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**Regulation of the renal Na<sup>+</sup>/H<sup>+</sup> exchanger by factors  
associated with diabetes mellitus**

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# Index of contents

<b>1 SUMMARY</b> .....	<b>1</b>
<b>2 ZUSAMMENFASSUNG</b> .....	<b>3</b>
<b>3 INTRODUCTION</b> .....	<b>5</b>
3.1 DIABETES MELLITUS.....	5
3.2 DIABETIC NEPHROPATHY .....	6
3.3 NEPHROTIC SYNDROME .....	7
3.3.1 <i>Proteinuria</i> .....	8
3.4 RENAL SODIUM TRANSPORT .....	9
3.4.1 <i>Na<sup>+</sup>/H<sup>+</sup> -Exchanger</i> .....	11
3.4.2 <i>NHE3</i> .....	14
3.5 CELL AND ANIMAL MODELS .....	17
3.5.1 <i>Opossum kidney (OKP) cells</i> .....	17
3.5.2 <i>The streptozotocin-induced diabetic model</i> .....	18
3.5.3 <i>Puromycin aminonucleoside-induced nephrosis</i> .....	19
3.6 INTRODUCTION TO METHODS USED .....	20
3.6.1 <i>Measurement of the Na<sup>+</sup>/H<sup>+</sup> exchange activity</i> .....	20
3.6.1 <i>Biotinylation assay</i> .....	21
<b>4 AIMS OF THE PROJECT</b> .....	<b>23</b>
<b>5 RESULTS</b> .....	<b>24</b>
5.1 INSULIN ACTIVATES THE NA <sup>+</sup> /H <sup>+</sup> EXCHANGER (NHE3): BIPHASIC RESPONSE AND GLUCOCORTICOID-DEPENDENCE .....	24
5.2 ALBUMIN REGULATES THE NA <sup>+</sup> /H <sup>+</sup> EXCHANGER 3 (NHE3) IN OKP CELLS.....	43
5.3 REGULATION OF THE PROXIMAL TUBULAR SODIUM/PROTON EXCHANGER NHE3 IN RATS WITH PUROMYCIN AMINONUCLEOSIDE (PAN) INDUCED NEPHROTIC SYNDROME .....	66
5.4 ACUTE AND CHRONIC EFFECTS OF STREPTOZOTOCIN INDUCED DIABETES ON THE REGULATION ON NA <sup>+</sup> /H <sup>+</sup> EXCHANGER NHE3 IN RAT PROXIMAL TUBULES .....	85
<b>6 DISCUSSION</b> .....	<b>103</b>
6.1 IN VITRO VERSUS IN VIVO MODELS .....	103
6.2 INSULIN ACTION ON NHE3 .....	104
6.3 ALBUMIN ACTION ON NHE3 .....	108
6.4 EFFECT OF PROTEINURIA ON NHE3 .....	110
6.5 EFFECT OF STZ-INDUCED DIABETES ON NHE3 .....	112
6.6 REMARKS ABOUT THE ANIMAL MODELS .....	114
<b>7 REFERENCES</b> .....	<b>115</b>
<b>8 ABBREVIATIONS</b> .....	<b>126</b>
<b>9 CURRICULUM VITAE</b> .....	<b>127</b>

## 1 Summary

The subject of this thesis was to examine the role of the factors associated with diabetes mellitus on the regulation of renal  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in both a cell culture and animal model. Diabetes has become the most common single cause of end stage renal disease in western world. Diabetic nephropathy in its early stage is characterized by the alteration of glomerular filtration, increased tubular sodium and water reabsorption and systemic volume expansion. The mechanisms that initially trigger this renal alterations are insufficiently understood. The proximal tubular sodium/proton exchanger NHE3 is responsible for most of the renal salt and water reabsorption from the glomerular ultrafiltrate and may be a potential mediator in a diabetic milieu to induce the functional and structural changes present in diabetic nephropathy. In order to examine the role of NHE3 in diabetic nephropathy, the effects of insulin, albumin and hydrocortisone in opossum kidney OKP cells, as well as the effects of diabetes mellitus and proteinuria *in vivo* on NHE3 was examined.

Insulin is an important regulator of renal salt and water excretion. One of the target proteins of insulin in the kidney is NHE3. Our experiments have shown that insulin activates the  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in OKP cells in a time- and concentration-dependent manner. This effect is biphasic, with mechanisms that involve increased activity of NHE3 on the cell surface followed by increased NHE3 transcript and total cellular and surface NHE3 protein. The insulin-stimulated increase in NHE3 is amplified by the presence of glucocorticoids.

Albuminuria is one of the hallmarks of renal dysfunction and a hallmark of diabetic nephropathy. Performed experiments have shown that albumin activates NHE3 in a time- and dose-dependent manner. The mechanism of the increase in apical membrane NHE3 activity includes three temporally distinct components. In the early phase of stimulation (6-12h), only NHE3 activity was increased. At 24 h, enhanced NHE3 activity was accompanied by an increase in cell surface NHE3 protein and NHE3 mRNA amount. The increase in surface NHE3 was due to a higher percentage of NHE3 protein reinserted from intracellular compartments into the cell membrane. At 48 h, total cell NHE3 protein amount was increased as well. The effects of albumin on NHE3 were enhanced by hydrocortisone.

The effect of proteinuria *in vivo* on NHE3 was investigated in rats with puromycin aminonucleoside (PAN)-induced nephrotic syndrome. We demonstrated that proteinuria in rats activates the Na<sup>+</sup>/H<sup>+</sup> exchanger in proximal tubule cells. Immunohistochemistry with a polyclonal antibody, which distinctively recognizes the megalin-associated storage pool of NHE3, revealed a decrease in NHE3 amount in PT cells of nephritic rats. In contrast, immunoreactivity for a monoclonal antibody, which specifically recognizes the non-megalin-associated, transport-competent pool of NHE3, was higher in PAN-treated rats than in controls, indicating that the activation of NHE3 is associated with a shift of NHE3 from an inactive, subapical pool to an active pool in the BBM of proximal tubule cells.

Regulation of the proximal tubular Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 was investigated in rats with streptozotocin (STZ) induced diabetes mellitus. STZ-induced diabetes in rats activates Na<sup>+</sup>/H<sup>+</sup> exchange in the proximal tubule. Inhibition experiments demonstrate that NHE3 is the responsible exchanger isoform for this effect. The change in activity is not followed by changes in NHE3 protein and mRNA amount and therefore implicates posttranslational regulation of NHE3.

In summary, the experiments performed in these studies suggest involvement of the proximal tubular Na<sup>+</sup>/Na<sup>+</sup> exchanger NHE3 in a diabetic milieu. With regard to salt and water retention in nephrotic syndrome, activation of NHE3 may even be of pivotal pathogenic importance.

## 2 Zusammenfassung

Im Rahmen der vorliegenden Dissertationsarbeit wurde die Regulation des renalen Natrium/Protonen-Austauschers NHE3 in einem diabetischen Milieu im Zellkultur- und Tiermodell untersucht. Diabetes ist die häufigste Ursache für die terminale Niereninsuffizienz (ESRD) in der industrialisierten Bevölkerung. Die diabetische Nephropathie im frühem Stadium ist durch veränderte glomeruläre Filtration, erhöhte tubuläre Natrium- und Wasser-Rückresorption sowie systemische Volumenexpansion gekennzeichnet. Die Mechanismen für die Auslösung dieser renalen Veränderungen sind zur Zeit noch ungenügend verstanden. NHE3, die renal tubuloapikale Isoform des  $\text{Na}^+/\text{H}^+$ -Austauschers (NHE), ist für den grössten Teil der renalen Salz- und Wasser-Rückresorption aus dem glomerulären Ultrafiltrat verantwortlich. Deshalb spielt NHE3 bezüglich den Funktions- und Strukturveränderungen, die während diabetischer Nephropathie auftreten, eine potentiell wichtige Vermittlerrolle. Um die Stellung von NHE3 in der Pathogenese der diabetischen Nephropathie zu untersuchen, wurde zuerst in Opossum Nieren (OKP) Zellen der Einfluss von einigen das diabetische Milieu definierenden Faktoren, wie Insulin, Albumin und Hydrokortison, auf den renalen  $\text{Na}^+/\text{H}^+$ -Austauscher NHE3 untersucht.

Insulin ist ein wichtiger Regulator der renalen Salz- und Wasserausscheidung. Eines der Zielproteine der Insulinwirkung in der Niere ist NHE3. In Rahmen dieser Arbeit wurde ermittelt, dass NHE3 in Abhängigkeit von der Zeitdauer der Insulinbehandlung von OKP-Zellen unterschiedlich reguliert werde: In der Frühphase der Insulinbehandlung war die NHE-Aktivität isoliert erhöht, während später eine Erhöhung des totalen und des auf der Zelloberfläche exprimierten NHE3-Proteins sowie eine Vermehrung der NHE3 mRNA Menge folgten. Die Resultate zeigen, dass Insulin die renale tubuläre NHE3 Aktivität mittels eines zweiphasigen Mechanismus anregt, der eine posttranslationale Modifikation sowie eine Erhöhung der NHE3 Genexpression einbezieht. Dieser Effekt von Insulin auf die NHE3-Regulation wird durch Hydrokortison verstärkt.

Die Exposition gegenüber erhöhten Albumin-Konzentrationen ist der zweite Faktor der im Zellkultur-Modell untersucht wurde. Albumin aktiviert den  $\text{Na}^+/\text{H}^+$ -Austausch in zeit- und konzentrationsabhängiger Weise. In der frühen Phase der Albuminexposition (6-12h) wurde nur die Aktivität von NHE3 erhöht. Nach 24 h Behandlung wurde diese erhöhte Aktivität des

NHE3 von einer Zunahme der NHE3-Proteinmenge auf der Zelloberfläche begleitet. Nach 48h waren totales zelluläres NHE3-Protein, wie auch die NHE3 mRNA erhöht. Die Zunahme des Oberflächen NHE3-Proteins beruht auf einer Verschiebung von NHE3-Molekülen aus intrazellulären Speichern zur Zellmembran. Hydrokortison moduliert die NHE3-Regulierung durch Albumin über verschiedene Mechanismen.

Die Regulation von NHE3 *in vivo*, in stark proteinurischen Ratten wurde in Puromycin Aminonukleosid (PAN) induzierter Nephrose untersucht. Die nephrotischen Ratten haben erhöhte NHE-Aktivität. Immunohistochemische Untersuchungen zeigten eine Abnahme an Megalin gebundenem, aktivem NHE3 im proximalen Tubulus nephrotischer Ratten. Im Gegensatz dazu, war die Menge an Megalin freiem, transportaktivem NHE3 in nephrotischen Ratten deutlich höher als in den Kontrollratten. Diese Resultate zeigen, dass die erhöhte Natriumreabsorption bei nephrotischem Syndrom die Folge einer Aktivitätserhöhung von NHE3 ist. Diese resultiert aus einer Verschiebung respektive Transformation von inaktivem NHE3 aus intrazellulären Speichern zu aktivem NHE3 in der Bürstensaum Membran (BBM).

Die Regulation von NHE3 bei diabetischer Stoffwechsellage wurde an Ratten mit Streptozotocin (STZ)-induziertem Diabetes mellitus untersucht. Die NHE-Aktivität in BBM von diabetischen Ratten war deutlich erhöht. Um zu bestätigen dass die beobachtete Veränderung der NHE-Aktivität auf einer Aktivierung von NHE3 basiert, wurden die Inhibitor-Experimente durchgeführt. Die Änderungen in der NHE-Aktivität in diabetischen Ratten war nicht von Änderungen der NHE3-Protein oder mRNA-Menge begleitet. Es ist wahrscheinlich, dass für die beobachtete Zunahme der NHE-Aktivität die posttranslationale Regulierung von NHE3 mit der Verschiebung von NHE3 aus dem Inneren der tubulären Zelle in die apikale Membran verantwortlich ist.

Zusammengefasst sprechen die Resultate dieser Studien dafür, dass Faktoren des diabetischen Milieus die Aktivität des proximal-tubulären  $\text{Na}^+/\text{H}^+$ -Austauschers NHE3 regulieren. Bezüglich der Salz- und Wasserretention im Rahmen des nephrotischen Syndroms spielt die Aktivierung von NHE3 in der Niere möglicherweise eine entscheidende pathogene Rolle.

## **3 Introduction**

### **3.1 Diabetes Mellitus**

Diabetes mellitus (DM) is characterized by hyperglycemia and other metabolic derangements. Different pathogenic processes are involved in the development of diabetes ranging from autoimmune destruction of the  $\beta$ -cells of the pancreas to impaired insulin action. The long-term effects of diabetes mellitus include damage, dysfunction and failure of various organs, in particular the eyes, kidneys, nerves, and the cardiovascular system. Specific complications are retinopathy with eventual blindness, nephropathy that may lead to renal failure, neuropathy with risk of foot ulcers and amputation, and features of autonomic dysregulation, including sexual dysfunction (Report of Am. Diabetes Association, 2003).

Diabetes mellitus can be classified into four categories: DM type 1 and type 2, specific types of diabetes, and gestational diabetes (Report of Am. Diabetes Association, 2003; Report of WHO, 1999). Type 1 diabetes (insulin dependent or juvenile diabetes) is triggered by a cellularly mediated autoimmune destruction of the  $\beta$ -cells of the pancreas resulting in absolute insulin deficiency. It is predominantly characterized by the presence of different autoantibodies like anti-glutamic acid decarboxylase, islet cell or insulin antibodies, which confer the autoimmune processes that lead to  $\beta$ -cell destruction. Some forms of type 1 diabetes have no known etiologies and are classified as type 1, or idiopathic diabetes. Type 2 (non insulin dependent or adult diabetes) is the most common form of diabetes and is characterized by disorders of insulin action with insulin resistance. Deficient insulin action results from insufficient insulin secretion and/or diminished tissue response to insulin. Insulin resistance is defined as impaired insulin efficacy in stimulating glucose uptake by skeletal muscle and in hepatic glucose production. Specific types of diabetes mellitus are less common, and are those in which the underlying defect or disease process can be identified in a relatively specific manner. They include, for example, genetic defects of  $\beta$ -cell function or insulin action, diseases of the exocrine pancreas, infections, or drugs. Gestational diabetes is characterized by glucose intolerance resulting in hyperglycemia of variable severity with onset or first diagnosis during pregnancy.

All these types of diabetes can be characterized by either fasting hyperglycemia or elevated levels of plasma glucose during an oral glucose tolerance test. A fasting venous plasma glucose concentration of less than 6,1 mM (110 mg · dl/1) has been defined as "normal".

The diabetic milieu is associated with complex changes of insulin secretion and sensitivity, hyperglycemia and glycosylation, and angiotensin II. Moreover, catecholamines, prostaglandins, glucocorticoids, AGE's, growth factor TGF- $\beta$ , ACE-polymorphism and protein kinase C play an important role in the pathogenesis of diabetes. Insulin is the key player in the control of intermediary metabolism. It has profound effects on both carbohydrate and lipid metabolism, and significant impact on protein and mineral metabolism. Consequently, derangements in insulin signaling have widespread and devastating effects on many organs and tissues.

### **3.2 Diabetic nephropathy**

Diabetic nephropathy is a common complication of diabetes mellitus. It affects more than one third of patients with type 1 diabetes (insulin-dependent DM) (Andersen *et al.*, 1983; Harvey *et al.*, 2001), and up to a quarter of all patients with type 2 diabetes (non-insulin-dependent DM) (Ruggenti and Remuzzi, 1998). Thus, diabetic nephropathy is the major cause of end stage renal disease (ESRD) in Western countries and is associated with a high mortality. The highest incidence of end stage renal disease attributed to hyperglycemia is found in patients with type 2 diabetes, (about 25%), with a steady increase over the last years. End stage renal disease in patients with type 1 diabetes is slightly lower, with an incidence of approximately 14% (Renal US Data System, 1998; Ritz and Orth, 1999).

Multiple factors contribute to the initiation and progression of diabetic nephropathy, including genetic and racial predisposition, glycemic and other metabolic abnormalities, alterations in systemic and renal hemodynamics, and various cytokines and growth factors (Parving *et al.*, 1996). The natural history of clinically detectable diabetic nephropathy in type 1 diabetes begins with the development of microalbuminuria, defined as albumin excretion ranging from 30 to 300 mg albumin per 24 hours. Patients at this stage of incipient nephropathy develop

glomerular hyperfiltration (GFR > 150 ml/min). Overt diabetic nephropathy is characterized by persistent proteinuria (excretion > 300 mg of albumin per 24 hours) and hypertension. Accordingly, the nephrotic syndrome develops with a progressive decline in glomerular filtration rate, resulting in end stage renal disease. Histopathological changes in diabetic nephropathy begin with glomerular hypertrophy and an increase in kidney size due to the thickening of the glomerular basement membrane and expansion of the mesangial matrix. End stage renal disease is characterized by small, atrophic kidneys with diffuse glomerulosclerosis (Nathan, 1993; Nelson, 1995).

### **3.3 Nephrotic Syndrome**

Nephrotic syndrome results from a disorder that is characterized by increased glomerular permeability and a disturbance in glomerular permselectivity. It is defined by a urinary protein excretion exceeding 3,5 g per 1,73 m<sup>2</sup> of body surface area per day, accompanied by sodium retention, edema, hyperlipoproteinemia, and thromboembolic and infectious complications. Damage of the glomerular barrier is responsible for the protein leak. The glomerular basement membrane is a size- and charge-selective filter. The size-selectivity is achieved by pores in the membrane meshwork and restricts the passage of large plasma proteins. The charge selectivity is based on polyanionic glycosaminoglycans in the glomerular basement membrane, which restrict the passage of smaller charge negative plasma proteins such as albumin (Orth and Ritz, 1998).

Nephrotic syndrome is categorized as primary and secondary disorders. Examples of primary or idiopathic disorders are minimal change glomerulopathy, focal segmental glomerulosclerosis, and membranous glomerulonephritis. Secondary glomerular diseases are associated with a specific pathogenesis or are the consequence of other diseases. Diabetic nephropathy is the most common cause of nephrotic syndrome.

### 3.3.1 Proteinuria

Proteinuria is one of the hallmarks of renal dysfunction. Urinary excretion of protein is elevated in a number of kidney diseases. Glomerular proteinuria is a frequent and early sign of renal disorders. The consequences of severe proteinuria are excessive tubular reabsorption of protein, disturbances of intracellular tubular signaling, interstitial inflammatory reactions, and finally renal insufficiency (Remuzzi and Bertani, 1998).

The healthy kidney excretes small amounts of protein, usually below 150 mg/day in adult humans. Urinary proteins contain mostly albumin (40%), other constituents include plasma immunoglobulins (15%), additional plasma proteins (5%), and different kinds of tissue proteins (40%) (Dennis and Robinson, 1985). Low molecular weight plasma proteins such as  $\alpha_2$ - and  $\beta_2$ -microglobulin, and high molecular weight proteins such as albumin are reabsorbed via receptor-mediated endocytosis pathways by proximal tubules and are then delivered to lysosomes for degradation. The luminal uptake of albumin requires the presence of binding proteins such as megalin. The transport of albumin and other low-molecular-weight proteins into endocytic vesicles also depends on appropriate endosomal acidification, which is accomplished by the transport of protons through a V-type  $H^+$ -ATPase and the sodium/proton exchanger. For the supply of the required amounts of counterions, channels like the chloride channel ClC-5 are necessary to allow for the compensatory chloride influx into endosomes (Marshansky *et al.*, 1997).

Renal diseases that affect glomerular permselectivity may lead to glomerulosclerosis as a result of modifications in mesangial, glomerular, epithelial and tubular cell function, involving increased production of extracellular matrix, scarring and breaking of tubular basement membranes (Remuzzi and Bertani, 1990). Results from experimental and human studies are indicating that abnormal protein traffic through the glomerular capillary barrier is potentially toxic by activating pathways of interstitial inflammation, albeit the exact mechanism of the renal toxicity of filtered albumin is not known. Some of the proposed models of action of this intrinsic toxicity of protein over-reabsorption are associated with proximal tubular cell alteration and leak of lysosomal enzymes into the renal interstitium, triggering interstitial inflammation, with the release of lipids with chemotactic activity accumulating in the interstitium, or with up-regulation of vasoactive and inflammatory genes

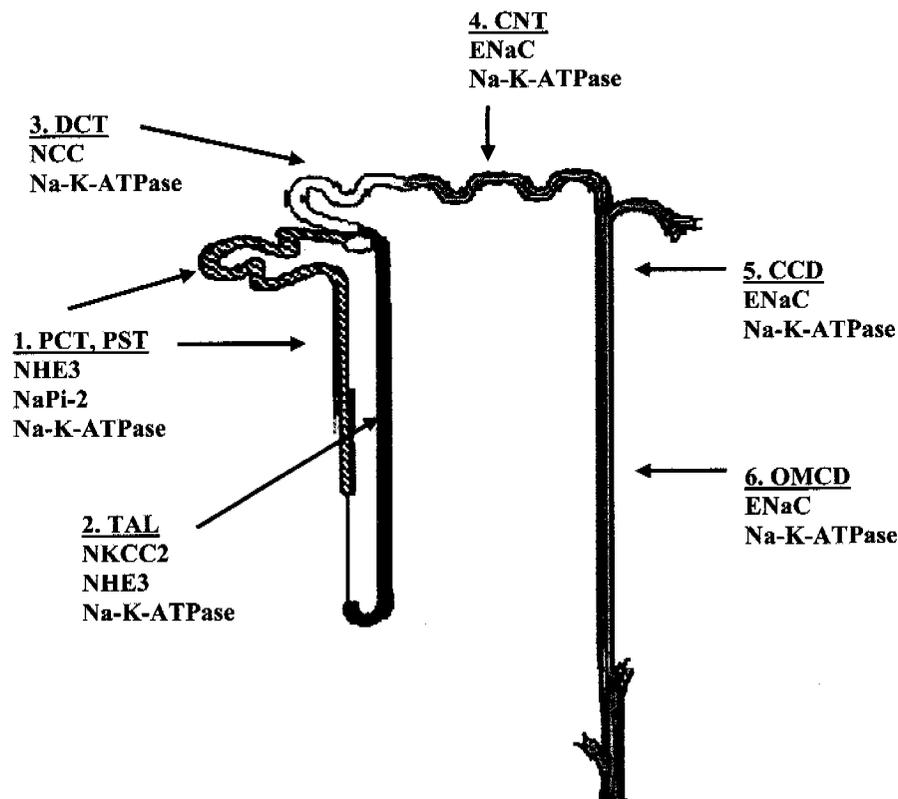
(Eddy and Giachelli, 1995; Kees-Folts *et al.*, 1994; Remuzzi, 1999; Remuzzi and Bertani, 1990).

Studies with experimental models and observations in patients with various progressive proteinuric nephropathies show that pharmacological treatment, i.e. with angiotensin-converting enzyme inhibitors (ACEi), can limit the glomerular permeability, protein traffic or the toxic effect of excessive tubular protein reabsorption, and, consecutively, prevent interstitial inflammation and slow renal disease progression (Zoja *et al.*, 2002).

### 3.4 Renal Sodium Transport

The renal tubule consists of several distinct segments, each expressing different sets of sodium transporters. The proximal tubule is responsible for the reabsorption of approximately 80% of the filtered  $\text{NaHCO}_3$  and 70% of the filtered  $\text{NaCl}$  and water load. Reabsorption is controlled by many regulatory factors, such as glomerulotubular balance, angiotensin II, endothelin, sympathetic innervation, parathyroid hormone, dopamine, acid base status and other mediators. The apical membrane  $\text{Na}^+/\text{H}^+$  exchanger NHE3 is responsible for quantitative transcellular  $\text{NaCl}$  reabsorption, and two thirds of the transcellular  $\text{NaHCO}_3$  reabsorption, as well as for 70% of  $\text{H}^+$  secretion in the proximal tubule (Alpern, 1990; Preisig *et al.*, 1987; Preisig and Rector, 1988). The rest of  $\text{H}^+$  secretion is likely mediated by a vacuolar H-ATPase. Another apical sodium transporter in proximal tubules is the sodium/phosphate cotransporter NaPi-2. At the basolateral side of the proximal tubular cells, the main transporter responsible for the maintenance of low intracellular  $\text{Na}^+$  concentration is the Na-K-ATPase. In the thick ascending limb of Henle's loop, 20 to 30% of the remaining sodium is reabsorbed via Na-K-2Cl cotransport driven by the Na-K-ATPase. The distal tubule reabsorbs 5-7% of filtered sodium through the thiazide sensitive  $\text{Na}^+-\text{Cl}^-$  cotransporter (NCC), which is also driven by the Na-K-ATPase. In the collecting duct, 3-5% of sodium crosses the luminal membrane through the epithelial amiloride sensitive sodium channel (ENaC) (Greger, 2000). The sodium transporter distribution is summarized in Figure 1. Regulation of renal sodium reabsorption is essential for maintenance of extracellular fluid volume, blood pressure, and acid/base homeostasis. Because the apical tubular  $\text{Na}^+/\text{H}^+$  exchanger reabsorbs

most of the filtered sodium load, it is likely to play a pivotal role in renal derangements of salt and volume homeostasis.



**Figure 1: Schematic representation of the nephron segments with different sodium transporters.** Adapted from (Brooks *et al.*, 2001).

Nephron segments: PCT, proximal convoluted tubule; PST, proximal straight tubule; TAL, thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD outer medullary collecting duct.

Sodium transporters: NHE3, type 3 sodium/hydrogen-exchanger; NaPi-2, sodium/phosphate transporter; NKCC2, bumetanide-sensitive sodium-potassium-chloride cotransporter; NCC, thiazide-sensitive sodium-chloride cotransporter; ENaC, epithelial sodium channel; Na-K-ATPase, sodium-potassium-ATPase.

### 3.4.1 Na<sup>+</sup>/H<sup>+</sup> -Exchanger

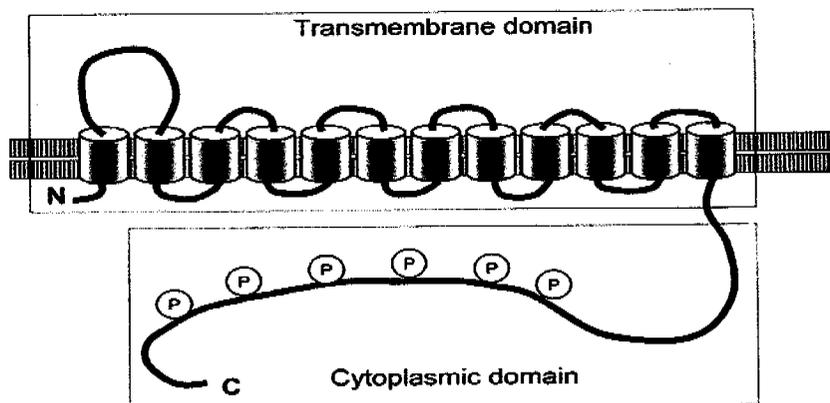
The sodium/hydrogen ion exchangers (NHE's) are a family of transmembrane proteins that mediate electroneutral transport of Na<sup>+</sup> for H<sup>+</sup> across cellular membranes. Chemical gradients of Na<sup>+</sup> and H<sup>+</sup> are the driving force of Na<sup>+</sup>/H<sup>+</sup> exchange. At neutral cytosolic pH, NHE is almost inactive. At decreasing pH, activity of the transporter gradually increases, although the sensitivity differs among isoforms. Eight currently known isoforms of the mammalian Na<sup>+</sup>/H<sup>+</sup> exchanger, NHE 1-8, mediate transepithelial sodium transport and regulate cellular and organellar pH and volume. The different NHE isoforms have distinct tissue and cellular distributions, membrane localization, pharmacological inhibitory profiles, and regulatory mechanisms and function.

NHE1 is present mainly on the basolateral membrane of epithelial cells and, as a housekeeping protein, has a broad tissue distribution (Biemesderfer *et al.*, 1992). NHE2, -3, -4 and -5 have restricted tissue distribution. NHE2 and NHE3 are apical membrane exchangers detected in small intestine, colon (NHE2, NHE3), gallbladder (NHE3), renal proximal tubule (NHE3), thick ascending limb of Henle (NHE2, NHE3), and renal distal collecting duct (NHE2) (Chow, 1999). NHE4 is basolateral transporter expressed in kidney, stomach, intestine, uterus, kidney, brain, and skeletal muscle as well as in stomach (Pizzonia *et al.*, 1998). NHE5 is found prevalently in the brain (Counillon and Pouyssegur, 2000). NHE6 and NHE7 are ubiquitously expressed proteins: NHE6 is localized in recycling endosomes, although initially suggested as being in the mitochondrial membrane, and NHE7 in the trans-Golgi network membranes (Brett *et al.*, 2002; Numata and Orłowski, 2001; Numata *et al.*, 1998). The recently cloned NHE8 is also found in kidney (Goyal *et al.*, 2003). Recently isolated isoforms NHE6-8 are still incompletely characterized with controversial findings. An overview of Na<sup>+</sup>/H<sup>+</sup> exchanger isoforms is given in Table 1.

**Table 1: Characteristics of the  $Na^+/H^+$  exchanger isoforms.** Adapted from (Chow, 1999).

Characteristics	Amino acid residues	Tissue distributions	Inhibitors: IC <sub>50</sub> (μM)
<b>NHE1</b>	815-822	Ubiquitous; basolateral	Amiloride: 1-5 EIPA: 0.02 HOE-642: 0.08 S1611: 4.7
<b>NHE2</b>	809-813	Kidney, GI tract, neuromuscular tissues; apical	Amiloride: 1-3 EIPA: 0.5 HOE-642: 1.6 S1611: 89
<b>NHE3</b>	831-834	Kidney, GI tract, other epithelial tissues; apical, subapical vesicles	Amiloride: 40-309 EIPA: 2-8 HOE-642: 100-1000 S1611: 0.05-1.03
<b>NHE4</b>	717	Stomach, collecting duct, intestine; basolateral	Amiloride: 180-813
<b>NHE5</b>	832	Brain, spleen, testis, skeletal muscle	Not known
<b>NHE6</b>	669	Ubiquitous (brain, skeletal muscle); recycling endosomes	Not known
<b>NHE7</b>	725	Ubiquitous; trans-Golgi network	Not known
<b>NHE8</b>	576	Ubiquitous (kidney, testis, skeletal muscle, liver); apical	Not known

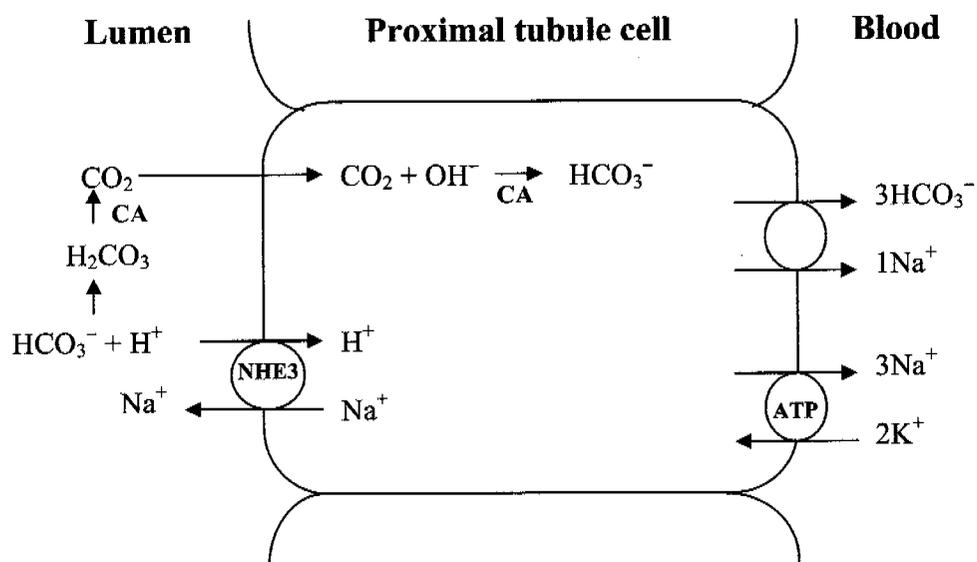
NHE 1-7 are proteins with 660-900 amino acids and share a similar primary structure with 30-60% sequence homology. The predicted molecular mass ranges from 81 to 93 kDa.  $\text{Na}^+/\text{H}^+$  exchangers contain two domains: a functional N-terminal membrane-spanning domain with 10-12 transmembrane regions, and a regulatory carboxy-terminal cytoplasmic domain. The hydrophobic N-terminal is a highly conserved part with ca. 60% homology among the isoforms and contains a core for ion transport across the membrane. The highly hydrophilic C-terminal has less similarity among isoforms (ca. 25-30%), and contains numerous phosphorylation sites that are targets for protein kinases, and binding domains for regulatory factors (Orlowski and Grinstein, 1997; Zizak *et al.*, 2000). Figure 2 shows a schematic representation of NHE.



**Figure 2:** *Proposed 3-dimensional structure of  $\text{Na}^+/\text{H}^+$  exchanger* with two domains: a functional N-terminal membrane-spanning domain with 12 transmembrane regions and a regulatory carboxy-terminal cytoplasmic domain with phosphorylation sites. Adapted from (Moe, 1999).

### 3.4.2 NHE3

NHE3 is the  $\text{Na}^+/\text{H}^+$  exchanger isoform highly expressed in the kidney. It is localized at the apical membrane of the proximal convoluted tubule and thick ascending limb (Amemiya *et al.*, 1995a). It reabsorbs a substantial part of  $\text{NaCl}$  and  $\text{NaHCO}_3$  in the proximal tubule by coupling to the parallel  $\text{Cl}^-$  and base exchanger. A schematic illustration of a tubule cell is shown in Figure 3. In the thick ascending limb, NHE3 mediates primarily  $\text{NaHCO}_3$  absorption.  $\text{H}^+$  secretion results in the regeneration of  $\text{HCO}_3^-$ , which is returned to the systemic circulation. Electroneutral transport of  $\text{Na}^+$  for  $\text{H}^+$  across cellular membranes occurs in a 1:1 stoichiometry (Moe, 1999).



**Figure 3: Schematic representation of the NHE3 dependent sodium and  $\text{HCO}_3^-$  reabsorption in the proximal tubule.** NHE3, apical sodium/hydrogen-exchanger type 3; circle with ATP, Na-K-ATPase; open circle, sodium/bicarbonate cotransporter; CA, carbonic anhydrase.

The presence of NHE3 is not restricted to the apical membrane. This exchanger is also found in intracellular vesicular compartments of the proximal tubule cells and can undergo rapid internalization and recycling back to the plasma membrane (Biemesderfer *et al.*, 1997).

Recent studies showed that the majority of NHE3 in OKP cells resides in an intracellular recycling endosomal compartment in multiple subdomains and in large heterogeneous multiprotein complexes ranging from ~400 to ~900 kDa, and/or in lipid rafts (Akhter *et al.*, 2002).

