Kinasen in der Regulation des epithelialen Transports

Kinases in the regulation of epithelial transport

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To my

Father Sri B Siva Rao,
Mother Srimati B Sujatha
Father-in-law 'Late' Sri R Rajeshwer Rao
Mother-in-law Srimati R Radha
my beloved husband and rest of the family

For all their support and encouragement

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SUMMARY

Insulin and insulin-like growth factor (IGF-1) regulate renal electrolyte excretion and the signalling includes PI3-kinase, PDK1, PKB and SGK isoforms. Thus SGK isoforms are expected to play a major role in the regulation of renal function which is evident from renal phenotype of *sgk1*$^{-/-}$ mice.

The end effect of PI3-kinase signalling is phosphorylation of GSK3 by PKB/SGK1 which results in inhibition of its activity. Accordingly, similar/parallel renal phenotype can be expected between *sgk1*$^{-/-}$ mice and *gsk3*^{KI} mice where GSK3 is resistant to PKB/SGK1 inhibition.

The first study was made to elucidate the role of GSK3 in renal electrolyte excretion and hormone release. The plasma aldosterone and corticosterone concentrations were significantly lower while 24-hour urinary aldosterone was significantly higher and urinary corticosterone tended to be higher in *gsk3*^{KI} mice than in *gsk3*^{WT} mice. The possibility of reduced salt appetite due to low aldosterone was checked in *gsk3*^{KI} mice .The *gsk3*^{WT} mice drank more saline over tap water while *gsk3*^{KI} mice drank similar amounts of tap and saline water. *gsk3*^{KI} mice display higher metabolic rate with significantly more food and fluid intake, fecal excretion, GFR, urinary flow rate and urinary Na^+, K^+ and urea excretion with lower plasma Na^+ and urea concentrations and significantly higher blood pressure.

Enhanced Na^+ excretion may at least partially be due to low aldosterone levels which should however, not increase but decrease the renal K^+ excretion. Thus, a renal mechanism involving enhanced ENaC activity presumably causes enhanced renal K^+ secretion as Lithium, an inhibitor of GSK3 is shown to downregulate ENaC expression. Higher fluid intake accounts for higher urinary flow rate but does not reflect decreased urine concentrating ability, as urinary osmolarity is increased in *gsk3*^{KI} mice. Water deprivation did not abrogate the differences in urine output. GSK3 may participate in the regulation of renal tubular water transport. The unrestrained GSK3 could downregulate NO-synthase which induces thirst. The role of GSK3 in eNOS regulation may further contribute to differences in BP. The results thus indicate that the renal phenotype of *gsk3*^{KI} mice is different from the *sgk1*$^{-/-}$ mice in several aspects, thereby suggesting a more direct role of GSK3 in renal electrolyte balance.

APC fosters degradation of ß-catenin which is known to upregulate a variety of proteins responsible for tumerogenesis. It was suggested and shown that SGK1 is among the proteins that are upregulated. SGK1 expression has also been shown recently to be enhanced in gastric glands of *apc* $^{Min/+}$ mice. A similar upregulation was expected in the kidneys of

these mice and as SGK1 regulates renal electrolyte homeostasis, a possible renal phenotype for APC mutant mice was expected. The second study was therefore conducted to explore the kidney function in APC mutant mice and thereby elucidate the renal phenotype of those mice. The body weight, food, fluid intake and fecal excretion were not significantly different between the genotypes. Urine flow tended to be lower in $apc^{Min/+}$ mice than $apc^{+/+}$ and $apc^{Min/+}/sgk1^{-/-}$ mice. The glomerular filtration rate and Na^+ excretion were decreased while fractional excretion of K^+ was enhanced in $apc^{Min/+}$ mice. The antinatriuresis and glomerular filtration tended to be partially reversed by additional lack of SGK1. Fecal sodium and potassium excretion were significantly higher in $apc^{Min/+}$ mice than in $apc^{+/+}$ mice. The additional lack of SGK1 could reverse the difference in fecal sodium excretion but not the fecal potassium excretion.

The plasma aldosterone and corticosterone concentrations were significantly higher in $apc^{Min/+}$ mice. While plasma corticosterone concentration was similar in $apc^{+/+}$ mice and $apc^{Min/+}/sgk1^{-/-}$ mice, plasma aldosterone was even higher in $apc^{Min/+}/\ sgk1^{-/-}$ mice than in $apc^{Min/+}$ mice. The hyperaldosteronism in $apc^{Min/+}$ mice was paralleled by elevated plasma volume and blood pressure. The difference in plasma volume and blood pressure were slightly reversed by additional lack of SGK1. The partial reversal of anti-natriuretic, hypervolemic and hypertensive effects in $apc^{Min/+}$ mice by additional lack of SGK1 implicates its role in the abnormal elelctrolyte homeostasis in $apc^{Min/+}$ mice. SGK1 defeciency augments the effect of defective APC on plasma aldosterone indicating a complex interaction of APC and SGK1 or independent regulations of APC and SGK1 in hormone release.

