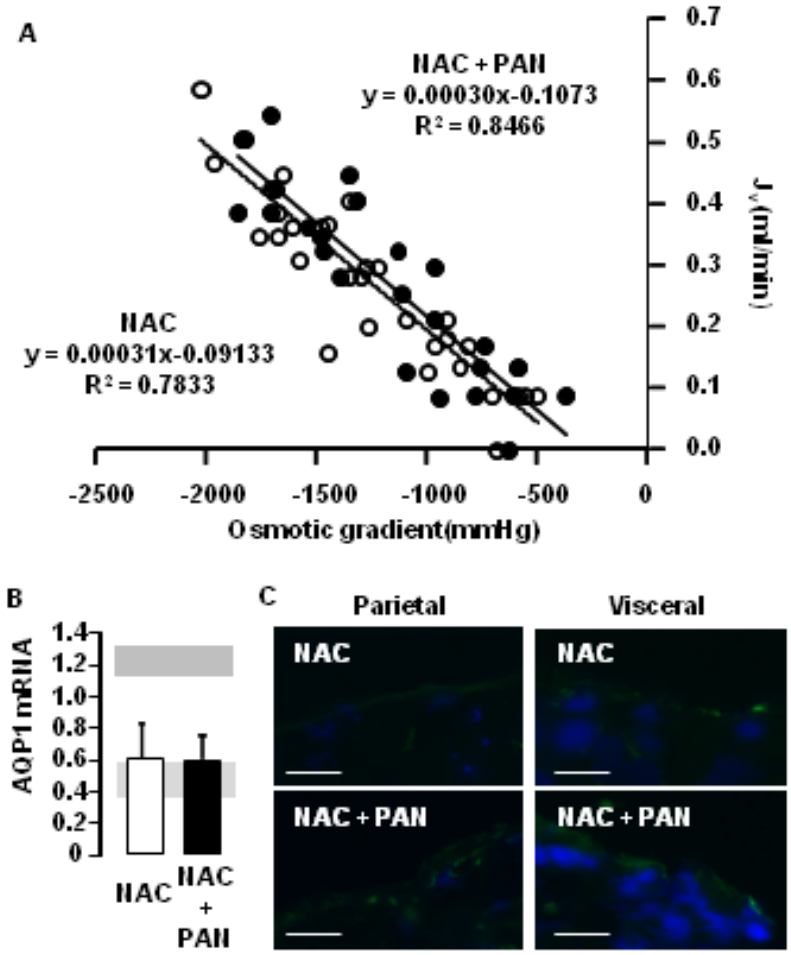

Figure 5. Effects ROS scavenging on filtration rate and AQP1 expression in the peritoneum of PAN animals. **A.** Water flux across the peritoneum of NAC-treated control and PAN rats as a function of the osmotic gradient. Values are individual measurements in 9 NAC and 6 NAC+PAN rats (3-4 measurements per rat). **B.** mRNA expression of AQP1 in the greater omentum of NAC-treated control and PAN rats. Values were normalized by Rps23 expression and data are means ± SE from 6 rats. Grey zones correspond to the mean ± SE domain of variation in NAC-untreated control (lower zone) and PAN rats (upper zone). **C.** AQP1 iImmuno-labelling (green) of parietal and visceral peritoneum from NAC-treated control and PAN rats. Scale bars: 10 µm.

Role of NF-κB pathway.

Because NF-κB is a target of ROS, we evaluated 1) whether NF-κB is activated in the peritoneum of nephrotic rats, 2) whether its activation is triggered by ROS and 3) whether it contributes to the increase in peritoneum permeability. Immunofluorescence revealed expression of the p50 active subunit of NF-κB in the parietal and visceral peritoneum of PAN rats but not in that of controls (Figure 6A).

RT-qPCR demonstrated over-expression of RANTES and TNFα, two targets of NF-κB, in the greater omentum (Figure 6B). The over-expression of both NF-κB and its targets was prevented in NAC-treated PAN rats (Figure 6A & 6B). Inhibition of NF-κB activity with JSH-23 (Figure 6A) prevented the over-expression of TNFα and RANTES in PAN rats (Figure 6B).

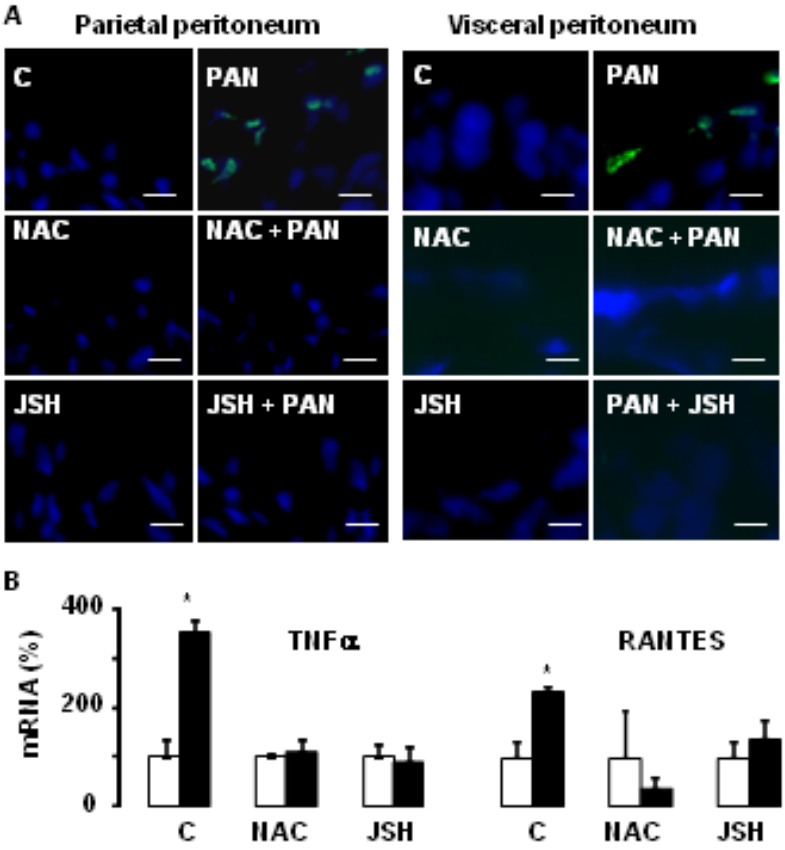

***Figure 6. Activation of NF-κB in the peritoneum of PAN rats*. A.** Immuno-labelling with anti NF-κB p50 subunit antibody (green) of parietal and visceral peritoneum from control and PAN rats. Rats were pre-treated or not with either NAC or JSH-23. Scale bars: 5 µm. **B.** mRNA expression of TNFα and RANTES in the greater omentum from untreated (C), NAC-treated or JSH-23-treated control (open bars) and PAN rats (black bars). Values were normalized by Rps23 expression and expressed as percent of controls. Data are means ± SE from 4-5 rats. *, $p<0.05$.

JSH23 also prevented the PAN-induced increases in J_v and in AQP1 expression in the peritoneum (Figure 7A & 7B). Accordingly, JSH-23 reduced the volume of ascites (Figure 7C) whereas it altered neither proteinuria nor hypoproteinemia (Figure 7D & 7E).

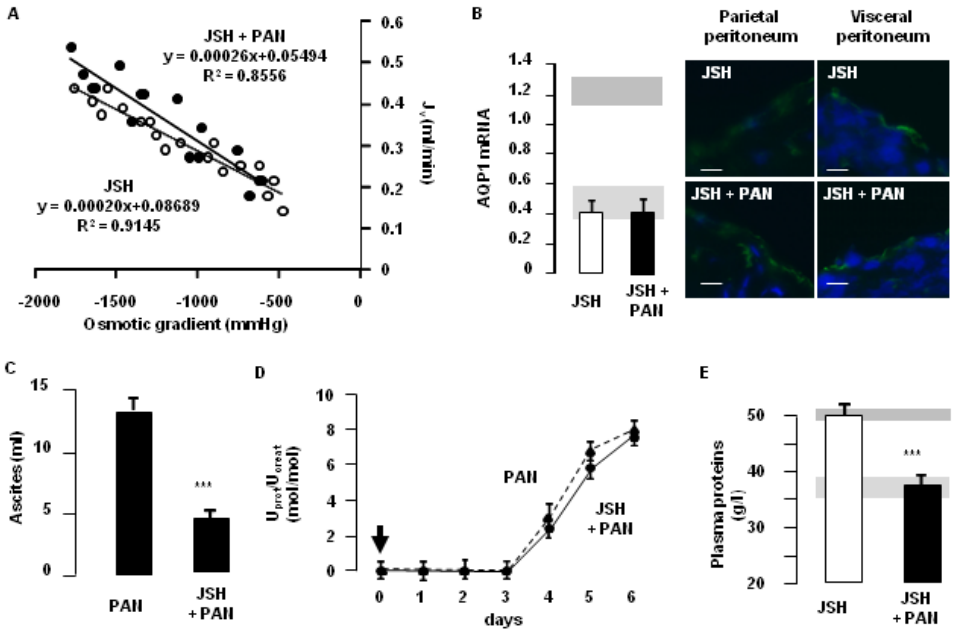

Figure 7. Effect of NF-κB inhibition on peritoneal filtration rate, protein homeostasis and ascites in PAN rats. **A.** Water flux across the peritoneum of control and PAN rats treated with the NF-κB inhibitor JSH-23 as a function of the osmotic gradients across the peritoneal fluid. Values are individual measurements in 6 rats per group (3-4 measurements per rat). **B.** Left. mRNA expression of AQP1 in the greater omentum of JSH-treated control and PAN rats. Values were normalized by Rps23 expression and data are means ± SE from 5 rats. Grey zones correspond to the mean ± SE domain of variation in JSH-untreated control (lower zone) and PAN rats (upper zone) (values from figure 1C). Right. AQP1 immuno-labelling (green) of parietal and visceral peritoneum from JSH-treated control and PAN rats. Scale bars: 5 µm. **C.** Volume of ascites in JSH-untreated and -treated PAN rats at day 6 post PAN injection. Values are means ± SE from 6-7 rats. ***, p<0.001. **D.** Urinary excretion of proteins before (day 0) and 1-6 days after PAN injection (arrow) in control and JSH-treated or -untreated PAN rats. Protein excretion is expressed relative to creatinine excretion and data are means ± SE from 6 rats. **E**. Plasma protein concentration in JSH-treated control and PAN rats as determined at the time of sacrifice, at day 6 after PAN or sham treatment. Values are means ± SE from 6 rats. Grey zones correspond to the mean ± SE domain of variation in untreated control (lower zone) and PAN rats (upper zone). ***, p<0.001.