NHE3 is regulated by a large variety of hormones, growth factors and physical parameters, including osmotic and hemodynamic factors. NHE3 is stimulated by hormones such as  $\alpha$ -adrenergic agonists, angiotensin II, endothelin and insulin, and is inhibited by dopamine, parathyroid hormone and angiotensin. The accurate regulation of the exchanger activity provides the adjustments necessary for the maintenance of systemic salt and water balance. It is possible to distinguish between acute and chronic regulation of NHE3. Acute regulation of NHE3 is a result of changes in intrinsic transport activity and changes in the amount of plasma membrane NHE3 protein. Regulation of activity can result from direct phosphorylation of the exchanger. NHE3 contains different sites for phosphorylation by the protein kinases PKA and PKC. Hormones and pharmacological agents that activate cAMP-dependent protein kinase A (PKA) are potent inhibitors of NHE3, due in part to increased intracellular cAMP levels and in part to direct phosphorylation of NHE3 (Moe, 1999).

NHERF proteins, as cofactors of PKA, are necessary for cAMP regulation of NHE3. NHERF 1 (or EBP50) and E3KARP (or NHERF 2) are two  $\text{Na}^+/\text{H}^+$  exchanger regulatory factors that contain two tandem PDZ (PSD-95/Discs large/ZO-1 protein interaction) domains and a C-terminal ezrin-radixin-moesin-merlin-binding domain (Shenolikar and Weinman, 2001). It is likely that NHERF protein together with ezrin serves as anchor to bring PKA to the vicinity of NHE3 and to promote NHE3 phosphorylation. Direct phosphorylation of NHE3 inhibits  $\text{Na}^+/\text{H}^+$  exchange, for example as a response to hormones that increase intracellular cAMP (Moe, 1999; Shenolikar *et al.*, 2002). It is probable that additional regulatory proteins affect inhibition of NHE3 through PKA, such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Ahn *et al.*, 2001; Bagorda *et al.*, 2002). Some other regulatory proteins are able to interact with NHE3, like calmodulin, calcineurin B homologous protein, dipeptidyl peptidase IV (DPPIV, also known as CD26) and megalin (Biemesderfer *et al.*, 1999; Girardi *et al.*, 2001). New studies suggest another regulation pathway of NHE3 inhibition by PKA, consisting in reorganization of the actin cytoskeletal structure (Szaszi *et al.*, 2001).

The Na<sup>+</sup>/H<sup>+</sup> exchanger is regulated by H<sup>+</sup> concentration, probably through an allosteric pH-sensitive site in the cytosolic part of NHE. The pH dependence of NHE activation indicates that the rate of transport is modulated by pH alteration, by immediate protonation/deprotonation of one or more side chains of the protein. This instantaneous reaction provides rapid and accurate regulation of intracellular pH. Recently, one group reported the occurrence of a slower secondary activation of the Na<sup>+</sup>/H<sup>+</sup> exchanger, that occurs over few minutes and was unique to NHE3 and the closely related isoform NHE5, but was not observed in other NHE isoforms. They propose that NHE3 undergoes a slow pH-dependent transition from a less active to a more active state, by changing its conformation or state of association, as the activation of NHE3 was not due to changes in phosphorylation or protein amount at the cell surface (Hayashi *et al.*, 2002). An overview of NHE3 regulation is given in Table 2.

**Table 2: Acute and chronic regulation of NHE3**

Process	Change
Acute regulation	intrinsic activity <ul style="list-style-type: none"> <li>→ phosphorylation</li> <li>→ change in the rate of transport</li> <li>→ conformational change by H<sup>+</sup></li> </ul>
	surface protein expression <ul style="list-style-type: none"> <li>→ endocytosis/ exocytosis</li> <li>→ regulatory proteins (NHERF1-2, megalin, esrin)</li> <li>→ actin cytoskeleton</li> </ul>
Chronic regulation	intrinsic activity
	surface protein
	total protein
	mRNA
	transcript

Another mechanism of acute regulation of NHE3 activity is by trafficking between an intracellular recycling compartment and the apical plasma membrane. It is probable that

exocytosis as well as endocytosis are used to acutely regulate the number of NHE3 protein on the cell surface. Different hormonal agents can modify NHE3 activity on the plasma membrane by the alteration of surface NHE3 protein amount. The amount of surface NHE3 can be modulated through regulation of insertion and/or retrieval of NHE3. Agents and states that can alter surface NHE3 amount are, for example, PTH, dopamine, endothelin-1, acidosis, and pressure natriuresis. In the present work we have demonstrated that insulin and proteinuria are also able to regulate surface NHE3 amount.

The mechanisms of chronic regulation of NHE3 may involve changes in apical membrane activity, amount of surface NHE3 protein, total cell NHE3 protein synthesis and/or stability, and NHE3 mRNA amount. Chronic metabolic acidosis, chronic potassium depletion, chronic hyperfiltration and poorly controlled diabetes mellitus are associated with chronic regulation of NHE3 (Alpern *et al.*, 1995). Regulation during diabetes mellitus may be coupled with changes in factors defining the diabetic milieu such as hyperglycemia, insulin and glucocorticoids, as well as with alterations in acid base homeostasis and renal hemodynamics.

### **3.5 Cell and animal models**

#### **3.5.1 Opossum kidney (OKP) cells**

Several renal cell lines are used as physiological models to study renal transport function and regulation: LLC-PK1, a porcine renal tubular cell line, and OKP, opossum kidney cells, as model systems for the proximal tubules, and MDCK, Madin-Darby canine kidney, a canine renal tubular cell line, as model systems for the distal tubules.

OKP cells are an established epithelial cell line, derived from the kidney of adult female American opossum (Koyama *et al.*, 1978). OKP cells have renal epithelial properties such as the polarized distribution of plasma membrane proteins, the expression of an apical brush border membrane with microvilli, and the expression of transport proteins that are characteristic of the proximal tubule. There are different clonal subpopulations that are morphologically and/or functionally different from the parental cell line (Cole *et al.*, 1989;

Gomes *et al.*, 2002). The apical membrane of OKP cells contains Na<sup>+</sup>-coupled transport systems for amino acids, sugars, protons and inorganic phosphate (Malstrom *et al.*, 1987).

OKP cells are widely used for studying renal proximal tubular transport. They express an apical membrane, EIPA and amiloride-resistant Na<sup>+</sup>/H<sup>+</sup> exchanger that is encoded by NHE3 (Amemiya *et al.*, 1995b), and therefore are well suited for the investigation of the role of NHE3 in renal transport.

### **3.5.2 The streptozotocin-induced diabetic model**

Streptozotocin (STZ), a carcinogenic glucose derivative of methyl nitrosourea, is used to induce diabetes mellitus in experimental animals, in the treatment of pancreatic islet cell tumors and as broad-spectrum antibiotic in cell culture. If given in high doses, it rapidly and specifically destroys pancreatic  $\beta$ -cells with resulting insulin deficiency.

The STZ-diabetic model is widely utilized in research studies associated with diabetes mellitus type 1, indicated by the fact that more than 3000 publications can be found in the NCBI database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Streptozotocin, as a glucose derivative, is internalized through the  $\beta$ -cell's glucose transporter GLUT2. Once the molecule is inside the cell, the nitrosourea moiety is released and induces methylation of DNA and the formation of DNA strand breaks in pancreatic islet  $\beta$ -cells, followed by activation of the nuclear poly(ADP-ribose) synthetase (PARP-1). This leads to a decrease in intracellular NAD<sup>+</sup> and NADP<sup>+</sup> content and a reduction in proinsulin concentration, along with an inhibition of insulin secretion, leading to hyperglycemia and a diabetic state (Bolzan and Bianchi, 2002; Murata *et al.*, 1999; Wilson *et al.*, 1984). Beta cells of the pancreas are more active than other cells in glucose uptake, as they continuously sample blood glucose. Thus, they are more susceptible to streptozotocin toxicity as compared to other cells, what makes them a more specific target to STZ. STZ is administrated by intra peritoneal injection. Alternatively, it can be given via intra venous injection. Rats are the most commonly used animals to study STZ induced DM, but studies with mice, rabbits, hamsters, dogs and monkeys have also been performed. Rats treated with STZ not only develop hypoinsulinemia but also other structural and hemodynamic changes comparable to those observed in patients with type 1 diabetes. Diabetes in STZ rats is associated with hyperglycemia, increases in

GFR, and kidney weight, induction of albuminuria, glucosuria and polydipsia (chronic excessive thirst) (Allen *et al.*, 1997; Jensen *et al.*, 1981; Michels *et al.*, 1984).

An alternative to STZ for induction of a diabetic milieu is alloxan. This chemical substance is also an activator of diabetes mellitus, but its action is not completely specific to  $\beta$ -cells, it also affects glucagon secretion in pancreatic cells. However, new models of diabetes mellitus based on genetic defects are increasingly used, for example Goto-Kakizaki rats (spontaneously non-obese diabetic rats), the db/db mouse model (obese diabetic mice), and Zucker and Zucker diabetic fatty rats. These genetic models are developed as equivalents of type II diabetes mellitus. Genetically determined models of type I diabetes mellitus are the Bio Breeding (BB) rat, and spontaneous autoimmune diabetes in mice of the nonobese diabetic (NOD) strain.

### **3.5.3 Puromycin aminonucleoside-induced nephrosis**

Puromycin is an aminonucleoside antibiotic that acts as an aminoacyl tRNA analogue and specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes. Puromycin binds to the A site on the ribosome, forms a peptide linkage with the growing chain, and then causes its premature termination. It is used to experimentally induce nephrosis in rats and also for selecting mammalian cell lines, which have been transformed by vectors that express puromycin-N-acetyl-transferase, a puromycin resistance gene.

Puromycin aminonucleoside-induced nephrosis (PAN) is an experimental model of nephrotic syndrome. In rats, it induces heavy proteinuria, strong renal salt retention, reduced glomerular filtration rate (GFR) and structural changes characteristic of nephrotic glomerulopathy (Caulfield *et al.*, 1976; Ryan and Karnovsky, 1975). Proteinuria in PAN nephrosis is primarily due to the glomerular lesions with podocyte foot process effacement. The latter is associated with a disaggregation and rearrangement of actin filaments and induction of  $\alpha$ -actinin, albeit the precise mechanisms of podocyte damage in PAN nephrosis are not well understood (Smoyer *et al.*, 1997; Whiteside *et al.*, 1993). It remains controversial whether the protein loss through the glomerular barrier is influenced by changes in glomerular size and/or charge selectivity. The latest studies show that puromycin destroys the glomerular size barrier with minimal effects on charge density (Hjalmarsson *et al.*, 2001). PAN nephrosis is mainly

induced in rats by single intra peritoneal injection of puromycin in a dose of 150 mg/kg body weight, but induction by repeated subcutaneous injection have been described (Grond *et al.*, 1985).

Another model of nephrotic syndrome (NS) is induced in rats by a single injection of adriamycin (doxorubicin), an anticancer drug. This model of glomerulopathy is characterized by heavy and persistent proteinuria with sodium retention (Bertani *et al.*, 1982). Rats injected with anti-nephrin antibodies directed against the extracellular domain of nephrin also develop massive though transient proteinuria and are a possible model for examining the effects of proteinuria (Orikasa *et al.*, 1988; Topham *et al.*, 1999). A new genetic model of idiopathic NS is the Buffalo/Mna rat model with inherited proteinuria and focal segmental glomerulosclerotic lesions (Nakamura *et al.*, 1988). Knockout mice lacking either the CD2-associated protein or NEPH1, a novel protein structurally related to nephrin, have been found to develop congenital nephrotic syndrome (Donoviel *et al.*, 2001; Shih *et al.*, 1999).

### **3.6 Introduction to methods used**

#### **3.6.1 Measurement of the $\text{Na}^+/\text{H}^+$ exchange activity**

$\text{Na}^+/\text{H}^+$  exchanger activity was determined by the acridine orange method in rat cortical brush border membrane (BBM) vesicles and by BCECF method in OKP cells. These methods are based on the measurement of pH dependent changes in fluorescence in cell cytoplasm or in BBM vesicles. Cytoplasmic pH measurement by pH-sensitive fluorescent dyes involves mostly fluorescein, which is naturally pH sensitive and has the highest quantum yield of any fluorophore. BCECF is its most widely used derivative. Uncharged BCECF diffuses into cells where is transformed in a charged compound that is trapped in the cell. Fluorescence signal is followed in a spectrofluorimeter, with emission measurement at 530 nm and excitation measurement at 450 nm and 500 nm. Fluorescence at around 500 nm excitation is dependent on the pH of the ambient fluid and the concentration of the dye, while fluorescence at 450 nm is dependent only on the concentration of the dye. The ratio of the fluorescence intensity at these two wavelengths is independent of dye concentration and pH is only determinant (Moe and Alpern, 1996). The BCECF excitation fluorescence ratio was calibrated intracellularly by maximal acidifying cell pH using sodium-free nigericin solution.  $\text{Na}^+/\text{H}^+$  exchange was

induced by exposing cells to isotonic NaCl, providing the sodium gradient to stimulate exchanger activity.  $\text{Na}^+/\text{H}^+$  exchange activity was assayed as the initial rate of the  $\text{Na}^+$ -dependent pH increase after an acid load, calculated by drawing a tangent to the initial deflection, and results are reported as  $\text{dpHi}/\text{dt}$ .

Acridine orange is a fluorescent amine and its fluorescence signal in extravesicular solution was measured for excitation at 493 nm and emission at 530. Addition of acid-loaded vesicles and the proton gradient-driven trapping of acridine orange in the vesicles caused a rapid quenching of fluorescence.  $\text{Na}^+/\text{H}^+$  exchanger activity was then assayed as the rate of increase in fluorescence occurring in response to  $\text{Na}^+$  addition to the extravesicular space. Specific activity of the  $\text{Na}^+/\text{H}^+$  exchanger was expressed as the slope of the initial  $\text{Na}^+$ -dependent fluorescence increase divided by the initial quench. The expression of exchanger specific activity as slope/quench is independent of vesicle protein amount and allows easier comparison between studies as it is independent of fluorimeter settings (Moe *et al.*, 1991b).

### 3.6.1 Biotinylation assay

The surface and intracellular forms of NHE3 were separated by biotinylation of membrane surface proteins and subsequent affinity binding of biotinylated membrane surface proteins to avidin-agarose. This assay permits selective labeling of proteins residing at either the apical or the basolateral membrane domains of polarized epithelial cells and is used to investigate endo-, exocytosis, recycling, and transcytosis of epithelial membrane proteins. Biotin is coupled to a highly reactive N-hydroxysuccinimide ester group (NHS-SS-biotin), which can react with free amino group to form an amide bond. NHS-SS-biotin should label any surface protein that contains either an unblocked  $\text{NH}_2$ -terminal amino acid or  $\epsilon$ -amino groups of reactive lysine residues (Gottardi *et al.*, 1995). After biotinylation, lysed cells were incubated with streptavidin agarose beads. Streptavidin selectively crosslinks biotinylated proteins to the agarose beads which may be separated by centrifugation from intercellular, non-biotinylated proteins. Biotinylated proteins are then dissolved from agarose beads by  $\beta$ -mercaptoethanol and heating and quantified by immunoblotting analysis. Biotinylation was performed at 4 °C to restrict the biotin labeling to the cell surface proteins, as at the higher temperatures internalization of biotin increases (Gottardi *et al.*, 1995). Binding of the NHS-SS-biotin to the protein is dependent of pH and ionic strength of the biotinylation buffer, and as well of disposability of protein surface, which may be diminish by protein glycosilisation. Although

the efficiency of biotinylation of the NHE3 in the present studies is not known, there are 11 lysine residues located on the putative extracellular surfaces of NHE3, and these lysine residues are theoretically able to react with biotin (Yip *et al.*, 1997). Some other successfully investigated renal membrane proteins by biotin labeling are for example aquaporin-2 (AQP2), Na-K-ATPase, and epithelial sodium channel (ENaC) (Fushimi *et al.*, 1997; Muth *et al.*, 1998).

## 4 Aims of the project

Diabetes mellitus is the leading cause of end-stage renal disease in industrialized countries. Early characteristics of diabetic nephropathy are alteration of glomerular filtration, increased tubular sodium and water reabsorption and systemic volume expansion (American Diabetes Association, 2003). These factors may be a major cause for the development of hypertension, continued hyperfiltration and renal hypertrophy. Understanding the factors that regulate renal sodium handling in diabetes may provide insight into the mechanisms that contribute to the progression and deterioration of renal function in diabetic patients.

Proximal tubular sodium reabsorption, mediated through the apical sodium/proton exchanger NHE3 is responsible for about 70 percent of renal salt and water handling (Greger, 2000). Therefore, the renal  $\text{Na}^+/\text{H}^+$  exchanger isoform NHE3 is a potential mediator of the functional and structural changes in diabetic nephropathy.

The purpose of this project was to examine the role of the renal  $\text{Na}^+/\text{H}^+$  exchanger in promoting functional and structural changes in diabetic nephropathy, in particular:

- The role of factors defining a diabetic milieu on the regulation of  $\text{Na}^+/\text{H}^+$  exchanger in a cell culture model. The specific effects of insulin, albumin and hydrocortisone on the regulation of the renal proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in OKP cells were investigated.
- The effects of diabetes, hyperglycemia and insulin on proximal tubular  $\text{Na}^+/\text{H}^+$  exchange and its molecular regulation in a rat model of type 1 DM. The acute and chronic effects of streptozotocin induced diabetes and of exogenous insulin treatment on  $\text{Na}^+/\text{H}^+$  exchange activity, sodium and water balance were studied.
- The interrelationship of renal albumin reabsorption and proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger activity in an animal model of proteinuria/nephrotic syndrome and the role of NHE3 in renal salt and water retention. The experiments were carried out in an established rat model of glomerular protein loss, induced by intra peritoneal administration of puromycin.

## 5 Results

### 5.1 Insulin activates the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE3): Biphasic response and glucocorticoid-dependence

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## Abstract

Insulin is an important regulator of renal salt and water excretion and hyperinsulinemia has been implicated to play a role in hypertension. One of the target proteins of insulin action in the kidney is the  $\text{Na}^+/\text{H}^+$  exchanger NHE3, a principal  $\text{Na}^+$  transporter responsible for salt absorption in the mammalian proximal tubule. The molecular mechanisms involved in activation of NHE3 by insulin have not been studied so far. In opossum kidney cells (OKP cells), insulin increased  $\text{Na}^+/\text{H}^+$  exchange activity in a time- and concentration-dependent manner. This effect is due to activation of NHE3 as it persisted after pharmacological inhibition of NHE1 and NHE2. In the early phase of stimulation (2-12h), NHE3 activity was increased without changes in NHE3 protein and mRNA. At 24 h, enhanced NHE3 activity was accompanied by an increase in total and cell surface NHE3 protein, and NHE3 mRNA abundance. All the effects of insulin on NHE3 activity, protein and mRNA were amplified in the presence of hydrocortisone. These results suggest that insulin stimulates renal tubular NHE3 activity via a biphasic mechanism involving post-translational factors and an increase in NHE3 gene expression and the effects are dependent on the permissive action of hydrocortisone.

## Introduction

Diabetes mellitus is associated with sodium and water retention and extracellular fluid volume expansion (Bank and Aynedjian, 1990; O'Hagan *et al.*, 1991). A principal site of renal salt and water reabsorption is the proximal tubule, where insulin receptors have been found in different species (Blanchard *et al.*, 1978; Nakamura *et al.*, 1983; Talor *et al.*, 1982). Insulin is present in the plasma and glomerular ultrafiltrate and is degraded in the proximal tubule (Hammerman, 1985). Several studies have provided evidence that insulin decreases urinary sodium excretion (Muscelli *et al.*, 1996; Nizet *et al.*, 1971; Saudek *et al.*, 1974; Skott *et al.*, 1991). Baum has shown that insulin directly stimulates volume absorption in rabbit proximal convoluted tubules (Baum, 1987). The stimulatory effect on the proximal tubule is associated with increased apical  $H^+$  secretion (Kubota *et al.*, 1988; Takahashi *et al.*, 1996) and ethylisopropyl amiloride-sensitive Na uptake (Gesek and Schoolwerth, 1991); findings compatible with increased apical membrane  $Na^+/H^+$  exchange activity. One postulate is that the peripheral insulin resistance may be associated with relatively preserved insulin sensitivity in the kidney and the price of hyperinsulinemia is renal NaCl retention and salt-sensitive hypertension (Reaven, 1997; Secchi, 1999).

In the mammalian proximal tubule, over 60% of the  $Na^+$  absorption is mediated by apical brush border membrane  $Na^+/H^+$  exchange. Of the 7 isoforms known to date, NHE3 is the only  $Na^+/H^+$  exchanger isoform definitively shown to be expressed in the brush border membrane of the renal proximal tubule based on antigenic (Amemiya *et al.*, 1995a; Biemesderfer *et al.*, 1993) and functional data (Choi *et al.*, 2000; Wang *et al.*, 1999; Wu *et al.*, 1996). NHE3 mediates proximal tubule transcellular NaCl absorption via coupled transport with chloride/base exchange (Aronson, 1997; Choi *et al.*, 2000; Wang *et al.*, 2001) as well as paracellular NaCl transport by lowering luminal  $[HCO_3^-]$  and elevating luminal  $[Cl^-]$  (Rector, 1983). The importance of NHE3 in sustaining extracellular fluid volume is evident by the hypovolemia and hypotension seen in NHE3 null mice (Schultheis *et al.*, 1998). Previous studies examining the effect of insulin on the proximal tubule did not specifically address the NHE3 isoform. The present study investigates the effects of insulin on the apical membrane NHE3 activity, surface protein, total protein and transcript levels in OKP cells, a cell line of the opossum kidney with proximal tubule characteristics. Since hydrocortisone has been shown to exert a permissive effect for the acid-induced activation of  $Na^+/H^+$  exchange activity

(Ambühl *et al.*, 1999), we also examined for glucocorticoid-dependence of insulin-induced activation.

## Materials and Methods

*Materials and supplies.* All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise noted as follows: acetoxymethyl derivative of 2'7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein from Molecular Probes Inc. (Eugene, Oregon, USA); NHS-ss-biotin and immobilized streptavidin from Pierce Chemical Co. (Rockford, Illinois, USA); and culture media from GIBCO BRL (Grand Island, New York, USA).

*Cell culture.* OKP cells (Cole *et al.*, 1989) were passaged in high glucose (450 mg/dl) DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Prior to study, confluent cells were rendered quiescent by incubation in serum-free media [1:1 mixture of low glucose (100 mg/dl) DMEM and Ham's F12 ±10<sup>-6</sup> M hydrocortisone] for 24 to 48 hours. Human insulin (10<sup>-6</sup> to 10<sup>-10</sup> M) was applied for the stated period of time prior to the assays.

*Measurement of intracellular pH and Na<sup>+</sup>/H<sup>+</sup> exchange activity.* Continuous measurement of cytoplasmic pH (pH<sub>i</sub>) was accomplished using the intracellularly trapped pH-sensitive dye BCECF, as described previously (Ambühl *et al.*, 1998). Cells were loaded with 10 µM acetoxymethyl ester of BCECF for 35 min at 37°C, and pH<sub>i</sub> was estimated from the ratio of fluorescence (λ<sub>ex</sub>: 500 and 450 nm, λ<sub>em</sub> 530 nm) in a computer-controlled spectrofluorometer (8000C, SLM Instruments Inc., Urbana, Illinois, USA; and a RF-5000, Shimadzu Corporation Kyoto, Japan). The BCECF excitation fluorescence ratio was calibrated intracellularly using K/nigericin as described (Alpern, 1985). Na<sup>+</sup>/H<sup>+</sup> exchange activity was assayed as the initial rate of Na<sup>+</sup>-dependent pH<sub>i</sub> increase after an acid load in the absence of CO<sub>2</sub>/HCO<sub>3</sub> and results are reported as dpH<sub>i</sub>/dt. Comparisons are always made between cells of the same passage studied on the same day. Intracellular buffer capacity was measured by pulsing with 20 mM NH<sub>4</sub>Cl. Buffer capacity β was then calculated according to the formula β = [NH<sub>4</sub>Cl]/ΔpH<sub>i</sub>. Results for control and insulin treated cells were not significantly different (34.5 vs. 34.4 mM, respectively).

*NHE3 antigen.* Cells were rinsed with ice-cold PBS three times and Dounce-homogenized in isotonic Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA) containing proteinase inhibitors (100 µg/ml PMSF, 4 µg/ml aprotinin, 4 µg/ml leupeptin). After nuclei removal (13,000 x g, 4°C, 5 min; Eppendorf 5415C, Hamburg, Germany), membranes were pelleted (109,000 x g, 4°C, 20 min; Sorvall RC M 120EX, rotor S120 AT2-0130, DuPont Company, Sorvall Products, Wilmington, Delaware, USA) and resuspended in Tris-buffered saline, and total protein content was determined by the method of Bradford. 15 µg of protein was diluted 1:5 in 5x SDS loading buffer (1 mM Tris-HCl [pH 6.8], 1% SDS, 10% glycerol, 1% 2-mercaptoethanol), size-fractionated by SDS-PAGE (7.5% gel), and electrophoretically transferred to nitrocellulose. After blocking (5% nonfat milk, 0.05% Tween-20 in PBS; 1 h), blots were probed in the same buffer with a polyclonal anti-opossum NHE3 antibody (antiserum 5683, generated against a maltose binding protein/NHE3 [aa 484–839] fusion protein) at a dilution of 1:300 (Ambühl *et al.*, 1998). Blots were washed in 0.05% Tween-20 in PBS one time for 15 min and two times for 5 min, incubated with a 1:10,000 dilution of peroxidase-labeled sheep anti-rabbit IgG, washed as above, and then visualized by enhanced chemiluminescence. NHE3 protein abundance was quantitated by densitometry (BioCapt software version 72.02s for Windows, Vilbert Lourmat, France, and Scion Image Beta 3b, 1998, Scion Corporation, Maryland, USA).

To measure plasma membrane NHE3, we used a surface biotinylation assay (Collazo *et al.*, 2000). Monolayers were rinsed with ice-cold PBS-Ca-Mg (PBS with 0.1 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>) three times. Membrane proteins were then biotinylated by incubation of cells in 1.5 mg/ml NHS-ss-biotin in 10 mM triethanolamine (pH 7.4), 2 mM CaCl<sub>2</sub>, and 150 mM NaCl for 90 min at 4°C. After labeling, plates were washed with 6 ml quenching buffer (PBS-Ca-Mg, with 100 mM glycine) for 20 min at 4°C. Cells were then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 5.0 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin), extracts were rocked for 30 min at 4°C, centrifuged at 12,000 x g at 2°C for 10 min, and the supernatant diluted to 3 mg/ml with RIPA buffer. Biotinylated proteins were then affinity-precipitated with streptavidin-conjugated agarose, released by β-mercaptoethanol, and subjected to immunoblotting with anti-NHE3 antisera as above.

*NHE3 transcript.* RNA was extracted using RNeasy (QIAGEN Inc., Valencia, California, USA). 15 µg of total RNA was size-fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes. The radiolabeled NHE3 probe was synthesized from a full-length OKP NHE3 cDNA (Amemiya *et al.*, 1995b) and the 18S probe from a 752-base *SphI/BamHI* fragment of the mouse 18S rRNA (No. 63178; American Type Culture Collection, Rockville, Maryland, USA) by the random hexamer method. Prehybridization, hybridization, and washing were performed as described previously (Ambühl *et al.*, 1998). Filters were exposed to film overnight at  $-70^{\circ}\text{C}$  and labeling was quantitated by densitometry. Changes in NHE3 abundance were normalized for changes in 18S rRNA abundance.

*Statistics.* All results are reported as means  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using ANOVA, unless stated otherwise. "n" refers to the number of plates studied.

## Results

*Insulin increases Na<sup>+</sup>/H<sup>+</sup> exchanger activity in OKP cells.* A typical tracing (Fig. 5.1.1A) shows that insulin stimulates Na<sup>+</sup>/H<sup>+</sup> exchange activity. Fig 1B shows a time course of the insulin effect. Acute incubation for 40 min does not significantly affect activity (+6%, N.S.). At 2-24 h of incubation, insulin increases Na<sup>+</sup>/H<sup>+</sup> exchange activity. This effect was dose-dependent (Fig. 5.1.1C) with a detectable effect down to 10<sup>-8</sup> M insulin and a half-maximal stimulation at around 10<sup>-7</sup> M for both the acute (2 hrs) and chronic (24 hrs) effect. OKP cells express an EIPA-resistant Na<sup>+</sup>/H<sup>+</sup> exchanger that is encoded by NHE3 (Amemiya *et al.*, 1995b). However, to exclude the possibility that the observed changes in dpH<sub>i</sub>/dt may be mediated through an effect of insulin on another NHE isoform, we performed experiments in the presence of 100 μM HOE642, which completely inhibits NHE1 or NHE2, but not NHE3. HOE642 does not affect the baseline or the insulin-stimulated Na<sup>+</sup>/H<sup>+</sup> exchange activity (Fig. 5.1.2) securing that the observed effect of insulin on Na<sup>+</sup>/H<sup>+</sup> exchange is exclusively on NHE3.

**Figure 5.1.1A**

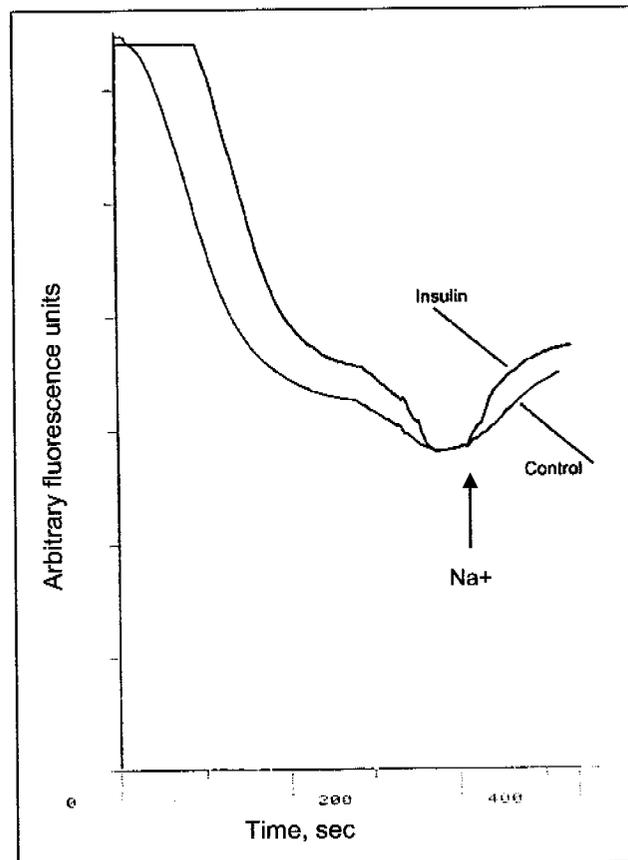


Figure 5.1.1B

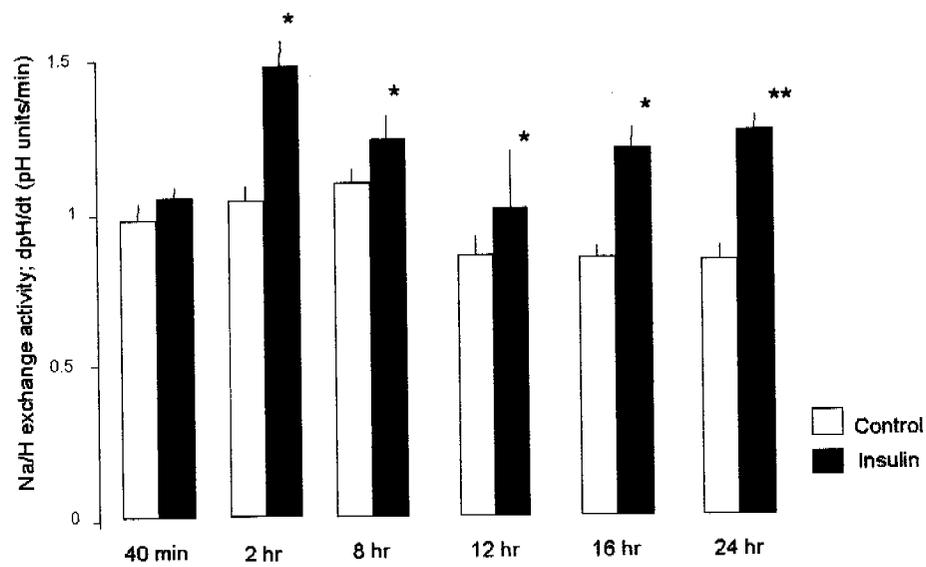
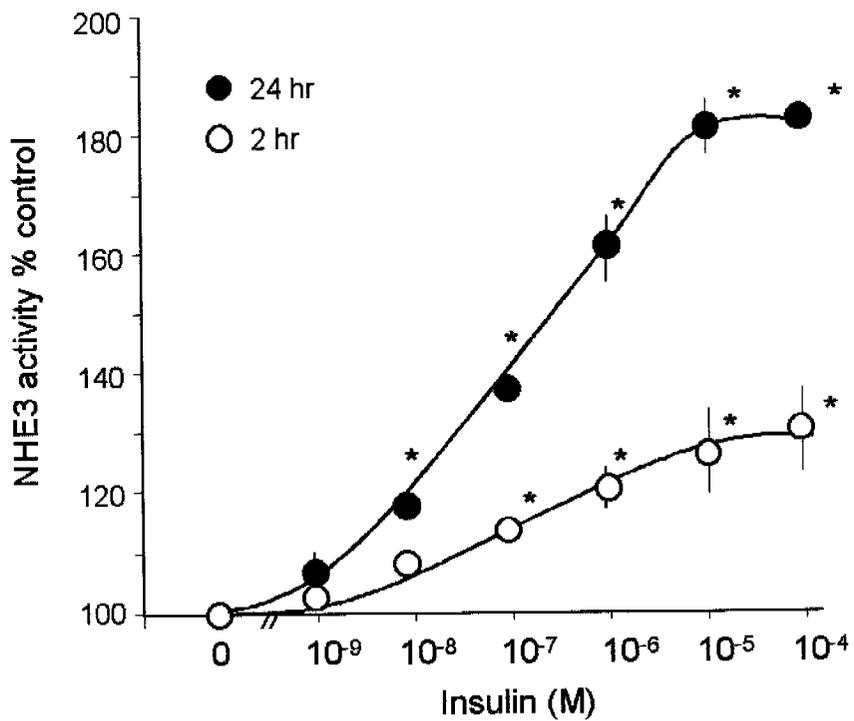
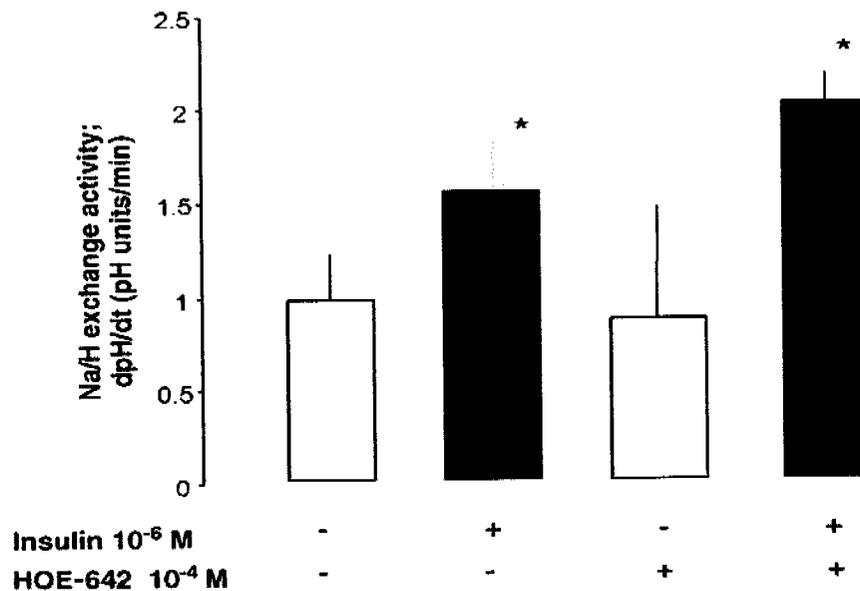


Figure 5.1.1C



**Figure 5.1.1: Effect of insulin on  $\text{Na}^+/\text{H}^+$  exchanger activity.** Cells were grown to confluence and serum deprived for 48 hours in the presence of  $10^{-6}$  M hydrocortisone and then kept in serum-deprived medium with hydrocortisone ( $10^{-6}$  M) and treated with insulin or vehicle. (A) *Representative BCECF fluorimetric tracing of control vs. insulin-treated cells ( $10^{-6}$  M x 24 hrs).* After acidification, extracellular  $\text{Na}^+$  was added (arrow) to stimulate exchanger activity. The initial slope of intracellular pH recovery represents the  $V_{\max}$  of  $\text{Na}^+/\text{H}^+$  exchanger activity. (B) *Time dependence.* Insulin ( $10^{-6}$  M) was given for 40 minutes (n=6), 2 hr (n=4), 8 hr (n=13), 12 hr (n=6), 16 hr (n=6), and 24 hr (n=13), respectively.  $\text{Na}^+/\text{H}^+$  exchange activity is expressed as  $\text{dpHi}/\text{dt}$ . Data are expressed as means  $\pm$  SEM. Unpaired t-tests of insulin vs. control: \*  $P < 0.05$ , \*\*  $P < 0.01$ . (C) *Dose dependence.* Insulin was added for 2 or 24 hrs. Results are expressed as % of controls (100%). Data are expressed as means  $\pm$  SEM. Each point represents 4-6 independent measurements. \*  $P < 0.05$  compared to control. The dose at half-maximal stimulation ( $K_{0.5}$ ) is about  $10^{-7}$  M at both 2 and 24 hrs.