The signalling cascade explaining insulin stimulated renal tubular phosphate reabsorption remains elusive. Renal phosphate reabsorption is regulated by membrane abundance of the phosphate transporter, NaPiIIa and PI3-kinase signalling possibly stimulates renal tubular reabsorption by increasing the membrane abundance of NaPiIIa. Again the possible role of SGK isoforms could be expected here and the third study thus focussed on the regulation of renal phosphate handling by SGK3 which is a downstream kinase in insulin signalling. Metabolic studies in $sgk3^{KO}$ mice showed significantly higher phosphate excretion in comparision to wild-type mice supporting this hypothesis. Invitro studies involving coexpression of SGK3 and NaPiIIa in *Xenopus* oocytes provided additional evidence by showing that phosphate induced current is significantly enhanced in SGK3 and NaPiIIa expressing *Xenopus* oocytes. Food intake was significantly higher in $sgk3^{KO}$ mice while the plasma PTH and plasma phosphate concentrations were similar to $sgk3^{WT}$ mice. Plasma

vitamin-D concentration and bone mass were significalty lower. Plasma and urinary calcium levels were not different between the genotypes.

These observations reveal a direct role of SGK3 in the phosphate transport and the higher phosphate loss and low plasma vit-D in *sgk3* ^{KO} mice could have contributed to the demineralization of the bones in *sgk3*^{KO} mice.

ZUSAMMENFASSUNG

Insulin und Insulin-like-Growth Factor (Insulinähnlicher Wachstumsfaktor, IGF-1) regulieren die Elektrolytausscheidung in der Niere. An dieser Signalkaskade beteiligen sich die PI3-Kinase, PDK1, PKB und verschiedene Sgk-Isoformen. Demzufolge spielen Sgk-Isoformen erwartungsgemäß eine bedeutende Rolle bei der Regulation der Nierenfunktion, die für den renalen Phänotyp von *sgk1*$^{-/-}$ Mäusen verantwortlich ist.

Am Ende der PI3-Kinase Signalkaskade steht die Phosphorylierung der GSK3 durch PKB/SGK1. Diese Phosphorylierung hat die Inaktivierung der Kinase zur Folge. Demnach ist ein ähnlicher Phänotyp bei *sgk1*$^{-/-}$ und *gsk3*^{KI} Mäusen zu erwarten.

Der erste Teil der Studie sollte die Funktion der GSK3 in Bezug auf Nierenfunktion und Hormonregulation aufklären. Die Aldosteron- und Kortisolplasmaspiegel waren signifikant erniedrigt. Gleichzeitig konnten signifikant erhöhte Aldosteronkonzentrationen bei den *gsk3*^{KI} im 24-Stunden-Urin gemessen werden. Die Kortisolausscheidung war bei *gsk3*^{KI} tendenziell höher als bei *gsk3*^{WT} Mäusen. Aufgrund der reduzierten Aldosteronplasmaspiegel wurde überprüft, ob *gsk3*^{KI} Mäuse möglicherweise einen gesteigerten Kochsalzkonsum aufweisen. Im Vergleich tranken *gsk3*^{WT} Mäuse mehr Kochsalzlösung als normales Trinkwasser, während *gsk3*^{KI} Mäuse keine Unterschiede im Trinkverhalten zeigten. *gsk3*^{KI} Mäuse zeichneten sich durch eine gesteigerte von Nahrungs- und Trinkwasseraufnahme, erhöhtes Fekalgewicht, sowie eine erhöhte GFR und Urinvolumen, gesteigerte Elektrolyt- (K^+ und Na^+) und Harnstoffausscheidung bei niedrigeren Plasmakonzentrationen von Na^+ und Kreatinin, sowie signifikant erhöhten Blutdruck aus.

Eine gesteigerte Na^+-Ausscheidung kann, zumindest teilweise, durch niedrige Aldosteronspiegel erklärt werden. Diese sollten ebenfalls die K^+-Ausscheidung vermindern und nicht erhöhen. Vermutlich ist dies ursächlich durch einen renal stimulierten Mechanismus zu erklären, der eine gesteigerte ENaC-Aktivität zur Folge hat. Für Lithium, einen GSK3-Inhibitor, wurde beispielsweise eine Herunterregulation der ENaC-Expression gezeigt. Eine vermehrte Flüssigkeitsaufnahme resultiert in einem größeren Urinvolumen, spiegelt jedoch nicht eine verminderte Konzentrationsfähigkeit des Urins durch die Niere wider. Die Osmolarität des Urins von *gsk3*^{KI} Mäusen war dementsprechend erhöht. Flüssigkeitsentzug glich den Unterschied in der Ausscheidungsmenge zwischen den Genotypen nicht aus. Möglicherweise spielt die GSK3 beim Wassertransport, der durch die Nierentubuli gewährleistet wird, eine Rolle. Die nicht-inhibierte GSK3 könnte die NO-Synthase, welche Durstgefühl vermittelt, herunterregulieren. Der Einfluss der Kinase auf die NO-Bildung