Discussion

It has been previously reported that the peritoneum from nephrotic patients displays increased capillary filtration (Lewis et al., 1998). The present study confirms this finding in the PAN model of nephrotic syndrome and demonstrates that increased filtration capacity stems from ROS-induced activation of NF-κB. In addition, it shows that nephrotic syndrome is associated with a decreased reflection coefficient to proteins, which enhances the driving force for water filtration through the paracellular pathways, and with an increased filtration coefficient of both the para- and trans-cellular pathways of water transport. Finally it confirms previous data showing that AQP1 only accounts for a minor fraction (1-3%) of the total filtration coefficient of the peritoneum.

The peritoneal filtration barrier is a complex structure that comprises the capillary endothelium, the interstitial tissue, and the mesothelium. In this study, it was considered as a simple layer for data analysis, and therefore we cannot determine the specific structures where the observed changes in filtration coefficient and reflection coefficient to proteins occurred. It seems, however, that the observed increase in the filtration coefficient of the trans-cellular pathway (K_1) mainly originates from the mesothelium, since AQP1 labelling observed in PAN rats was mostly restricted to the most superficial layer of the peritoneum. It should be noted that there is a controversy in the literature regarding the presence of AQP1 in the mesothelium, some reports concluding that it is exclusively expressed in the capillary endothelium (Devuyst and Ni, 2006) whereas other describe it in both the capillaries and the mesothelium (Ota et al., 2002). Our finding that mesothelial over-expression of AQP1 accompanies the increase in K_1 in PAN rats suggest that the mesothelium represents a limiting barrier to trans-cellular water transport.

The filtration coefficient K is the product of the hydraulic conductance (L_p) and the surface area (S) of the filtration barrier. An increased in K may therefore stem from an increase in L_p and/or in S. The surface area of the filtration barrier is difficult to estimate but we did not observe any macroscopic change that would have suggested a surface area increase in PAN rats. In addition, the simultaneity of the variations in K and AQP1 expression in the peritoneum suggests that the K increase originates from an increase in L_p rather than in S, at least for the trans-cellular pathway. Results also show that the peritoneum of PAN rats displayed a decreased coefficient of reflection to proteins. This indicates that the disease is associated not

only with an increased number of pores but also with structural changes that render pores more permeable to proteins.

Peritoneal oxidative stress was attested by the transcriptional induction of NADPH oxidase, the main cellular supplier of ROS, and the down-regulation of catalase, which catalyzes the decomposition of hydrogen peroxide to water and O_2, and a trend towards the down-regulation of SOD, which catalyzes the generation of hydrogen peroxide from superoxide. Interestingly, we observed a marked over-expression of NOX4 and its associated stabilizing partner p22phox, whereas over-expression of NOX2 was borderline significant. In opposition to NOX2 which generates superoxide, NOX4 directly releases hydrogen peroxide (Serrander et al., 2007). This isotypic specificity of NADPH oxidase induction in the peritoneum of nephrotic rats likely accounts for the weakness of the DHE labeling, since DHE is oxidized by superoxide but not by hydrogen peroxide. The NOX4-p22phox complex is also unique among NADPH oxidases in that its activity is constitutive, and is therefore determined by the NOX4 expression level (Martyn et al., 2006). The stimulus for peritoneal NOX4 over-expression during nephrotic syndrome remains to be determined. However, once it is triggered, over-expression of NOX4 might be maintained through positive feedback regulation since we showed that ROS activate NF-κB which in turn may induce NOX4 (Zhang et al.). NF-κB may also exert a positive feedback on hydrogen peroxide levels by promoting SOD production (Wong et al., 1989).

While this study demonstrated the causal role of peritoneal oxidative stress on increased water permeability and AQP1 over-expression during nephrotic syndrome, it did not address the mechanism responsible for oxidative stress. In the plasma, albumin is a potent anti-oxidant agent through its dual action as a ROS scavenger and a Cu^{2+} chelating agent (Evans, 2002). Thus, the hypoalbuminemia observed in PAN nephrotic rats likely increases ROS in blood, but it cannot account for peritoneal oxidative stress. Sphingosine 1 phosphate (S1P) is a bioactive lipid with increasingly recognized functions, especially on capillary endothelia (reviewed in (Proia and Hla, 2015)). In particular, S1P released from red blood cells maintains a low vascular permeability (Minnear et al., 2005; Camerer et al., 2009). Recently, it was reported that plasma albumin facilitates the release of S1P from red blood cells and its delivery to the endothelium (Adamson et al., 2014). Although several authors reported a stimulatory role of S1P on NADPH oxidase activity and/or ROS production

(Catarzi et al., 2011; Schenten et al., 2011; Harijith et al., 2013), a recent report showed that S1P inhibits NADPH oxidase and ROS production in vascular cells (Tolle et al., 2008). Thus, hypoalbuminemia would decrease S1P availability to the endothelium and might thereby induce oxidative stress and increase vascular permeability.

We did not address the mechanism by which activation of NF-κB increases water permeability and AQP1 expression in the peritoneum. Because AQP1 is not a known direct target of NK-κB (http://www.bu.edu/nf-kb/gene-resources/target-genes/), its induction requires an intermediate NF-κB-induced mediator. TNFα is a known target gene of NF-κB (Shakhov et al., 1990) which is induced in the peritoneum of nephrotic rats and has been reported to increase capillary hydraulic conductance *in vitro* (Skinner et al., 1999). However this is an acute effect which cannot account for the transcriptional induction observed in the present study. Moreover, several reports suggest that TNFα would repress rather than induce AQP1 expression (Su et al., 2004; Ma and Liu, 2013). Another possible candidate is cyclooxygenase 2 (COX2), a gene target of NF-κB (Kaltschmidt et al., 2002). Prostaglandin E2, a product of COX2, increases microvascular permeability (Malik et al., 1985) and activation of COX2/PGE2 pathway was reported to increase AQP1 mRNA expression in human umbilical vein endothelial cells (Zhao et al., 2012).

It is noteworthy that the reversion of K_1, K_2 and σ_{pl} to control levels in response to NAC treatment reduced the volume of ascites by 60% but did not suppress it. This suggests that 60% of ascites stems from changes in the intrinsic filtration properties of the peritoneum whereas the remaining 40% is due to changes in the driving force for water filtration, i.e. the decreased oncotic pressure of plasma. Calculation shows that the observed changes in K_1, K_2 and σ_{pl} in the peritoneum of PAN rats per se would increase water filtration by 42.6 µl/min, whereas the 6 mmHg decrease in the oncotic gradient resulting from the decreased plasma protein concentration observed in PAN rats by itself would increase the water flux by 16.4 µl/min. According to these calculations, changes in the intrinsic properties of the filtration barrier and in the oncotic pressure of plasma respectively account for 72% and 28% of the increased water flux, which is consistent with experimental findings. The total increase in filtration rate calculated above, namely 85 ml/day, largely exceeds the volume of

ascites (~13 ml in 3-4 days), meaning that lymphatic drainage of the peritoneum cavity also increases drastically during nephrotic syndrome.

In summary, results show that PAN-induced nephrotic syndrome in rats is associated with marked increases in the water permeability and a decreased reflection coefficient to proteins of the peritoneal barrier. These changes, which are triggered by oxidative stress and subsequent activation of NF-κB, account for approximately two-third of the volume of ascites.

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Table 1. Sequence of primers used for qPCR

Gene	Forward	Reverse
AQP1	5'-cacttgccattggcttgt-3'	5'-ggccaggatgaagtcatagat-3'
RANTES	5'-ccattctgatcctgtatagctttc-3'	5'-ggccataggagaggacac-3'
Rps23	5'-ctcacgcaaagggaattgt-3'	5'-caatgaagttcaagcaaccg-3'
TNFα	5'–cggagctaaactaccaggtat-3'	5'-cagaggttcagtgatgtagcga-3'

Table 2. Filtration coefficients and reflection coefficient to proteins.

				p vs Control
K_1	Control	σ_{man} = 0.02	0.068 ± 0.009	
		σ_{man} = 0.01	0.068 ± 0.009	
	PAN	σ_{man} = 0.02	0.199 ± 0.018	0.0002
		σ_{man} = 0.01	0.199 ± 0.018	0.0002
	PAN + NAC	σ_{man} = 0.02	0.057 ± 0.005	NS
		σ_{man} = 0.01	0.057 ± 0.005	NS
K_2	Control	σ_{man} = 0.02	2.50 ± 0.07	
		σ_{man} = 0.01	4.94 ± 0.14	
	PAN	σ_{man} = 0.02	3.83 ± 0.34	0.0038
		σ_{man} = 0.01	7.63 ± 0.71	0.0041
	PAN + NAC	σ_{man} = 0.02	2.15 ± 0.13	NS
		σ_{man} = 0.01	4.28 ± 0.26	NS
σ_{pl}	Control	σ_{man} = 0.02	0.63 ± 0.08	
		σ_{man} = 0.01	0.85 ± 0.04	
	PAN	σ_{man} = 0.02	0.08 ± 0.01	0.0007
		σ_{man} = 0.01	0.70 ± 0.01	0.0191
	PAN + NAC	σ_{man} = 0.02	0.45 ± 0.04	NS
		σ_{man} = 0.01	0.91 ± 0.02	NS

K_1 and K_2 are the water filtration coefficients of the $HgCl_2$-sensitive (AQP1) and -insensitive filtration pathways, respectively (in µl/min/mmHg), and σ_{pl} is the reflection coefficient to protein. Values were calculated from experimental data and the equations described in the method section, assuming a ΔP of 16.7 mmHg and a reflection coefficient to mannitol (σ_{man}) of 0.02 or 0.01.