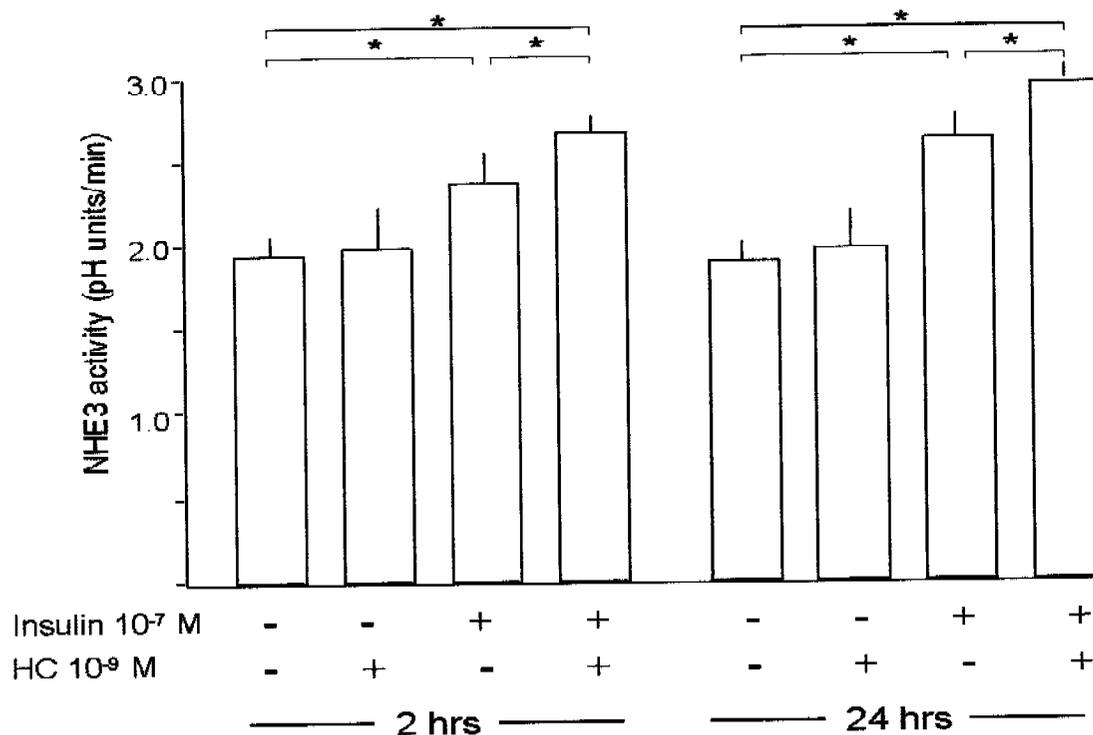
**Figure 5.1.2**



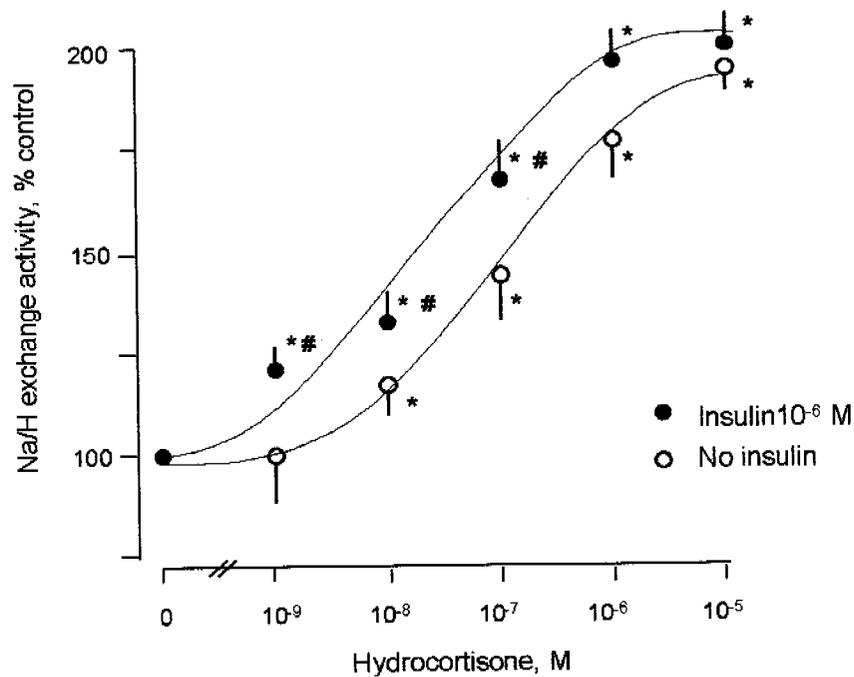
**Figure 5.1.2: Effect of insulin on  $\text{Na}^+/\text{H}^+$  exchanger activity in the presence and absence of HOE-642.** Cells were treated with insulin ( $10^{-6}$  M) for 24 h.  $\text{Na}^+/\text{H}^+$  exchange activity (expressed as  $\text{dpHi}/\text{dt}$ ) was measured in the absence or presence of  $10^{-4}$  M HOE-642.  $P < 0.01$  vs. control (n=6).

We have previously shown that the activation of NHE3 in response to chronic acid incubation requires the synergistic effect of hydrocortisone (Ambühl *et al.*, 1999). To test for glucocorticoid-dependence, we examined the interaction between insulin and hydrocortisone. Hydrocortisone was supplemented into the incubation medium during both the periods of serum deprivation (for 48 h) and insulin treatment (4-24 h). As shown in Figure 5.1.3, hydrocortisone alone ( $10^{-9}$  M) has no effect on NHE3 activity but when given with insulin ( $10^{-7}$  M), stimulated NHE3 activity to a level that is higher than insulin ( $10^{-7}$  M) alone. Likewise, insulin itself ( $10^{-7}$  M) has a small stimulatory effect on NHE3 activity but the effect is much greater in the presence of  $10^{-9}$  M hydrocortisone (Fig 5.1.3A). Figure 5.1.3B shows the effect of varying doses of hydrocortisone added to OK cells for 24 hrs with and without  $10^{-6}$  M insulin. Insulin augmented the effect of hydrocortisone from  $10^{-9}$  through  $10^{-7}$  M. At saturating dose of hydrocortisone, insulin had no further effect. These findings are in accordance with a synergistic effect between hydrocortisone and insulin on NHE3. The synergistic effect of hydrocortisone on the insulin-induced increase in  $\text{Na}^+/\text{H}^+$  exchange is only visible when hydrocortisone was added at least 24 hrs prior to insulin addition (during the period of serum deprivation). If hydrocortisone was added simultaneously with insulin, minimal or no difference is observed between the insulin vs. insulin + hydrocortisone group (data not shown).

**Figure 5.1.3A**



**Figure 5.1.3B**

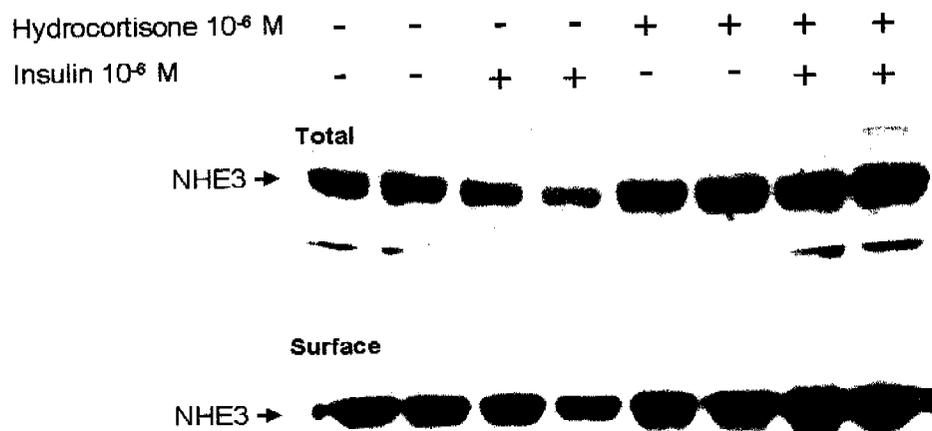


**Figure 5.1.3: Interaction of insulin and hydrocortisone on NHE3 activity.** Cells were grown to confluence and serum deprived for 24 to 48 hours in the presence or absence of hydrocortisone. Cells were then kept in serum-deprived medium +/- hydrocortisone, and treated with insulin or vehicle. NHE3 activity was measured fluorimetrically as  $\text{Na}^+$ -dependent cell pH recovery. (A) Effect of  $10^{-7}$  M insulin and  $10^{-9}$  M hydrocortisone on  $\text{Na}^+/\text{H}^+$  exchange activity. Bars and error bars are means and SEM each from 5-6 independent measurements. \*  $P < 0.05$  by ANOVA. (B) Effect of hydrocortisone on NHE3 activity: Dose-dependence. Hydrocortisone was added for 24 hrs in the absence and presence of  $10^{-6}$  M insulin. Symbols and error bars are means and SEM each from 5-8 independent measurements. \*  $P < 0.05$  by unpaired t test compared to no hydrocortisone. #  $P < 0.05$  insulin vs. no insulin.

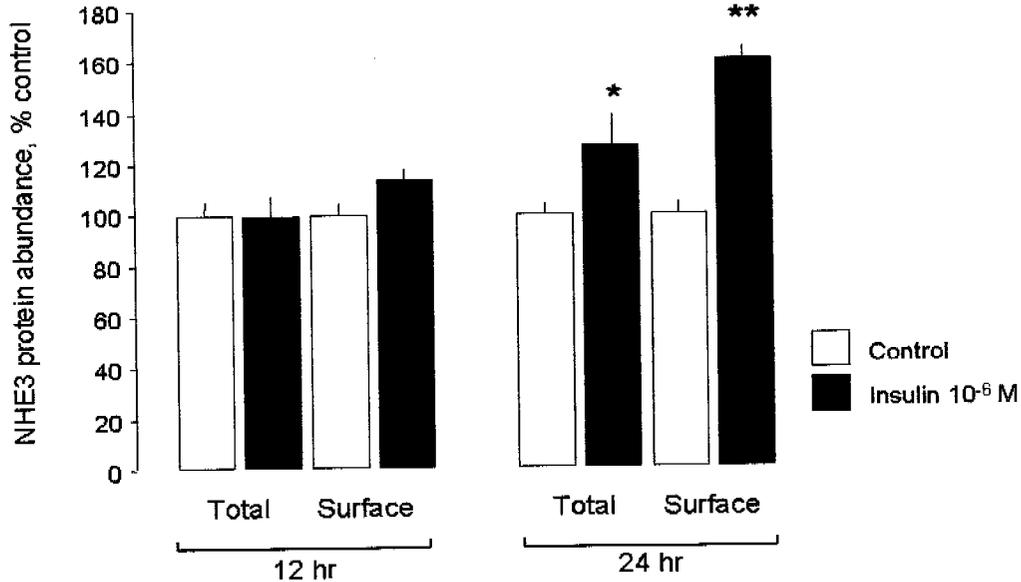
*Insulin increases total and cell surface NHE3 protein abundance.* Changes in NHE3 activity can be associated with changes in total cellular NHE3 protein and/or changes in surface plasma membrane NHE3 protein. Figure 5.1.4A shows a typical blot of the effect of insulin in

the presence or absence of  $10^{-6}$  M hydrocortisone on OKP NHE3 total and surface protein abundance. Insulin does not affect total cell or surface NHE3 protein abundance after 12 hrs when NHE activity is clearly stimulated. In contrast at 24 hrs, insulin increased total cellular NHE3 antigen by 27% surface NHE3 by 60%. The results are summarized in Figure 5.1.4B. These results indicate that the early (8-12 h) and late (24 h) stimulation of NHE is mediated by distinct mechanisms. In the absence of hydrocortisone, the increase in cellular and surface NHE3 is variable and much less pronounced (Fig 5.1.4A). In the presence of hydrocortisone, the increase in NHE3 activity observed at 24 hrs is associated with increased cell and surface antigen.

**Figure 5.1.4A**



**Figure 5.1.4B**



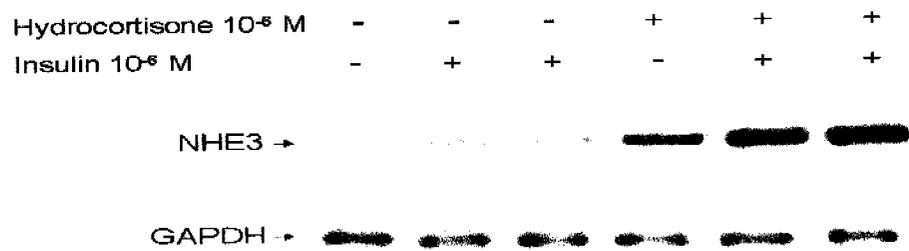
**Figure 5.1.4: Effect of insulin and hydrocortisone on NHE3 protein abundance.**

Cells were grown to confluence and serum deprived for 24 to 48 hours in the presence or absence of hydrocortisone ( $10^{-6}$  M). Cells were then kept in serum-deprived medium +/- hydrocortisone, and treated with insulin ( $10^{-6}$  M) or vehicle for 24 hrs and NHE3 protein abundance was measured by immunoblot and their relative abundance was quantified by densitometry. (A) *Representative blot*. NHE3 protein as indicated. (B) *Summary of results*. Number of experiments: 12 hr (total: n=4) and 24 hr (insulin only; n=6, insulin + hydrocortisone n=4). \*  $P < 0.05$ , \*\*  $P < 0.0001$  vs. controls.

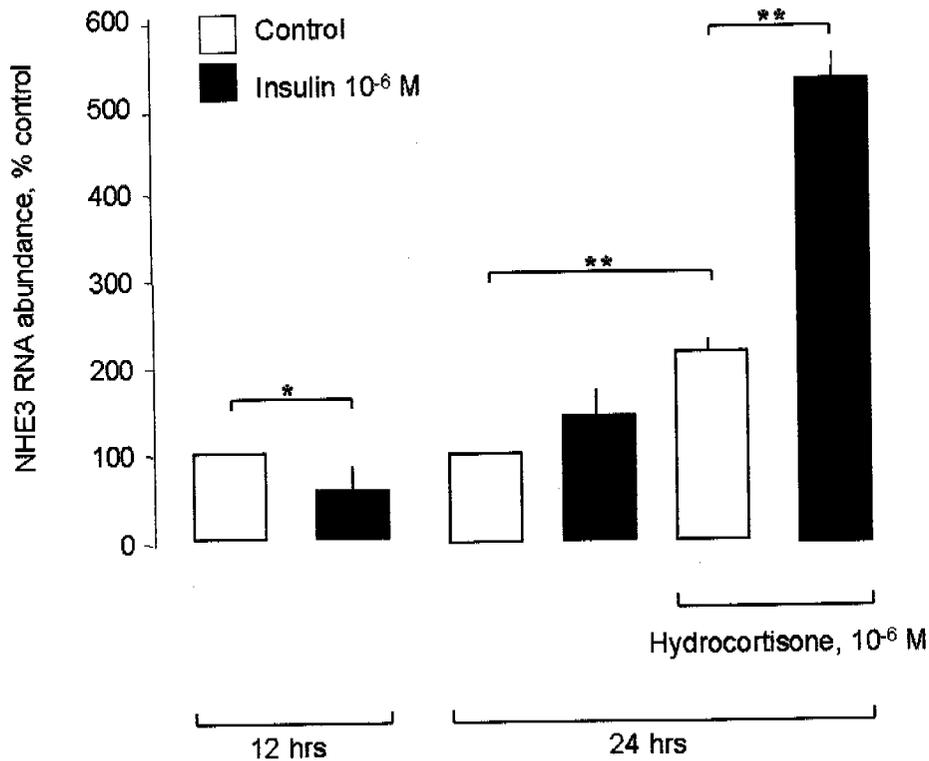
*Insulin increases NHE3 transcript.* Insulin treatment of OKP cells for 24 hrs increases NHE3 transcript abundance (Fig 5.1.5A). In contrast, insulin treatment for 12 hrs actually slightly decreases NHE3 transcript ( $P = 0.021$ ). Again, we determined the hydrocortisone-dependence of the insulin effect on NHE3 transcript level at 24 h. Insulin alone increases NHE3 transcript slightly by about 43 percent. As shown before (Ambühl *et al.*, 1999), hydrocortisone ( $10^{-6}$  M) by itself approximately doubles NHE3 transcript level. Combined treatment with insulin and

hydrocortisone results in another 2.4-fold increase in NHE3 mRNA compared to hydrocortisone alone.

**Figure 5.1.5A**



**Figure 5.1.5B**



**Figure 5.1.5: Effect of insulin and hydrocortisone on NHE3 transcript.** Cells were grown to confluence and serum deprived for 24 to 48 hours in the presence or absence of hydrocortisone ( $10^{-6}$  M). Cells were then kept in serum-deprived medium +/- hydrocortisone, and treated with insulin ( $10^{-6}$  M) or vehicle for 24 hrs and NHE3 transcript abundance was measured by RNA blot and their relative abundance was quantified by densitometry. (A) *Representative RNA blot.* (B) *Summary of results.* The graph shows the percentage of NHE3 mRNA vs. controls (n=8). \*  $P < 0.05$  vs. control; \*\*  $P < 0.002$  vs. hydrocortisone alone or control, respectively.

## Discussion

The role of insulin on renal tubular salt and water handling has been previously implicated from clinical observations (DeFronzo *et al.*, 1975; Nizet *et al.*, 1971; Saudek *et al.*, 1974) and tubule perfusion studies in animals (Baum, 1987; Kubota *et al.*, 1988; Takahashi *et al.*, 1996). The causal relation between hyperinsulinemia and hypertension is still an issue of debate (Hall *et al.*, 1995). Reaven has suggested that even in states of hyperinsulinemia, additional factors other than insulin likely contribute to the hypertension (Reaven, 1997). Although it is controversial whether hyperinsulinemia leads to salt-sensitive hypertension, the current body of data is strongly supportive of a salt-retaining action of insulin on the kidney. Insulin stimulates  $\text{Na}^+$  transporters and  $\text{Na}^+$  absorption both at the proximal tubule (Baum *et al.*, 1996; Gesek and Schoolwerth, 1991; Kubota *et al.*, 1988; Takahashi *et al.*, 1996) and thick ascending limb (Kirchner, 1988; Takahashi *et al.*, 1996).

The molecular mechanisms of insulin-induced increase in  $\text{Na}^+$  transport have not been examined. The present study demonstrates that insulin directly stimulates  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in OKP cells in a time- and concentration-dependent manner. The concentrations used in our experiments were higher than the circulating plasma levels. Therefore, we can not exclude that some of the effects on the  $\text{Na}^+/\text{H}^+$  exchanger NHE3 are mediated through the IGF-1 receptor. However, for NHE3 activity, a significant stimulation by insulin was detectable down to a concentration of  $10^{-8}$  M. The NHE1 isoform is ubiquitously expressed in the kidney (Biemesderfer *et al.*, 1992) and there is indirect evidence supporting stimulation of NHE1 by insulin in cultured renal cells (Fine *et al.*, 1985a). Although NHE2 is expressed in the kidney (Chambrey *et al.*, 1998; Sun *et al.*, 1997), its functional role is still enigmatic (Choi *et al.*, 2000) and may only be activated under certain circumstances. We have ruled out the role of both NHE1 and NHE2 in mediating the insulin-induced increase in  $\text{Na}^+/\text{H}^+$  exchange activity. We showed that insulin specifically up-regulates proximal tubule NHE3 which likely mediates the increased proximal tubule  $\text{Na}^+$  absorption in response to insulin. The stimulation of NHE3 by insulin has two characteristics. First, it occurs in a biphasic fashion. Second, it is amplified by glucocorticoids.

$\text{Na}^+/\text{H}^+$  exchangers are regulated by a wide variety of agonists through vastly different mechanisms. Regulation at the level of transcription (Ambühl *et al.*, 1998; Ambühl *et al.*,

1999; Amemiya *et al.*, 1995b; Baum *et al.*, 1996; Cano, 1996; Cano *et al.*, 1999; Kandasamy and Orłowski, 1996), translation (Wu *et al.*, 1996), protein trafficking (Akhter *et al.*, 1990; Chow *et al.*, 1999; Collazo *et al.*, 2000; D'Souza *et al.*, 1998; Fan *et al.*, 1999; Hu *et al.*, 2001; Janecki *et al.*, 2000; Janecki *et al.*, 1998; Kurashima *et al.*, 1998; Magyar *et al.*, 2000; Peng *et al.*, 2001; Yang *et al.*, 2000; Yip *et al.*, 1998; Zhang *et al.*, 1998; Zhang *et al.*, 1999), phosphorylation (Kurashima *et al.*, 1997; Peng *et al.*, 1999; Weinman *et al.*, 2000 May 23; Wiederkehr *et al.*, 2001; Wiederkehr *et al.*, 1999; Zhao *et al.*, 1999; Zizak *et al.*, 1999), binding to protein (Biemesderfer *et al.*, 2001) or lipid regulators (Aharonovitz *et al.*, 2000) have been implicated or proven. A single condition or agonist can regulate NHE3 at more than one step. This has been shown for acid incubation (Ambühl *et al.*, 1998; Ambühl *et al.*, 1999; Amemiya *et al.*, 1995b; Wu *et al.*, 1996; Yang *et al.*, 2000), parathyroid hormone (Collazo *et al.*, 2000; Fan *et al.*, 1999) and dopamine (Hu *et al.*, 2001; Wiederkehr *et al.*, 2001). The induction of NHE3 activation by insulin is time-dependent, as a significant increase in  $\text{dpH}_i/\text{dt}$  was detectable only at 2 hrs and beyond. After 12 h, NHE3 activity is clearly increased while surface NHE3 protein abundance is still unchanged in insulin treated cells. The possibility remains that the  $\text{dpH}_i/\text{dt}$  assay is more sensitive than the biotinylation assay. Alternatively, a more plausible explanation is that other post-translational mechanisms may be operative and contribute to the stimulation of  $\text{Na}^+/\text{H}^+$  exchange by insulin (Collazo *et al.*, 2000; Fan *et al.*, 1999; Moe, 1999). A biphasic response has previously been described for PTH (Collazo *et al.*, 2000; Fan *et al.*, 1999) and dopamine (Hu *et al.*, 2001; Wiederkehr *et al.*, 2001) involving changes in transport activity of surface NHE3 followed by internalization of NHE3 protein. However in those two situations, the decrease in NHE3 surface protein commences after a relatively short time. In the case of insulin, surface NHE3 activity is increased without changes in surface NHE3 protein for over 12 hrs. At present the mechanism of how insulin induces and sustains this suppression of surface NHE3 transporters is unknown. After 24 hrs of incubation with insulin, one can see a concomitant increases in surface and total NHE3 protein abundance which approximate but are not equal to the magnitude of increase in NHE3 activity. The slightly higher increase in surface NHE3 compared to total NHE3 may reflect an additional step while the increased cellular pool of NHE3 is preferentially targeted to the cell membrane. Moreover, increase in NHE3 protein is associated with an increase in NHE3 mRNA at 24 hrs. This pattern of coordinated up-regulation at the levels of activity, surface protein, total protein, and mRNA is reminiscent of the effects of thyroid hormone on NHE3 (Cano *et al.*, 1999).

The stimulation of intrinsic NHE3 activity in the early phase and the increase in NHE3 activity, protein and mRNA in the late phase are all enhanced by hydrocortisone. At  $10^{-9}$  M, where glucocorticoid itself has no effect on NHE3 activity (Baum *et al.*, 1996), glucocorticoid's presence allows insulin to exert its full action on NHE3 hence befitting the classic permissive role described by the pioneering manuscript of Ingle half a century ago (Ingle, 1952). At  $10^{-7}$  and  $10^{-8}$  M when corticosteroids themselves activate NHE3, the presence of insulin further increases NHE3 activity. At this point, hydrocortisone acts more like a biologic amplifier as discerned by (Granner, 1979). In a saturating dose of hydrocortisone ( $10^{-5}$  M), addition of insulin no longer leads to further stimulation. Whether this is synergism, permission, or amplification, the interactive relationship (both positive and negative) between glucocorticoids and a variety of other agonists is pervasive in mammalian biology (Sapolsky *et al.*, 2000). In the liver, the ability of glucocorticoids to promote hepatic glycogen synthesis is "pro-insulin" (Stalmans *et al.*, 1987). In contrast, in skeletal muscle, glucocorticoid decreases insulin's ability to stimulate glycogen synthesis (Bjorntorp, 1999). In the kidney, the acid-induced increase in  $\text{Na}^+/\text{H}^+$  exchange can be abolished by adrenalectomy (Kinsella *et al.*, 1984). We have shown that this is a direct effect of glucocorticoids because the acid-induced increase in NHE3 is dependent on the presence of hydrocortisone in the cell culture media during serum deprivation and acid incubation (Ambühl *et al.*, 1999; Hamm *et al.*, 1999). Glucocorticoids may represent a more general permissive agent for regulation of NHE3 in the kidney. The mechanism of the permissive effect of glucocorticoids is currently unknown.

In summary, we have shown that insulin activates the  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in OKP cells. This effect is biphasic in nature with distinct mechanisms that involve increased activity of existing NHE3 proteins on the cell surface followed later by increased NHE3 transcript, total cellular and surface NHE3 protein. In both phases, the insulin-stimulated increase of NHE3 is enhanced by the presence of glucocorticoids. In conjunction with data from clinical and tubule perfusion studies, we propose that insulin stimulates NHE3 and proximal tubule  $\text{Na}^+$  absorption and contributes to the volume expansion and hypertension seen in insulin resistance states.

## **Acknowledgments**

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## **5.2 Albumin regulates the Na<sup>+</sup>/H<sup>+</sup> exchanger 3 (NHE3) in OKP cells**

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## Abstract

Albumin filtered by the glomerulus is reabsorbed in the proximal tubule. We have shown previously that proteinuria stimulates the proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in nephrotic rats. Activation of NHE3 may be a pathophysiologically important factor in the development of renal salt and water retention observed in the nephrotic syndrome. In order to examine whether albumin is a specific inducer of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange and to determine the molecular mechanisms by which it regulates  $\text{Na}^+/\text{H}^+$  exchange, we examined the effect of albumin on NHE3 in opossum kidney (OKP) cells. Albumin activated  $\text{Na}^+/\text{H}^+$  exchange in a time- and dose-dependent manner up to 100% in 48 h. In the early phase of stimulation (2-12 h), NHE3 activity was increased without changes in NHE3 protein and mRNA. At 24 h, increased NHE3 activity was accompanied by an increase in cell surface NHE3 protein. At 48 h, total cell NHE3 protein abundance and mRNA was increased as well. The increase in surface NHE3 was associated with increased bi-directional trafficking of NHE3 protein between intracellular compartments and the cell surface. The effects of albumin on NHE3 protein abundance were modified by hydrocortisone in a complex pattern. These results indicate that albumin directly regulates proximal tubular NHE3 at multiple levels.

## Introduction

Albuminuria is a common manifestation of renal disease. Glomerular damage results in variable amounts of urinary protein loss and renal salt and water retention (Orth and Ritz, 1998). Permselectivity of an intact glomerulus ensures retention of most of the serum proteins in the glomerular capillary (Brenner *et al.*, 1978). However, up to 5 grams of protein per day may be filtered by the glomeruli even under normal conditions, which then are reabsorbed by the renal tubule (Maack, 2000; Straus, 1957). In a diseased kidney, substantial amounts of protein (mainly albumin) are filtered through the damaged glomeruli and into the urinary space and increasing quantities of the filtered protein are reabsorbed by the renal tubule in order to minimize renal protein loss (Blantz *et al.*, 1997). Other than a hallmark of glomerular disease, proteinuria may be an independent factor that induces and perpetuates renal damage (Remuzzi and Bertani, 1998). One theory is that enhanced tubular protein reabsorption triggers inflammation and fibrosis by induction of several cytokines and growth regulating factors such as TGF $\beta$  (Brunskill, 1998). The reabsorption of albumin by the proximal tubule is achieved predominantly by endocytosis (Maack, 2000; Straus, 1957). Several recent studies have suggested an interrelation of transcellular albumin transport by endocytosis, and acidification of lysosomes (Mukherjee *et al.*, 1997; Schmid *et al.*, 1989) through endosomal Na<sup>+</sup>/H<sup>+</sup> exchange (Gekle *et al.*, 1999; Gekle *et al.*, 1998). Besides proteinuria, the nephrotic syndrome (NS) is also accompanied by various degrees of salt and water retention and represents a major clinical problem in the management of patients with nephrosis (Palmer and Alpern, 1997a; Vande Walle and Donckerwolcke, 2001). One mechanism of salt retention is systemic interstitial volume sequestration due to hypoalbuminemia. This is unlikely to be sufficient as congenital analbuminemia is not accompanied by disturbances in volume (Russi and Weigand, 1983). An alternative but not mutually exclusive explanation is that primary renal salt retention *per se* may contribute substantially to systemic volume expansion in NS (Palmer and Alpern, 1997a; Vande Walle and Donckerwolcke, 2001). Regulation of sodium transport in the nephrotic state has been demonstrated to occur in the collecting duct through activation of the Na/K-ATPase (Deschenes *et al.*, 2001; Zolty *et al.*, 1999). However, we have shown recently, that proximal tubule NHE3, is activated in rats with puromycin aminonucleoside (PAN) induced proteinuria [chapter 5.3]. This finding suggests, that the proximal tubular Na<sup>+</sup>/H<sup>+</sup> exchange may not only be a regulator of transtubular protein reabsorption through endosomal acidification but may also be affected by tubular protein

concentration and contributes to transcellular sodium and volume reabsorption. The increase in proximal tubular  $\text{Na}^+/\text{H}^+$  exchange may be secondary to either hemodynamic factors or non-protein substances that are lost in the glomerular ultrafiltrate. The direct effects of albumin have not yet been tested.

In the mammalian proximal tubule, over 60% of the  $\text{Na}^+$  absorption is mediated by apical brush border membrane  $\text{Na}^+/\text{H}^+$  exchange. Of the 8 isoforms known to date, NHE3 and NHE8 are the only NHE isoforms definitively shown to be expressed in the brush border membrane of the renal proximal tubule based on antigenic (Amemiya *et al.*, 1995a; Biemesderfer *et al.*, 1993) and functional data (Choi *et al.*, 2000; Wang *et al.*, 1999; Wu *et al.*, 1996). NHE3 mediates proximal tubule transcellular NaCl absorption via coupled transport with chloride/base exchange (Aronson, 1997; Wang *et al.*, 2001) as well as paracellular NaCl transport by lowering luminal  $[\text{HCO}_3^-]$  and elevating luminal  $[\text{Cl}^-]$  (Rector, 1983). The importance of NHE3 in sustaining extracellular fluid volume is evident by the hypovolemia and hypotension seen in NHE3 null mice (Schultheis *et al.*, 1998). To further specify the role of proteins on proximal tubular NHE3 and to study the mechanisms by which its activity is regulated, we examined the direct effects of albumin on NHE3 in OKP cells, an opossum kidney cell line with proximal tubular characteristics. Since hydrocortisone has been shown to exert a permissive effect for the acid- and insulin-induced activation of  $\text{Na}^+/\text{H}^+$  exchange (Ambühl *et al.*, 1999; Klisic *et al.*, 2002), we also examined for glucocorticoid-dependence of albumin-induced activation.

## Methods

*Materials and supplies.* All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise noted as follows: acetoxymethyl derivative of 2'7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein from Molecular Probes Inc. (Eugene, Oregon, USA); NHS-ss-biotin and immobilized streptavidin from Pierce Chemical Co. (Rockford, Illinois, USA); and culture media from GIBCO BRL (Grand Island, New York, USA).