könnte darüber hinaus eine Ursache für den erhöhten Blutdruck der *gsk3*^{KI} Mäuse sein. Der Konsens dieser Ergebnisse deutet auf einen nachweisbaren Unterschied des Nierenphänotyps zwischen *gsk3*^{KI} und *sgk1*$^{-/-}$ Mäusen hin und lässt die Rolle der GSK3 bezüglich des Elektolythaushaltes als bedeutender erscheinen.

APC fördert den Abbau von ß-Catenin, welches für die Hochregulation einer großen Anzahl an Proteinen, die für die Tumorgenese von Bedeutung sind, wichtig ist. SGK1 befindet sich nachweislich unter diesen hochregulierten Proteinen. Ebenso konnte bewiesen werden, dass die Expression der SGK1 in den Magendrüsen von apcMin/+ Mäusen erhöht ist. Ähnliche Regulationsmechansimen hätte man in der Expression der Nieren dieser Mäuse erwartet, und, da bekannt ist, dass SGK1 die renale Elektrolytausscheidung reguliert, auch einen möglicher relevanter Nierenphänotyp. Der zweite Teil dieser Studie wurde daher der Exploration der Nierenfunktion von APC-defekten Mäusen gewidmet. Köpergewicht, Nahrungs- und Trinkverhalten und Fekalvolumen war nicht unterschiedlich. Das Urinvolumen erschien bei den *apc*$^{Min/+}$ Mäusen niedriger als bei den *apc*$^{Min/+}$ und *apc*$^{Min/+}$*/sgk1*$^{-/-}$ Mäusen. Die glomeruläre Filtrationsrate und Na^+-Ausscheidung waren erniedrigt während die fraktionelle K^+-Ausscheidung bei den apcMin/+ erhöht war. Die verminderte GFR und Exkretion von Na^+ wurde teilweise durch die Abwesenheit der SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$) aufgehoben. Der Na^+ und K^+-Gehalt in den Feces waren bei den *apc*$^{Min/+}$ im Vergleich zu den *apc*$^{Min/+}$ Mäusen signifikant erhöht. Die verminderte Na^+-Ausscheidung wurde durch den zusätzlichen Mangel an SGK1 egalisiert während die K^+-Ausscheidung im Stuhl unverändert blieb.

Die Konzentrationen an gemessenen Aldosteron- und Kortisolspiegeln im Plasma waren bei den *apc*$^{Min/+}$ signifikant erhöht. Die Kortisolplasmakonzentrationen der *apc*$^{Min/+}$ und *apc*$^{Min/+}$*/sgk1*$^{-/-}$ Mäusen waren gleich, die Aldosteronkonzentration war bei den *apc*$^{Min/+}$*/sgk1*$^{-/-}$ sogar höher als bei den *apc*$^{Min/+}$ Mäusen. Der Hyperaldosteronismus der *apc*$^{Min/+}$ Mäuse ging einher mit einem gesteigerten Plasmavolumen sowie einem erhöhtem Blutdruck, beides wurde durch einen zusätzlichen Mangel an SGK1l zu einem gewissen Grad normalisiert. Die partielle Aufhebung der antinatriuretischen, hypervolämischen und hypertensiven Effekte bei den *apc*$^{Min/+}$*/sgk1*$^{-/-}$ Mäusen lässt einen Zusammenhang zwischen einem SGK1-knock-out und dem veränderten Elektrolythaushalt der *apc*$^{Min/+}$ Mäuse vermuten. Das Fehlen von SGK1 erhöht die Auswirkung von nicht-funktionellem APC auf die Plasmaaldosteronkonzentration und deutet auf eine komplexe Interaktion zwischen APC und SGK1 hin. Eine unabhängige Hormonregulation durch APC und SGK1 ist ebenfalls denkbar und daher in Betracht zu ziehen.