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2. Role of albumin in ROS production and sodium retention in nephrotic syndrome

2.1. Introduction

Up to now the pathogenesis of nephrotic sodium retention is not fully elucidated. Classically it was thought to stem from the stimulation of the renin-angiotensin-aldosterone system (RAAS) resulting from hypovolemia secondarily to the leakage of fluid from the vascular bed to the interstitium as a consequence of decreased oncotic pressure. However, it is now admitted that sodium retention is a primary alteration which is independent of volemia and aldosterone in both patients and PAN nephrotic rats [92]. In addition, the fact that only the treated kidney retains sodium in rats with unilateral PAN nephrosis [10] demonstrates that sodium retention is not brought about by a circulating factor.

Alternatively, it is now proposed that increased sodium reabsorption in CCD is triggered by a factor abnormally present in the luminal urinary fluid of nephrotic rats and patients, secondarily to the alterations of the glomerular filtration barrier. Albumin is the most abundant protein that is abnormally present in the urine of nephrotic patients. In addition, we previously reported that albumin is endocytosed in the CCD of PAN nephrotic rats and albumin endocytosis in the proximal tubules induces oxidative stress [24]. Furthermore, ROS are known to participate in the control of the expression of various sodium transporting proteins. We therefore explored the role of albuminuria in sodium retention in PAN nephrotic rats.

Our results show that albumin triggers oxidative stress in a CCD cell line as well as in CCDs from PAN nephrotic rats through a mechanism that relies on PI3 kinase activation and induction of NADPH oxidase. Treatment with the ROS scavenger NAC blunted the over expression of both ENaC and Na,K-ATPase in the CCD of PAN rats. Reversal of Na,K-ATPase induction stems from inhibition of ROS production in CCD whereas that of ENaC is secondary to the reduction of aldosterone plasma level, owing from the blockade of aldosterone secretion by ROS scavenging in adrenals. Despite these changes in expression of ENaC and Na,K-ATPase, NAC treatment prevented neither sodium retention nor the increase in sodium reabsorption in CCD. This last effect stems in part from the induction of ASIC2, a channel previously found to palliate the defect in ENaC in aldosterone-clamped PAN rats (to be published, see summary below).

PAN nephrotic rats display high plasma aldosterone level whereas most nephrotic patients do not. Therefore, we recently evaluated the mechanism of sodium retention in a model of aldosterone-clamped PAN rats. These corticosteroid-clamped (CC) PAN rats still display amiloride-sensitive sodium retention that originates from the distal nephron, but they display no expression of ENaC [15, 16], suggesting the existence of an alternate pathway for apical sodium entry in the CCD. Using *in vitro* microperfusion of CCD from AC-PAN nephrotic rats, we found that this alternative pathway shares with ENaC its sensitivity to amoliride, but differs by its insensitivity to extracelluar Zn^{++} and acid pH.. We isolated from kidneys of CC-PAN rats a truncated variant of the 2b regulatory subunit of acid sensing ion channels (ASIC) [93, 94] lacking most of its intracellular N-terminal domain (t-ASIC2b). Co-expression of t-ASIC2b with the conductive ASIC2a subunit in Xenopus ovocytes generated sustained acid-stimulated sodium currents instead of transient ones. Besides the apical pole of ASDN cells from CC-PAN rats, ASIC2 was detected in the ASDN of a majority of nephrotic patients. Collectively, these results show that a new truncated variant of ASIC2b converts transient neuronal ASIC2a into a long-lasting epithelial sodium channel involved in renal sodium retention and edema formation in normo-aldosteronemic nephrotic patients and PAN-rats. This work has not been published yet. For this reason, the second manuscript presented in this document, although completed, has not been submitted for publication.

2.2. Article #2

Endocytosis of albumin induces oxidative stress and over-expression of Na,K-ATPase in the collecting duct of nephrotic rats

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Running headline: ROS and retention in nephrotic syndrome

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Introduction

Nephrotic syndrome is a multifactorial kidney disorder characterized by abnormal urinary excretion of plasma proteins (>50 mg/kg/day) and hypoalbuminemia (<30 g/l). It is usually associated with sodium retention and edema formation. Early *in vivo* micropuncture experiments [8] in rats with unilateral puromycin aminonucleoside (PAN) nephrosis demonstrated that sodium retention originates from the distal part of the nephron. More recently, *in vitro* studies in nephron segments isolated from bilateral PAN nephrotic rats showed that sodium retention stems from over-expression of Na,K-ATPase and of the sodium channel ENaC in the connecting tubule and cortical collecting duct (CCD) [9, 10].

The pathogenesis of nephrotic sodium retention is not fully elucidated. In PAN nephrotic rats, the over-expression of ENaC stems from hyper-aldosteronemia, the origin of which remains unclear, whereas that of Na,K-ATPase is independent of aldosterone [13]. When plasma aldosterone is clamped to control values, PAN nephrotic rats still retain sodium in their distal nephron because a variant of acid-sensing ion channel 2 (ASIC2) substitutes for ENaC (manuscript in preparation). The fact that in unilateral PAN nephrotic rats only the PAN-treated kidney retains sodium [8] indicates that sodium retention is not triggered by a circulating factor. Alternately, it has been proposed that sodium reabsorption is stimulated by a substance abnormally filtered and present in the fluid delivered to the distal nephron. Thus, high levels of plasmin were detected in the urine of nephrotic patients and rats owing to the filtration of plasminogen and the secretion of plasminogen activator. In addition, plasmin was shown to activate ENaC through moderate proteolysis [15]. However, this mechanism can account neither for sodium retention in the absence of functional ENaC, as in nephrotic patients and rats with normal plasma aldosterone, nor for the over-expression of Na,K-ATPase.

The most abundant substance abnormally present in nephrotic urine is albumin. In healthy individuals, the low fraction of plasma albumin filtered is reabsorbed by glomeruli (<0.1%) [4, 16, 17], thereby delivering a protein-free luminal fluid to the distal nephron [18]. Conversely, in nephrotic patients, the high rate of protein filtration saturates the proximal tubule endocytotic absorption pathway and exposes the distal part of nephron to albumin. It is well established that albuminuria is associated with progression of chronic kidney disease [19-21] and *in vitro* albumin

treatment induces oxidative stress [22-24]. Albumin endocytosis also occurs in CCDs from PAN nephrotic rats where it induces phosphorylation of ERK and inhibition of the potassium channel ROMK and of potassium secretion [26]. More recently, studies using a murine CCD cell line showed that luminal endocytosis of albumin is mediated by a receptor and activates the canonical NF-κB pathway leading to expression of pro-inflammatory cytokines [25]. Whether it also induces an oxidative stress has not been evaluated yet.

Reactive oxygen species (ROS) play an essential role in several models of renal diseases [65]. In nephrotic syndrome, ROS have been involved in the pathogenesis of proteinuria [69]. Moreover, it was observed that superoxide anion induces an oxidative injury of the glomerular basement membrane and increases the glomerular permeability [70, 71]. In addition, several clinical studies have documented oxidative stress and impaired antioxidant defense during acute nephrotic syndrome [67, 72]. Recently, we reported that oxidative stress is responsible for increased peritoneal water permeability and decreased reflection coefficient to albumin in PAN nephrotic rats, and thereby accounts for two-third of ascites. Oxidative stress in the proximal tubules influences a number of physiologic processes including renal sodium handling and regulation of Na^+,K^+-ATPase activity [29, 30]. In the medullary thick ascending limb of the loop of Henle, both exogenous and endogenous superoxide stimulates the Na-K-2Cl cotransporter and sodium reabsorption [31, 32]. Moreover, ROS produced through NADPH oxidase system have a stimulatory effect on ENaC activity in murine CCD cell line [33].

The purposes of this study were to determine whether 1) albumin endocytosis in the distal nephron generates oxidative stress, and 2) oxidative stress triggers the over-expression of Na,K-ATPase and/or ENaC and participates in sodium retention.

Material and methods

Ethical approval. All animal experimental protocols were approved by the Charles Darwin Ethic Committee (#Ce5/2012/041) and performed according to the French legislation for animal experimentation.

Animals. Male Sprague–Dawley rats (Charles Rivers, L'Abresles, France) weighing 150–170 g at the onset of the study were fed a standard laboratory chow (A04, Safe, Augy, France) with free access to water. Rats were anaesthetized by intraperitoneal injection of either a mix of Xylazine (Bayer, 13.2 mg/kg body wt) and ketamine

(Vibrac France, 86 mg/kg body wt) or sodium pentobarbital (Ceva France, 55 mg/kg body wt), before surgery or sacrifice respectively. NS was induced by intra-jugular injection of puromycin aminonucleoside (PAN, Sigma-Aldrich, 150mg/kg body wt). The antioxidant N-acetyl cysteine (NAC) was given by daily gavage (0.5g/kg body wt) starting the day before PAN injection. Control rats were treated with NAC or water using the same protocol.

Rats were sacrificed at day 6 post PAN injection, when sodium retention, proteinuria and ascites are maximal [9]. The left kidney was immediately dry frozen in liquid nitrogen and stored at -80°C for protein extraction. The right kidney was used for microdissection of CCDs after perfusion with collagenase or not (see below).

Metabolic studies. Animals were housed in individual metabolic cages with free access to food and water, starting 3 days before the onset of experimentation for acclimatization. 24 h urine was collected starting 1 day before the onset of the experimental period. Urine creatinine and sodium concentrations were measured in an automatic analyzer (Konelab, Thermo, France) and by flame photometry (Instrumentation Laboratory) respectively. Urinary excretion of sodium was expressed as a function of creatinine excretion.

Microdissection of CCDs. CCDs were dissected either from fresh kidney slices (for microperfusion) or after collagenase treatment (for RNA extraction, determination of Na,K-ATPase activity and ROS detection). For RT-PCR experiments, microdissection was performed under RNase-free conditions. For collagenase treatment, the left kidney was infused via the abdominal aorta with incubation solution (Hank's solution supplemented with 1 mM pyruvate, 0.1% bovine serum albumin (BSA), 0.5 mM $MgCl_2$, 1 mM glutamine and 20 mM Hepes, pH 7.4) containing liberase (Blendzyme 2, Roche Diagnostics, Meylan, France, 125 µg/ml). Small pieces of kidney were incubated for 25 min at 30°C in incubation solution containing liberase 50 µg/ml. CCDs were dissected under stereomicroscopic observation at 4°C.