*Cell culture.* OKP cells (Cole *et al.*, 1989) were cultured in high glucose (450 mg/dl) DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Prior to study, confluent cells were rendered quiescent by incubation in serum-free media [1:1 mixture of low glucose (100 mg/dl) DMEM and Ham's F12 ±10<sup>-6</sup> M hydrocortisone] for 24 to 48 hours. Bovine serum albumin, fraction V, from Fluka (Buchs, St. Gallen, Switzerland), was applied for the stated period of time prior to the assays. The albumin preparation is of high purity grade, processed by the manufacturer using absorptive charcoal and extensive dialysis to reduce contamination with low molecular substances. To further increase purity, albumin was dialyzed again before use in pilot experiments. However, as the results were comparable irrespective of pretreatment albumin as provided by the manufacturer was used for the bulk of experiments.

*Measurement of intracellular pH and Na<sup>+</sup>/H<sup>+</sup> exchange activity.* Continuous measurement of cytoplasmic pH (pH<sub>i</sub>) was performed using the intracellularly trapped pH-sensitive dye BCECF, as described previously (Ambühl *et al.*, 1998). Cells were loaded with 10 µM acetoxymethyl ester of BCECF (35 min at 37°C), and pH<sub>i</sub> was estimated from the ratio of fluorescence (λ<sub>ex</sub>: 500 and 450 nm, λ<sub>em</sub> 530 nm) in a computer-controlled spectrofluorometer (RF-5000, Shimadzu Corporation Kyoto, Japan). The intracellular BCECF excitation fluorescence ratio was calibrated using K/nigericin as described (Alpern, 1985). Na<sup>+</sup>/H<sup>+</sup> exchange activity was assayed as the initial rate of Na<sup>+</sup>-dependent pH<sub>i</sub> increase (dpH<sub>i</sub>/dt) after intracellular acidification (Nigericin H<sup>+</sup>/K<sup>+</sup> exchange) in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. Comparisons were always made between cells of the same passage studied on the same day and results are reported as percent change from the dpH<sub>i</sub>/dt of the relative controls. Intracellular buffer capacity was measured by pulsing with 20 mM NH<sub>4</sub>Cl. Buffer capacity β

was then calculated according to the formula  $\beta = [\text{NH}_4\text{Cl}]/\Delta\text{pH}_i$ . Results for control and albumin treated cells were not significantly different ( $\beta = 24.2$  vs.  $26.1$  mM, respectively).

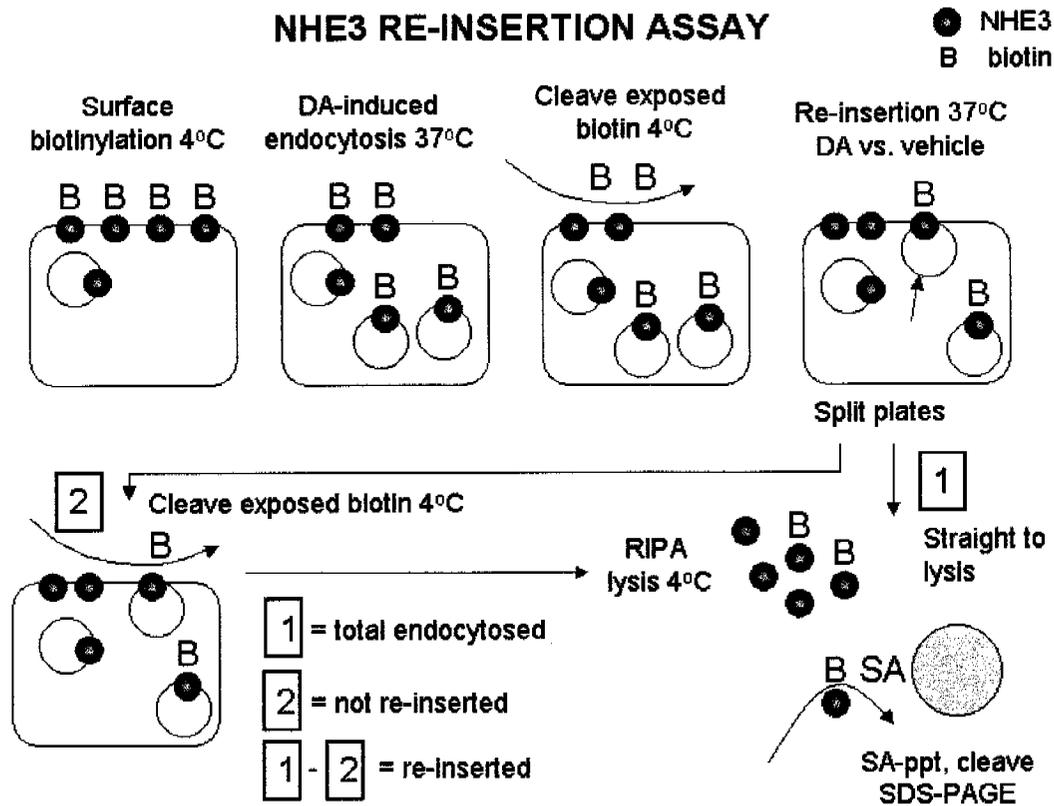
*NHE3 antigen.* Cells were rinsed with ice-cold PBS three times and Dounce-homogenized in isotonic Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA) containing proteinase inhibitors (100  $\mu\text{g}/\text{ml}$  PMSF, 4  $\mu\text{g}/\text{ml}$  aprotinin, 4  $\mu\text{g}/\text{ml}$  leupeptin). After nuclei removal (13,000  $\times$  g, 4°C, 5 min; Eppendorf 5415C, Hamburg, Germany), membranes were pelleted (109,000  $\times$  g, 4°C, 20 min; Sorvall RC M 120EX, rotor S120 AT2-0130, DuPont - Sorvall, Wilmington, Delaware, USA) and resuspended in Tris-buffered saline, and total protein content was determined by the method of Bradford. 15  $\mu\text{g}$  of protein was diluted 1:5 in 5x SDS loading buffer (1 mM Tris-HCl [pH 6.8], 1% SDS, 10% glycerol, 1% 2-mercaptoethanol), size-fractionated by SDS-PAGE (7.5% gel), and electrophoretically transferred to nitrocellulose. After blocking (5% nonfat milk, 0.05% Tween-20 in PBS; 1 h), membranes were probed in the same buffer with a polyclonal anti-opossum NHE3 antibody (antiserum #5683, generated against a maltose binding protein/NHE3aa 484–839 fusion protein) at a dilution of 1:300 (Ambühl *et al.*, 1998). Blots were washed in 0.05% Tween-20 in PBS one time for 15 min and two times for 5 min, incubated with a 1:10,000 dilution of peroxidase-labeled sheep anti-rabbit IgG, washed as above, and then visualized by enhanced chemiluminescence. NHE3 protein abundance was quantitated by densitometry (BioCapt software version 72.02s for Windows, Vilbert Lourmat, France, and Scion Image Beta 3b, 1998, Scion Corporation, Maryland, USA).

*Surface biotinylation assay.* To measure plasma membrane NHE3, we used a surface biotinylation assay (Collazo *et al.*, 2000). Monolayers were rinsed with ice-cold PBS-Ca-Mg (PBS with 0.1 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ ) three times. Membrane proteins were then biotinylated by incubation of cells in 1.5 mg/ml NHS-ss-biotin in 10 mM triethanolamine (pH 7.4), 2 mM  $\text{CaCl}_2$ , and 150 mM NaCl for 90 min at 4°C. After labeling, plates were washed with 6 ml quenching buffer (PBS-Ca-Mg, with 100 mM glycine) for 20 min at 4°C  $\times$  2. Cells were then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 5.0 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 100  $\mu\text{g}/\text{ml}$  PMSF, 5  $\mu\text{g}/\text{ml}$  aprotinin, and 5  $\mu\text{g}/\text{ml}$  leupeptin), extracts were rocked for 30 min at 4°C, centrifuged at 12,000  $\times$  g at 2°C for 10 min, and the supernatant diluted to 3 mg/ml with RIPA buffer. Biotinylated proteins were then affinity-precipitated with streptavidin-conjugated agarose, released by  $\beta$ -mercaptoethanol, and subjected to immunoblotting with anti-NHE3 antisera as above.

*Endo-and exocytosis assay.* Measurement of NHE3 endocytosis was performed as previously described (Hu *et al.*, 2001). OKP cells were treated with either albumin or vehicle for 48 hrs, surface labeled with NHS-SS-biotin and quenched as described above and then warmed to 37°C to allow endocytosis to occur over 30 minutes. Surface biotin was cleaved with the small cell-impermeant reducing agent TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) (100 mM in 50 mM Tris pH 7.4). The freshly endocytosed proteins bearing biotin were protected from TCEP cleavage. Cells were then solubilized in RIPA and biotinylated proteins were retrieved and assayed for NHE3 as described above. Exocytic insertion of NHE3 was measured as previously described (Peng *et al.*, 2001). Cells were rinsed with PBS-Ca-Mg × 3 at room temperature. OKP cells were treated with albumin or vehicle for 48 hrs. The apical surface was then exposed to 1.5 mg/ml sulfo-NHS-acetate in 0.1 M sodium phosphate (pH 7.5), to saturate NHS reactive sites on the cell surface and 0.15 M NaCl for 2 h at 4°C. After quenching for 20 min as described above, cells were warmed to 37°C to permit trafficking. Cells were then surface-labeled with 1.5 mg/ml sulfo-NHS-SS-biotin and lysed with RIPA buffer. The biotinylated fraction, which represents newly inserted surface proteins, was precipitated with streptavidin-coupled agarose, and the precipitate was subjected to SDS-PAGE and blotting with anti-NHE3 antibodies, as above.

*Re-insertion assay.* The re-insertion assay was modified from that described by Ehlers (Ehlers, 2000). The schematic representation is shown in Fig 5.2.1. Confluent quiescent OKP cells were treated with either albumin or vehicle for 24 hrs prior to the start of the experiment. Cells were biotinylated, rinsed, and quenched at 4° C exactly as described above. Cells were subsequently warmed to 37° C in serum-free cell culture medium with or without 5 mg/ml albumin for 1 hr to allow protein trafficking. Plates were afterwards rinsed with ice cold TBS × 3 and the surface biotin was cleaved with 50 mM glutathione-SH (G-SH) for two rounds (4°C × 15 mins each) of cleavage. One set of plates were lysed at this stage and biotinylated proteins were retrieved with streptavidin precipitation as described above. This represents the total endocytosed fraction over 1 hr (Fraction 1 in Fig. 5.2.1). A second set of plates were subjected to a second round of warming in culture medium containing either 5 mg/ml albumin or vehicle to permit trafficking. Reinserted biotinylated proteins were cleaved again as described above with G-SH. The remaining biotinylated proteins were affinity-precipitated from a RIPA lysate. This represents the endocytosed fraction that was not reinserted (Fraction 2 in Fig. 5.2.1). The difference between fractions 1 and 2 yields the NHE3 that was endocytosed and then reinserted.

Figure 5.2.1



**Figure 5.2.1: Effect of albumin on NHE3 reinsertion into the plasma membrane.**

Confluent cells were rendered quiescent by serum removal (48 hrs), treated  $\pm$  5 mg/ml albumin for 48 hrs and then subjected to the reinsertion assay. Schematic summary of the reinsertion assay. Two parameters are determined: Fraction **1** = total endocytosed NHE3, Fraction **2** = endocytosed NHE3 that is not re-inserted. The difference between the two yields gives the amount of re-inserted NHE3.

*NHE3* transcript. RNA was extracted using RNeasy (QIAGEN Inc., Valencia, California, USA). 15  $\mu$ g of total RNA was size-fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes. The radiolabeled NHE3 probe was synthesized from a full-length OKP NHE3 cDNA (Amemiya *et al.*, 1995b) and the 18S probe from a 752-base *SphI/BamHI* fragment of the mouse 18S rRNA (No. 63178; American Type Culture Collection, Rockville, Maryland, USA) by the random hexamer method. Prehybridization,

hybridization, and washing were performed as described previously (Ambühl *et al.*, 1998). Filters were exposed to film overnight at  $-70^{\circ}$  C and labeling was quantitated by densitometry. Changes in NHE3 abundance were normalized for changes in 18S rRNA abundance.

*Statistics.* Statistical analysis was performed using ANOVA, unless stated otherwise. "n" refers to the number of plates studied.

## Results

*Albumin activates Na<sup>+</sup>/H<sup>+</sup> exchanger activity.* Figure 5.2.2 summarizes the functional data. At both 1 mg/ml and 5 mg/ml, albumin increased Na<sup>+</sup>/H<sup>+</sup> exchange activity. As shown for 1 mg/ml, the effect became apparent at 6 h of incubation (+ 50% vs. control;  $P=0.02$ ) and persisted at 24 h (+ 40%;  $P=0.018$ ) and 48 h (+ 44%;  $P=0.019$ ). After 24 h of treatment 5 mg/ml albumin had a comparable effect to 1 mg/ml (+ 47% vs. control;  $P=0.025$ ), whereas after 48 hours activity was increased with 5 mg/ml of albumin as compared to 1 mg/ml (+97% vs. control;  $P=0.0013$ ). We have previously shown that the activation of NHE3 in response to chronic acid incubation requires the presence of hydrocortisone (Ambühl *et al.*, 1999). We examined the albumin effect in the presence or absence of hydrocortisone (HC) during the periods of serum deprivation (48 h prior to albumin) and albumin treatment (24 and 48 h, respectively). As shown previously (Ambühl *et al.*, 1999), HC ( $10^{-6}$  M) *per se* significantly activates NHE3 by about twofold at 24 h (Fig. 5.2.3). Combined treatment with albumin and HC resulted in a further increase in activity of 48 and 58 percent with 1 and 5 mg/ml respectively, compared to hydrocortisone alone. The percentage increase in NHE activity induced by albumin is approximately the same in the presence or absence of HC. HC appears to exert an additive rather than a synergistic effect on albumin induced activation of the NHE3 activity.

Figure 5.2.2

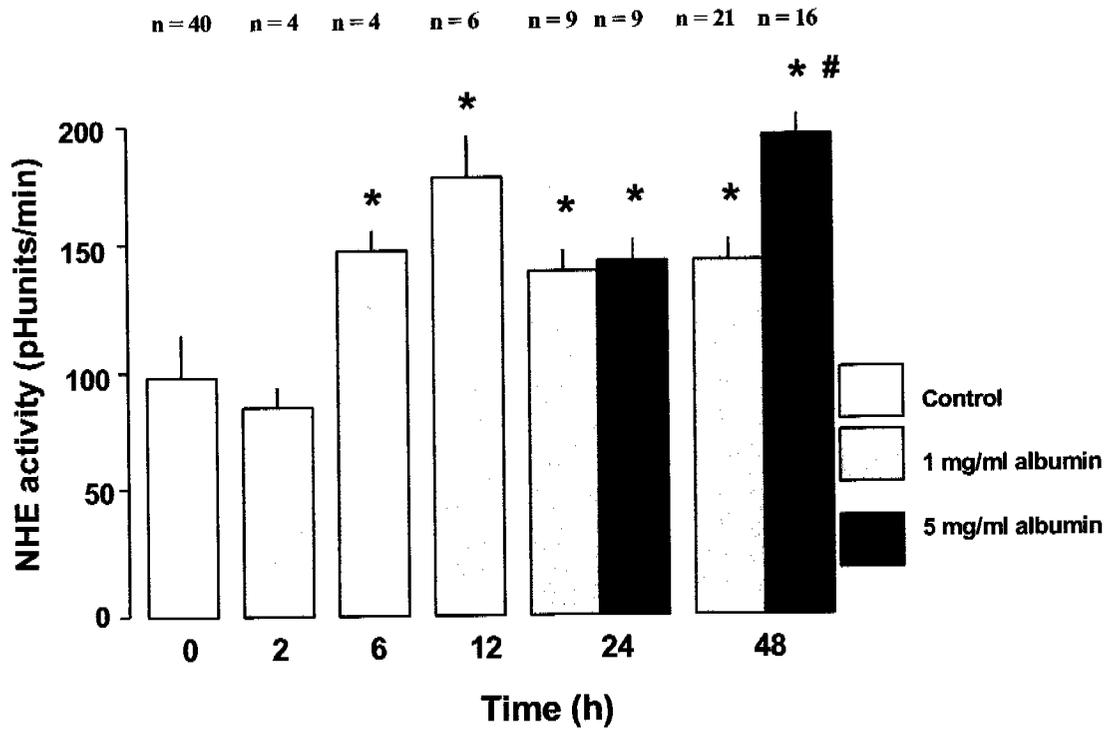
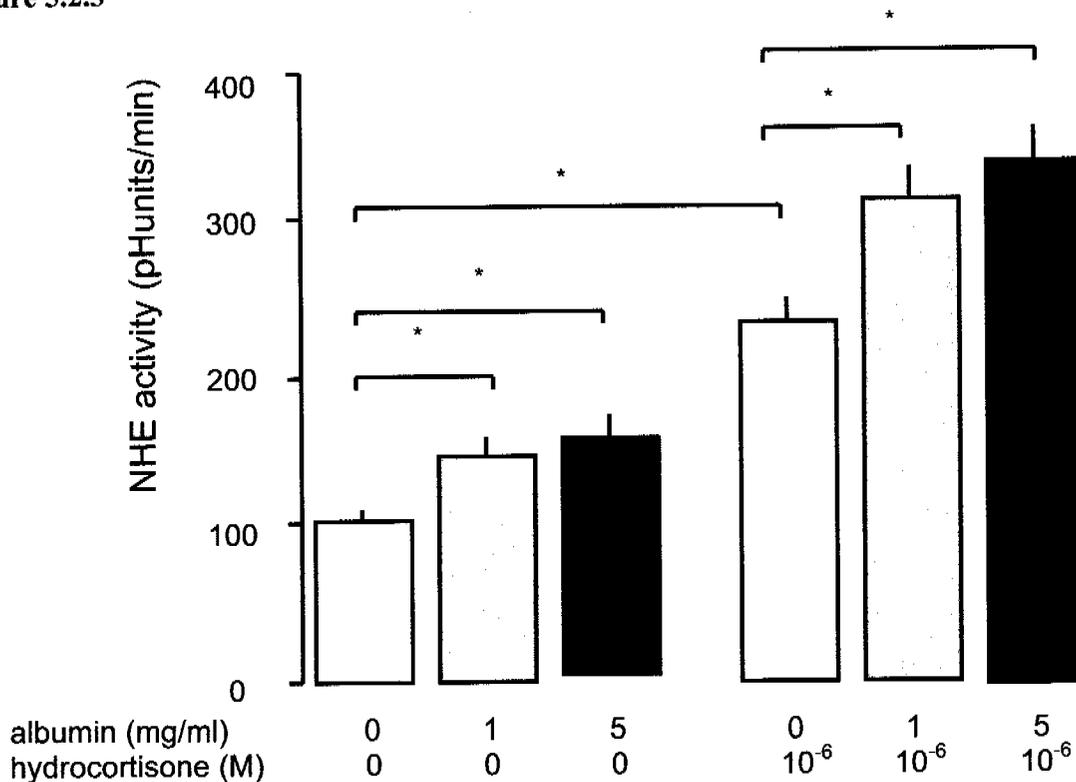


Figure 5.2.2: *Effect of albumin on Na<sup>+</sup>/H<sup>+</sup> exchanger activity.* OK cells were grown to confluence and serum deprived for 24 to 48 hours. Cells were then kept in serum-deprived medium and treated +/- albumin 1 mg/ml and 5 mg/ml, respectively. Na<sup>+</sup>/H<sup>+</sup> exchange activity was measured fluorimetrically under Vmax conditions as Na<sup>+</sup>-dependent cell pH recovery and is expressed as dpHi/dt. Bars represent mean ± SE. \* *P* < 0.05 vs. control (0 time); # *P* < 0.05 vs. albumin, 1 mg/ml at the same 48 hr time point. Unpaired t test.

Figure 5.2.3



**Figure 5.2.3: Effect of albumin  $\pm$  hydrocortisone,  $10^{-6}$  M, on  $\text{Na}^+/\text{H}^+$  exchanger activity.** Cells were grown to confluence and serum deprived for 24 to 48 hours in the presence or absence of  $10^{-6}$  M hydrocortisone. Cells were then kept in serum-deprived medium  $\pm$  hydrocortisone, and treated  $\pm$  albumin (1 or 5 mg/ml, for 24 h).  $\text{Na}^+/\text{H}^+$  exchange activity was measured as indicated in methods. Each bar represents mean  $\pm$  SE for 9 experiments. \*  $P < 0.05$ ; ANOVA.

*Albumin increases total and cell surface NHE3 protein abundance.* Changes in NHE3 activity can be associated with changes in total cellular NHE3 protein and/or changes in surface plasma membrane NHE3 protein. Albumin increased NHE3 total protein abundance by 30 and 37% after 48 h of incubation at the concentration of 1 ( $P=0.04$ ) and 5 mg/ml ( $P=0.05$ ) respectively (Figs. 5.2.4A and B). Despite changes in NHE3 activity, no significant effect can be detected at earlier time points for either concentration. The interaction between albumin and HC on total cellular NHE3 appears to depend on the concentration of albumin. The addition of HC did not amplify the magnitude of the albumin-induced increase in total cellular

NHE3 at 1 mg/ml albumin (not shown) but the stimulation by 5 mg/ml albumin was higher in the presence (77% increase,  $P < 0.01$ , not shown) compared to absence of HC.

Figure 5.2.4A

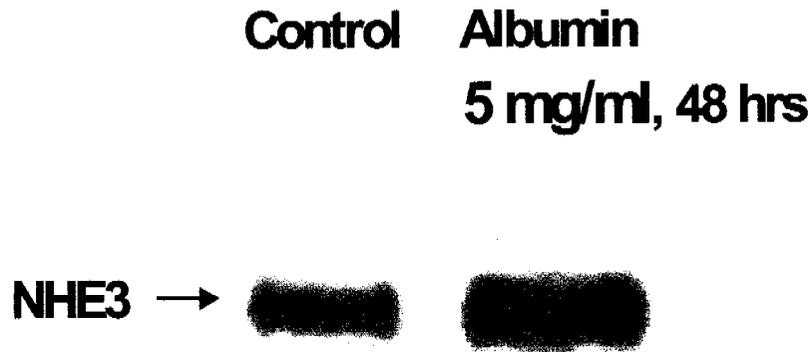


Figure 5.2.4B

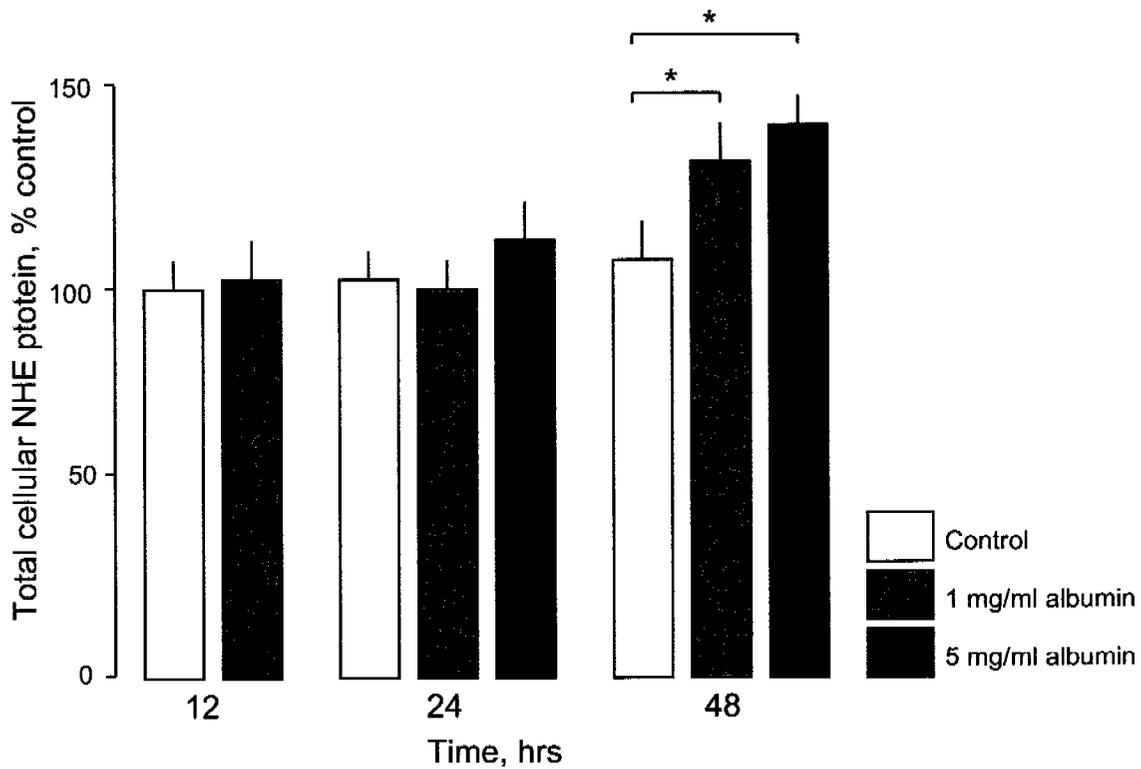


Figure 5.2.4: *Effect of albumin, on NHE3 protein amount.* Cells were grown to confluence and serum deprived for 24 to 48 hours. Cells were then kept in serum-

deprived medium and treated  $\pm$  albumin. Equal quantities of total cell membranes were prepared for immunoblot with anti-opossum NHE3 antiserum. NHE3 protein amount was quantified by densitometry. (A) Representative immunoblot after 48 h of 5 mg/ml albumin treatment. (B) Summary of results. Bars represent mean  $\pm$  SE of a number of experiments: Albumin 1 mg/ml, for 24 h (n=11) or 48 h (n=24); 5 mg/ml, for 24 h (n=9) or 48 h (n=16). \*  $P < 0.05$  vs. control; unpaired t test.

We next examined the effect of albumin on NHE3 surface protein abundance. At 5 mg/ml, albumin caused an increase in NHE3 surface protein of 96% at 48 h of incubation ( $P < 0.0001$ ; Fig. 5.2.5A and B), which is more than the 37% increase in total NHE3 protein abundance (Fig. 5.2.4). At 1 mg/ml, albumin has no detectable effect on NHE3 surface protein abundance. Surprisingly, the interaction between albumin and hydrocortisone for surface NHE3 is quite different from that observed with total NHE3. While 1 mg/ml of albumin did not increase surface NHE3, in the combination with HC, albumin induced a significant increase of 61% ( $P=0.0063$ ) and 116% ( $P=0.0002$ ) in NHE3 surface protein abundance at 24 and 48 h, respectively (Fig. 5.2.5B). At 5 mg/ml albumin in the presence of HC, albumin increased the NHE3 surface fraction by 64% at 48 h ( $P=0.038$ ) which is not higher than the albumin-induced increase in surface NHE3 in the absence of HC (Fig. 5.2.5C).

**Figure 5.2.5A**

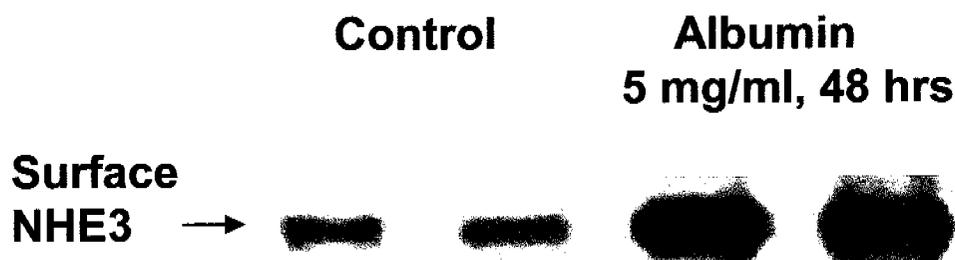


Figure 5.2.5B

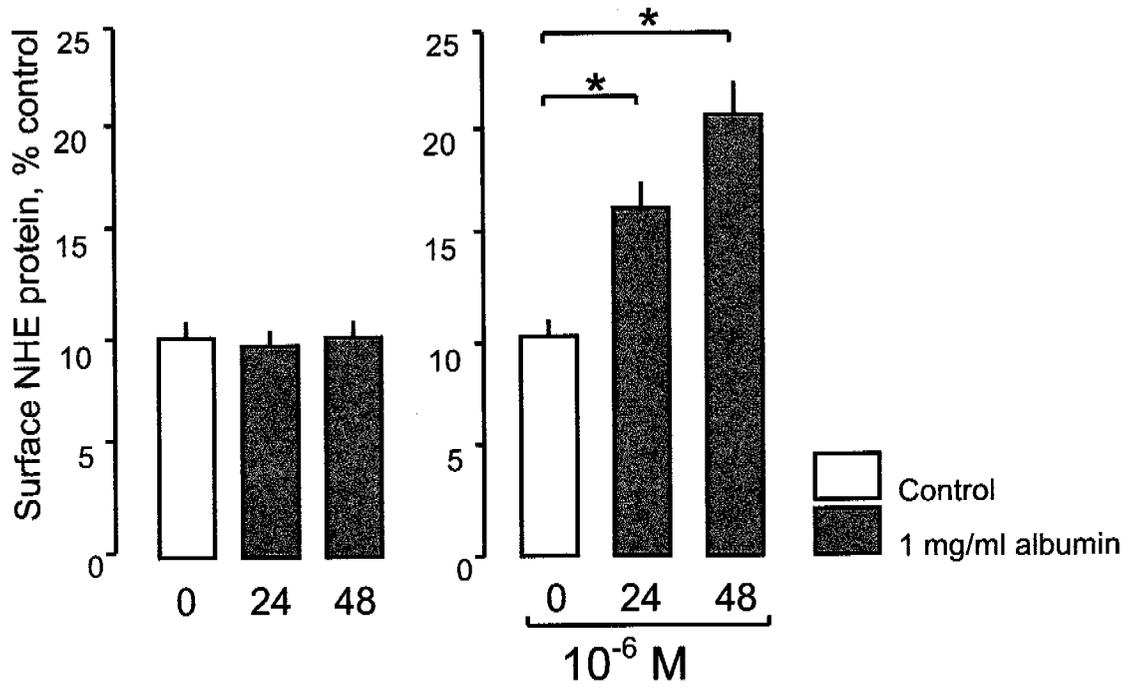
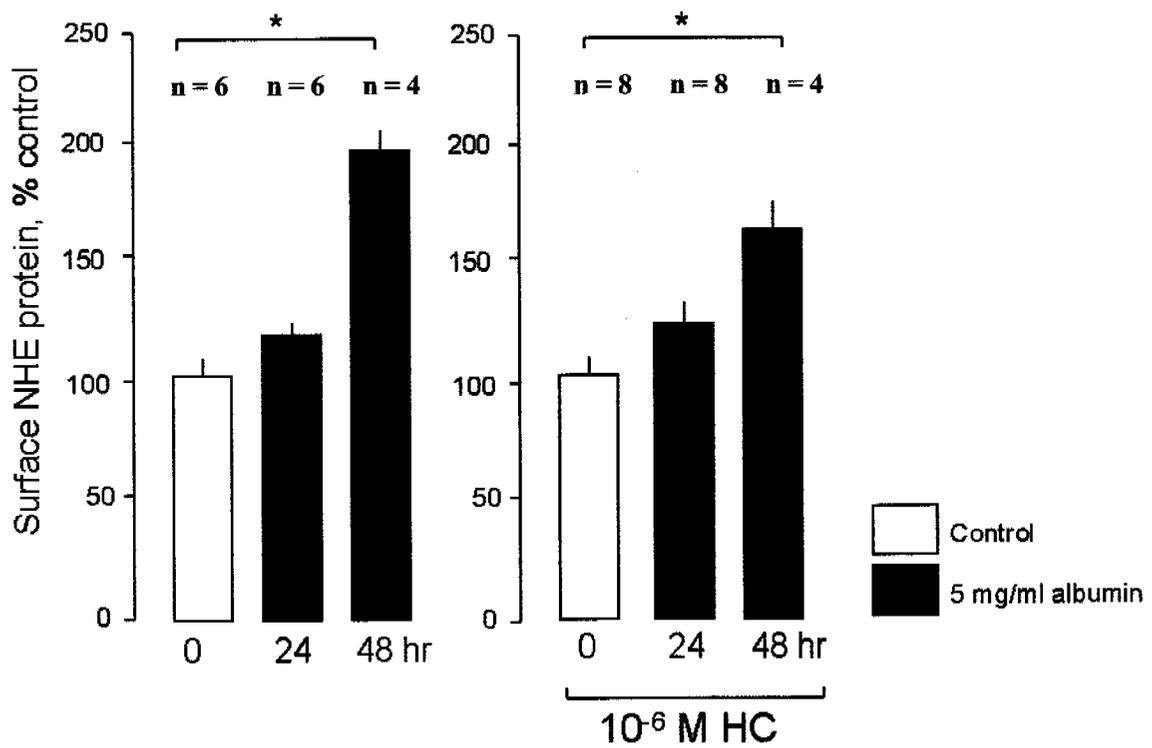


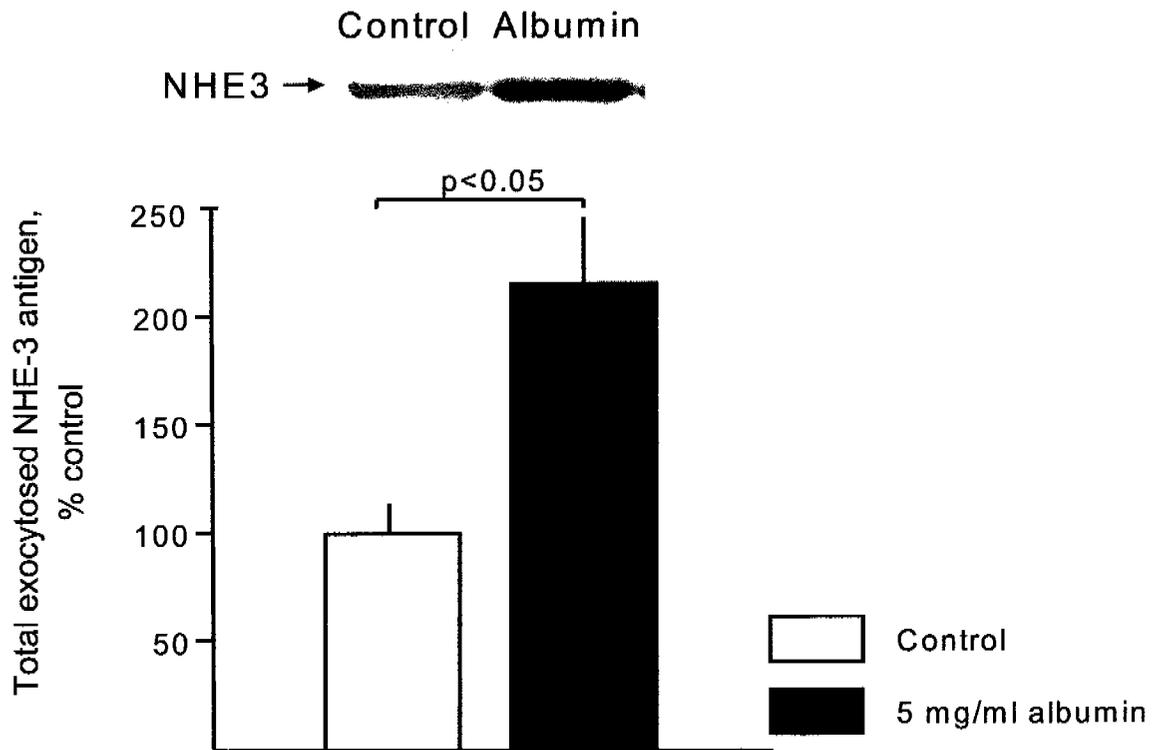
Figure 5.2.5C



**Figure 5.2.5:** *Effect of albumin on cell surface NHE3 protein.* Cells were grown to confluence and serum deprived for 24 to 48 hours. Cells were then kept in serum-deprived medium, and treated  $\pm$  albumin. Surface proteins (biotin-accessible) from equal amount of cell lysates were immunoblotted for NHE3. (A) Representative immunoblot after 48 h of 5 mg/ml albumin treatment. (B) Summary of results. Bars represents mean  $\pm$  SE: 1 mg/ml albumin  $\pm$  hydrocortisone after 0h, 24 h and 48 h. n = 4 for all groups. \*  $P < 0.05$  vs. control, unpaired t test. (C) Summary of results. Bars represents mean  $\pm$  SE: 5 mg/ml albumin  $\pm$  hydrocortisone after 0h, 24 h and 48 h. \*  $P < 0.05$  vs. control, unpaired t test.