Die Signalkaskade, welche die Insulin stimulierte renal-tubuläre Phosphatrückresorption erklären könnte, bleibt unklar. Die Phosphatrückresorption in der Niere wird durch die Anwesenheit von Phosphattransportern (NaPiIIa) in der Membran reguliert. Die PI3-Kinasekaskade stimuliert möglicherweise diese Rückresorption, indem die Menge des NaPiIIa in der Membran hochreguliert wird. Auch hier wäre ein möglicher Einfluss der SGK-Isoformen denkbar, daher fokussierte der dritte Teil der Studie die Wirkung der SGK3, eine Kinase innerhalb der Insulinsignalkaskade, auf den renalen Phosphattransport. Untersuchungen an SGK3-defizienten (*sgk3*$^{-/-}$) Mäusen zeigten eine signifikant erhöhte Phosphatausscheidung. Dieser Befund unterstützte die These einer möglichen Beteiligung der Insulinsignalkaskade an der renalen Phosphatregulation. Voltage clamp Untersuchungen, die in vitro Experimente an Oozyten beinhalteten, zeigten, dass die Koexpression von SGK3 und NaPiIIa in den Oozyten den Phosphatausstrom aus den Zellen signifikant erhöhte. Die Nahrungsaufnahme war bei *sgk3*$^{-/-}$ Mäusen signifikant erhöht. Die Konzentrationen von Parathormon und Phosphat im Plasma ähnlich deren von *sgk3*$^{+/+}$ Mäusen. Die Plasmakonzentration von Vitamin D und die Knochenmasse waren bei den *sgk3*$^{+/+}$ Mäusen signifikant vermindert. Die Calciumkonzentration im Plasma und Urin unterschieden sich nicht.

Diese Befunde decken einen direkten Einfluss der SGK3 auf den Phosphathaushalt und dessen Regulationsmechansimen, der den Vitamin D-Haushalt und die Knochenmineralisierung beeinträchtigt, auf.

INTRODUCTION

KIDNEY

Kidneys are primarily responsible for the maintenance of body fluid homeostasis, which is achieved by regulating the volume and concentration of body fluids by selectively filtering and reabsorbing materials from the blood and urine. Neural and endocrine systems acting on kidneys from outside the excretory system along with auto-regulatory mechanisms of kidneys regulate the functional processes and thus help in the homeostasis.

The main functions of kidney include:

- Regulation of water and electrolyte content of the body.
- Retention of substances vital to the body such as protein and glucose
- Maintenance of acid/base balance.
- Excretion of waste products, water soluble toxic substances and drugs.
- Endocrine functions

Water and electrolyte balance

Water content of the body is maintained by regulating the osmalality of body fluids. Loss of fluid through lungs or skin increases while fluid intake decreases the osmalality. Kidney regulation of water excretion through antidiuretic hormone (ADH) maintains the osmotic pressure of the extracellular fluid (ECF) by negative feedback mechanism. The osmoreceptors located in the anterior hypothalamus are sensitive to the changes in intracellular volume or changes in osmotic concentration. With a rise in ECF osmotic concentration, the impulses from the receptors are transmitted to the secretory neuron endings in the posterior pituitary which triggers ADH release. The released ADH increases water reabsortion and continues solute excretion reducing the osmotic concentration of the ECF providing the negative feedback to osmoreceptors which in return induce fall in the rate of ADH release.

The hormones interact when blood loss or dehydration occurs to maintain intravascular volume.

Excretion of waste products

Filtration occurs as blood flows through the glomerulus. Metabolic wastes and drugs that cannot be filtered by the glomerulus are secreted into the tubule and excreted in the urine.

Regulation of Acid-Base balance

The body is very sensitive to its pH level. Alterations in the pH causes protein denaturation and loss of function of enzymes. The kidneys maintain acid-base homeostasis by regulating the pH of the blood plasma.

The lungs and kidneys work together to maintain constant pH of 7.35 - 7.45 (34-46 nmol.l-1 H^+ concentration) in the extracellular fluid and arteries. The two buffer systems are in dynamic equilibrium with the same hydrogen ion concentration (pH). The lungs assist in maintaining a constant blood pH by removing CO_2, while the kidney excretes acid in the form of $H_2PO_4^-$ and NH_4^+ and alkali in the form of HCO_3^-.

Regulation of BP

The kidney influences blood pressure by:

* Constriction and dilatation of arteries and veins
* Increasing the circulating blood volume

Specialized cells called macula densa located in a portion of the distal tubule and in the wall of the afferent arteriole sense the Na^+ in the filtrate and the arterial cells (juxtaglomerular cells) sense the blood pressure. When the blood pressure drops the juxtaglomerular cells sense it and convey to the macula densa cells leading to decrease in amount of Na^+ filtered. The juxtaglomerular cells then release an enzyme called renin. Renin converts angiotensinogen into angiotensin I which is then converted to angiotensin II by an angiotensin-converting enzyme (ACE) that is found mainly in the lungs. Angiotensin II causes blood vessels to contract and thus elevates the blood pressure Figure 1.