***In vitro microperfusion*.** CCDs were perfused at 37 °C under symmetrical conditions [26], with bath and perfusate containing (in mM): 118 NaCl, 23 $NaHCO_3$, 1.2 $MgSO_4$, 2 K_2HPO_4, 2 calcium lactate, 1 sodium citrate, 5.5 glucose, 5 alanine, 12 creatinine, pH 7.4 (bath continuously gassed with 95% O_2/5% CO_2). Transepithelial voltage (V_{te}) was measured via microelectrodes connected to an electrometer. Concentrations of Na^+ and creatinine were determined by HPLC, and sodium flux (J_{Na^+}) was calculated as

$$J_{Na+} = [[Na^+]_p \times V_p - [Na^+]_c \times V_c] / L \times t$$

where $[Na^+]_p$ and $[Na^+]_c$ are the concentration of Na^+ in the perfusate and collection, respectively, V_p and V_c are the perfusion and collection rates, respectively, L is the tubule length, and t is the collection time. V_p was calculated from the measured V_c and the change in creatinine concentration. For each tubule, J_{Na+} was calculated as the mean of four successive 10 to15-min collection periods.

Na,K-ATPase hydrolytic activity. Na,K-ATPase activity was determined by a radiochemical assay based on the measurement of phosphate released from [γ-^{32}P] ATP by the ATPase contained in pools of 2 to 5 mm of CCD, as previously described [9]. To study the role of albumin on Na,K-ATPase activity, the CCDs were successively pre-incubated in dissection medium at 30°C for 1hr, incubated with or without NAC (5mM) or apocynin (100µM) at 37°C for 1hr, and thereafter treated with or without BSA (1mg/ml) at 37°C for 3hr,always in presence of NAC or apocynin when required, before measurement of Na,K-ATPase activity.

RNA extraction and RT-qPCR. RNAs were extracted from pools of 40–60 CCDs using the RNeasy micro kit (Qiagen, Hilden, Germany) and reverse transcribed using a first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics), according to the manufacturers' protocols. Real-time PCR was performed using a cDNA quantity corresponding to 0.1 mm of CCD with LightCycler 480 SYBR Green I Master qPCR kit (Roche Diagnostics) according to the manufacturer's protocol. Specific primers (Table 1) were designed using ProbeDesign (Roche Diagnostics).

Cell culture. $mCCD_{cl1}$ cells, a mouse collecting duct principal cell line [112], were cultured as described in the original study. Cells were grown in Transwell filter cups (Corning, Cambridge, MA) in supplemented DMEM/F-12 medium (1:1 vol/vol, Invitrogen) containing 50 nM dexamethasone (Amersham Bioscience) for 5 days (growth medium), and then 3 nM dexamethasone (differentiation medium) for at least additional 5 days. Before experiments, the mCCDcl1 cells were kept for 24 h in a minimal DMEM/F12 medium without serum and dexamethasone. Albumin (1 mg/ml) was added to the apical solution whereas NAC (5 mM), apocynin (100 µM), or LY294002 (50 µM) were added to both sides.

DHE staining. For mCCD cell studies, DHE (5 µM in PBS) was added to the apical side of the cells in six-well plates and incubated for 30 minutes at 37°C. Filters were then rinsed 3-time with PBS and fixed for 5 min with paraformaldehyde (4% in PBS). Afterwards, filters were rinsed thrice with PBS, cut, mounted on Superfrost + glass

slides and observed on a fluorescence microscope (×40, Zeiss observer Z1, LSM710).

For microdissected CCDs, tubules were transferred to Superfrost Gold+ glass slides, rinsed twice with PBS and fixed for 5 min with paraformaldehyde (4% in PBS), rinsed twice with PBS. Afterwards, they were incubated 30 min at 37°C with 5µM DHE in PBS, rinsed thrice in PBS, and observed on a fluorescence microscope.

For *in vivo* studies, DHE was injected intravenously (5 mg/kg body wt) 2 hours before sacrifice. Thereafter, kidneys were frozen in OCT and kept at -80C till the time of use. 5µm cryosections were transferred to Superfrost Gold+ glass slides and rinsed twice with PBS, treated with 0.2% triton in PBS for 10min, rinsed thrice with PBS, blocked for 10min with 2% BSA in PBS, and then incubated for 1hr at room temperature with AQP2 antibodies (Santa Cruz, AQP2 C-17 1:300), and revealed with a Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA). Slides were mounted and observed on a fluorescence microscope.

Immunoblotting. Kidneys were homogenized in lysis buffer containing 50 mM Tris buffer, 150 mM NaCl, 1 mM EDTA, and a protease inhibitor cocktail (EDTA-free, EASYpack, Roche Diagnostics), pH 7.4. The homogenates were centrifuged at 10000 x g for 15 min and the protein content of the supernatant was measured with the BCA method (Thermo Scientific Pierce). Fourty µg of protein were denaturated, resolved by SDS-PAGE (10% poyacrylamide) and transferred onto a nitrocellulose membrane. Western blots were performed with standard procedure with an anti-ENaC γ subunit antibody (kindly provided by G. Crambert, Centre de recherche des Cordeliers, 1/1000) or an anti-ASIC2 antibody (Abcam, ab77384, 1/1000) and anti-rabbit IgG antibody coupled to horseradish peroxidase (Promega France, W401B, 1/7000) and revealed with the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences). After stripping, membranes were incubated with anti-β actin antibody (Sigma Aldrich, A-1978, 1/1000) and post-treated as above. Densitometry of the bands was quantified with ImageJ.

Statistics. Results were expressed as means ± SE from several animals. Comparison between groups was performed by unpaired Student's *t* test, p value <0.05 were considered significant.

Results

Albumin induces an oxidative stress in the CCD of PAN nephrotic rats. Dihydroethidium (DHE) staining on kidney cortex sections revealed marked accumulation of superoxide anion in most tubules from proteinuric PAN nephrotic rats whereas no labeling was detected in control rats. Presence of ROS in the CCD was confirmed by co-labeling kidney sections with an anti-AQP2 antibody, a specific marker for principal cells in CCD and connecting tubules (Figure 1A), and by DHE labeling on isolated CCDs (Figure 1B). In addition, RT-qPCR analysis revealed over-expression of NADPH oxidase subunits (NOX2, p22phox, p40phox) and decreased expression of several superoxide detoxifying enzymes (catalase, superoxide dismutase, thioredoxin and thioredoxin reductase) in CCDs from PAN rats (Fig 1C-D).

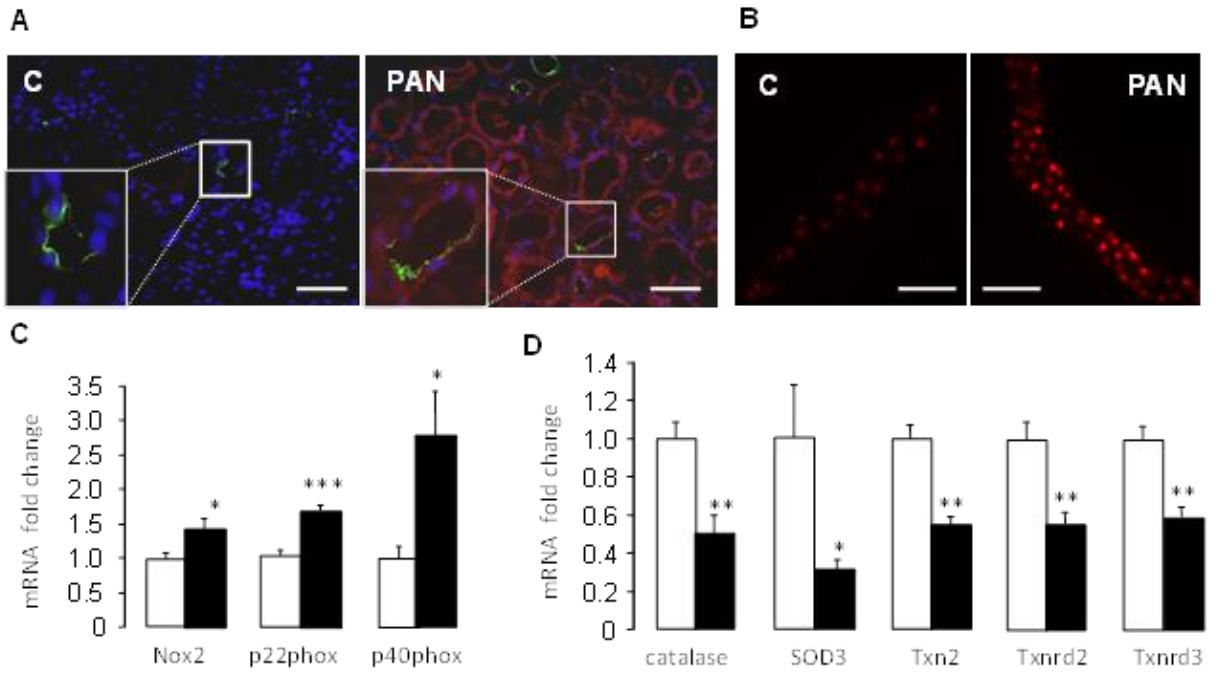

Figure 1. Oxidative stress in the CCD of PAN rats. A. *Labeling with dihydroethidium (DHE, red), anti-aquaporin 2 antibody (green) and DAPI (blue) of 5µm cryosections of kidney cortex from control and PAN rats (bars: 50µm).* ***B.*** *DHE labeling of CCDs dissected from a control and a PAN rat (bars: 50µm).* ***C & D.*** *mRNA expression of NADPH oxidase subunits (B) and ROS detoxifying enzymes (C) in CCDs from control (open bars) and PAN rats (black bars). SOD3, super oxide dismutase 3; Txn2, thioredoxin 2; Txnrd2 and 3, thioredoxin reductase 2 and 3. Values were normalized as a function of the Rps23 expression and expressed as percent of controls. Data are means ± SE from 8 control and 9 PAN rats. *, p<0.05; **, p<0.005; ***, p<0.001.*

The role of albumin on ROS production was first evaluated on mCCD cells. Addition of albumin (1mg/ml) to the apical side of mCCD cells cultured on filters time-dependently induced the accumulation of ROS, as detected by DHE labeling (Figure 2A). Pre-treatment with apocynin (100 µM) or LY294004 (50 µM) abolished albumin-

induced ROS accumulation, demonstrating its dependence on NADPH oxidase activity and on the PI3 kinase system (Figure 2B). DHE labeling was fully prevented by the ROS scavenger N-acetylcysteine (NAC). Pre-incubation of CCD isolated from control rats with albumin (1mg/ml, 3hrs) also induced ROS accumulation (Figure 2C).