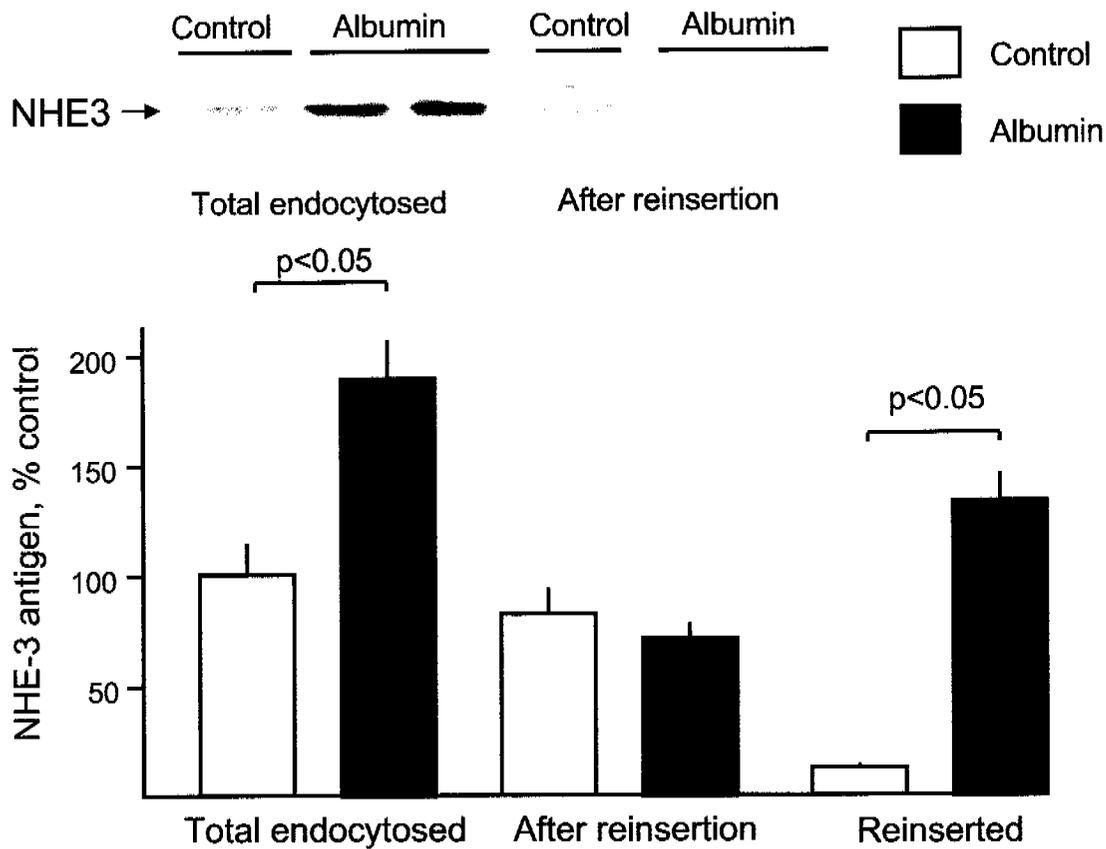
*Regulation of NHE3 trafficking by albumin.* The increase in surface NHE3 in response to albumin exposure can be caused by changes in exocytotic insertion or endocytotic retrieval. In order to determine the mechanisms that regulate increase in NHE3 surface protein content we studied the effect of albumin on rates of NHE3 endocytosis, exocytosis, and re-insertion. Treatment of cells with albumin, 5 mg/ml, for 48 h increased exocytosis of NHE3 protein by  $116 \pm 26\%$  (n=5) (Fig. 5.2.6A). Endocytosis on the other hand was increased by  $80 \pm 23\%$  (n=4) compared to control cells (Fig 5.2.6B). As this two assays does not distinguish between trafficking of de novo synthesized protein from the endoplasmic reticulum vs. recycling of protein between subapical storage compartments and the cell surface, the percentage of NHE3 protein reinsertion was determined by a modified biotinylation assay (described in “methods” and outlined in figure 5.2.1). As shown in figure 5.2.6B the percentage of reinserted NHE3 protein was increased over 5-fold in albumin treated cells.

Figure 5.2.6A



**Figure 5.2.6A: Effect of albumin on exocytosis of NHE3.** Cells were grown to confluence and serum deprived for 48 hours and then treated  $\pm$  albumin (5 mg/ml) for another 48 hrs and then subjected to the exocytosis assay as described in Methods. Inset shows one typical experiment. Bars represents mean  $\pm$  SE of four independent experiments. P value was from unpaired t test.

**Figure 5.2.6B**



**Fig. 5.2.6B: Effect of albumin on endocytosis of NHE3 and reinsertion into the cell membrane:** for methods refer to legend of figure 1.

*Regulation of NHE3 transcript by albumin.* We next examined whether the increase in total cellular NHE3 was accompanied by increase in NHE3 transcript. Incubation of OKP cells with 5 mg/ml of albumin increased NHE3 transcript by about 2-fold in the absence of hydrocortisone (Fig. 5.2.7A and B). In the presence of hydrocortisone, 5 mg/ml of albumin induced a similar increase in NHE3 transcript abundance.

Figure 5.2.7A

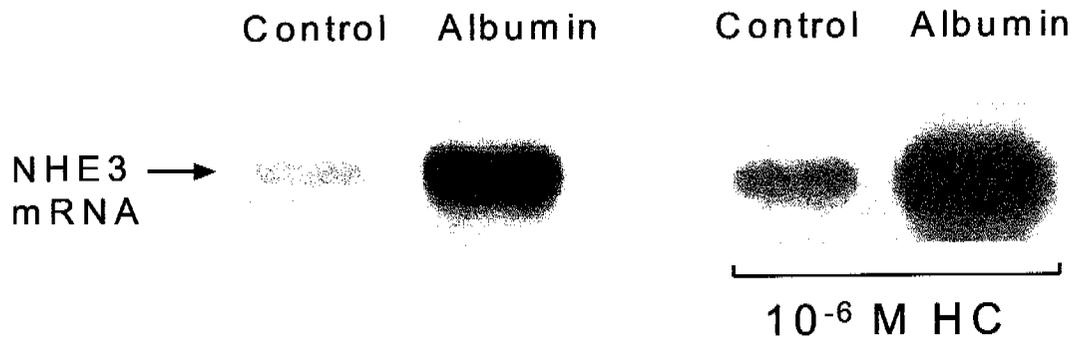
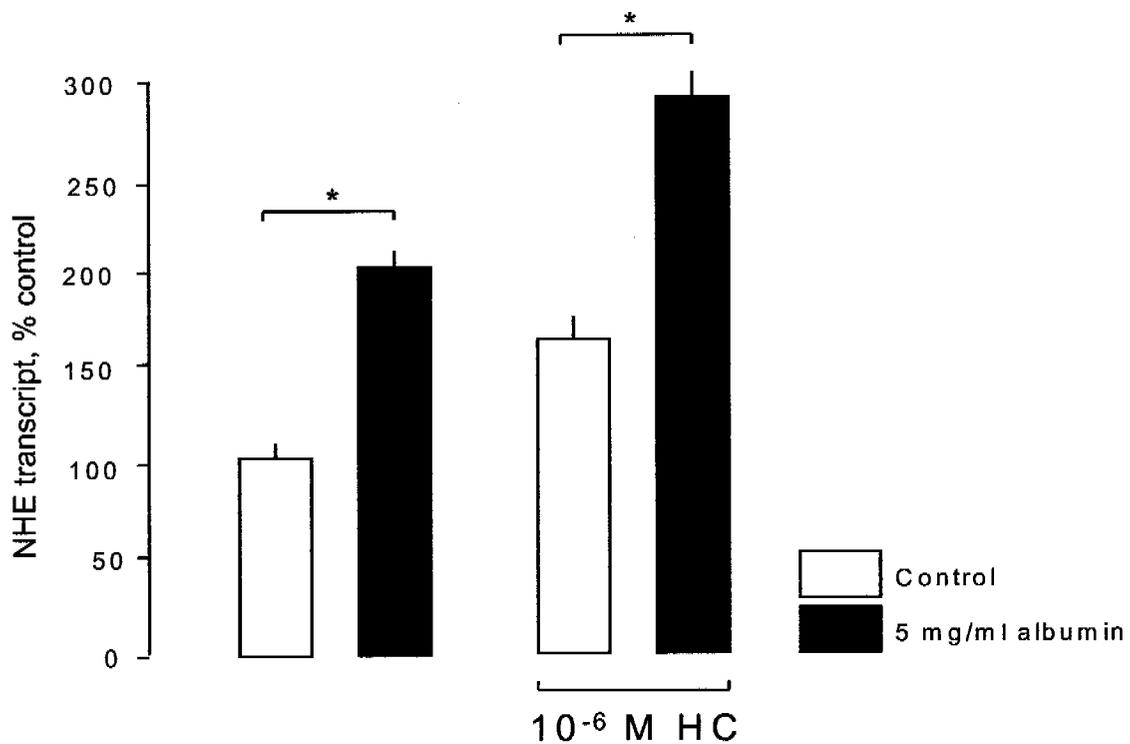


Figure 5.2.7B



**Figure 5.2.7: Effect of albumin on NHE3 transcript.** Cells were grown to confluence and serum deprived for 24 to 48 hours. Cells were then kept in serum-deprived medium, and treated  $\pm$  albumin. Hydrocortisone ( $10^{-6}$ M) were included or omitted from the culture medium. NHE3 transcript was quantified in total cellular RNA by RNA blot. (A) Representative RNA. (B) Summary of data. Bars represent mean  $\pm$  SE: Albumin (5 mg/ml) without hydrocortisone, n=4, albumin (5 mg/ml) with  $10^{-6}$  M hydrocortisone, n=4.

## Discussion

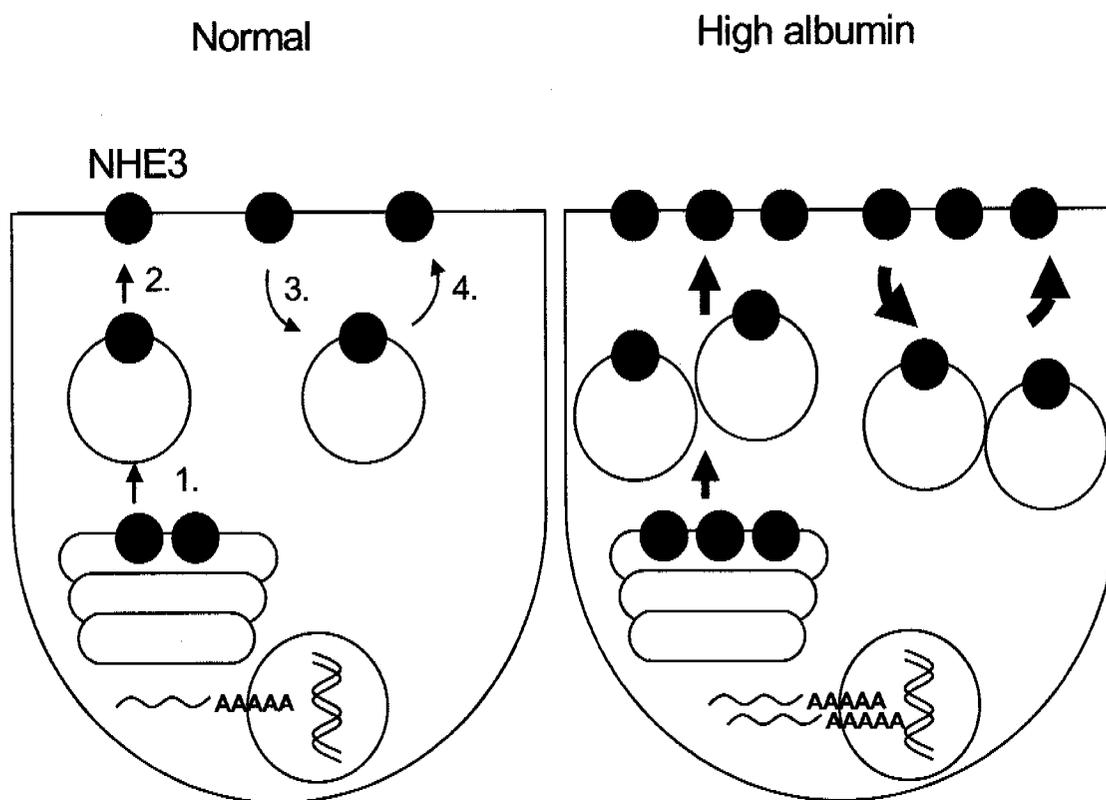
Proteinuria has been implicated both in clinical as well as in animal studies to be an important factor in the progression of renal damage partially mediated via an inflammatory reaction leading to tissue scarring and functional impairment of the kidney (Brunskill, 1998; Remuzzi and Bertani, 1998). Transtubular albumin reabsorption is mainly achieved by lysosomal uptake (Mukherjee *et al.*, 1997; Schmid *et al.*, 1989), which depends partially on endosomal acidification by the  $\text{Na}^+/\text{H}^+$  exchanger NHE3 (Gekle *et al.*, 1999; Gekle *et al.*, 1998). Moreover, experiments in puromycin aminonucleoside (PAN) induced nephrotic syndrome have shown an increase in proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger activity [chapter 5.3]. These findings suggest that proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger is induced by albumin possibly in response to increased demand in tubular protein reabsorption and the increase in apical membrane  $\text{Na}^+/\text{H}^+$  exchanger may contribute to renal sodium and fluid volume retention. The present studies in OKP cells support the notion that albumin directly stimulates NHE3.

This study in a cell culture model highlights several points. First,  $\text{Na}^+/\text{H}^+$  exchanger function increased after 6 hrs of incubation with albumin before any detectable changes in surface NHE3 protein. This was unlikely to be due to differential sensitivity of the assays as the surface biotinylation method can detect as little as ~25% changes in surface NHE3. There are examples where changes in NHE3 activity is dissociated from surface NHE3 protein (Fan *et al.*, 1999; Janecki *et al.*, 1998; Moe, 1999; Szaszi *et al.*, 2001; Wiederkehr *et al.*, 2001). Our previous study with the PAN nephrosis model also suggests that NHE3 activity is increased per brush border membrane NHE3 antigen [chapter 5.3]. We propose this to be due to changes in the megalin-bound vs. free NHE3 pool. The mechanisms by which albumin modifies NHE3 activity are not known presently.

Second, after 24 hrs of incubation with albumin, an increase in surface NHE3 was detectable but increased total NHE3 protein and NHE3 mRNA were not observed until after 48 hrs. Since the magnitude of increase in surface NHE3 (96%) exceeded and preceded that of total NHE3 (37%), albumin must alter trafficking of NHE3 protein. Indeed, NHE3 exocytosis was increased by ~115% and endocytosis was stimulated by ~80%. However, of the endocytosed NHE3, almost all of it was reinserted back into the cell surface with a 500% increase in recycling rate. This change in insertion and retrieval kinetics results in an increase in steady state surface NHE3 without any expansion of the total cellular pool.

Third, after 48 hrs of albumin incubation, total cellular NHE3 and mRNA were increased. There are multiple examples of regulation of NHE3 at the mRNA and protein levels (Ambühl *et al.*, 1996; Baum *et al.*, 1996; Cano *et al.*, 1999; Loffing *et al.*, 1998). The mechanism by which albumin increases NHE3 mRNA and protein remains to be determined. These mechanisms are schematically summarized in Fig. 5.2.8.

**Figure 5.2.8**



**Figure 5.2.8: Proposed model for albumin effect on NHE3 in the proximal tubule.** 1. Synthesis of NHE3. 2. Exocytotic insertion of newly synthesized NHE3. 3. Endocytosis of NHE3; possibly coupled to albumin. 4. Recycling of endocytosed NHE3 back to the plasma membrane.

Fourth, there is evidence of interaction between albumin and glucocorticoid on NHE3. Because of previous findings of glucocorticoid dependence of the regulation of NHE3 by acid

incubation and insulin (Ambühl *et al.*, 1999), [chapter 5.1], we wanted to see whether such a permissive effect exists for albumin. The combined action of albumin and HC on NHE3 is complex. The modifying effect of hydrocortisone on regulation of NHE3 by albumin is different for activity, surface protein vs. cellular protein, and dosage of albumin used. HC appeared to have a simple additive effect with albumin on NHE3 activity and total NHE3 protein. For surface protein, it appears that at 1 mg/ml of albumin, the presence of HC is absolutely required to increase surface NHE3 protein whereas at 5 mg/ml, supplementation with HC did not seem to make a difference. The complexity of these findings does not permit the construction of a simple paradigm to account for how glucocorticoids interact with albumin.

The apical uptake of albumin from the proximal tubule has been shown to be coupled to megalin and cubilin (Birn *et al.*, 2000; Cui *et al.*, 1996; Zhai *et al.*, 2000), as well as other albumin binding proteins located in the proximal tubule (Brunskill, 1998). Activity of cortical brush border Na/H exchange activity seems to be regulated by its binding to megalin. (Biemesderfer *et al.*, 2001) have demonstrated that NHE3 occurs in two different pools; a 21 S, megalin-associated, inactive form and a 9.6 S active form present in brush border microvilli unassociated with megalin. They postulated that partitioning of NHE3 between these two pools can operate as regulatory mechanism for NHE3. Data from proteinuric rats showing an increase in cortical brush border NHE3 immunofluorescence with an antibody that preferentially detects the megalin-free apical fraction of NHE3 [chapter 5.3], but a generalized decrease in total apical membrane NHE3 is compatible with the hypothesis of Biemesderfer *et al.* The increase in NHE3 activity without changes in surface NHE3 protein may represent another example of shifting fractions of apical membrane NHE3 although this remains to be proven.

Although the increase in surface NHE3 (96%) is larger than the increase in total NHE3 (37%), there is about 4 times more intracellular than cell surface NHE3 in OKP cells (Peng *et al.*, 2001) which means that the absolute increase in intracellular NHE3 will be much more than cell surface NHE3. The increase in endocytosis of NHE3 with albumin incubation is compatible with the hypothesis that the megalin/NHE3 complex serves as a mediator of albumin endocytosis and processing (Birn *et al.*, 2000; Zhai *et al.*, 2000). Recycling of NHE3 from the endosomes back to the cell surface has been described in other cell culture models (Kurashima *et al.*, 1998). After processing of albumin, NHE3 is recycled back to the apical

membrane instead of being targeted for degradation, perhaps as a mechanism to economize and conserve NHE3 proteins for the proximal tubule cell. The purpose of having higher levels of apical membrane NHE3 in response to albumin load is unclear, but one potential effect is enhanced transepithelial Na<sup>+</sup> absorption and contribution to extracellular fluid volume expansion.

If proteinuria *per se* inflicts damage and contributes to progression of renal disease, then to understand tubular toxicity of albumin, one needs to understand the mechanism of its processing. In the future, therapeutic measures may include antagonists of NHE3 and the megalin/NHE3 complex to reduce volume expansion as well as reduction of tubular toxicity from protein overload.

### **Acknowledgments**

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### **5.3 Regulation of the proximal tubular sodium/proton exchanger NHE3 in rats with puromycin aminonucleoside (PAN) induced nephrotic syndrome**

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## Abstract

Excessive proteinuria due to loss of glomerular permselectivity in nephrotic syndrome can cause disturbances in renal salt and water handling with edema formation. Apart from oncotic and hydrostatic mechanisms associated with hypoalbuminemia, primary derangements in renal tubular sodium transport may contribute to the pathogenesis of nephrotic edema. Whereas there is evidence for an increase of cortical collecting duct sodium reabsorption in nephrotic rats, it remains controversial whether proximal tubule sodium transport may also be activated in this condition. We investigated the regulation of the cortical  $\text{Na}^+/\text{H}^+$  exchanger NHE3, the main pathway for Na reabsorption in the proximal tubule (PT), in rats with puromycin aminonucleoside (PAN) induced nephrotic syndrome. PAN rats developed reduced GFR, severe proteinuria and sodium retention within 3 days. After 10 days, immunoblots of brush border vesicles revealed a decreased abundance of NHE3 in nephrotic animals. However, the  $\text{Na}^+/\text{H}^+$  exchanger activity in the same vesicle preparations was not significantly altered. Exchanger activity normalized for NHE3 protein was increased by 88% in nephrotic animals ( $P=0.025$ ). Immunohistochemistry with the same polyclonal antibody as for immunoblots revealed a decrease of NHE3 abundance in PT. In contrast, immunoreactivity for the monoclonal antibody 2B9, that specifically recognizes the non megalin-associated, transport-competent pool of NHE3, was higher in PAN-treated rats than in controls. We propose that increased sodium reabsorption might be associated with a shift of NHE3 from an inactive pool to an active pool, thus contributing to sodium retention in a state of proteinuria.

## Introduction

The nephrotic syndrome (NS) is a common manifestation of renal disease and is characterized by urinary protein loss due to glomerular damage and renal salt and water retention (Orth and Ritz, 1998). Under regular conditions, permselectivity of intact glomerular structures ensures retention of most of the serum proteins in the vascular space (Brenner *et al.*, 1978). Up to 5 grams of protein per day may be filtered by the glomeruli even under normal conditions, which then are reabsorbed by the renal tubule (Maack, 2000; Straus, 1957). In glomerular diseases, two pathophysiologically important events occur: First, substantial amounts of protein (mainly albumin) are filtered through the damaged glomeruli and are lost into the urine; second, increasing quantities of the filtered protein are reabsorbed by the renal tubule (Blantz *et al.*, 1997). Besides from being a hallmark of glomerular disease proteinuria has been shown to be an independent factor that induces and maintains renal damage (Remuzzi and Bertani, 1998). The current understanding is that protein reabsorption by the proximal tubule triggers inflammation and scarring by induction of several cytokines and growth factors (for example TGF $\beta$ ) (Brunskill, 1998). The reabsorption of albumin is achieved predominantly by endocytosis. Several recent studies have suggested an interrelation of transcellular albumin transport by endocytosis, and acidification of lysosomes (Mukherjee *et al.*, 1997; Schmid *et al.*, 1989) through endosomal Na<sup>+</sup>/H<sup>+</sup> exchange (Gekle *et al.*, 1999; Gekle *et al.*, 1998). The nephrotic syndrome (NS) is also accompanied by various degrees of salt and water retention and represents a major clinical problem in the management of patients with nephrosis (Palmer and Alpern, 1997b; Vande Walle and Donckerwolcke, 2001). One proposed mechanism is systemic interstitial volume sequestration due to hypoalbuminemia and secondary Na<sup>+</sup> retention. In addition, primary salt retention per se may contribute substantially to systemic volume expansion in NS. The nephrotic state has been associated with activation of the Na/K-ATPase in the collecting duct (Deschenes *et al.*, 2001; Zolty *et al.*, 1999). The effect of nephrotic syndrome on proximal tubule Na<sup>+</sup> transporters has not been explored. The bulk of sodium reabsorption in the proximal tubule is mediated by the apical sodium/proton exchanger NHE3 (Aronson, 1996). Given the potential role of proximal tubular sodium/proton exchange in both tubular protein reabsorption and salt retention, we investigated the regulation of the cortical brush border membrane Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 in rats with nephrotic syndrome induced by puromycin amino nucleoside (PAN).

## Methods

### *Materials and supplies*

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) unless stated otherwise.

### *Animals*

Male Sprague-Dawley rats, average 200 gr., were injected with either puromycin aminonucleoside (n=9, PAN 150 mg per kg body weight i.p.), or an identical volume of vehicle (H<sub>2</sub>O, n=9). Animals were then placed separately into metabolic cages, with free access to distilled water. Control and PAN rats were pair fed standard rat chow (No 9331 25 W10; Eberle Nafag AG, Gossau SG, Switzerland). Daily 24 hour urine collections were performed in all animals. Urinary sodium concentration, as well as serum and urinary creatinine concentrations were measured with an AVL984 electrode electrolyte analyzer (AVL Medical Instruments, Schaffhausen, Switzerland). Urine protein concentration was determined using the Bradford assay on a Merck Vitalab "Eclipse" filter photometer. Creatinine clearance was calculated from the urinary creatinine excreted over the last 24 h prior to sacrifice and the serum creatinine concentration from venous blood taken at sacrifice. Ten days after injection of PAN or vehicle, rats were anesthetized with Dormitor<sup>®</sup> Pfizer (medetomidini hydrochloridum 1mg/ml), 0.25ml/kg, and Hypnorm<sup>®</sup> Janssen (fentanyl citrate 0.315 mg/ml), 0.25ml/kg, and kidneys were excised, weighed, and placed in ice-cold phosphate-buffered saline (PBS).

### *Cortical brush border membrane vesicle preparation*

Renal cortical apical membrane vesicles were prepared by Mg<sup>2+</sup> aggregation, as previously described (Levi *et al.*, 1991). Dissected kidney cortex was homogenized in membrane buffer (Mannitol, 300 mM, HEPES, 18 mM, EGTA, 5 mM, PMSF; pH 7.50) at 4°C with a Brinkman Polytron. MgCl<sub>2</sub> was added to the homogenate to obtain a final concentration of 15 mM, and the homogenate pelleted at 4°C by centrifugation at 2,500 g for 30 min (Polytron, Kinematica GmbH, Kriens, Switzerland). Supernatant was transferred, an additional MgCl<sub>2</sub> precipitation was performed, and membranes were pelleted from the final supernatant at 48,000 g, 4°C, for 30 min (RC 5c Plus, Du Pont Company, Sorvall Products, Wilmington, Delaware, USA). Enrichment of brush border membrane vesicles was not affected by PAN

treatment, as evidenced by comparable ratios of BBM to total cortical homogenate leucine aminopeptidase activities in control and PAN animals ( $\times 16 \pm 3$  vs.  $\times 13 \pm 2$ , respectively,  $P = \text{n.s.}$ ).

#### *Immunoblot*

Cortical brush border membrane pellets from the above preparation were resuspended in membrane buffer (see above), and protein content was assessed according to Bradford. Thirty micrograms of protein were diluted 1:5 in 5 $\times$  sodium dodecyl sulfate (SDS) loading buffer [1 mM Tris  $\cdot$  HCl, pH 6.8, 1% SDS, 10% glycerol, 1% (v:v) 2-mercaptoethanol], boiled for 10 min, size fractionated by SDS-polyacrylamide gel electrophoresis on 7.5% gels, and electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, blots were probed in the same buffer for 1 h with a polyclonal anti-rat NHE3 antibody directed against amino acids 809-822 of the COOH-terminal cytoplasmic domain of rat NHE3 (kindly provided by Dr. Orson W. Moe, University of Texas Southwestern Medical Center, Dallas, Texas, USA) at a dilution of 1:10'000. Blots were washed in 0.05% Tween 20 in PBS one time for 15 min and two times for 5 min., incubated with a 1:10,000 dilution of peroxidase-labeled sheep anti-rabbit IgG in 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, washed as above, and then visualized by enhanced chemiluminescence (Nitro-Block II, Applied Biosystems, Foster City, California, USA; CDP-Star detection reagent, Amersham, Piscataway, New Jersey, USA). NHE3 protein abundance was quantitated by densitometry (BioCapt software version 72.02s for Windows, Vilbert Lourmat, France, and Scion Image Beta 3b, 1998, Scion Corporation, Frederick, Maryland, USA). Signal was measured as an integrated volume with correction for a defined background.

#### *Na<sup>+</sup>/H<sup>+</sup> Exchanger Activity Assay*

Na<sup>+</sup>/H<sup>+</sup> exchanger activity was determined by the acridine orange method as described by Moe et al. (Moe *et al.*, 1991b). Cortical brush border membrane vesicles were used as prepared for western blotting, with the exception that after the final precipitation step at 48,000 g the pellet was resuspended in 140 mM NMDG gluconate and 5 mM MES at pH 5.5. The acid-loaded vesicles were then added to a solution containing 120 mM NMDG-gluconate, 20 mM HEPES at pH 7.50, and 6  $\mu$ M acridine orange (Molecular Probes, Eugene, Oregon, USA). Fluorescence signal was followed in a spectrofluorometer ( $\lambda_{\text{ex}} = 493$  nm,  $\lambda_{\text{em}} = 530$  nm; Shimadzu 5000, Japan). The proton gradient-driven trapping of acridine orange in the

vesicles provoked a fluorescence quenching.  $\text{Na}^+/\text{H}^+$  exchanger activity was then assayed as the rate of increase in fluorescence (acridine orange efflux) occurring in response to  $\text{Na}^+$  addition to the extravascular space. Specific activity of the  $\text{Na}^+/\text{H}^+$  exchanger was expressed as the slope of the initial  $\text{Na}^+$ -dependent fluorescence increase divided by the initial quench.

#### *Tissue preparation for light microscopy and immunohistochemistry*

An additional set of animals ( $n = 3$  for both PAN and control) undergoing identical treatment as described above (except for the following modifications) was analyzed by light microscopy and immunohistochemistry. Nine days after injection of PAN or vehicle, rats were anesthetized with an intraperitoneal injection of thiopental (Pentothal, 0,5 mg/kg; Abbott, Abbott Park, Illinois, USA) and kidneys were fixed by vascular perfusion via the abdominal aorta as described previously (Dawson *et al.*, 1989). The fixative consisted of 3% paraformaldehyde and 0.05% picric acid in 0.06 M cacodylate buffer (pH 7.4; containing 3 mM  $\text{MgCl}_2$  and adjusted to 300 mOsm with sucrose) and 10% hydroxyethyl starch (HAES, Fresenius, Switzerland). After fixation the left renal artery was clamped and the fixative in the right kidney was washed out by perfusion with 0.1 M cacodylate buffer. Both kidneys were then removed.

For light microscopy the left unwashed kidney was cut in coronal slices and immersed for at least 24 hours in the 3% paraformaldehyde solution, to which 0.1% glutardialdehyde (Fluka Chemie AG, Buchs, Switzerland) was added. Thereafter, the tissue was postfixed in 1%  $\text{OsO}_4$  and embedded in epoxy resin. Then, sections of 1  $\mu\text{m}$  thickness were cut from the epoxy-resin embedded tissue and stained with azur II-methylene blue. Coverslips were applied with DPX mounting medium (Agar Scientific, Stansted, Essex, UK).

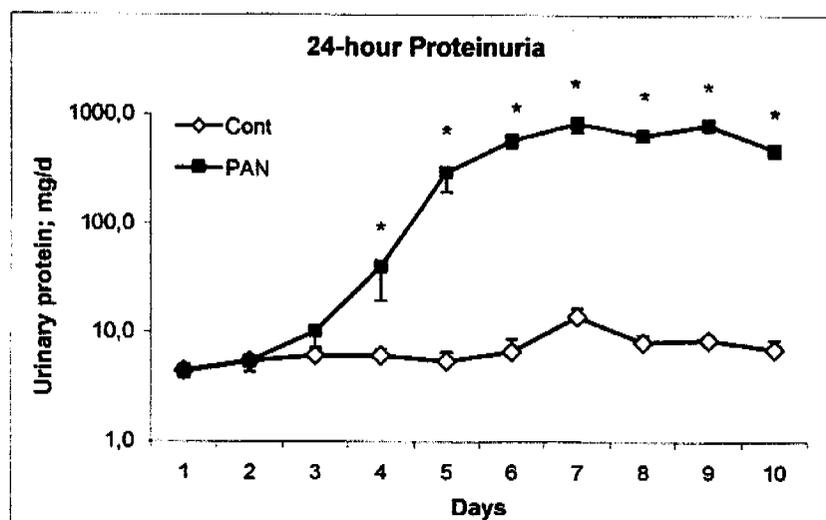
For immunofluorescence coronal slices of the right kidney were mounted on cork disks, frozen in liquid propane cooled by liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until use. 4  $\mu\text{m}$  thick cryosections were placed on chromalum/gelatine-coated slides. Sections were pretreated with SDS 1% in PBS for four minutes. After rinsing with PBS they were covered with 10% normal goat serum in phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA) for ten minutes. Sections were then incubated overnight at  $4^\circ\text{C}$  with a mouse anti-rabbit NHE3 monoclonal antibody directed against a Maltose Binding Protein fusion protein containing C-terminal 131 amino acids of rabbit NHE3 (clone 2B9; Chemicon International, Temecula, California) diluted 1:50, with a polyclonal anti-rat NHE3 antibody (see in "immunoblot")

diluted 1:500 in PBS/BSA or with a polyclonal anti-ecto-5'-nucleotidase antibody (Dawson *et al.*, 1989) 1:5000 for staining the brush border (in this case without SDS-pretreatment). Sections were then rinsed three times with PBS and covered for one hour at room temperature with FITC-conjugated goat anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted 1:50 and Cy3-conjugated goat anti-rabbit IgG (Jackson) diluted 1:1000 in PBS/BSA together with normal rat serum 1:100. Finally, the sections were rinsed with PBS, coverslips were applied with DAKO-Glycergel (Dakopatts, Glostrup, Denmark) containing 2,5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO; Sigma) as a fading retardant, and the sections were examined by epifluorescence microscopy. The same protocol was followed for double labeling but the two primary antibodies as well as the two second antibodies were mixed.