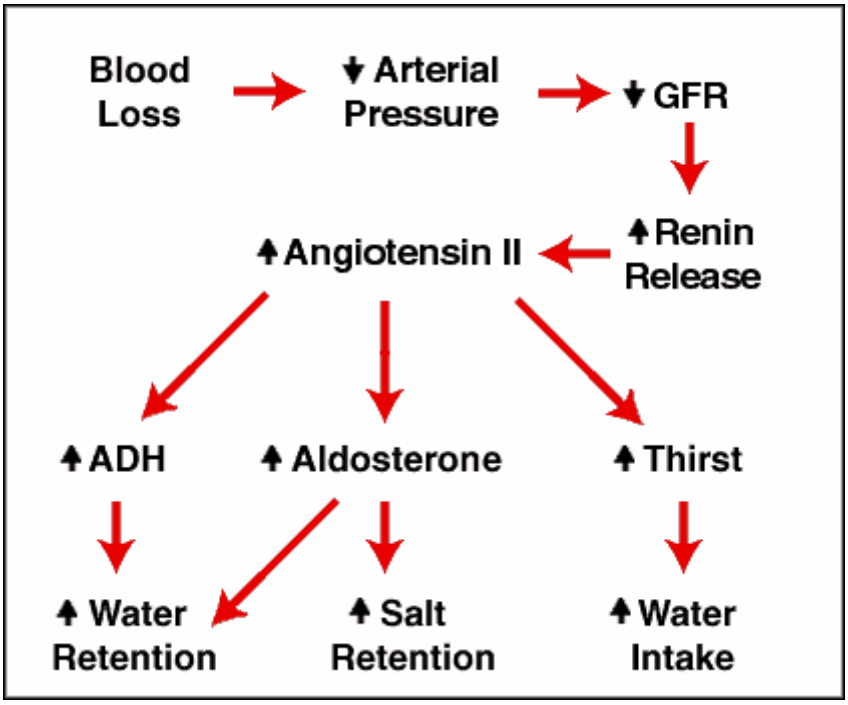

Figure 1 A simplified flow chart of blood pressure regulation by the kidney.

Endocrine regulation and homeostasis

Aldosterone hormone regulation of sodium and potassium

The kidneys play a central role in the maintenance of Na^+ homeostasis. It is important to tightly regulate the urinary Na^+ excretion in order to maintain a constant extracellular volume during varying dietary Na^+ intake and extrarenal Na^+ losses. The final concentration of renal Na^+ that is excreted is controlled by the ASDN (aldosterone sensitive distal nephron) which includes the late distal convoluted tubule, the connecting tubule and the cortical (CCD) as well as the medullary (MCD) collecting ducts [1]. Na^+ entry into the epithelial cells via the epithelial Na^+ channel (ENaC) in the luminal membrane and exit through the Na^+,K^+-ATPase in the basolateral plasma membrane accomplishes the transepithelial Na^+ transport in these segments. The rate-limiting step in this process is ENaC which is highly regulated [2]. It is composed of three subunits (α, β, and γ) [3-6] with a stoichiometry of 2α1β1γ [7], although other stoichiometries have also been suggested (octa- or nonamers) [8;9]. Mutations in the genes encoding ß- and γ-ENaC leads to Liddle's syndrome, an inherited form of salt-sensitive hypertension [10]. The PY motifs of ENAC sub-units are the binding sites for ubiquitin protein

ligases of Nedd4/Nedd4-2 like family [11-13]. Binding to these motifs ubiquitylates ENAC on α and γ sub-units consequently leading to the internalization and degradation of ENaC in the endosomal/lysosomal system [14;15].

Apart from its stimulatory effect on renal Na^+ reabsorption, aldosterone has strong kaliuretic action. The renal outer medullary K^+ channel (ROMK) regulates the K^+ secretion occurring in ASDN. ROMK is coexpressed with ENaC in the ASDN cells and the necessary driving force for K^+ secretion is provided by the Na^+ reabsorption. Inhibition or genetic loss of function of ENAC lowers renal K^+ secretion leading to hyperkalemia. However, it remains unresolved whether the kaliuretic effect of aldosterone is through direct regulation of ROMK function or by Na^+ reabsorption via ENAC activation. No measurable effect of acute aldosterone administration on K^+ channel number, open probability or conductance was found by patch-clamp studies on rat CCDs [16;17], but some data suggested that aldosterone induces renal K^+ secretion already at aldosterone concentrations that do not exhibit any measurable effect on urinary Na^+ excretion [18]. Moreover, high K^+ intake results in more efficient increase in ROMK activity in intact rats than in adrenalectomized animals, suggesting that aldosterone may have at least a permissive effect on ROMK activation [16].

Parathyroid hormone and calcium and phosphate regulation

Parathyroid hormone (PTH) is responsible for the endocrine regulation of calcium and phosphate. Decrease in blood levels of calcium stimulates the production of PTH, which has physiological effects on the kidneys.

In the kidneys the parathyroid hormone increases calcium reabsorption in the renal distal tubules, while it inhibits phosphate reabsorption in proximal tubules and thus forces renal phosphate wasting.