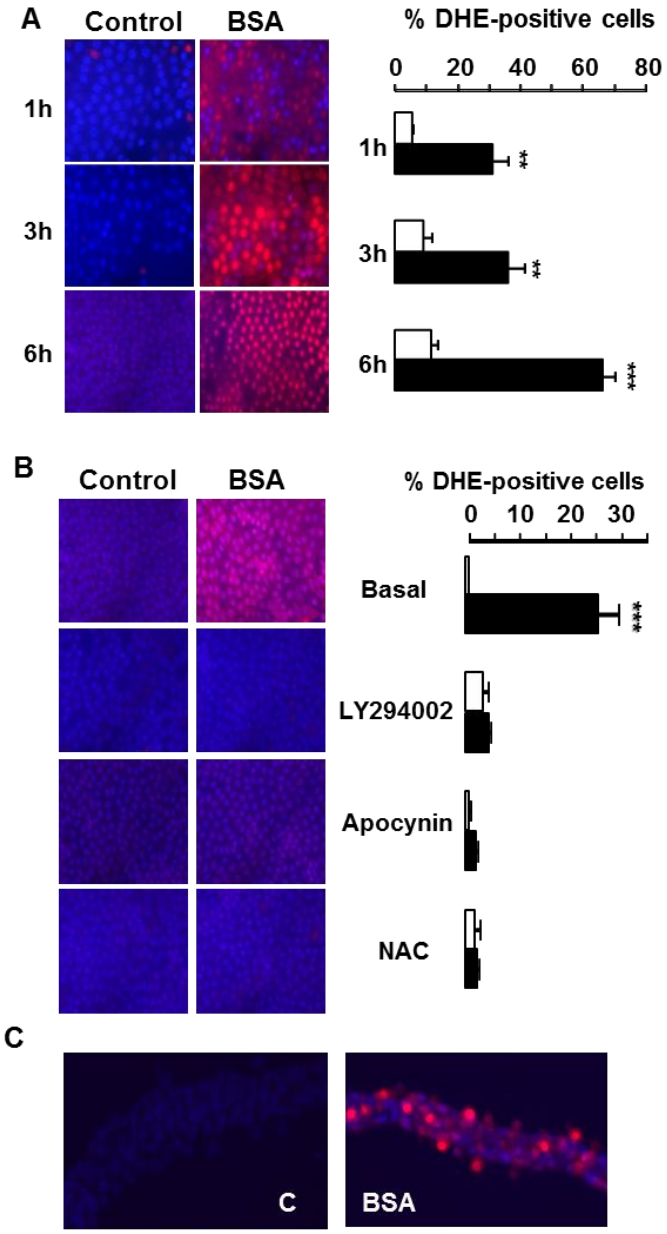

***Figure 2. BSA-induced ROS production in mCCD cells and rat CCD**. **A.** Representative DHE labeling and quantification of DHE-positive cells of mCCD cells grown at confluence and thereafter exposed for 1-6 h to an apical medium containing BSA (1mg/ml) or not (black and open bars respectively). Values are means ± SE from 6 filters. **, p<0.01; ***, p<0.001. **B.** Same as above except that starting before exposure to BSA (1mg/ml, 6h), cells were pre-treated or not (Basal) for with the PI3 kinase inhibitor LY294002 (50 µM), NADPH oxidase inhibitor apocynin (100 µM), or the ROS scavenger N-acetylcysteine (5 mM). Treatment started 1h before BSA addition and throughout the 6h with or without BSA. Values are means ± SE from 6 filters. ***, p<0.001. **C.** DHE labeling of CCDs from a control rat incubated in the presence or absence of BSA (1 mg/ml) for 3h.*

ROS scavenging prevents the induction of ENaC and Na,K-ATPase in CCD from PAN nephrotic rats. Per os administration of NAC (0.5g/kg body wt per day) throughout the study reduced drastically ROS accumulation in the kidney and the CCD of PAN nephrotic rats (Figure 3).

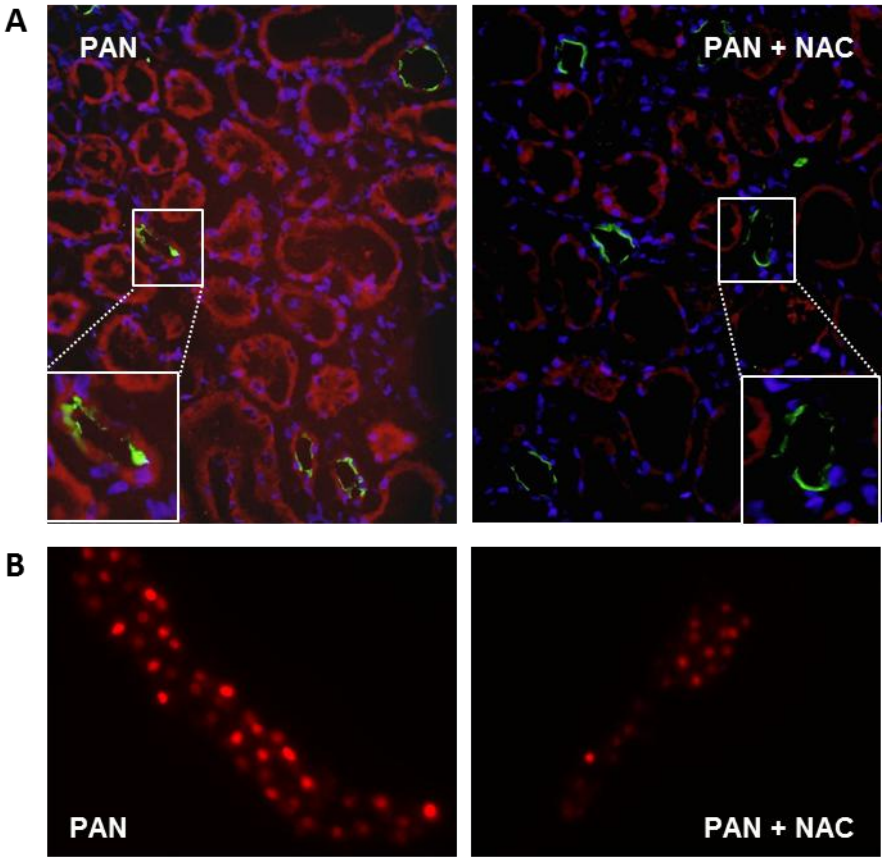

Figure 3. Efficiency of in vivo treatment with the ROS scavenger N-acetyl cysteine. A. *Labeling with dihydroethidium (DHE, red), anti-aquaporin 2 antibody (green) and DAPI (blue) of 5µm cryosections of kidney cortex from NAC-untreated or -treated PAN rats (bars: 50µm).* ***B.*** *DHE labeling of CCDs dissected from NAC-untreated or -treated PAN rats (bars: 50µm).*

Six days after administration of PAN, when urinary sodium excretion reaches its nadir, rat CCD display increased Na,K-ATPase activity and mRNA expression of its catalytic subunit as well as increased activity of ENaC and over-expression of its α and β subunit mRNA[113-115] .Here we found that NAC treatment reduced the activity of Na,K-ATPase and the level of its α subunit mRNA in CCDs from PAN rats down to levels in control rat CCD (Figure 4A). It also reduced the mRNA expression of αENaC down to control level whereas that of βENaC was slightly but not significantly decreased. The activity of ENaC was not determined but we evaluated the degree of cleavage of its γ subunit which is associated with the channel activation, we found that NAC treatment significantly decrease the cleavage of γ subunit of ENac in the CCD compared to PAN (Figure 4 B and C).

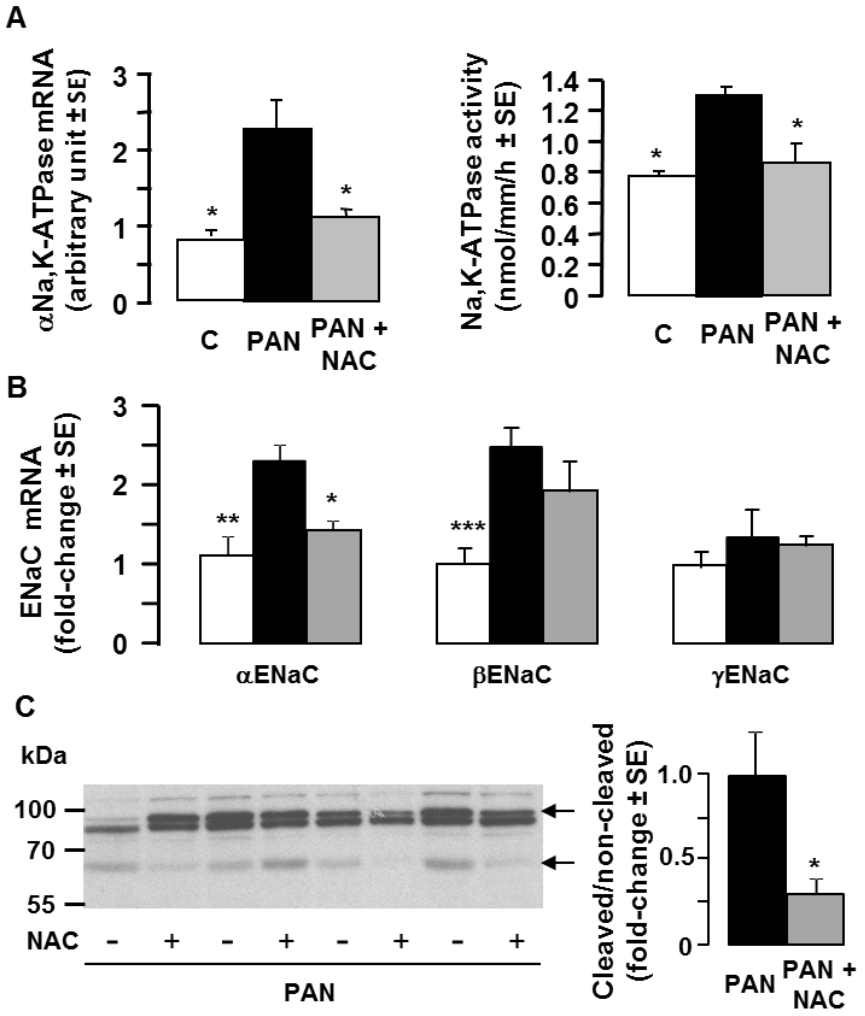

Figure 4. Effect of NAC treatment on the expression and activity of Na,K-ATPase and ENaC. A. *mRNA expression of Na,K-ATPase catalytic α subunit (left) and Na,K-ATPase activity (right) in CCDs from control rats (C) and PAN nephrotic rats treated or not with NAC. Values are means ± SE from 5-7 animals. *, p<0.025 as compared to the PAN group.* ***B.*** *mRNA expression of α, β and γ subunits of ENaC in CCDs from control rats (open columns) and PAN nephrotic rats treated or not with NAC (grey and dark columns respectively). Values are means ± SE from 6 animals. *, p<0.01; **, p<0.005; ***, p<0.001 as compared to the PAN group.* ***C.*** *Immunoblots of γ-ENaC subunits in kidney cortex from NAC-treated or –untreated PAN rats. The specific non-cleaved (~90 kDa) and cleaved (~65 kDa) bands are indicated by arrows. The right part shows the ratio of densitometry of the cleaved above non-cleaved band. Values are means ± SE of 4 animals. *, p<0.05.*