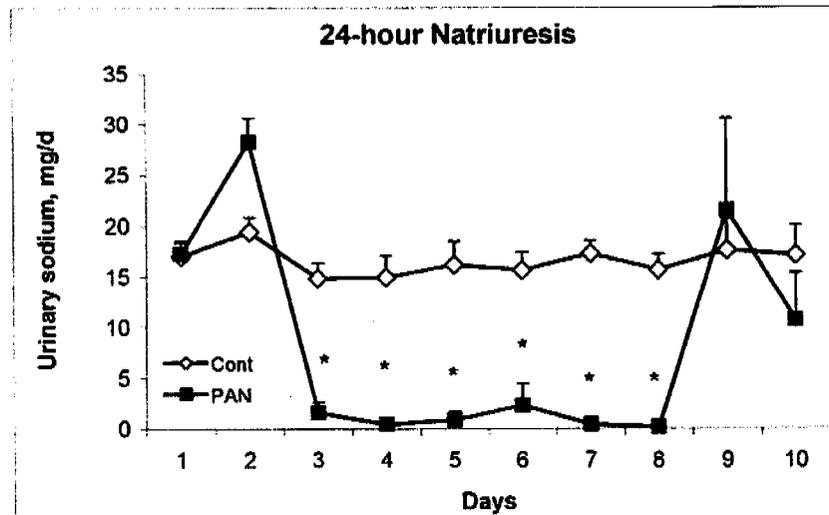
## Results

### *Renal sodium and protein excretion; creatinine clearance*

As shown in figure 5.3.1, PAN-treated rats developed severe proteinuria on day 4 and beyond, whereas renal protein excretion was minimal in control animals. Similarly, PAN rats showed a significant decrease in urinary sodium excretion compared to control animals within three days after administration of the drug (Fig. 5.3.2). A new steady state in sodium balance was achieved on day 9 in PAN animals, with a urinary sodium excretion no longer statistically different from rats not treated with puromycin. Most of the PAN rats also exhibited ascites on the day of sacrifice. Taken together, administration of puromycin resulted in the clinical equivalent of nephrotic syndrome with proteinuria as well as salt and volume retention. Creatinine clearance was significantly lower in nephrotic versus control rats ( $0.67 \pm 0.09$  vs.  $1.65 \pm 0.15$  ml/min, respectively;  $P=0.0074$ ).



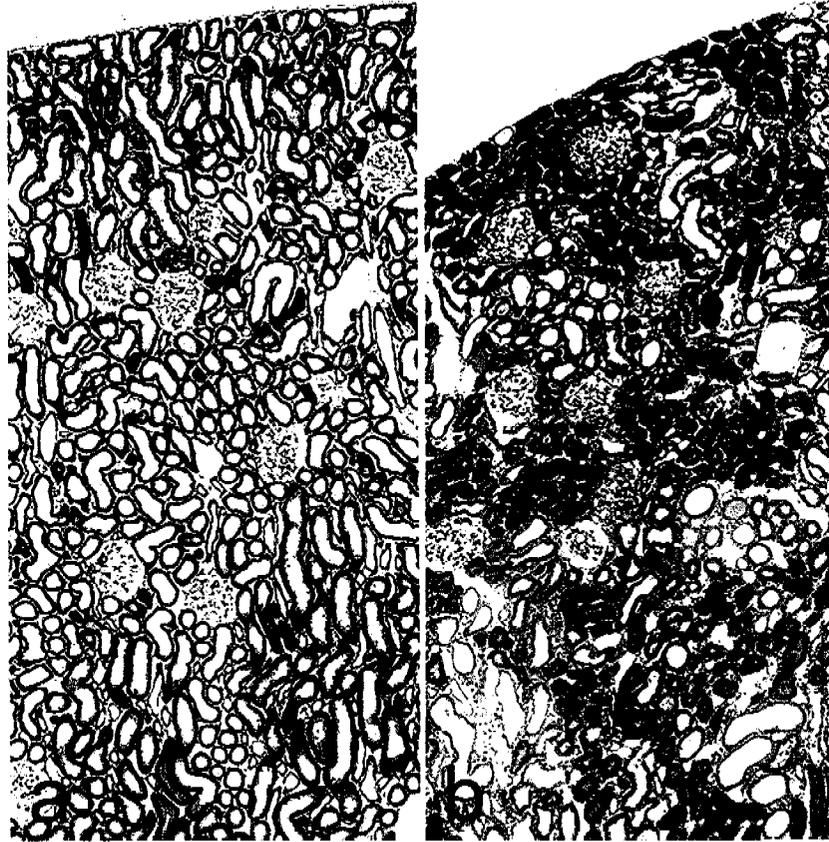
**Figure 5.3.1: 24 hour urinary protein excretion.** Protein excretion was determined from 24 hour urine collections of control and PAN treated rats. Experimental rats developed severe proteinuria 4 days after administration of puromycin aminonucleoside, which persisted throughout the whole study period. Note the logarithmical scale of the y-axis. \*)  $P < 0.001$



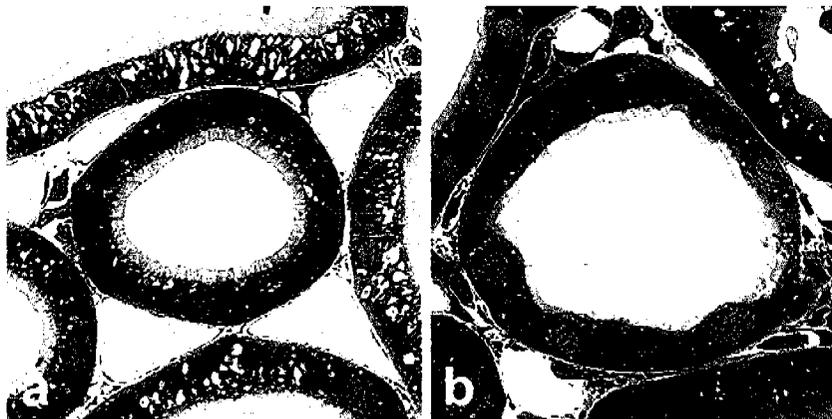
**Figure 5.3.2: 24 hour urinary sodium excretion.** Sodium excretion was determined from 24 hour urine collections of control and PAN treated rats. Whereas control rats were in sodium balance, as revealed by constant natriuresis over the whole study period, PAN rats retained sodium from day 3 through 8, as evidenced by almost undetectable sodium excretion during this period. However, natriuresis in PAN animals returned back to control levels at day 9. \*)  $P < 0.001$

#### *Light microscopy*

The overview of control kidneys (Fig. 5.3.3a) shows intact cortex and outer stripe of medulla with open tubules. In contrast, the kidneys of rats treated with puromycin aminonucleoside (PAN) exhibit an irregular pattern with many partially collapsed tubules (Fig. 5.3.3b). Moreover, many dilated proximal tubules, especially in the medullary rays, can be detected. At higher magnification the proximal tubules of control rats (Fig. 5.3.4a) manifest with an intact brush border. In PAN rats (Fig. 5.3.4b), a widespread shortening of the brush border takes place in proximal tubules. The brush border also undergoes focal destruction, especially in S2 segments. The proximal tubular cells display some variability in size with many cells being smaller than in control animals, but some cells also with an increase in size.



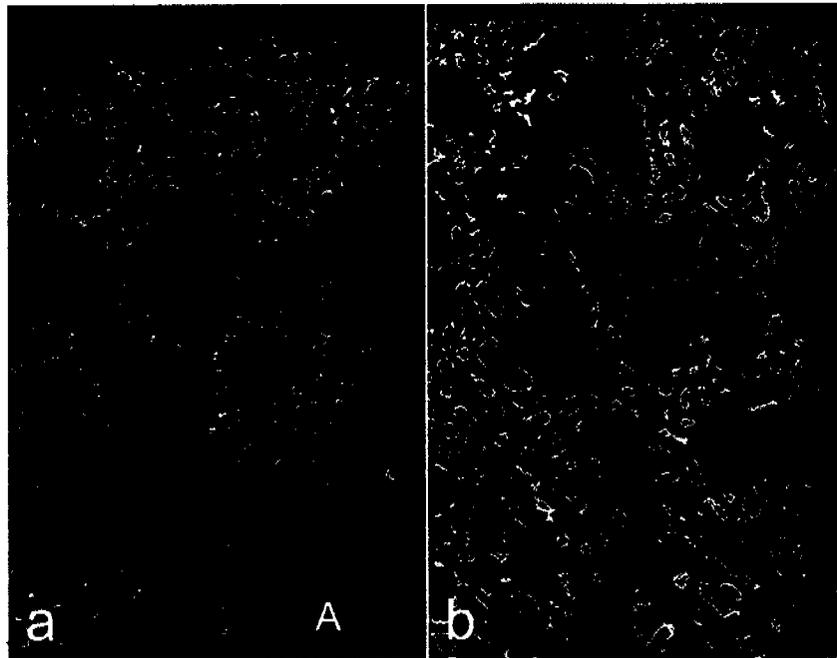
**Figure 5.3.3:** *Light microscopic overview of cortex.* 1 $\mu$ m thick epon sections. *a)* Control kidney; *b)* PAN-treated animal showing some collapsed tubules, whereas many tubules are dilated in the cortical medullary rays.



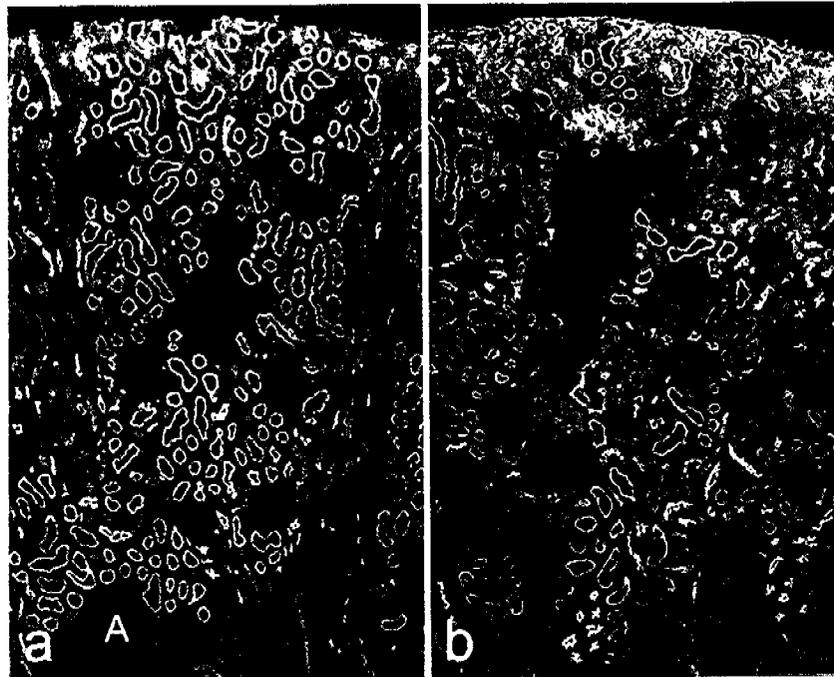
**Figure 5.3.4:** *Light microscopic view of S2-segment of proximal tubule.* 1 $\mu$ m thick epon sections. The profiles are from the sections shown in Fig. 1. *a)* Control, *b)* PAN treated rat with partial disruption of the apical brush border membrane.

### *Immunofluorescence*

Two different antibodies were used for detection of NHE3 in this study. With the monoclonal antibody 2B9 immunoreactivity was homogeneously increased in PAN treated animals (Fig. 5.3.5). In contrast, with the polyclonal antibody there was an overall decrease in PAN treated animals. The levels of immunofluorescence were similar to controls in some tubules, whereas in other tubules they were very low (Fig. 5.3.6). The decrease of immunoreactivity with the polyclonal antibody may be related to the structural alteration of the brush border described above. Indeed, the immunoreactivity for two unrelated proteins of the luminal membrane, ecto-5'-nucleotidase and NaPi-IIa, decreased also (not shown).



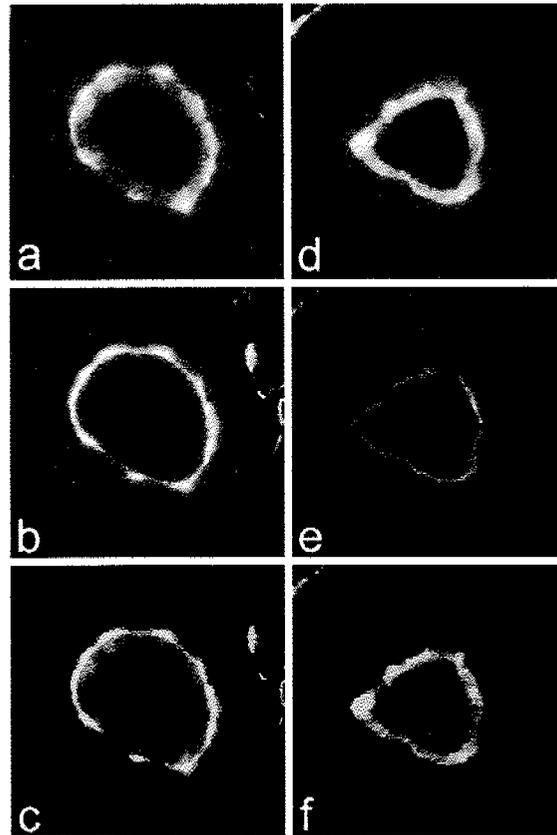
**Figure 5.3.5: *NHE3* immunoreactivity with the monoclonal antibody 2B9 in the cortex.** Cryosections were labeled with the 2B9 monoclonal antibody. In comparison to control animals (*a*), the immunofluorescence signal of the monoclonal antibody was increased in treated animals (*b*). A: arcuate artery.



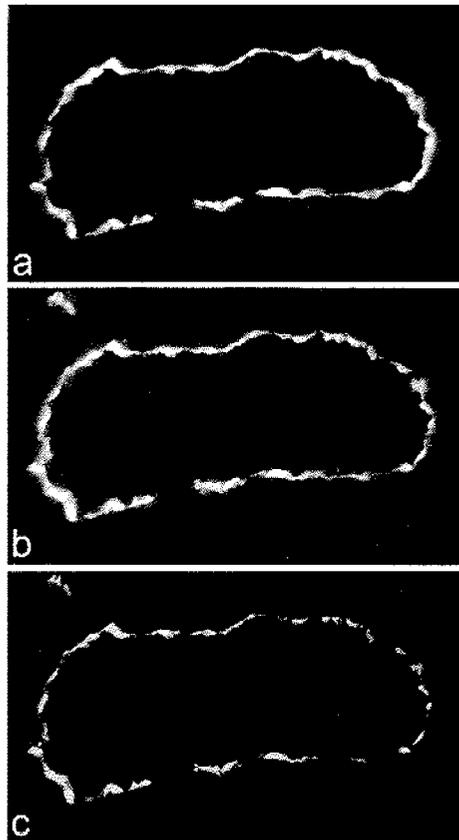
**Figure 5.3.6: NHE3 immunoreactivity with the polyclonal antibody in the cortex.** Cryosections were labeled with a polyclonal antibody against NHE3. In comparison to control animals (*a*), the immunofluorescence signal obtained with the polyclonal antibody was decreased in treated animals (*b*). A: arcuate artery.

Both in control and PAN-treated animals the intracellular distribution of NHE3 immunoreactivity was strikingly different with the two antibodies (Fig. 5.3.7). NHE3 is mainly localized at the base of the brush-border in the so-called intermicrovillar compartment (Ambühl *et al.*, 1996; Biemesderfer *et al.*, 2001; Kwon *et al.*, 1999), which the polyclonal antibody labeled almost exclusively in the present study (Fig. 5.3.7). In contrast, strong labeling was detected with antibody 2B9 up to the tip of the microvilli, as described previously (Biemesderfer *et al.*, 2001). With 2B9 the intracellular distribution pattern of immunoreactivity was somewhat altered by PAN treatment. Whereas in control animals the intermicrovillar compartment showed a distinctly higher immunofluorescence than the brush border there was little difference between the two compartments in PAN treated animals (Fig. 5.3.7). Although the overall immunoreactivity with 2B9 was stronger in treated animals, its pattern often appeared irregular within tubular profiles at high magnification. This probably reflects the focal damage in the brush border, which has been observed also by light

microscopy (see above). Indeed, the same irregular pattern was seen when adjacent sections were double-labeled with 2B9 and with an antibody against the brush border enzyme ecto-5'-nucleotidase (Fig. 5.3.8).



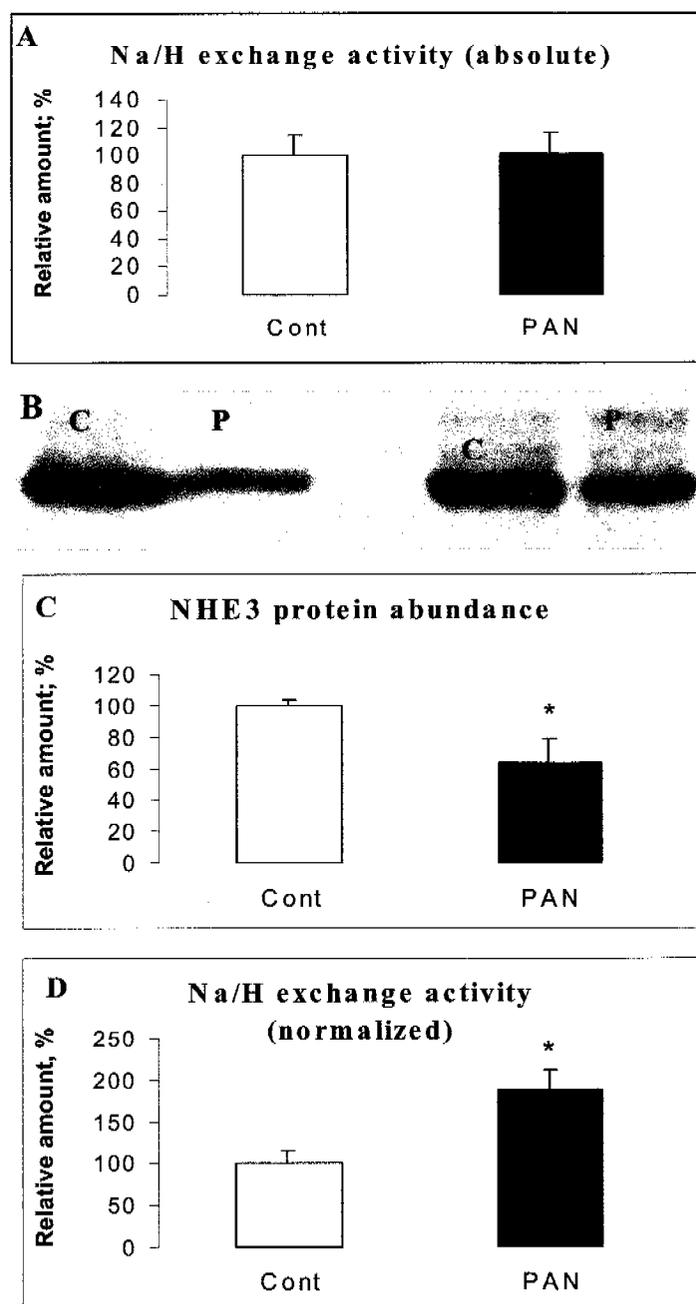
**Figure 5.3.7: *NHE3* immunoreactivity with monoclonal and polyclonal antibody at high magnification.** Cryosections were double-labeled with monoclonal and polyclonal anti-NHE3 antibodies. Two cross-sections of S1 segments of the proximal tubule in a control (*a-c*) and a treated rat (*d-f*) are shown. The two cryosections were processed together. In panels *a* and *d* the immunofluorescence signal for monoclonal anti-NHE3 and in *b* and *e* for polyclonal anti-NHE3 antibody are shown separately. In panels *c* and *f* the data from both channels are superimposed, with the signal obtained with monoclonal antibody shown in red and with polyclonal antibody in green. With the monoclonal antibody 2B9 the intermicrovillar membrane compartment was distinctly visible in controls only. The immunoreactivity in the brush border appeared slightly higher in the tubules of PAN treated rats. With the polyclonal antibody immunoreactivity was mostly restricted to the intermicrovillar compartment and was markedly lower in the tubules of treated animals.



**Figure 5.3.8:** *Colocalization of immunoreactivities with the anti-NHE3 monoclonal antibody and the brush border enzyme ecto-5'-nucleotidase.* A profile of proximal tubule in a treated animal is shown. The cryostat section was double-labeled with a polyclonal antibody against the ecto-5'-nucleotidase (*a*, green in *c*) and with 2B9 (*b*, red in *c*). The damaged brush border shows short and discontinuous microvilli. The immunoreactivities with the two antibodies are congruent.

*Na<sup>+</sup>/H<sup>+</sup> exchange activity and NHE3 protein abundance of renal cortical brush border membrane vesicles.* Na<sup>+</sup>/H<sup>+</sup> exchange activity in cortical brush border membrane (BBM) vesicles was not significantly different between PAN and control rats (Figure 5.3.9A). However, NHE3 immunoblot showed that NHE3 protein abundance (per unit of total BBM vesicle protein) was decreased by 38 percent in animals that had been treated with puromycin (p=0.003; figure 5.3.9B and C). This can be explained mainly by the partial disruption of the cortical brush border from PAN toxicity. To estimate specific Na<sup>+</sup>/H<sup>+</sup> exchange activity of

intact tubules, activity measurements were normalized to cortical brush border NHE3 protein abundance, as  $\text{Na}^+/\text{H}^+$  exchange activity and NHE3 protein abundance were always determined from the same brush border vesicle preparation. Specific  $\text{Na}^+/\text{H}^+$  exchange activity was significantly increased in PAN rats by 88 percent vs. control animals ( $p=0.032$ ; figure 5.3.9D). As an internal control western immunoblot experiments were performed using an antibody directed against the rat sodium/phosphate cotransporter NaPi-2 (kindly provided by Dr. Jürg Biber, Institute of Physiology, University of Zurich-Irchel, Zürich, Switzerland). Unlike for NHE3 no relevant changes in NaPi-2 cortical BBM protein abundance could be detected between control and PAN treated animals (data not shown).



**Figure 5.3.9: Proximal tubular Na<sup>+</sup>/H<sup>+</sup> exchange activity and NHE 3 protein abundance.** Panel *A* depicts results of Na<sup>+</sup>/H<sup>+</sup> exchange activity as determined from changes in intravesicular pH of cortical brush border membrane vesicles by acridine orange. No apparent difference exists in unadjusted measurements between control (white bar) and PAN treated animals (black bar) regarding Na<sup>+</sup>/H<sup>+</sup> exchange activity. *B*. Typical western immunoblot from the same vesicle preparations as used for activity measurements showing substantially reduced NHE3 protein abundance in PAN rats compared to controls. *C*. Quantification of NHE3 protein abundance in control and PAN treated animals as determined by densitometry from western immunoblot chemifluorescence signals. *D*. Proximal tubular brush border Na<sup>+</sup>/H<sup>+</sup> exchange activity normalized for the amount of NHE3 protein abundance depicted in panel *B* and *C*. The adjusted Na<sup>+</sup>/H<sup>+</sup> exchange activity was increased by 88% in nephrotic rats (PAN) versus normal controls. \*)  $P < 0.05$

## Discussion

It is still a matter of debate whether nephrosis is a state of underfill due to hypoalbuminemia or the result of primary renal salt and water retention from an activated renin-angiotensin-aldosterone system. Although our study was not designed to resolve this question, the findings point to a novel mechanism for primary renal sodium retention in a model of nephrosis in the rat. Puromycin aminonucleoside (PAN) in a dose of 150 mg per kg body weight resulted in salt retention within three days of administration (Fig. 5.3.1), preceding overt proteinuria by one day (Fig. 5.3.2). Besides a clear reduction in urinary sodium excretion, PAN rats also exhibited volume retention as evidenced by the development of ascites. Current evidence suggests that the collecting duct is one of the primary sites of salt retention in nephrotic syndrome (Bernard *et al.*, 1978; Bohrer *et al.*, 1977; Ichikawa *et al.*, 1983). The Na/K-ATPase seems to be the major regulator of sodium reabsorption in this tubular segment in rats with proteinuria (Deschenes *et al.*, 2001; Zolty *et al.*, 1999). However, other studies have also shown increased sodium reabsorption to occur in the proximal tubule (Allon *et al.*, 1990). Varying levels of intravascular volume status and GFR at time of study may account for the diverse findings with regard to the principal site of sodium reabsorption in nephrotic subjects. As the bulk of sodium absorption occurs in the proximal tubule it seems reasonable to postulate that this segment contributes at least in part to salt and volume retention in the nephrotic syndrome.

The combined functional and immunohistochemical data of our study point towards an increase in activity of the Na/H exchanger in proximal tubules in PAN induced nephrotic syndrome. Na/H exchange activity normalized to NHE3 antigen was increased by 88% in nephrotic versus control rats. As other BBM proteins, like the cortical sodium/phosphate cotransporter NaPi-2 and the ecto-5'-nucleotidase, are not affected in PAN treated animals we postulate a specific effect of proteinuria on proximal tubular NHE3. Furthermore, our immunohistochemical data along with recent studies by Biemesderfer and colleagues provide the basis for a possible explanation how NHE3 activity may be regulated in the nephrotic syndrome (Biemesderfer *et al.*, 2001). They demonstrated that NHE3 occurs in two different pools. The majority of NHE3 is found as a 21 S, megalin-associated, inactive form in the intermicrovillar microdomain at the base of the apical plasma membrane, whereas the remainder is present mainly in a 9.6 S active form present in brush border microvilli unrelated to megalin. There are two separate lines of evidence in the present study which suggest that

the relative distribution of NHE3 is shifted towards the megalin-free, active pool in nephrotic animals. First, in BBM vesicles the  $\text{Na}^+/\text{H}^+$  exchange activity per unit NHE3 protein increased in PAN treated rats. Second, in tissue sections the abundance of NHE3 detected with the polyclonal antibody decreased, whereas increased immunoreactivity could be noted with the monoclonal antibody 2B9, which is specific for the megalin-free, active form of NHE3. Since megalin is a receptor for filtered proteins, increased protein filtration might represent the link between PAN treatment and the postulated dissociation of NHE3 from the complexes with megalin. In order to substantiate this proposed link between proteinuria and activation of the  $\text{Na}^+/\text{H}^+$  exchanger it will be necessary to examine further models of proteinuria. However, regulation of NHE3 activity in OKP cells via trafficking of NHE3 protein between the microvillar plasma membrane and a subapical compartment has been shown previously by various stimuli such as acid (Ambühl *et al.*, 1996), osmolarity (Ambühl *et al.*, 1998), endothelin-1 (Peng *et al.*, 2001), parathyroid hormone (Collazo *et al.*, 2000) and dopamine (Hu *et al.*, 2001). Finally, we have demonstrated that albumin increases  $\text{Na}^+/\text{H}^+$  exchange activity and apical cell surface NHE3 antigen in OKP cells [chapter 5.2].

The immediate signal for  $\text{Na}^+/\text{H}^+$  exchanger stimulation could be activation of the renin-angiotensin system. Indeed,  $10^{-11}$  M angiotensin II activates  $\text{Na}^+/\text{H}^+$  exchange as shown in cultured proximal tubule cells. However, the literature on changes in local and systemic angiotensin II concentrations in PAN nephrosis is controversial. Whereas some investigators found increased angiotensin II levels in nephrotic animals, others did not observe such changes. PAN treated rats had a decreased whole animal glomerular filtration rate as revealed by the significantly lower creatinine clearance compared to control animals. Given the reduced total kidney GFR, the “normal” total cortical BBM NHE3 activity suggests a heightened level of proximal tubule reabsorption reflecting a reset level of glomerulotubular balance. The second implication of our findings relates to proteinuria. Albumin enhances  $\text{Na}^+/\text{H}^+$  exchange activity in OKP cells as shown in a preliminary report [chapter 5.2]. Since NHE3 participates in tubular albumin uptake (Gekle *et al.*, 1999; Gekle *et al.*, 1998), the increase in NHE3 specific activity may be in response to the increased albumin load presented to the proximal tubule.

In conclusion, in a model of nephrotic syndrome the abundance of NHE3, its specific activity and its subcellular localization were altered, suggesting a novel mechanism of control of  $\text{Na}^+/\text{H}^+$  exchanger in the proximal tubule *in vivo*.

## **Acknowledgments**

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## **5.4 Acute and chronic effects of streptozotocin induced diabetes on the regulation on Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 in rat proximal tubules**

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Manuscript in preparation

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## Abstract

Early stages of diabetic nephropathy are characterized by alterations of glomerular filtration, increased tubular sodium and water reabsorption, and systemic volume expansion, which may be a major cause for the development of hypertension, continued hyperfiltration and renal hypertrophy. As most of renal salt and water transport is mediated by the proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger NHE3 we have investigated its involvement and regulation in rats with STZ induced diabetes mellitus. Male Sprague Dawley rats were injected +/- streptozotocin (STZ), 60 mg/kg, and sacrificed after 2, 7 or 14 days. Compared to controls STZ rats were clearly hyperglycemic at all time points studied. Renal cortical BBM vesicles were prepared to measure  $\text{Na}^+/\text{H}^+$  exchange (NHE) activity, determined by  $\text{Na}^+$ -dependent changes in intracellular pH, and NHE3 protein abundance. NHE activity was significantly increased by 40 and 37 percent in diabetic rats after 7 and 14 days, respectively, but not after 2 days. Administration of exogenous insulin to diabetic rats resulted in lower blood sugars, but not NHE activity. Moreover, serum glucose concentration did not correlate with NHE activity in any subgroup nor in all animals analyzed together. However, in STZ rats supplemented with exogenous insulin NHE activity was positively correlated with serum insulin concentrations ( $r=0.86$ ,  $P < 0.01$ ). The increase in  $\text{Na}^+/\text{H}^+$  exchange activity was not inhibited by pharmacological inhibitor of NHE1 and NHE2, HOE-642, 3  $\mu\text{M}$ , but was abolished by NHE3 inhibitor S1611, 2  $\mu\text{M}$ , indicating NHE3 to be the isoform conferring the observed changes in exchanger activity. *In vivo*, the increase in NHE activity induced by STZ could completely be inhibited when rats were fed 6ppm of HOE-642 with the diet over 14 days. The changes in  $\text{Na}^+/\text{H}^+$  exchange activity were not paralleled by changes in NHE3 protein or mRNA abundance in diabetic rats at any of the time points investigated. These results suggest that NHE3 is the mediator of renal salt and volume retention observed in the early stage of diabetes mellitus. The increase in NHE activity probably occurs from posttranslational modification with translocation of NHE3 from intracellular compartments into the apical brush border membrane. Mediators that induce NHE activation are to be identified, as it seems not to be related to serum glucose concentration. Exogenous insulin may be a modifier of NHE activity but not an independent factor for its increase in diabetes mellitus (DM).

## Introduction

Diabetes mellitus is accompanied by many characteristic long-term complications. Among those, diabetic nephropathy and renal failure are of paramount importance as they account for about one third of patients with end stage renal disease in the western world. However, only a subset of diabetic patients is susceptible to renal damage and the factors that confer the liability to or protection from renal disease of diabetic patients remain unknown. The early phase of diabetic nephropathy is characterized by alterations of glomerular filtration, increased tubular sodium and water reabsorption and systemic volume expansion, which may be a major cause for the development of hypertension, continued hyperfiltration and renal hypertrophy. Understanding the factors that regulate renal sodium handling in diabetes may provide insight into the mechanisms that contribute to the progression and deterioration of renal function in diabetic patients. It has been suggested earlier, that proximal tubular sodium/exchange is altered in a diabetic milieu (El-Seifi *et al.*, 1987; Harris *et al.*, 1986; Jensen *et al.*, 1981). Fifty percent of filtered NaCl and 70 to 80% of filtered NaHCO<sub>3</sub> are reabsorbed by the renal proximal tubule, whereby the apical Na<sup>+</sup>/H<sup>+</sup> exchanger mediates two thirds of the transcellular NaHCO<sub>3</sub> and all of the transcellular NaCl reabsorption (Alpern *et al.*, 1995). NHE3 is the Na<sup>+</sup>/H<sup>+</sup> exchanger isoform that mediates proximal tubule Na<sup>+</sup>/H<sup>+</sup> exchange activity (Amemiya *et al.*, 1995a; Biemesderfer *et al.*, 1993), and NHE3 knockout mice show disturbed acid/base handling and impaired systemic fluid homeostasis (Schultheis *et al.*, 1998). Regulation of NHE3 has been shown to be increased by chronic metabolic acidosis and low salt diet *in vivo* (Ambühl *et al.*, 1996; Moe *et al.*, 1991b). Moreover, Na<sup>+</sup>/H<sup>+</sup> exchange has been implicated as a mediator of mitogenic stimuli in the development of hypertrophy of renal proximal tubular cells (Fine *et al.*, 1985b). In accordance, hypertrophy in a remnant kidney model of uninephrectomized rats is accompanied by an increase in the Na<sup>+</sup>/H<sup>+</sup> exchanger in the luminal membrane of the proximal tubule (Nord *et al.*, 1985).

The aims of the present study were to assess the role of proximal tubular sodium/proton exchange (NHE) in rats with streptozotocin (STZ) induced diabetes, to identify the NHE isoform responsible for changes in proximal tubular salt and volume transport, and to study its molecular regulation.

## Materials and Methods

### *Materials and Supplies*

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise.

### *Induction of STZ-induced diabetes in rats*

Male Sprague-Dawley rats weighing approximately 200 g each were injected intraperitoneally with either streptozotocin (STZ, 60 mg/kg body weight, dissolved in citrate buffer) or an identical volume of vehicle (fresh citrate buffer, pH 4.5). One or two days later, tail-vein blood glucose levels were measured to check for the onset of hyperglycemia (Glucometer Elite<sup>®</sup>, Bayer, Germany). Rats with glucose levels beyond 10 mM were considered as diabetic. A subgroup of diabetic rats was supplemented with exogenous insulin every other day (2 units/rat, Insulatard<sup>®</sup> HM 100 IE/UI/ml, subcutaneously, Novo Nordisk<sup>®</sup>, Bagsvaerd, Denmark). Animals were housed separately in metabolic cages and allowed free access to distilled water. Control and STZ rats were pair fed a standard rat chow (No 9331 25 W10; Eberle Nafag AG, Gossau SG, Switzerland). Daily 24 h urine collections were performed in all animals. Hyperglycemia and ketosuria were controlled by measuring urine glucose and ketones using Keto-Diabur-Test<sup>®</sup> 5000 strips (Roche, Switzerland). Urinary sodium concentration, as well as serum and urinary creatinine concentrations, were measured with an AVL984 electrode electrolyte analyzer (AVL Medical Instruments, Schaffhausen, Switzerland). Urine protein concentration was determined using the Bradford assay on a Merck Vitalab Eclipse filter photometer. Creatinine clearance was calculated from the urinary creatinine excreted over the last 24 h before sacrifice and the serum creatinine concentration from venous blood taken at sacrifice. Two, seven and fourteen days after injection of STZ or vehicle, rats were anesthetized with 0.25 ml/kg Dormitor<sup>®</sup> (medetomidini hydrochloridum, Pfizer, Sandwich, Kent, UK) and 0.25 ml/kg Hypnorm<sup>®</sup> (fentanyl citrate, Janssen, New Brunswick, NJ), and kidneys were excised, weighed, and placed in ice-cold phosphate-buffered saline (PBS). During sacrifice blood from the vena cava inferior was taken and glucose levels were measured. Endogenous insulin levels in serum were measured, as well as insulin content in 0.2 g of pancreas excised and homogenized in ice-cold 0.18 N HCl/70% ethanol.