Phosphate homeostasis

Phosphate (Pi) homeostasis in higher organisms depends on the coordinated transport of Pi across intestinal and renal epithelia. Transport of Pi across the apical membrane is mediated by the three members of the SLC34 family of solute carriers referred commonly as 'NaPi' [19]. NaPiIIa (SLC34A1) and NaPiIIc (SLC34A3) are specifically expressed in the brush border membrane (BBM) of renal proximal tubules whereas NaPi-IIb (SLC34A2) has a broader pattern of expression and is highly abundant in the BBM of small intestine. The transporter mediating the basolateral Pi exit in both renal and intestinal epithelia is unidentified. Na^+-

dependent electroneutral anion exchanger has been proposed to be at least partially responsible for Pi exit in the proximal tubule [20].

NaPiIIa and NaPiIIc mediate the reabsorption of Pi from the urine by using the free energy provided by the electrochemical gradient for Na^+. NaPiIIa is electrogenic and it preferentially transports divalent Pi with a strict Na^+-Pi stoichiometry of 3:1, which results in the net inward movement of one positive charge per cotransport cycle. In contrast, NaPiIIc is electroneutral and functions with a 2:1 stoichiometry (Figure 2) [21;22]. In mice, NaPi-IIa is the main phosphate transporter in the adult kidney, whereas NaPiIIc appears to be more important in weaning animals. This was further confirmed by the phenotype of NaPiIIa knockout mice which suggested that this cotransporter is responsible for the bulk of renal Pi reabsorption with a very small percentage potentially attributed to NaPi-IIc [23]. The expression of NaPiIIa and NaPiIIc is regulated to adapt the renal reabsorption of Pi to the needs of the organism. Thus, the phosphaturic effect associated with parathyroid hormone (PTH) is due to the membrane retrieval of both cotransporters, whereas in conditions of Pi deprivation their expression is increased [24-26].

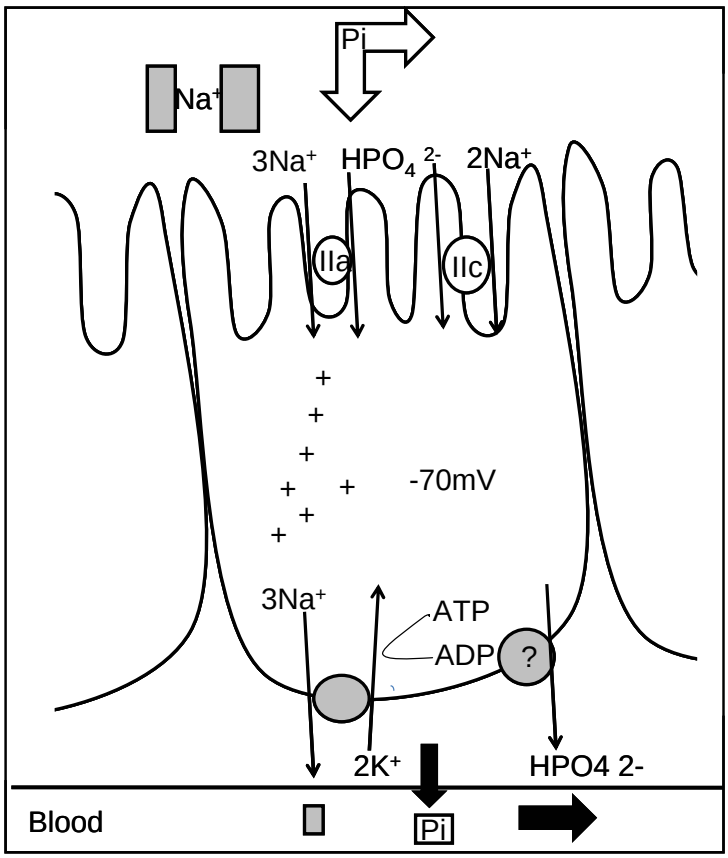

Figure 2: Mechanisms of phosphate transport.

Many hormonal and non-hormonal factors also regulate renal Pi reabsorption and the effect of PTH and dietary Pi on NaPi-IIa has been investigated in detail [27]. These studies suggest that regulation of NaPi-IIa depends on its shuttling to/from the BBM. This contrasts with many other transporters, whose activity is modulated by modification of the transport protein itself (e.g. phosphorylation, dimerization etc). This means that the body's requirements for a higher Pi reabsorption (i.e. after low Pi-diet) are met by increasing the expression of NaPiIIa [24;28;29] and NaPiIIc [21] at the BBM. Acute upregulation of NaPiIIa is thus independent of changes in transcription or translation and the increased expression of NaPiIIa must be due to either the stabilization of the transporter at the BBM [30] or to an increased rate of insertion at the membrane [28].

On the other hand, reduced reabsorption of Pi (i.e. upon PTH release or high Pi-diet) is achieved via downregulation of NaPiIIa [25;28;31] and NaPiIIc [26] at the BBM. PTH-induced downregulation of NaPiIIa has been studied extensively and the identifiable steps are summarized in Figure 2.