The question rises whether the inhibitory effects of NAC on Na,K-ATPase and ENaC in the collecting duct stem from ROS scavenging in the CCD or elsewhere in the kidney or in another organ. Importantly, ROS are essential for angiotensin 2-induced aldosterone synthesis[116], and PAN nephrotic rats display an activation of the renin-angiotensin-aldosterone system which is responsible for the induction of ENaC in the CCD. Figure 5A shows that increased urinary excretion of aldosterone observed in PAN nephrotic rats, which reflects increased plasma aldosterone level,

was blunted by NAC treatment. Accordingly, NAC treatment also blunted the increased expression of the aldosterone-induced kinase Sgk mRNA in CCDs from PAN nephrotic rats (Figure 5B). This normalization of aldosterone level is sufficient to account for the normalization of ENaC expression in NAC-treated rats (Figure 4b and 4c).

In contrast, this normalization of aldosterone cannot account for the normalization of Na,K-ATPase expression since over expression of Na,K-ATPase in PAN rats is independent of aldosterone[13]. We confirmed that induction of Na,K-ATPase in the CCD of PAN nephrotic rats is accounted for by albumin-induced generation of ROS by showing that *in vitro* exposure of CCDs from normal rats to albumin increased Na,K-ATPase activity and that this stimulation was prevented by NAC as well as by the NADPH oxidase inhibitor apocynin (Figure 5C).

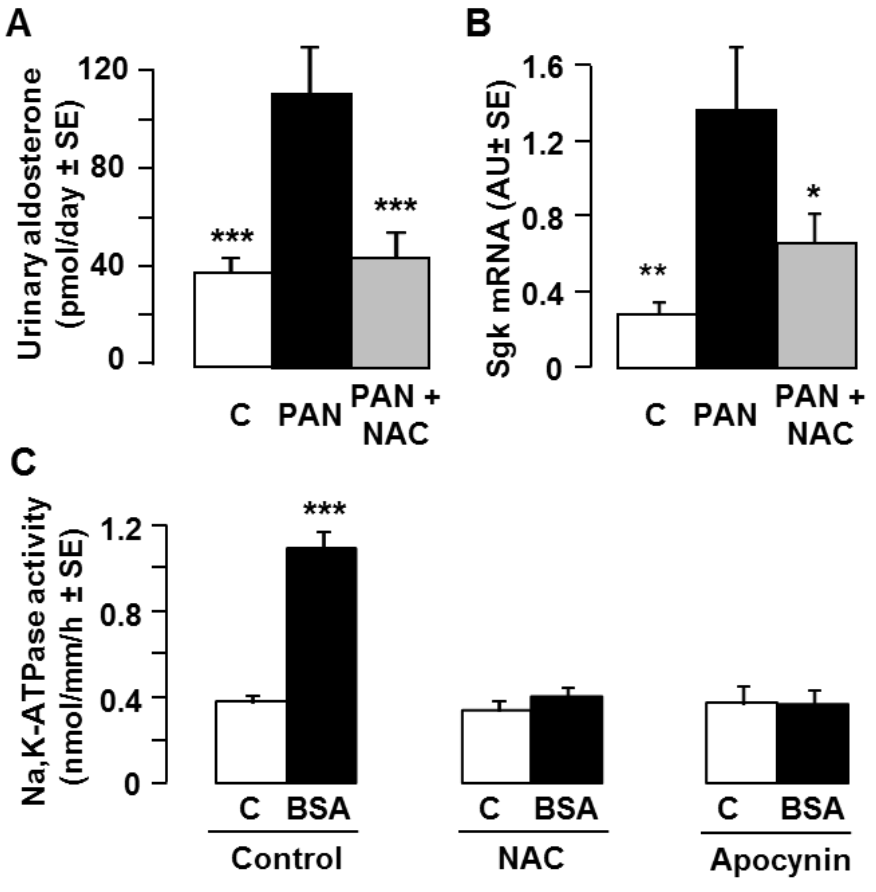

Figure 5. ROS-induced stimulation of Na,K-ATPase is independent of aldosterone. A. Urinary excretion of aldosterone in control and NAC-treated or -untreated PAN rats. Values are means ± SE of 7-9 animals. ***, p<,001 as compared to PAN group. **B.** mRNA expression of Sgk in CCDs from control and NAC-treated or –untreated PAN rats. Values are means ± SE of 7-9 animals. **, p<,001 and *, p<,005 as compared to PAN group. **C.** Na,K-ATPase activity was determined in CCDs from control rats incubated with or without BSA (1 mg/ml) and with or without N-acetylcysteine (5 mM) or apocynin (100 µM). Values are means ± SE from 7 (Control groups) or 4 animals (NAC and apocynin groups). ***, p<0.001 as compared to the absence of BSA.

ROS scavenging does not prevent sodium retention. Despite the lack of stimulation of ENaC and Na,K-ATPase in the CCD of NAC-treated PAN nephrotic rats, urinary sodium excretion decreased with the same time course and amplitude as in NAC-untreated rats (Figure 6A). To determine whether the distal nephron is a site of sodium retention in NAC-treated PAN rats as in untreated PAN nephrotic (PAN) rats, we measured net sodium transport ($J_{Na}+$) and transepithelial voltage (V_{te}) by *in vitro* microperfusion of isolated CCD. In contrast with CCD from control rats which do not transport sodium, CCD from both NAC-treated and -untreated PAN rats displayed significant and similar $J_{Na}+$ and V_{te} (figure 6B).

We recently reported that CCDs from PAN nephrotic rats with clamped plasma level of aldosterone express a variant of ASIC2 which compensates for the absence of ENaC and allows sodium retention ( ). Because NAC treatment blunted hyper aldosteronemia in PAN rats, we evaluated whether it might trigger ASIC2 expression. RT-qPCR showed that expression of ASIC2b mRNA was increased in CCD from NAC-treated PAN rats as compared with Control and NAC-untreated PAN rats (Figure 6C), and western blot confirmed increased expression of ASIC2 in the kidney of NAC-treated PAN rats (Figure 6D).

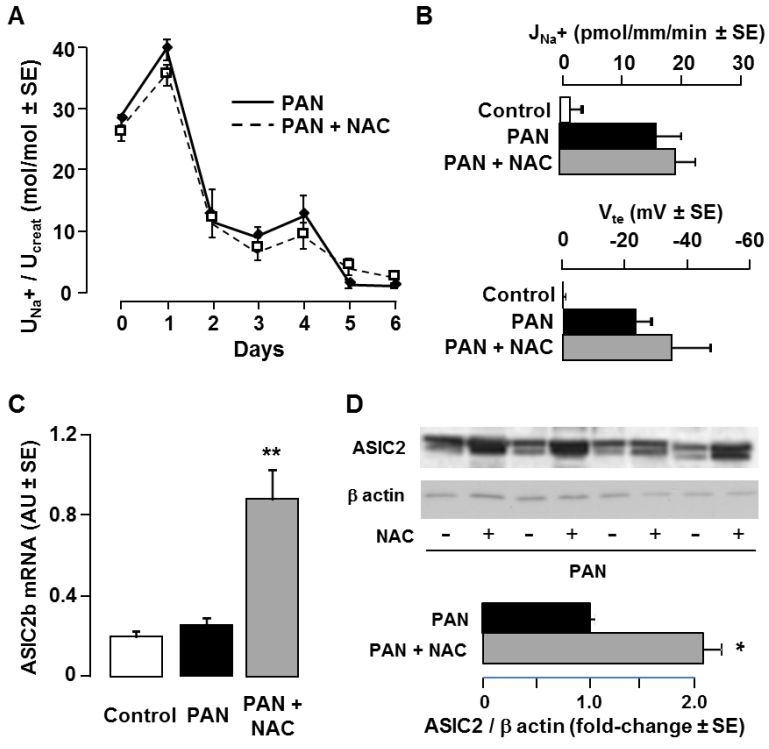

Figure 6. ROS scavenging does not prevent sodium retention. A. *Urinary excretion of sodium in NAC-treated and -untreated rats following administration of PAN. Excretion of sodium is expressed as a function of creatinine excretion. Values are means ± SE from 6 animals. There was no statistically significant differences between the two groups of rats at any time.* ***B.*** *Sodium reabsorption ($J_{Na}+$) and transepithelial voltage (V_{te}) in CCDs from control rats and NAC-treated and –untreated PAN rats microperfused in vitro. Values are means ± SE from 4 control, 7 PAN and 8 PAN + NAC animals.* ***C.*** *mRNA expression of ASIC2b in CCDs from control rats and NAC-treated or -untreated PAN rats. Values are means ± SE from 8-9 animals. ***, p<0.001 as compared to PAN group.* ***D.*** *Immunoblot of ASIC2 in kidney cortex from NAC-treated or –untreated PAN rats. The lower part shows the quantification of ASIC2 expressed as a function of β-actin Values are means ± SE of 4 animals. *, p<0.05.*

Discussion

Sodium retention in nephrotic syndrome was initially thought to stem from the activation of the renin-angiotensin-aldosterone system secondarily to plasma volume constriction brought about by hypoalbuminemia and fluid leakage towards the interstitium [117]. However, many experimental and clinical observations have invalidated this underfill theory (reviewed in [83]) and sodium retention is now considered as independent of aldosterone. In the PAN model of nephrotic rat, sodium retention appears independent of any factor/hormone circulating in the blood stream since in a unilateral model of PAN nephrotic syndrome, only the proteinuric kidney displays sodium retention [8]. Therefore, it is now proposed that sodium retention is triggered by a substance abnormally filtered by the protein-leaky glomeruli and acting on the distal nephron. Thus, it has been shown that urine from nephrotic patients and PAN rats shows abnormally high levels of plasmin and can activate ENaC (reviewed in [118]). This is the first demonstration for the presence of a sodium reabsorption-stimulating factor in nephrotic urine. However, the pathophysiological importance of urinary plasmin must be tempered by two facts: firstly, ENaC, the described target of plasmin, is not mandatory for sodium retention, at least in PAN rats with normal aldosterone level [13], and secondly the presence of luminal plasmin can hardly account for activation of basolateral Na,K-ATPase, the motor for sodium reabsorption. In the present study, we report that intra-tubular albumin induces ROS production in the collecting duct that in turn increases Na,K-ATPase activity.