### ***Cortical Brush Border Membrane Vesicle Preparation***

Renal cortical apical membrane vesicles were prepared by  $Mg^{2+}$  aggregation, as described previously (Levi *et al.*, 1991). Dissected kidney cortex was homogenized in membrane buffer (300 mM mannitol, 18 mM HEPES, 5 mM EGTA, 100  $\mu$ g/ml PMSF; pH 7.50) at 4° C with a Polytron® (Polytron, Kinematica, Kriens Switzerland).  $MgCl_2$  was added to the homogenate to obtain a final concentration of 15 mM, and the homogenate was pelleted at 4° C by centrifugation at 2500 g for 30 min (RC 5C Plus centrifuge, DuPont, Sorvall Products, Wilmington, USA). Supernatant was transferred, an additional  $MgCl_2$  precipitation was performed, and membranes were pelleted from the final supernatant at 48000 g at 4° C for 30 min (RC M1 20EX micro ultracentrifuge; DuPont, Sorvall Products, Wilmington, USA).

### ***Immunoblot***

Cortical brush border membrane pellets from the above preparation were resuspended in membrane buffer, and protein content was assessed according to Bradford. Ten micrograms of protein were diluted 1:5 in 5x sodium dodecyl sulfate (SDS) loading buffer (1 mM Tris HCl, pH 6.8, 1% SDS, 10% glycerol, 1% [vol:vol] 2-mercaptoethanol), boiled for 10 min, size fractionated by SDS-polyacrylamide gel electrophoresis on 7.5% gels, and electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, blots were probed in the same buffer for 1 h with a polyclonal anti-rat NHE3 antibody directed against amino acids 809 to 822 of the COOH-terminal cytoplasmic domain of rat NHE3 (kindly provided by Dr. Orson W. Moe, University of Texas Southwestern Medical Center, Dallas, TX) at a dilution of 1:10,000. Blots were washed in 0.05% Tween 20 in PBS one time for 15 min and two times for 10 min, incubated with a 1:5,000 dilution of peroxidase-labeled sheep anti-rabbit IgG in 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, washed as above, and then visualized by enhanced chemiluminescence (Nitro-Block II®, Applied Biosystems, Foster City, CA, CDP-Star® Chemiluminescent Substrate solution, Sigma-Aldrich Corporation, St. Louis, MO). NHE3 protein abundance was quantitated by densitometry (BioCapt software version 97.02 s for Windows, Vilbert Lourmat, France; Scion Image Beta 3b 1998, Scion Corporation, Frederick, MD). Signal was measured as an integrated volume with correction for a defined background.

### ***Na<sup>+</sup>/H<sup>+</sup> Exchange Activity Assay***

Na<sup>+</sup>/H<sup>+</sup> exchange activity was determined by the acridine orange method as described by Moe et al. (Moe *et al.*, 1991b). Cortical brush border membrane vesicles were used as prepared for western blotting, with the exception that after the final precipitation step at 48,000 g, the pellet was resuspended in 140 mM NMDG gluconate and 5 mM MES at pH 5.5. The acid-loaded vesicles were then added to a 37° C warm extravesicular solution containing 120 mM NMDG-gluconate, 20 mM HEPES at pH 7.50, and 6 μM acridine orange (Molecular Probes, Eugene, OR). Fluorescence signal was followed in a spectrofluorometer (ex = 493 nm, ex = 530 nm; Shimadzu 5000, Japan). The proton gradient-driven trapping of acridine orange in the vesicles provoked fluorescence quenching. Na<sup>+</sup>/H<sup>+</sup> exchange activity was then assayed as the rate of increase in fluorescence (acridine orange efflux) occurring in response to Na<sup>+</sup> addition to the extravesicular space. Sodium was given as sodium gluconate solution (final concentration 30 mM). Specific activity of Na<sup>+</sup>/H<sup>+</sup> exchange ((FU/t)/Q) was expressed as the slope of the initial Na<sup>+</sup>-dependent fluorescence increase divided by the initial quench.

### ***Inhibition of Na<sup>+</sup>/H<sup>+</sup> Exchanger Activity in Vitro***

*In vitro* inhibition of Na<sup>+</sup>/H<sup>+</sup> exchange activity was performed with the pharmacological agents S1611 and HOE-642 (kindly provided by Aventis Pharma, Frankfurt, Germany), which, in the concentrations used, selectively affect NHE3 and NHE1/NHE2, respectively. Na<sup>+</sup>/H<sup>+</sup> exchange activity was determined by the acridine orange method as described above. S1611, 2 μM, or an identical volume of vehicle DMSO, was added together with sodium gluconate to the vesicle solution. HOE-642 (final concentrations 3 μM and 30 μM), or an identical volume of vehicle DMSO was added to the extravesicular solution and Na<sup>+</sup>/H<sup>+</sup> exchange activity measured as described above.

### ***Inhibition of Na<sup>+</sup>/H<sup>+</sup> Exchanger Activity in Vivo***

To inhibit Na<sup>+</sup>/H<sup>+</sup> exchange activity *in vivo*, diabetic and control rats were fed rat chow with or without 6ppm HOE-642 (Cariporide, Aventis Pharma, Frankfurt, Germany), for 14 days (0,6 mg daily per kg of body weight). Analyses of serum parameters and measurement of Na<sup>+</sup>/H<sup>+</sup> exchange activity were determined as described above.

### ***NHE3 transcript***

Total RNA was extracted by the modified method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Slices of kidney cortex were homogenized in 4 M guanidium thiocyanate/0.75 M sodium citrate/0.1 M p-mercaptoethanol/10% sarcosyl. RNA was extracted with phenol/sodium acetate/chloroform and isopropanol precipitated at 12,000 g at 4° C for 10 min (RC 5C Plus centrifuge, DuPont, Sorvall Products, Wilmington, USA). The resulting pellet was washed with 80% ice-cold ethanol, centrifuged again and dissolved in TE buffer, pH 7.4 (10mM tris chloride/1mM EDTA). RNA was quantified by spectrophotometric absorbance at 260 nm (GeneQuant<sup>®</sup>, Pharmacia Biotech, Cambridge, England). Fifteen micrograms of total RNA were size fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes (Zeta Probe Blotting Membranes, Bio-Rad, Hercules, CA), followed by ultraviolet crosslinking (UV Crosslinker; Hoefer Instruments, SF). Membranes were prehybridized for 30 min at 68° C in hybridisation solution (QuikHyb<sup>®</sup>, Stratagene, TX), hybridized in the same solution containing 0.2 mg/ml herring sperm DNA (Roche, Switzerland) and a radiolabeled probe at 68° C for 2 hours, washed at low stringency (2 x SSC, 0.1 SDS, where SSC is standard sodium citrate) at room temperature three times for 10 min; and washed again at high stringency (0.1 x SSC, 0.1 x SDS) three times for 20 min at 60° C. Radioactive labeled probes were synthesized from the appropriate cDNA (full-length rat NHE3 (Orlowsky *et al.*, 1992)) and [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; Hartmann Analytic, (Zurich, Switzerland) by the random prime labeling system (Rediprime II<sup>®</sup>, Amersham Pharmacia, England). After quantification of the NHE3 mRNA signal, the membranes were stripped and reprobbed with the radioactive labeled 18S rRNA oligonucleotide probe synthesized as described previously (Moe *et al.*, 1991a). Hybridization signals were visualized by autoradiography and quantitated by densitometry (BioCapt 97.02<sup>®</sup> s, Vilbert Lourmat, France; Scion Image Beta 3b 1998, Scion Corporation, Frederick, MD). Changes in NHE3 abundance were normalized for changes in 18S rRNA abundance.

### ***Statistics***

All results are reported as means  $\pm$  SEM. Statistical analysis was performed using ANOVA (One-way ANOVA, two-way ANOVA or ANOVA for repeated measurements, as appropriate) unless stated otherwise, and "n" refers to the number of rats. Significance was assigned at  $P < 0.05$ .

## Results

### *Characteristics of streptozotocin treated animals*

One or two days after administration of STZ injection rats developed a state of insulin deficient diabetes mellitus as defined by hyperglycemia, and low pancreas insulin concentration. As shown in Table 5.4.1, diabetic rats but not insulin treated diabetic rats had significantly elevated blood glucose levels with mean blood sugar concentrations of  $20.3 \pm 2.5$ ,  $11.2 \pm 1.7$  and  $12.8 \pm 2.9$  mM after 2, 7 and 14 days, respectively (vs.  $7.6 \pm 0.9$ ,  $5.7 \pm 0.2$  and  $5.7 \pm 0.4$  mM in control animals). The insulin content in pancreas was notably decreased in STZ and insulin treated diabetic rats after one and two weeks. Furthermore, diabetic rats presented with polydipsia, polyuria, glucosuria (control rats: 0 mM; STZ rats: 111-280 mM; STZ/insulin rats: 16-56 mM), and slight proteinuria, independent of exogenous insulin administration. However, all groups were negative for urinary ketone. Similarly, none of the different groups were acidotic based on arterial blood gas analyses (data not shown). These findings exclude diabetic ketoacidosis in STZ treated rats.

**Table 5.4.1** *Physiological characteristics of control and diabetic rats with and without insulin supplementation.* Blood for glucose and insulin concentration, pancreas for insulin quantification and rat body weight were taken at the day of sacrifice. Values are expressed as mean  $\pm$  SE; \* P < 0.05; § P < 0.01; ¶ P < 0.0001 versus control.

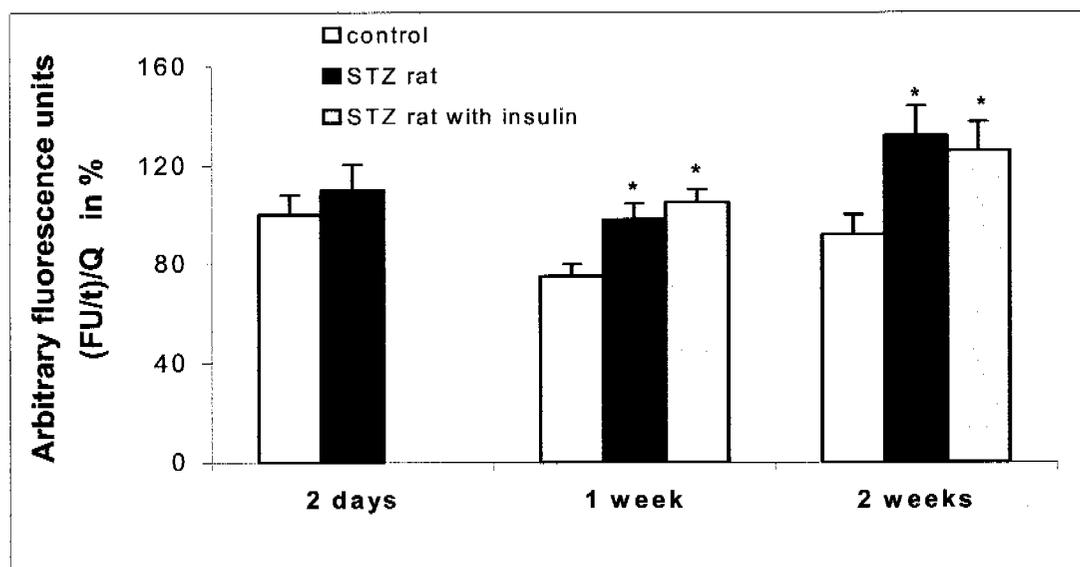
Number of animals	2 days	7 days	14
Control	12	9	9
STZ	11	7	12
STZ + insulin		6	11
Blood glucose (mM)			
Control	$7.6 \pm 0.9$	$5.7 \pm 0.2$	$5.7 \pm 0.4$
STZ	$20.3 \pm 2.5$ §	$11.2 \pm 1.7$ *	$12.8 \pm 2.9$ *
STZ + insulin		$7.8 \pm 1.9$	$9.0 \pm 1.4$

Pancreatic insulin (nM)		
Control	1 697.6 ± 69.8	2 063.5 ± 315.0
STZ	197.1 ± 103.5 <sup>¶</sup>	154.7 ± 48.6 <sup>¶</sup>
STZ + insulin	72.5 ± 43.3 <sup>¶</sup>	146.8 ± 37.7 <sup>¶</sup>
Body weight (g)		
Control	265 ± 17	254 ± 4
STZ	216 ± 25	202 ± 18 <sup>§</sup>
STZ + insulin	242 ± 28	213 ± 12 <sup>§</sup>

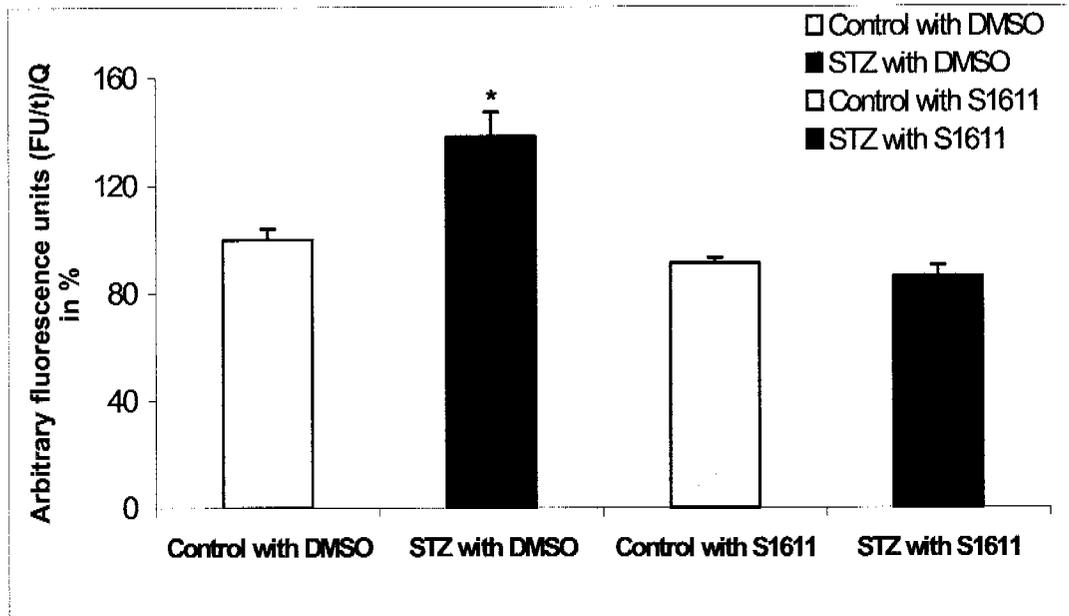
*Time course of Na<sup>+</sup>/H<sup>+</sup> exchange activity in renal cortical brush border membrane vesicles and inhibitor experiments*

Na<sup>+</sup>/H<sup>+</sup> exchange activity was measured in brush border membranes at 2, 7 and 14 days after STZ administration. Activity was significantly increased in diabetic rats after 7 days (+ 30%) and 14 days (+ 34%) as shown in Figure 5.4.1 (105 ± 6 % vs. 75 ± 5 % in controls at 1 week, and 126 ± 12 % vs. 92 ± 8 % in controls at 2 weeks; all results given as artificial fluorescence units). Na<sup>+</sup>/H<sup>+</sup> exchange activity was not affected by the administration of insulin to diabetic rats. In order to determine the isoform specificity of the observed increase in Na<sup>+</sup>/H<sup>+</sup> exchange activity we performed activity measurements in the presence of two pharmacological NHE inhibitors. S1611, a NHE3 inhibitor, in a concentration of 2 μM (IC<sub>50</sub> 0.69 μM for rat NHE3) (Schwark *et al.*, 1998), completely returned increased Na<sup>+</sup>/H<sup>+</sup> exchange activity in samples of STZ treated rats back to the control levels (Figure 5.4.2). In contrast, no significant inhibition was detected in samples from control rats. Furthermore, the dose specific sensitivity of different NHE isoforms to HOE-642 (IC<sub>50</sub> 0.033 μM, 4.5 μM, and 1 mM in isolated system for NHE1, NHE2, and NHE3, respectively) [(Schwark *et al.*, 1998), and unpublished observation from M. Bleich from Adventis] was used to further determine their contribution to the observed increase in Na<sup>+</sup>/H<sup>+</sup> exchange activity in diabetic rats. NHE activity in vesicles from diabetic rats in the absence of inhibitor was increased by 79% vs. controls (Figure 5.4.3). Vesicle exposure to a low concentration of HOE-642, 3 μM, that inhibits NHE1, had no effect on pH recovery after vesicle acidification in both control and STZ samples (Figure 5.4.3). Similarly, 30 μM HOE-642, which blocks prevalently NHE2, did

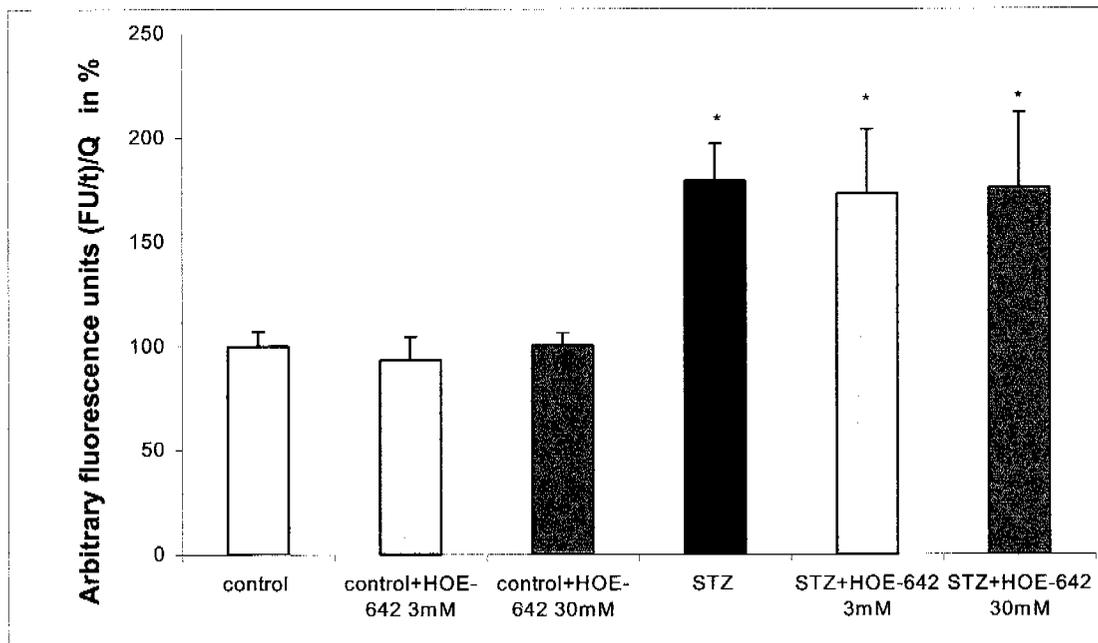
not affect  $\text{Na}^+/\text{H}^+$  exchange activity in both groups. However, at higher concentration, 1 mM that block also NHE3, HOE-642 diminished  $\text{Na}^+/\text{H}^+$  exchange activity in control samples by 21% and in STZ vesicles by 50% (data not shown), indicating that the increase in NHE activity in diabetic animals is due to NHE3.



**Figure 5.4.1 Proximal tubular  $\text{Na}^+/\text{H}^+$  exchange activity.** Results of  $\text{Na}^+/\text{H}^+$  exchange activity ((FU/t)/Q) as determined from changes in intravesicular pH of cortical brush border membrane vesicles by acridine orange. Data are given as arbitrary fluorescent units ((FU/t)/Q) as percent of control sample after 2 days.  $\text{Na}^+/\text{H}^+$  exchange activity was significantly increased in diabetic rats after 7 days and 14 days, but not after 2 days. Data are expressed as mean  $\pm$  SE. \*  $P < 0.05$  vs. control.

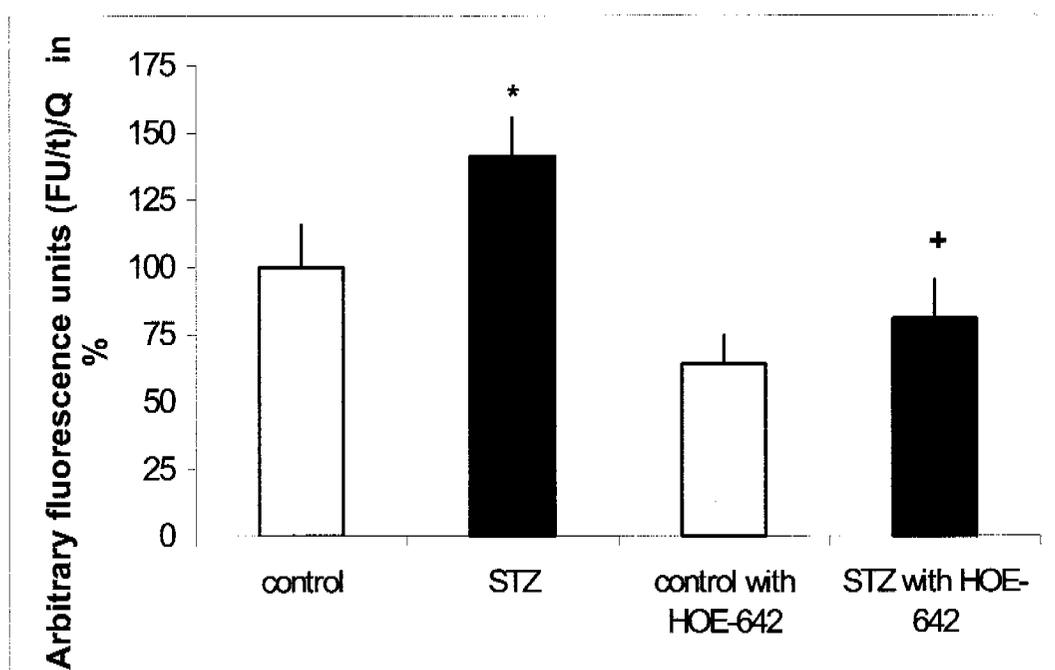


**Figure 5.4.2 Inhibition of proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger activity by S1611 *in vitro*.** S1611, a specific inhibitor of NHE3 in the given dose, was applied in a concentration of 2  $\mu\text{M}$  to the BBM vesicles from control and diabetic rats (14 days,  $n = 8$ ). DMSO was added as vehicle control. Samples from diabetic rats show significantly increased  $\text{Na}^+/\text{H}^+$  exchange activity versus control animals. S1611 significantly diminishes  $\text{Na}^+/\text{H}^+$  exchange activity in diabetic rats. Data are expressed as mean  $\pm$  SE, as percent of control with DMSO. \*  $P = 0.0150$  for STZ versus control rats;  $^+P = 0.0009$  for STZ versus STZ + S1611.



**Figure 5.4.3 Inhibition of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange activity by HOE-642 *in vitro*.** HOE-642 was applied in concentration of 3  $\mu\text{M}$  and 30  $\mu\text{M}$  to BBM vesicles from control and diabetic rats after 14 days of STZ injection ( $n = 3$ ). Vesicle exposure to a lower (3  $\mu\text{M}$ ) and higher (30  $\mu\text{M}$ ) concentration of HOE-642 had no effect on pH recovery after vesicle acidification in both control and STZ samples. All activities from diabetic animals with or without inhibitor were significantly higher vs. their respective controls. Data are expressed as mean  $\pm$  SE, as percent of control. \*  $P < 0.05$  vs. control.

Control and diabetic rats were fed a diet containing 6ppm HOE-642 over 14 days. HOE-642 is a dose specific inhibitor of NHE1 and NHE2 *in vitro*. However, the exact effect *in vivo* on NHE activity in the kidney is not known. In rats fed HOE-642, the increase in renal cortical brush border membrane vesicle NHE activity induced by STZ was returned back to control level, as shown in Figure 5.4.4. Control animals fed a diet containing HOE-642 did not significantly differ with regard to NHE activity from control rats fed a normal rat chow. Physiological characteristics of control and diabetic rats treated with HOE-642 are given in Table 5.4.2.



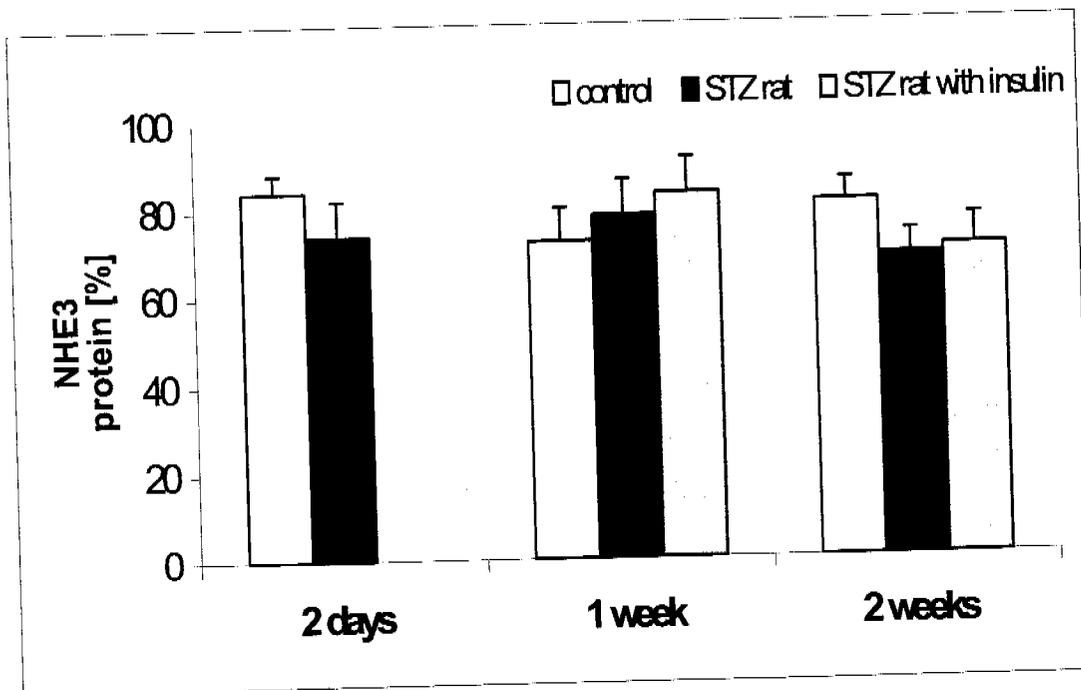
**Figure 5.4.4 Inhibition of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange activity by HOE-642 *in vivo*.** HOE-642 given with rat chow in a concentration of 6ppm for 14 days

(n=5 and 6) abolished increased Na<sup>+</sup>/H<sup>+</sup> exchange activity in vesicles from diabetic rats. Data are expressed as mean ± SE, as percent of control. \* P < 0.05 vs. control, + P < 0.05 vs. STZ.

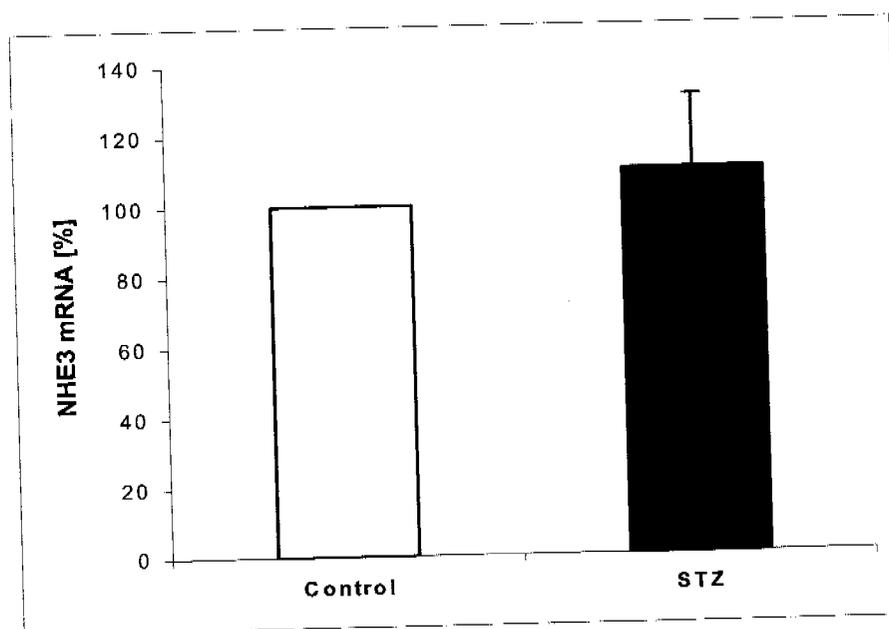
**Table 5.4.2 Physiological characteristics of control and diabetic rats treated with the NHE inhibitor HOE-642, 6ppm for 2 weeks.** Data are expressed as mean ± SE. \* P < 0.05 versus control, \*\* P < 0.05 versus control and control with HOE-642. § P < 0.001 versus control; ¶ < 0.0001 versus control.

Group (n)	Blood glucose (mM)	Body weight (g)
Control (6)	12.5 ± 0.7	260 ± 6
STZ (5)	23.5 ± 3.5 *	185 ± 12 §
Control + HOE-642 (6)	11.7 ± 1.0	267 ± 7
STZ + HOE-642 (6)	22.7 ± 3.3 **	196 ± 8 ¶

Changes in NHE3 activity can be due to changes in protein synthesis. However, western immunoblot analysis of BBM vesicles from diabetic and control animals using a polyclonal anti-rat NHE3 antibody revealed no differences in the protein abundance between groups at 2 days, 1 week and 2 weeks (Figure 5.4.5). Similar findings for NHE3 were obtained from diabetic and control rats fed a diet containing HOE-642. Also, NHE3 abundance from total cortical cell extracts was not different between control and diabetic animals, as was brush border membrane NHE2 abundance (data not shown). Finally, NHE3 mRNA amount was comparable for control and diabetic rats at 14 days (Figure 5.4.6).



**Figure 5.4.5** *Quantification of NHE3 protein abundance in BBM vesicles of control and STZ-treated animals as determined by densitometry from western immunoblot chemifluorescence signals. No difference was seen in NHE3 antigen at all time points between groups. Data are expressed as mean  $\pm$  SE.*



**Figure 5.4.6** *Quantification of NHE3 mRNA abundance in control and STZ-treated animals after 14 days of STZ administration (n = 8). Data are expressed as mean  $\pm$  SE.*

## Discussion

Diabetes mellitus is associated with changes in renal tubular transport and renal hemodynamics, which may be the trigger for renal hypertrophy, glomerular hyperfiltration and progression of renal disease as seen in diabetic nephropathy. Increased reabsorption of salt and water in proximal tubules is mainly due to activation of the apical  $\text{Na}^+/\text{H}^+$  exchanger, encoded by NHE3, as immunohistochemical (Amemiya *et al.*, 1995a; Biemesderfer *et al.*, 1993; Biemesderfer *et al.*, 1997), pharmacokinetic (Wu *et al.*, 1996) and knockout experiments confirm (Schultheis *et al.*, 1998). It has been suggested in previously, that proximal tubular  $\text{Na}^+/\text{H}^+$  exchange may confer renal salt and water retention in a state of diabetes (El-Seifi *et al.*, 1987; Harris *et al.*, 1986). In the present study we have investigated the regulation of NHE3, an apical brush border isoform of  $\text{Na}^+/\text{H}^+$  exchanger, in diabetic rats. We demonstrate that  $\text{Na}^+/\text{H}^+$  exchange in brush border membrane vesicles of proximal tubules of STZ-treated diabetic rats is increased through activation of NHE3. This increase is most likely due to posttranslational modification of the NHE3 protein.

In our studies we have used a model of streptozotocin (STZ) induced diabetes mellitus (DM) to examine proximal tubular  $\text{Na}^+/\text{H}^+$  exchange. Within two days after STZ administration experimental rats developed hyperglycemia and glucosuria. Furthermore, pancreatic insulin content was decreased at time of sacrifice due to destruction of  $\beta$  cells. Thus, STZ induced diabetes resembles type 1 (or insulin deficient) DM in humans.  $\text{Na}^+/\text{H}^+$  exchange activity in diabetic rats was significantly increased by about 40% at one and two weeks after STZ administration, but was not different between groups at two days. Several different isoforms of the  $\text{Na}^+/\text{H}^+$  exchanger have been cloned as of today. Based on current evidence, NHE3 is the most abundant isoform expressed in the proximal tubular apical brush border membrane. Experiments using different pharmacological agents in isoform specific inhibitory concentrations clearly reveal NHE3 to be the isoform that confers the increase in  $\text{Na}^+/\text{H}^+$  exchange activity induced by a diabetic milieu in rats. Interestingly, by oral administration of the NHE-inhibitory drug HOE-642 to diabetic rats the increase in BBM vesicle  $\text{Na}^+/\text{H}^+$  exchanger activity could completely be abolished, whereas the inhibitor had no effect on  $\text{Na}^+/\text{H}^+$  exchanger activity in control rats. The  $\text{IC}_{50}$  of HOE-642 *in vitro* is much lower for NHE1 than for NHE3. Therefore, one would expect a predominant inhibitory effect on NHE1 activity *in vivo* too, when given by oral administration. As NHE1 is ubiquitously expressed as a housekeeping protein its inhibition would possibly result in toxic effects, which, however,

were not notable in experimental animals. Studies by others have shown that oral administration of HOE-642 has a cardioprotective effect in ischemia induced reperfusion injury (Scholz *et al.*, 1995), and a hepatoprotective effect during liver fibrosis (Di Sario *et al.*, 2003). In preclinical studies HOE-642 was well tolerated and showed a favorable pharmacokinetic profile as well as an excellent bioavailability (Scholz *et al.*, 1999; Theroux *et al.*, 2000). Plasma concentrations from rats fed with chow containing 3ppm cariporide are about 300-400 ng/ml (Kusumoto *et al.*, 2001; Yoshida and Karmazyn, 2000), what is equivalent to the concentration of 1  $\mu$ M in rat blood and about 2  $\mu$ g/mL, or 8  $\mu$ M, for a 6ppm diet (Di Sario *et al.*, 2003). We did not measure HOE-642 plasma levels in our studies, but it can be assumed that they are in a comparable range. Based on *in vitro* data an inhibitory effect of HOE-642 on NHE3 was not to be expected in these concentrations, however, little is known about local concentrations of the substance in renal tissue.