SGK1

Serum and glucocorticoid-inducible kinase, an inducible Ser/Thr Kinase, (*sgk1*) was originally isolated in a differential screen searching for glucocorticoid-induced transcripts in a mammary tumor cell line [32]. It was found to be induced within 30 minutes, either by glucocorticoids or by serum and in both mammary epithelial cells and fibroblasts. SGK1 belongs to AGC kinases family, which include protein kinase A (PKA), protein kinase G (PKG), protein kinase C (PKC), and protein kinase B/Akt/rac (PKB/Akt). Its catalytic domain shares 54% identity with those of Akt/PKB/rac kinases, 48% with that of PKC-β, 50% with that of rat p70S6K kinase and 45% with that of PKA.

SGK1 is also induced by a very large spectrum of stimuli distinct from glucocorticoids and serum. These include aldosterone [33;34], cell shrinkage [35;36], cell swelling [37], TGF-β [38-41], ischemic injury of the brain [42;43], neuronal excitotoxicity [44], memory consolidation [45], chronic viral hepatitis [40], DNA-damaging agents [46], vitamin D_3 [47], psychophysiological stress [48], iron [49], glucose [41], endothelin-1 [41], granulocyte-macrophage colony–stimulating factor (GM-CSF) [50], fibroblast growth factor (FGF) [51], platelet-derived growth factor (PDGF) [51], phorbolesters [51], follicle-stimulating hormone (FSH) [52], sorbitol [46;53], heat shock, oxidative stress, UV irradiation and p53 [46;53].

SGK1 is phosphorylated on Ser422 in the C-terminal region by a so far unknown kinase termed the hydrophobic motif (H-motif)/PDK2 that is dependent on PI3 Kinase signalling. SGK1 is an unstable protein with a rapid turnover and a half-life of approximately 30 minutes. Rapid degradation of SGK1 involves its ubiquitylation followed by degradation by the proteosome. Moreover, it appears that ubiquitylated SGK1 is preferentially associated with intracellular membranes [54]

Target Proteins

SGK1 was shown to phosphorylate a variety of proteins. The first demonstrated substrate for SGK1 was glycogen synthase kinase 3 (GSK3), a kinase that is involved in the regulation of glycogen and protein synthesis by insulin and that is also a substrate of PKB/Akt [55;56]. Phosphorylation of GSK3 by both SGK1 and PKB/Akt leads to an increase in the synthesis of glycogen.

Role of SGK1 in Aldosterone-Dependent Na^+ Reabsorption

SGK1 can directly interact with ENaC [57] and increase ENaC channel activity by phosphorylating the α-ENaC subunits. The action of SGK1 on ENaC is complex and likely involves (a) increase in the subunit abundance in the plasma membrane and (b) activation of channels already in the plasma membrane combined with an increase in ENaC opening probability.

As an aldosterone-induced protein, SGK1 is thought to mediate at least some of the physiological effects of aldosterone on ENaC and Na^+,K^+-ATPase. The stimulatory effect of aldosterone (or of dexamethasone) on SGK1 expression has now been firmly documented and extensively reviewed [58]. Corticosteroids rapidly (within 30 minutes) induce SGK1 at the mRNA and/or protein levels. This induction coincides with enhanced phosphorylation of Nedd4-2 and reduced renal Na^+ secretion in intact animals. The physiological importance of aldosterone in SGK induction is supported by the fact that dietary Na^+ restriction, which physiologically increases plasma aldosterone, also induces SGK1 mRNA in the renal cortex [59]. The aldosterone-dependent induction of SGK1 occurs specifically in the ENaC-positive cells of the ASDN, whereas in other nephron portions such as the thick ascending limb or the proximal tubule SGK1 expression is not increased by aldosterone [58].