We found that albumin-induced ROS production in mCCD cells is inhibited by LY294002 and apocynin, indicating that it relies on the activity of PI3 kinase and NADPH oxidase, and that CCDs from PAN nephrotic rats display increased mRNA expression of NAPDH oxidase 2. In proximal tubule, endocytosis of albumin is mediated by megalin-cubulin, a receptor which is not expressed in the distal nephron. A recent report indicates that endocytosis of albumin in mCCD cells is mediated in part by the lipocalin receptor 24P3R [25]. In proximal tubule cells, receptor-mediated endocytosis of albumin requires the activity of PI3 kinase [119]. Albumin-induced activation of NADPH oxidase also requires the activation of a protein kinase C [120], possibly through its role on membrane targeting of p47phox [121]. Phosphorylation of ERK and/or activation of NF-κB, two targets of ROS in kidney [22, 122] may be

responsible ROS-induced activation of Na,K-ATPase, as observed in mCCD cells [28, 123].

The effect of ROS accumulation on ENaC activity seems dependent on the tissue. In lung alveolar epithelium, ROS inhibit ENaC activity [124, 125], whereas they activate it in collecting duct cell lines [33, 126]. Thus, both hyperaldosteronemia and ROS could stem for ENaC activation in CCD from PAN rats. However, the previous report that clamping plasma aldosterone to control level fully abolishes ENaC activity in CCD from PAN rats indicates that ROS accumulation is not sufficient per se to activate ENaC in PAN rats. The decrease activation of ENaC observed in NAC-treated rats therefore stems likely from the low aldosterone plasma level rather than from ROS scavenging in CCD.

NAC-induced alteration of aldosterone status in PAN rats is also an original finding. The inhibitory role of ROS scavenging on aldosterone secretion may stem from two complementary actions of ROS. Firstly, angiotensin II increases ROS level in several cell types including renal mesangial cells [127], cardiac fibroblasts [128], vascular smooth muscles [129] and adrenal cortical cells [116]. In the latter, angiotensin II-induced H_2O_2 production stimulates the activity of CYP11B2 (the aldosterone synthase) and aldosterone production. Secondly, ROS also stimulates the renal renin-angiotensin system [120]. This last effect might account for the paradoxical stimulation of the renin-angiotensin-aldosterone system observed in PAN nephrotic rats.

CCDs from NAC-treated PAN rats differ from those of aldosterone-clamped PAN rats in that they do not display an increased activity of Na,K-ATPase. However, despite this absence of stimulation of both ENaC and Na,K-ATPase, they nonetheless display a high rate of sodium reabsorption. As in aldosterone-clamped PAN rats, the absence of ENaC is likely palliated by the expression of the variant of ASIC2 previously characterized (manuscript in preparation). It is noteworthy that this channel was found not only at the apical pole of principal cells but also of intercalated cells (manuscript in preparation). In the latter, ASIC2-mediated sodium reabsorption might be energized by the V-type H-ATPase rather than Na,K-ATPase, as recently reported for electrogenic thiazide-sensitive sodium transport in B-intercalated cells [130].

In summary, this study shows for the first time that endocytosis of albumin abnormally present in the distal nephron of nephrotic rats triggers oxidative stress that in turn activates Na,K-ATPase activity and participates in sodium retention. It also suggests that ROS production in kidney cells and/or in adrenal glands triggers aldosterone secretion which is responsible for over-expression of ENaC which, in turn can be activated by luminal plasmin.

Table 1. Sequence of primers used for qPCR

	Forward	**Reverse**
ASIC 2b	5‘-ctcaccacggtcaaggg-3’	5‘-tcttggatgaaaggcggc-3’
Catalase	5’-cgagatggcacactttgaca-3’	5’-aataggagtcctcttcccaatatg-3’
αENaC	5‘-cagggtgatggtgcatggt-3’	5‘-ccacgccaggcctcaag-3’
βENaC	5‘-cataatcctagcctgtctgtttgga-3’	5‘-cagttgccataatcagggtagaag-3’
γENaC	5‘-tgcaagcaatcctgtagctttaag-3’	5‘-gaagcctcagacggccatt-3’
α_1Na,K-ATPase	5’-tcccttcaactccaccaacaa-3’	5-tttgggctcagatgcatttg-3’
Nox2	5’-agctgaacgaattgtacgtg-3’	5’-atttccaagtcataggagggt-3’
p22phox	5’-cagatcgagtgggccat-3’	5’- ctccagcagacagatgagc-3’
p40phox	5’-gcaagaacagcccattcac-3’	5’-ggtccatcagcacgcaga-3’
Rps23	5’-ctcacgcaaagggaattgt-3’	5’-caatgaagttcaagcaaccg-3’
Sod3	5’-tagcagaccggcttgac-3’	5’-gcatggctgagggctgta-3’
Txn2	5’-ggccagcctgtagtcac-3’	5’-cttgttcagcttcttgtttcctt-3’
Txnrd2	5’-catagtcatcgctacaggagg-3’	5’-cacactccagggccacataa-3’
Txnrd3	5’-gtcaagttccaaaggaaattcacc-3’	5’-ggagtcacgaccaattgc-3’

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2.3. Additional data

In the course of this study, we addressed two additional questions regarding 1) the potential role of the distal convoluted tubule (DCT) in sodium retention, and 2) the site of sodium storage in NAC-treated PAN rats which retain sodium but display reduced ascites.

2.3.1. Sodium handling in DCT from PAN nephrotic rats.

The possible role of DCTs in sodium retention was investigated *in vivo* by evaluating the natriuretic effect of thiazide, the well-known diuretic that inhibits the sodium chloride co-transporter (NCC) responsible for apical sodium entry in DCT cells, in PAN nephrotic rats. For this purpose, hydrochlorothiazide (HCTZ, 50mg/kg, IP) was injected at day 4 after PAN administration or in control rats, and urine was collected 2 hours later for determination of sodium and creatinine excretion.

As expected, HCTZ induced a significant increase in sodium excretion in control animals, and this natriuretic effect was not affected by NAC treatment (Figure 9). In contrast, the effect of HCTZ was blunted in the PAN nephrotic rats, suggesting an inhibition of sodium reabsorption along the DCT.

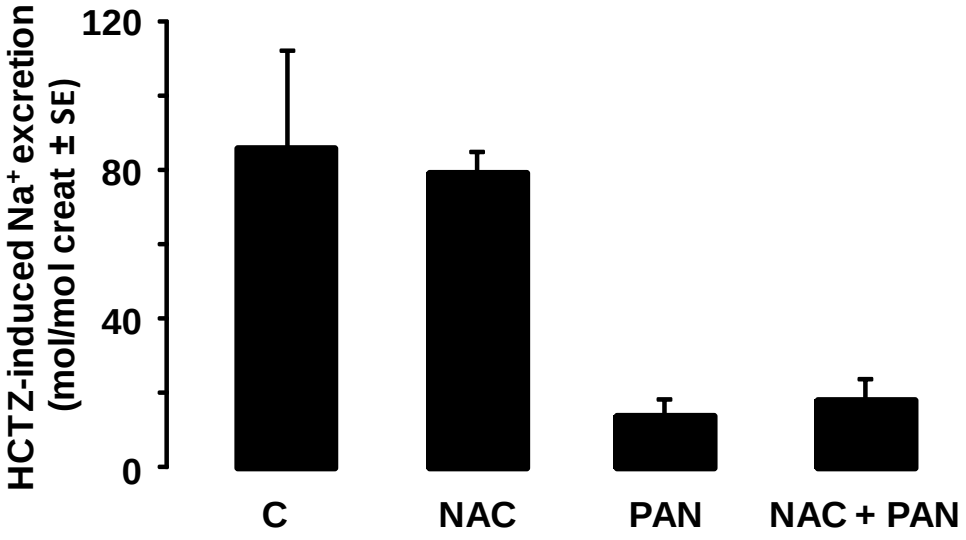

Figure 9. HCTZ-induced natriuresis in NAC-reated and –untreated control and PAN nephrotic rats. *Urine was collected during the 2 hours following administration of HCTZ (50 mg/kg). Excretion of sodium is expressed as a function of creatinine excretion. Values are means ± SE from 6 animals.*

This blunted effect of thiazide treatment in PAN nephrotic rats was associated with a significant decrease in the expression of NCC at the protein level (figure 10).