Increased activity of  $\text{Na}^+/\text{H}^+$  exchanger can be the result of increased turnover rate of the exchanger with or without changes in protein expression. In our experiments the increased BBM vesicle  $\text{Na}^+/\text{H}^+$  exchanger activity was not accompanied by changes in NHE3 protein abundance as evidenced by western immunoblot studies in cortical BBM's and total cortical homogenates. In accordance, no increase in cortical NHE3 mRNA was found. These results imply posttranslational regulation of NHE3. We have recently shown regulation of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange activity due to translocation of NHE3 from subapical intermicrovillar storage compartments into the apical brush border membrane in rats with puromycin aminonucleoside (PAN) induced proteinuria. This translocation was accompanied by changes in the abundance of NHE3 protein bound to megalin, a recycling protein with regulatory function (Besse-Eschmann *et al.*, 2002). In analogy, and in view of the results in our present study, a similar mechanism may be operative in the regulation of NHE3 in a diabetic milieu. Rapid internalization of NHE3 with recycling back to the plasma membrane has been demonstrated in cell culture for diverse stimuli such as parathyroid hormone (Collazo *et al.*, 2000), dopamine (Hu *et al.*, 2001), endothelin-1 (Peng *et al.*, 2001), acid (Yang *et al.*, 2000) and albumin [chapter 5.2]. The mode of activation of NHE3 in a diabetic milieu is different from what we have found earlier for chronic metabolic acidosis in rats (Ambühl *et al.*, 1996). Induction of acidosis by adding  $\text{NH}_4\text{Cl}$  to the diet resulted in an increase in brush border membrane NHE3 abundance after 14 days by about 90 percent.

The current study did not address the question of how a diabetic milieu results in an increase of proximal tubular sodium/proton exchange. Several factors associated with diabetes can be discussed as possible mediators for the regulation of NHE3. Insulin, as a principal determinant of the diabetic milieu, is a probable contributor, although the mechanisms by which it induces  $\text{Na}^+/\text{H}^+$  exchange do not seem to be straightforward. The results of the present animal studies may appear contradictory to our earlier findings on the effect of insulin in isolated proximal tubule cells [chapter 5.1].  $\text{Na}^+/\text{H}^+$  exchange is increased both in STZ treated animals with decreased serum insulin concentrations and in OKP cells supplemented with insulin in the culture serum. The latter results suggest, that insulin may play a role for the induction of the  $\text{Na}^+/\text{H}^+$  exchanger mainly in type 2 diabetes characterized by hyperinsulinemia and insulin resistance. Interestingly, it has been speculated, that the kidney may be more sensitive to insulin compared to other tissues, which would be compatible with the stimulating effect of insulin on NHE3 observed in OKP cells. Similarly, Gesek *et al.* found stimulation of  $\text{Na}^+/\text{H}^+$  exchange by insulin in isolated proximal segments from normotensive and spontaneously hypertensive rats (Gesek and Schoolwerth, 1991). In accordance with this finding, insulin reduces sodium excretion in animals (Finch *et al.*, 1990; Hall *et al.*, 1991) and humans (Endre *et al.*, 1994; Herlitz *et al.*, 1996; Stenvinkel *et al.*, 1992). In type 1 DM pancreatic  $\beta$ -cell mass is diminished, resulting in insulin depletion. The fact, that  $\text{Na}^+/\text{H}^+$  exchange activity was increased nevertheless in our animal model of STZ induced type 1 diabetes suggests, that other factors besides of insulin are operative in the regulation of proximal tubular NHE3 activity. This hypothesis is compatible with our finding that administration of exogenous insulin to STZ rats did not affect  $\text{Na}^+/\text{H}^+$  exchange activity. Another explanation for the apparently opposite effects is that insulin may have different ways of action in diabetic and non-diabetic subjects. Hyperglycemia is another feature of DM, and it has been shown previously, that glucose has a stimulatory effect on NHE3 in OKP cells (Ambühl *et al.*, 1998). Again, in our STZ rat model, lowering serum glucose concentration by administration of exogenous insulin did not significantly diminish  $\text{Na}^+/\text{H}^+$  exchange activity. However, this may be a dose dependent effect, as others have shown that lowering serum glucose to normal levels does result in a less pronounced increase in renal cortical  $\text{Na}^+/\text{H}^+$  exchange activity. Taken together, the mechanism(s) responsible for regulation of NHE3 in a diabetic state can not be determined based on the available data. Besides of the factors mentioned before, other hormonal, neuronal and hemodynamic effects may be of importance, and may also explain the discrepancies between isolated cell culture and whole animal models.

Our experiments clearly demonstrate the involvement of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange in a diabetic milieu. However, the question remains whether and how activation of the proximal tubular exchanger contributes to the induction and progression of diabetic nephropathy. Besides the development of renal salt and water retention a typical feature of diabetic kidney disease is the occurrence of glomerular hyperfiltration and renal hypertrophy early in the stage of diabetes. The sequence of events it is still a matter of debate. (Bak *et al.*, 2000) have recently shown that renal hypertrophy occurs prior to salt retention and hyperfiltration. As tubular reabsorption in diabetes has been shown to increase more than GFR - resulting in a rise in fractional salt and water reabsorption - it is more likely to be the cause rather than the consequence of glomerular hyperfiltration. Thus, proximal tubular sodium retention, mediated via NHE3, may be the cause of glomerular hyperfiltration, resulting in chronic hyperfiltration damage of the renal tissue. Alternatively, the sodium/proton exchanger could directly be involved in renal hypertrophy. Fine *et al.* have suggested years ago, that the exchanger is a mediator of growth regulatory hormones. To solve these questions further studies will be needed that can be conducted using selective NHE.

In summary and conclusion, the present studies demonstrate regulation of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange, mediated through NHE3, in rats with features of type 1 diabetes mellitus. The increase in NHE3 activity is independent of changes in NHE3 transcription and protein abundance, and is most likely regulated by translocation of NHE3 from intracellular subapical compartments to the apical brush border membrane. These results suggest that NHE3 is the mediator of renal salt and volume retention observed in the early stage of diabetes mellitus.

## 6 Discussion

### 6.1 In vitro versus in vivo models

Complementary *in vivo* and *in vitro* studies are needed to identify and characterize the exact pattern of NHE3 regulation in a diabetic milieu. *In vitro* experiments were performed in cell culture model, an isolated system examining the “cellular level of complexity” in a defined physiochemical environment (growth media, pH, temperature, O<sub>2</sub>, etc.). Cell culture models are ideal to investigate isolated effects of single parameters, like insulin or albumin, in defined concentrations, and to exclude complex systemic effects present in whole animal studies. OKP cells are a well suited *in vitro* model with regard to several aspects: They grow in monolayers as a defined cell population which can be studied in many ways: microscopically on a structural basis, functionally with regard to transport activity, and on a molecular level to study mechanisms of regulation. However, cell culture models have the disadvantage that they do not always reflect phenomena occurring in an intact organism, as they are not part of systemic regulatory factors such as the nervous, endocrine, vascular and hemodynamic system. Moreover, isolated cells are devoid of some of their specific functions, like transcellular transport of reabsorbed substances through the basolateral membrane, as these cells grow as adherent monolayers on solid substrate. Nevertheless, the OKP cells used in our studies share many morphological and physiological similarities with proximal tubular cells and are ideal model to investigate singular parameters of diabetes mellitus.

Whole animal *in vivo* experiments, instead, reflect the complex interactions that occur in the body. Rat models of diabetes mellitus and nephrosis used in this project allow us to obtain an integral picture of the multifaceted relations among parameters that are involved in the regulation of sodium transport. The general disadvantage of these animal models is that they do not exactly match the pattern of pathologies encountered in human disease. In addition, animal experiments are time consuming, expensive and raise ethical problems.

## 6.2 Insulin action on NHE3

We have treated OKP cells with insulin to simulate hyperinsulinemia as it occurs in type 2 diabetes mellitus, and have shown that insulin activates the  $\text{Na}^+/\text{H}^+$  exchanger NHE3 in OKP cells. Hyperinsulinemia regulates NHE3 in a time-dependent manner, which implicates distinct regulatory mechanisms. After short insulin treatment time (2-12 h) the NHE3 activity was increased, without any detectable change in total NHE3 protein mRNA level, thus presumably due to regulation of NHE3 protein on a posttranslational level. We have demonstrated that the acute regulation of NHE3 is due to its trafficking from subapical vesicles to the brush border membrane (BBM). Alternatively, an increase in the turnover rate of the sodium and hydrogen exchanger may be an additional regulatory factor. At later time points of stimulation increases in NHE3 protein and mRNA amount are involved. Based on our experiments it is not possible to distinguish between increased synthesis and decreased degradation of NHE3 protein.

Our studies suggest an important role for insulin and insulin resistance in the regulation of renal tubular salt and water handling in diabetes mellitus. This observation fits the concept that other pathological conditions, such as hypertension, hyperlipidemia and atherosclerosis are also related to insulin resistance. The epidemiological association of insulin levels and blood pressure has raised the possibility of a role for insulin in the pathogenesis of essential hypertension. Studies performed by (Landsberg, 1986) established a relationship between insulin resistance and high blood pressure: hyperinsulinemia stimulates sympathetic nervous system (SNS) activity through dietary induced mediators, and, via effects on blood vessels, the heart and the kidneys, exerts a prohypertensive effect that causes hypertension. The effect of insulin to stimulate the SNS is readily demonstrable in humans (Anderson *et al.*, 1991; Hausberg *et al.*, 1995) as well as in rodents (Moreau *et al.*, 1995; Muntzel *et al.*, 1995). Another mechanism of insulin induced hypertension is via direct stimulation of sodium reabsorption in the renal proximal tubule. Numerous publications provide evidence for insulin stimulated sodium reabsorption in humans (DeFronzo, 1981; Quinones-Galvan and Ferrannini, 1997). Animal studies revealed that insulin stimulates sodium reabsorption by activating the basolateral sodium/potassium ATPase in rat collecting duct (Feraille *et al.*, 1995), in rat proximal convoluted tubule (Feraille *et al.*, 1994) and in proximal tubule cells (Feraille *et al.*, 1999). One animal model of type 2 DM, Obese Zucker rats, also present with high insulin levels in blood and increased amount of renal sodium transporters as well as high blood pressure (Bickel *et al.*, 2001). (Gesek and Schoolwerth, 1991) showed that  $\text{Na}^+/\text{H}^+$

exchange is increased in proximal tubules of spontaneously hypertensive rats (SHR) after insulin treatment. Our present study in OKP cells provides direct evidence for insulin dependent stimulation of NHE3. As NHE3 is responsible for the bulk of sodium reabsorption in proximal tubules, its selective activation may increase sodium retention in the kidneys and therefore play an important role in the volume expansion and salt dependent hypertension seen in insulin resistant diabetes.

In our experiments the effects of insulin were amplified in the presence of hydrocortisone. Hydrocortisone (HC) in combination with insulin enhances NHE3 activity, as well as protein, and mRNA amount in both early and late phase action. Using low concentrations of hydrocortisone only ( $10^{-9}$  M) we noticed no effect on NHE3 activity but, when given with insulin in the lowest stimulating dose ( $10^{-7}$  M), the effect on NHE3 activity was much greater than with insulin alone. This effect of hydrocortisone can be described as permissive. In contrast, at higher concentrations of HC, where HC itself has an effect on NHE3, its action together with insulin can be characterized as synergistic.

Hydrocortisone is a glucocorticosteroid hormone that modulates a large number of metabolic, cardiovascular, and immune functions throughout the body. In the fasted state, cortisol stimulates several processes that collectively serve to increase and maintain normal concentrations of glucose in the blood. These effects include stimulation of gluconeogenesis, mobilization of amino acids from extrahepatic tissues, inhibition of glucose uptake in muscle and adipose tissue and stimulation of fat breakdown in adipose tissue. Glucocorticoids also play a role in fat and protein metabolism, maintenance of arterial blood pressure, alteration of the connective tissue response to injury, reduction in the number of circulating lymphocytes, and functions of the central nervous system. Chronic corticosteroid overload increases the urine excretion of different ions, as ammonium, phosphate, and acid (Hulter *et al.*, 1980). The increased excretion of acid results at least in part from increased proximal tubule proton secretion and bicarbonate reabsorption (Baum and Quigley, 1993), due to the glucocorticoid-dependent stimulation of apical the  $\text{Na}^+/\text{H}^+$  exchanger in rat BBM vesicles (Freiberg *et al.*, 1982). The administration of dexamethasone in adrenalectomized and normal adult rats respectively has been shown to stimulate the rate of  $\text{Na}^+/\text{H}^+$  exchange activity without altering the affinity of the exchanger for sodium or protons (Kinsella *et al.*, 1985). Finally, the regulation of the amount of NHE3 protein in rat brush border membranes by dexamethasone was confirmed directly by immunoblot and immunohistochemistry (Loffing *et al.*, 1998).

HC has been shown to have a modulating effect on stimulation of NHE3 also by other agents, e.g. proximal tubule acidification (Baum and Quigley, 1993). Administered dexamethasone was equally efficient in stimulating  $\text{Na}^+/\text{H}^+$  exchange in normal and acidotic rats, but acidosis alone did not increase  $\text{Na}^+/\text{H}^+$  exchange activity in the absence of glucocorticoid treatment (Kinsella *et al.*, 1984). This indicates that the glucocorticoid effect was independent of acidosis, whereas the effect of acidosis was mediated by glucocorticoids. However, in cell culture, acid incubation in OKP cells did not require the presence of glucocorticoids for  $\text{Na}^+/\text{H}^+$  exchange activation (Amemiya *et al.*, 1995b), although glucocorticoids synergistically enhance the ability of acidosis to increase NHE3 translation and trafficking to the apical membrane (Ambühl *et al.*, 1999).

In this study we did not investigate the mechanisms of glucocorticoid action on NHE3. From what is known, glucocorticoids regulate  $\text{Na}^+/\text{H}^+$  exchange at different levels. There is a transcriptional effect of glucocorticoids, as shown by the direct increase in NHE3 mRNA amount in the proximal tubules from rabbits treated for 2 days with dexamethasone (Baum *et al.*, 1994) and in cell culture (Baum *et al.*, 1996), where the transcription rate is increased as well. In addition, the promoter of the rat NHE3 gene contains multiple DNA sequence elements which are recognized by the glucocorticoid receptor. Moreover, glucocorticoid treatment induced luciferase activity in OKP and LLC-PK1 cells transiently transfected with a chimera made of the 5'-regulatory region of NHE3 gene coupled to the luciferase gene (Cano, 1996; Kandasamy and Orłowski, 1996).

Glucocorticoid action is not only concentration but also time dependent, as we noticed in our experiments with insulin and albumin. Several studies indicate that dexamethasone can exert short term (few hours) *in vitro* effects on proximal tubule  $\text{Na}^+/\text{H}^+$  exchanger (Kinsella *et al.*, 1985). In rabbit proximal tubule cells, stimulation of  $\text{Na}^+/\text{H}^+$  exchanger activity was observed as soon as 1 h after dexamethasone addition (Bidet *et al.*, 1987). No change in the NHE3 mRNA level *in vivo* was observed at early time points (Baum *et al.*, 1994), suggesting that dexamethasone might induce transcription and synthesis of a protein that regulates  $\text{Na}^+/\text{H}^+$  exchanger activity, or shift NHE3 to the brush border membrane. In contrast, in OKP cells dexamethasone increased within 4 h both the activity of the  $\text{Na}^+/\text{H}^+$  exchanger (Baum *et al.*, 1993), the amount of NHE3 mRNA, and its transcription rate (Baum *et al.*, 1996), suggesting a direct transcriptional effect of glucocorticoids on NHE3.

It is not entirely clear whether glucocorticoids affect NHE3 translational or posttranslational factors that, in turn, may affect NHE3 activity. However, at early time points, glucocorticoids in combination with other activators of NHE3, such as acid, insulin or albumin, stimulate NHE3 without an effect on NHE3 transcription, which indicates a glucocorticoid dependent step in trafficking of NHE3 to the apical membrane.

A recent article presents new evidence for glucocorticoid activation of NHE3, which is different from NHE3 gene activation (Yun *et al.*, 2002a). Comparable to the *in vivo* activation of NHE3 in proximal tubules, dexamethasone treatment of the human colon carcinoma cell line CaCo-2 stimulates NHE3 activity within 4 h without changes in the NHE3 mRNA level, as analyzed by Northern blot and semiquantitative RT-PCR. Glucocorticoid activation of NHE3 required the presence of NHERF2 (E3KARP), a scaffold protein which clusters NHE3 and the cytoskeletal protein ezrin (Yun *et al.*, 1998). Ezrin itself can bind filamentous actin, serving as a bridge between membrane associated proteins and cytoskeleton. The second finding of Yun *et al.* is the activation of SGK1 (serum- and glucocorticoid-inducible kinase 1), a serine/threonine kinase, ubiquitously expressed in a wide variety of tissues, including intestine and kidney. During the stimulation of NHE3 activity by glucocorticoids, SGK1 likely interacts with NHERF2 via the PDZ domains to activate NHE3, which may facilitate translocation of NHE3 to the plasma membrane. In addition to glucocorticoids, SGK1 is induced by various stimuli such as follicle stimulating hormone, aldosterone, hyperosmolarity, protein kinase A, expression of p53, and injury to the brain (Kobayashi *et al.*, 1999; Park *et al.*, 1999; Waldegger *et al.*, 1997). Insulin is also a stimulator of SGK activity by a mechanism requiring the participation of phosphatidylinositol 3 kinase (PI 3-kinase) (Kobayashi *et al.*, 1999; Park *et al.*, 1999; Perrotti *et al.*, 2001). Albeit the study from Yun *et al.* was performed in intestinal cells, it opens a new view on how NHE3 regulation may occur. In kidneys, SGK1 activates the epithelial sodium channel (ENaC) in response to aldosterone (Chen *et al.*, 1999), and in a recent report, an interaction of SGK1 and NHERF2 in the regulation of ROMK1 (renal outer medullary K<sup>+</sup> channel) activity has been shown (Yun *et al.*, 2002b). It will be interesting to investigate whether insulin or glucocorticoid regulation of NHE3 in the kidneys is also connected to the SGK1 kinase pathway.

### 6.3 Albumin action on NHE3

In the second paper included in this thesis it was shown that albumin overload activates NHE3 in OKP cells in a time- and dose-dependent manner. The mechanisms of the activation include increase in NHE3 activity alone, increase in surface NHE3 due to a reinsertion of NHE3 protein from intracellular compartments into the apical cell membrane, and finally, increase in NHE3 mRNA and total cell NHE3 protein amount. The effects of albumin on NHE3 were enhanced by hydrocortisone.

Proteinuria is a cardinal feature of advanced diabetic nephropathy. Excessive protein filtration through the damaged glomeruli per se may lead to renal injury mediated by interstitial inflammatory reactions after protein reabsorption by the proximal tubules. Salt and water retention are common complications of the nephrotic syndrome. There are few mechanisms proposed for the pathophysiology of sodium retention. Hypoalbuminemia is a consequence of severe proteinuria and can cause secondary sodium retention due sequestration of salt and water into the interstitium. However, clinical and experimental findings are suggesting that systemic volume expansion in most nephrotic patients is the result of primary salt retention. A recent study suggested that renal interstitial inflammation followed by vasoconstriction may result in decreased sodium filtration and increased net sodium reabsorption (Rodriguez-Iturbe *et al.*, 2002). Primary salt retention in nephrotic syndrome is connected to the activation of the collecting duct  $\text{Na}^+/\text{K}^+$  ATPase (Deschenes and Doucet, 2000; Zolty *et al.*, 1999). The present results in OKP cells demonstrate that albumin stimulates apical brush border membrane  $\text{Na}^+/\text{H}^+$  exchange, indicating that proteinuria may lead to primary renal salt retention and systemic volume expansion due to increased proximal tubular  $\text{Na}^+/\text{H}^+$  exchange activity mediated through NHE3.

We have found that albumin increases cell surface NHE3 protein amount through an increase in reinsertion of endocytosed NHE3. Albumin reabsorption in proximal tubular cells occurs by receptor mediated endocytosis. NHE3 is present not only in the apical BBM but also in recycling endosomes of PT cells where, in addition to the  $\text{H}^+$ -ATPase, it regulates endosomal pH (D'Souza *et al.*, 1998; Hilden *et al.*, 1990). Vesicular acidification plays an essential role in the internalization of ligand/receptor complexes into endosomes during receptor mediated endocytosis. NHE3 appears to recycle between the plasma membrane and the early endosomal compartment. (Janecki *et al.*, 1998; Kurashima *et al.*, 1998). Gekle *et al.* have

suggested that endosomal albumin uptake depends on proper acidification of lysosomes by NHE3 (Gekle *et al.*, 1999). It is probable that in our study albumin overload stimulates NHE3 endocytosis because of increased demand for NHE3 in endocytotic vesicles.

A recent study suggests that the state of actin organization plays a role in the regulation of NHE3 activity and trafficking between intracellular compartments and the luminal membrane (Chalumeau *et al.*, 2001). The possible mechanism of NHE3 trafficking is based on alterations of protein/protein interactions, probably related to ezrin and F-actin proteins. Ezrin itself is able to bind F-actin and is a link between actin and the apical membrane. Regulatory proteins NHERF, E3KARP and EBP50 bind to NHE3 but also to ezrin (Kurashima *et al.*, 1999) and are possibly important elements in the regulation of the NHE3 endo-/exocytosis pathway.

At early time points of albumin treatment we observed an increase in NHE3 activity without a change in surface or total NHE3 protein amount. Apart from phosphorylation, shift and conversion of megalin-bound NHE3 from subapical membrane regions to the unbound NHE3 fraction in brush border membranes may control NHE3 activity. This hypothesis is supported by findings that a significant pool of NHE3 exists in association with megalin in the intermicrovillar microdomain of the brush border of proximal tubule cells (Biemesderfer *et al.*, 1999). The NHE3/megalín interaction will be discussed in detail in the next paragraph. Albumin also binds megalin (Cui *et al.*, 1996) and cubulin (Birn *et al.*, 2000) in PT, and eventually some other low molecular weight receptors (Brunskill *et al.*, 1997). It is unclear what relationship exists between endosomal NHE3 and megalin bound NHE3, and whether the megalin/NHE3 complex plays a role in albumin endocytosis. Taken together, NHE3 plays an important role in receptor mediated endocytosis of albumin due to its regulation of endosomal pH. Moreover, albumin per se is an activator of NHE3 in proximal tubule cells.

The interaction of hydrocortisone and albumin is quite complex and somewhat inconsistent. The modifying effect of hydrocortisone on the regulation of NHE3 by albumin is mechanism dependent, and, therefore, different for activity, surface protein vs. cellular protein, and it depends on the albumin concentration as well. Unlike in combination with acid (Ambühl *et al.*, 1999) or insulin, hydrocortisone has more likely an additive effect in combination with albumin on  $\text{Na}^+/\text{H}^+$  exchange activity in OKP cells.

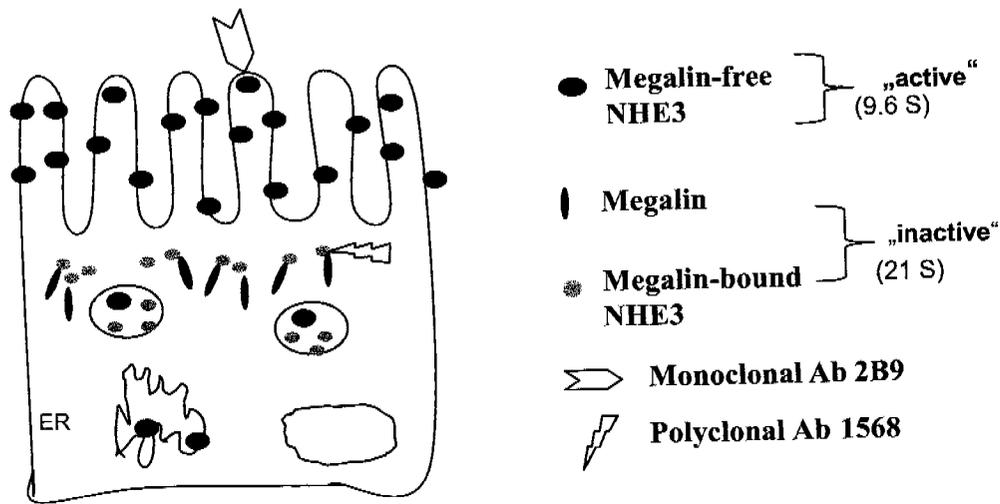
## 6.4 Effect of proteinuria on NHE3

Our paper on PAN induced nephrotic syndrome is the first to describe regulation of NHE3 in a proteinuric rat model. Proteinuria in rats activates  $\text{Na}^+/\text{H}^+$  exchange in proximal tubule cells. As detected by immunofluorescence experiments, the activation of NHE3 is associated with a shift of NHE3 from an inactive, megalin-bound, subapical pool to an active, megalin-free pool in the BBM of PT cells.

Diabetic nephropathy is the most common cause of nephrotic syndrome. Nephrotic syndrome is characterized by marked albuminuria due to loss of glomerular permselectivity and resultant disturbance in renal salt and water handling with edema formation. Other studies proposed the collecting duct and not the proximal tubule as the place of origin of sodium retention. This is based on micropuncture studies of the distal nephron, and on the increase in  $\text{Na}^+/\text{K}^+$  ATPase activity found in the collecting duct of nephrotic animals (Deschenes and Doucet, 2000; Ichikawa *et al.*, 1983; Zolty *et al.*, 1999). However, activation of proximal tubule sodium transport in nephrotic syndrome has not been systematically investigated, yet. In PAN induced nephrotic syndrome we demonstrate activation of proximal tubule NHE3, which can be correlated with enhanced salt and water reabsorption.

Deschenes *et al.* hypothesized that sodium retention may be independent of the development of proteinuria, since animals in several different nephrosis models exhibit different patterns of changes in protein and sodium excretion (Deschenes and Doucet, 2000). In our PAN experiments proteinuria appears only one day after the appearance of sodium retention. Along with the results from our experiments of albumin overload in cell culture with increased  $\text{Na}^+/\text{H}^+$  exchange activity, proteinuria induced salt retention seems very likely.

The mechanism of increased  $\text{Na}^+/\text{H}^+$  exchange activity was investigated by immunofluorescence studies, using antibodies that recognize different NHE3 pools. Biemesderfer *et al.* suggested that NHE3 is present in two states in PT cells: an active, "free" fraction of NHE3 in the BBM, and a megalin bound, inactive or storage fraction in subapical vesicles (Biemesderfer *et al.*, 2001). A scheme of the proposed mechanism is outlined in Figure 6.1.



**Figure 6.1: Trafficking of NHE3.** The apical, active fraction of NHE3 is not linked to megalin and is recognized by the monoclonal antibody # 2B9. NHE3 in the subapical compartment is prevalently bound to megalin and is recognized by the polyclonal antibody # 1568. Adapted graph courtesy of P. Ambühl.

In immunohistochemistry analyses of renal cortex from rats with PAN induced nephrotic syndrome, we clearly observe a shift of the megalin bound part of NHE3 from intracellular stores to the apex of the brush border, where it appears in its active, megalin free form. These findings clearly indicate a trafficking mechanism coupled with an interaction between megalin and NHE3 as regulatory factors for the activation of proximal tubular  $\text{Na}^+/\text{H}^+$  exchange.

## 6.5 Effect of STZ-induced diabetes on NHE3

In the present work, streptozotocin induced diabetes mellitus in rats activates  $\text{Na}^+/\text{H}^+$  exchange. Experiments with different NHE inhibitors clearly identified NHE3 as the isoform responsible for the increase in  $\text{Na}^+/\text{H}^+$  exchange observed in diabetic animals. This change in activity is not followed by changes in NHE3 protein and mRNA amount and therefore implicates posttranslational regulation of NHE3.

Streptozotocin induced diabetes is a commonly used model for type 1 DM. Comparable to humans, STZ treated animals present with high blood glucose levels, insulin deficiency and reduced sodium and water excretion. In contrast, proteinuria as an essential feature of advanced diabetes, is not inducible in this animal model. Sodium retention, along with water reabsorption and systemic volume expansion, may be a major cause for the development of hypertension, continued hyperfiltration and renal hypertrophy. Therefore, we investigated the regulation of the proximal tubular  $\text{Na}^+/\text{H}^+$  exchanger NHE3 and observed its up-regulation in rats with STZ induced diabetes mellitus.

Our experiments showed enhanced NHE3 activity without alteration of protein and mRNA amount. Considering our findings from cell culture and PAN induced nephrosis, we propose a shifting of NHE3 from the intercellular compartment to the apical membrane of proximal tubules responsible for NHE3 activation. Other animal studies have suggested trafficking as the major mechanism responsible for altered apical membrane NHE3 amount too, for example during acute hypertension (Yang *et al.*, 2002), and regulation by parathyroid hormone (Fan *et al.*, 1999, Zhang, 1999 #254).

Sixteen years ago, El-Seifi *et al.* found that tubular  $\text{Na}^+/\text{H}^+$  exchange in STZ rats is increased (El-Seifi *et al.*, 1987). We could demonstrate for the first time, using different pharmacological inhibitors, that NHE3 is the isoform responsible for the increase in  $\text{Na}^+/\text{H}^+$  exchange present in diabetic rats. However, only recently a new isoform, NHE8, was detected in the kidney's proximal tubule (Goyal *et al.*, 2003). As there are no data available yet on the pharmacological profile of NHE8 towards different inhibitors, we can not exclude some contribution of NHE8 to the changes in  $\text{Na}^+/\text{H}^+$  exchange activity observed in diabetic rats.

It remains an interesting question what factor(s) of the diabetic milieu is responsible for the regulation of NHE3. Regarding the effect of insulin, as a principal determinant of the diabetic milieu, we found contradictory findings comparing our cell culture and animal studies.  $\text{Na}^+/\text{H}^+$  exchange was increased both in cells treated with insulin, as well as in animals with decreased serum insulin concentrations. These results suggest, that insulin may play a role mainly in type 2 diabetes. As mentioned earlier, type 2 DM is characterized by hyperinsulinemia and insulin resistance as represented in our cell culture setting. Interestingly, it has been speculated, that the kidney may be more sensitive to insulin compared to other tissues, which would be compatible with the stimulating effect of insulin on NHE3 observed in OKP cells. Conversely, in type 1 DM pancreatic  $\beta$ -cell mass is diminished, resulting in insulin depletion. The fact, that  $\text{Na}^+/\text{H}^+$  exchange activity was increased nevertheless in our animal model of STZ induced type 1 diabetes suggests, that other factors besides of insulin are operative in the regulation of proximal tubular NHE3 activity. This hypothesis is compatible with our finding that administration of exogenous insulin to STZ rats did not affect  $\text{Na}^+/\text{H}^+$  exchange activity. Hyperglycemia is another feature of DM, and it has been shown previously, that glucose has a stimulatory effect on NHE3 in OKP cells (Ambühl *et al.*, 1998). Again, in present STZ rat model, lowering serum glucose concentration by administration of exogenous insulin did not significantly diminish  $\text{Na}^+/\text{H}^+$  exchange activity. This, however, may be a concentration dependent effect, as others have shown that lowering serum glucose to normal levels does result in a less pronounced increase in renal cortical  $\text{Na}^+/\text{H}^+$  exchange activity (El-Seifi *et al.*, 1987). Taken together, the mechanism(s) responsible for regulation of NHE3 in a diabetic state can not be determined based on the available data. Besides of the factors mentioned before, other hormonal, neuronal and hemodynamic effects may be of importance, and may also explain the discrepancies between isolated cell culture and whole animal studies.

In summary, our studies suggest that proximal tubular  $\text{Na}^+/\text{H}^+$  exchange is regulated both in a diabetic milieu as well as in nephrotic syndrome, and may be implicated in the pathogenesis of renal salt and water retention as well as in the progression of renal disease in these clinical settings. Future studies should be directed towards more detailed characterization of the mechanisms involved in the regulation of NHE3 in diabetes and proteinuria. This knowledge will help to develop strategies to interfere with the processes that lead to functional and structural damage of the kidneys in patients suffering from diabetes mellitus and other protein losing disorders.

## 6.6 Remarks about the animal models

Diabetes mellitus in rats was induced by single injection of streptozotocin, 60 mg/kg of body weight. In few rats blood sugar two days after injection was below 10.0 mM these rats were subjected to a second injection of STZ, and subsequently developed hyperglycemia. Titration experiments using different doses of STZ showed that streptozotocin administration at concentrations exceeding 40 mg/kg resulted in long-term, stable hyperglycemia with no insulin response to glucose and a marked derangement of islet morphology after 3 months of diabetes. In contrast, at 30 and 40 mg/kg, streptozotocin induced transient hyperglycemia with blood glucose levels returning to normal within 10 days after streptozotocin administration, and normal islet morphology at 3 months after induction of diabetes (Ar'Rajab and Ahren, 1993). As we used STZ in a concentration of 60 mg/kg, it is safe to assume that rats developed stable hyperglycemia. This is supported by detection of hyperglycemia at the day of sacrifice in our studies. The difference in the severity of diabetes was manifested by the range of blood glucose levels which varied between 13 mM to 33.3 mM and beyond. This is probably due to variable absorption of STZ from the peritoneal cavity, as well as the individual capacity for regeneration of the rat pancreas. This variation in the extent of hyperglycemia may have affected  $\text{Na}^+/\text{H}^+$  exchanger activity. However, we did not notice any correlation between blood sugar and NHE3 activity.

The administration of puromycin results in the clinical equivalent of nephrotic syndrome with proteinuria as well as salt and volume retention. The kidneys of rats treated with PAN exhibit partially collapsed and dilated proximal tubules and generalized shortening of the brush border in proximal tubules. Microscopical evaluation of cortical tissue of PAN treated rats indeed revealed focal damage of mainly early proximal tubular segments. PAN induced nephritis is primarily characterized as a damage of the glomerular barrier, but it has been reported that puromycin not only affects glomerular structures but that it has also tubulotoxic effects (Diamond and Anderson, 1990). A decrease of NHE3 protein amount that we noticed in BBM of PAN treated rats could be partially due puromycin toxicity, although other BBM proteins such as NaPi-2 showed no relevant difference in there amount among control and PAN treated animals.

## 7 References

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## 8 Abbreviations

BBM	brush border membrane
BP	blood pressure
Cl <sup>-</sup>	chloride ion
DM	diabetes mellitus
ESRD	end stage renal disease
GFR	glomerular filtration rate
H <sup>+</sup>	hydrogen ion
HC	hydrocortisone
HOE 642	cariporide, selective NHE1 (IC <sub>50</sub> 0.08 μM) and NHE2 (IC <sub>50</sub> 1.6 μM) inhibitor
Na <sup>+</sup>	sodium ion
NaCl	sodium chloride
NaHCO <sub>3</sub>	sodium bicarbonate
NHE	sodium/proton exchanger
NHE3	sodium/proton exchanger type 3
NHERF	sodium/proton exchanger regulatory factor proteins
NS	nephrotic syndrome
OKP	opossum kidney cells (also as OK cells in literature)
PAN	puromycin aminonucleoside
PT	proximal tubule
S1611	selective NHE3 inhibitor (IC <sub>50</sub> 0.05-1.03 μM)
S3226	selective NHE3 inhibitor (IC <sub>50</sub> 0.02-0.71 μM)
SDS	sodium dodecyl sulfate
SSC	standard sodium citrate
STZ	streptozotocin

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