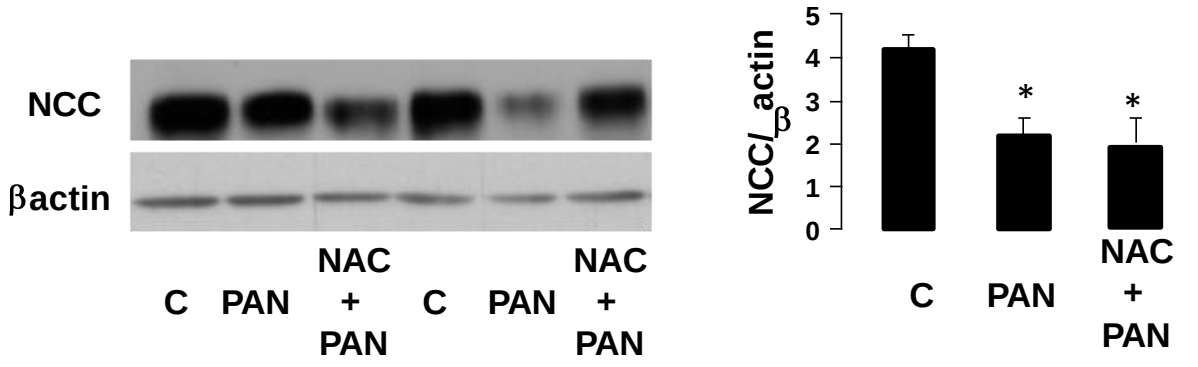

Figure 10. Expression of NCC in kidney cortex from control and NAC-treated and –untreated rats. *Immunoblot of NCC and β actin in kidney cortex from control (C) and NAC-treated or –untreated PAN rats. The right part shows the quantification of NCCexpressed as a function of β-actin Values are means ± SE of 6 animals. *, p<0.05.*

These observations may hint to the presence of a compensatory mechanism in the kidney aimed at compensating the increased reabsorption of sodium in the CCD. These changes in NCC and its possible role as a compensatory mechanism should be explored more, and a possible mechanism of cross-talk between ENaC and NCC should be taken into consideration.

2.3.1. Site of sodium storage in NAC-treated PAN rats

NAC-treated PAN rats display the same sodium retention as NAC-untreated PAN rats but they did not show ascites and edema. This raises the question of localization of the retained sodium. A first possibility would be that it is retained in the blood compartment, and this would increase blood pressure. However, we measured blood pressure in NAC-treated and untreated PAN rats and found no difference (figure 11).

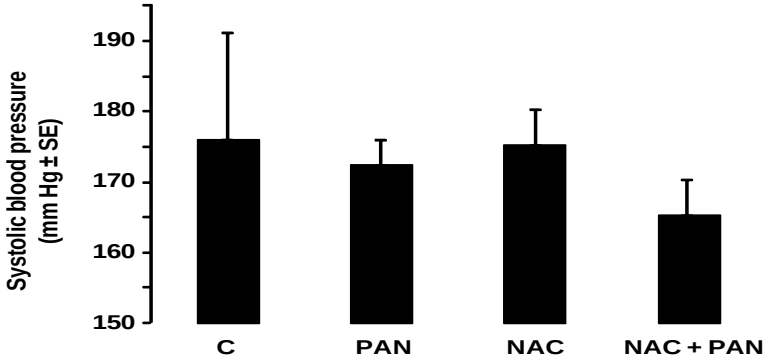

Figure 11. Systolic blood pressure in NAC-treated or –untreated control and PAN nephrotic rats. *Blood pressure was measured by the tail cuff method at day 6 after PAN injection. Values are means from 6 rats.*

It has been proposed that the skin can be an osmotically inactive site of sodium retention [95]. We therefore measured sodium content in the skin of the different groups of rats. We found that PAN rats displayed statistically significantly higher sodium content in their skin but observed no difference between NAC-treated and -untreated rats (figure 12).

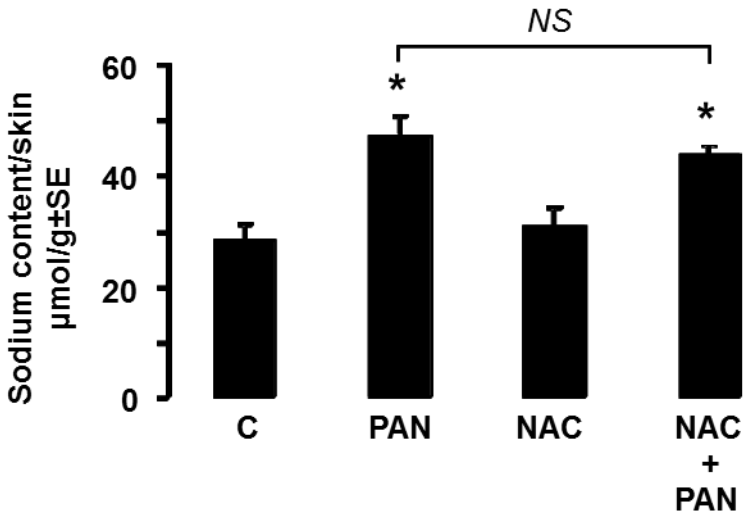

Figure 12. Sodium content in the skin. *A small piece of the skin (≈1cm2) was dissected from the back and homogenized (polytron) in 2 ml of distilled water. 100 µl of homogenate were mixed with 100 µl TCA, vortexed, kept for 1h at 4°C, centrifuge 10,000 rpm for 3 min. Sodium content of the supernatant was determined by flame photometry. Values are expressed as a function of the dry tissue weight and are means from 4-5 rats. *, p<0.005 as compared to the relevant controls.*

Thus, we have presently no answer to the last question.

Summary and perspectives

These studies provided pieces of evidence for a critical role of oxidative stress and reactive oxygen species in the hydro-electrolytic disorders associated with nephrotic syndrome. In the kidney, endocytosis of abnormally filtered albumin in the distal nephron induces an oxidative stress which is responsible for the up-regulation of Na,K-ATPase. In the peritoneum, an oxidative stress, the origin of which has not been characterized, increases water permeability and decreases the reflection coefficient to albumin, and thereby accounts for two third of ascites. Finally, in the adrenal glands, oxidative stress participates in the angiotensin-stimulated secretion of aldosterone. ROS scavenging drastically reduced the volume of ascites and the hyper-aldosteronemia whereas it did not alter renal retention of sodium indicating that oxidative stress has a causal role in the alterations of the peritoneal filtration barrier but not in those of kidney sodium transport. These results also raise numbers of questions which were not addressed.

Regarding the alterations of the peritoneal filtration barrier, the main three questions are: 1. What is the origin of oxidative stress, 2. What are the molecular targets responsible for the increased paracellular water permeability, and 3. What modulates these targets beyond ROS-dependent activation of NF-κB.

As discussed in the manuscript, decreased albuminemia may trigger oxidative stress in the plasma, owing to the ROS scavenging role of albumin, but not in peritoneal cells. A candidate for coupling hypo-albuminemia to intracellular oxidative stress might be sphingosine 1 phosphate (S1P), a circulating and signaling metabolite of ceramide [96]. Firstly, it has been shown that decreased albuminemia increases endothelial water permeability by decreasing the delivery of S1P from red blood cells to the endothelium of microvessels [97]. Secondly, inhibition of sphingosine kinases, the S1P-generating enzymes, induces ROS [98]. Thus, hypoalbuminemia may reduce S1P availability and thereby promote ROS formation which in turn activate NF-□B and increase water permeability. This hypothesis could be tested experimentally by treating PAN rats with S1P receptor agonists or antagonists of S1P lyase (reviewed in [96]), the enzyme that degrades S1P.

Claudins are main effectors of paracellular permeability, and in the peritoneum, claudin 5 limits water permeability [99]. We could therefore evaluate the expression and localization of claudin 5 in the peritoneum of nephrotic rats.

We found that ROS-induced activation of NF-κB is responsible for the changes in water permeability. AQP1, which is the effector of increased trans-cellular water permeability, is not a direct target of NF-κB. Therefore its induction requires an intermediate

NF-□B-induced mediator. A possible candidate is cyclooxygenase 2 (COX2), a gene target of NF-κB [100]. Prostaglandin E2, a product of COX2, increases the microvascular permeability [101] and activation of COX2/PGE2 pathway was reported to increase AQP1 mRNA expression in human umbilical vein endothelial cells [102]. This hypothesis could be tested by treating nephrotic rats with an inhibitor of PGE2 synthesis like indomethacin.

Regarding renal sodium retention, our results showed that endocytosis of albumin is responsible for the up-regulation of Na,K-ATPase, but that this effect is not a pre-requisite for sodium retention. They also confirm that in absence of hyper-aldosteronemia, ENaC is no longer up-regulated but that its absence is compensated by the expression of a variant of ASIC2. These findings raise several questions including: 1. In absence of up-regulation of Na,K-ATPase, what system energizes increased sodium reabsorption, 2. Does albuminuria play a role in the stimulation of sodium transport, 3. If so, why does normal sodium transport resumes 7-8 days after PAN injection when proteinuria remains massive.

As discussed in the manuscript, sodium reabsorption in CCD from NAC-treated nephrotic rats may originate from both principal and intercalated cells, where ASIC2 has been localized. In principal cells it may be energized by residual Na,K-ATPase whereas in intercalated cells it may be energized by H-ATPase. This hypothesis could be tested *in vitro* by evaluating the effect of ouabain and bafilomycin A, which inhibit Na,K-ATPase and H-ATPase respectively, on sodium transport in microperfused CCDs.

To evaluate the causal role of luminal albumin on sodium transport, one might evaluate the effect of PAN treatment on sodium transport in analbuminemic Nagase rats [103]. These rats develop proteinuria in response to PAN administration but whether or not they retain sodium and develop ascites has not been evaluated.

Regarding the reversal of sodium retention in presence of sustained proteinuria, a possible explanation is that the expression of albumin endocytosis receptor 24p3R is down regulated by TGFβ, itself triggered by albumin endocytosis [27]. This hypothesis could be tested by evaluating albumin endocytosis and 24p3R expression in rats CCDs 2 weeks after PAN injection.

The fact that NAC treatment prevents ENaC and Na,K-ATPase over-expression but does not reduce sodium reabsorption in CCD and sodium retention raises a more general question: is there some advantage for nephrotic rats to adapt and find a way to retain sodium. A possible answer is that sodium retention protects against the hypovolemia stemming from fluid leakage from the blood compartment towards the interstitium. This hypothesis reverses the paradigm of sodium retention in nephrotic syndrome: it is not triggered by hypovolemia,

in accordance with the underfil theory, but it develops to prevent hypovolemia. In other words, it would not be part of a feedback regulatory loop but would participate in a feed-forward regulation process.

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