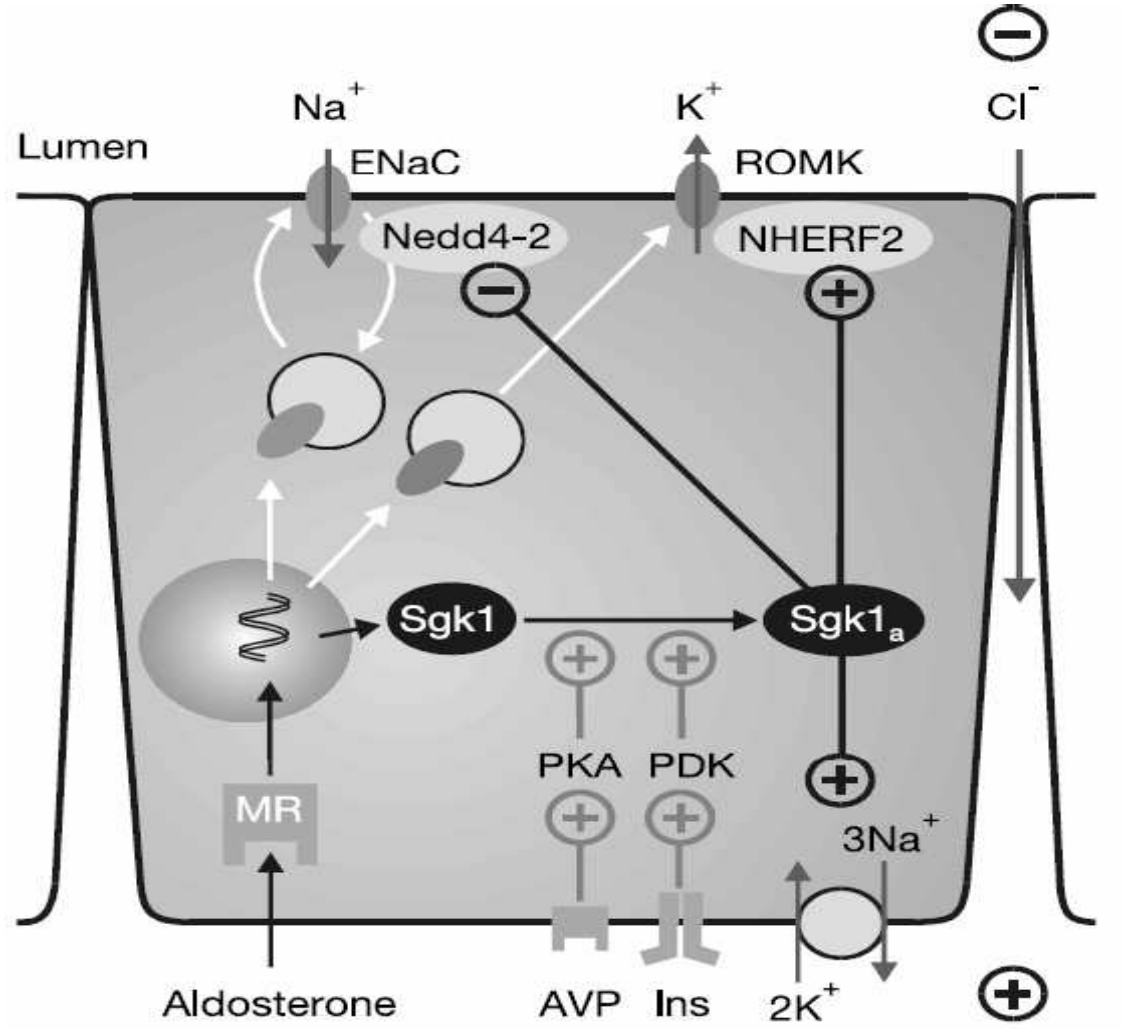

Figure 3: Proposed interaction between SGK1, epithelial Na^+ channel (ENaC) and renal outer medullary K^+ channel (ROMK) in aldosterone-sensitive distal nephron. [60]

Aldosterone stimulates the expression of ENaC, ROMK and SGK1. Activation of SGK1 (Sgk1a) requires phosphorylation of the kinase, which, in addition to other factors, can be induced by binding of insulin (Ins) or arginine vasopressin (AVP) to their basolateral receptors. Sgk1 is not absolutely required for insertion of ENaC or ROMK into the apical membrane, explaining the mild phenotype of SGK1-deficient mice under standard NaCl and K^+ diet. SGK1-dependent upregulation of Na^+ reabsorption, however, is required under reduced dietary NaCl intake as well as for upregulation of renal K^+ excretion in response to increased dietary K^+ intake. SGK1a increases Na^+ reabsorption by activating Na^+-K^+-ATPase and enhancing the abundance in the cell membrane of ENaC through inhibition of ubiquitin ligase Nedd4–2 mediated internalization of ENaC. Effects of SGK1a on ENaC and Na^+-K^+-ATPase increase the electrical driving force for paracellular Cl^- reabsorption as well as the electrochemical driving force for K^+ secretion through ROMK. In addition, SGK1 may enhance the abundance of ROMK in the apical membrane by synergizing with NHERF2. MR, mineralocorticoid receptor; PKA, protein kinase A; PDK, 3-phosphoinositide-dependent kinase.

Mouse phenotype

The SGK1-deficient mice exhibiting rather a mild phenotype points to a certain degree of redundancy in SGK1-dependent signal transduction cascades. SGK1 mice have no obvious defect [61]. Closer analysis reveals the decreased ability of SGK1 knockout mice to retain salt under a salt-deficient diet [61] or to adequately enhance renal K^+ output during a K^+ load [62]. Presumably due to salt depletion, plasma aldosterone concentration is enhanced [61] and renal Ca^{2+} excretion is decreased [63]. The mice are relatively resistant to the hypertensive effect of a high-fructose diet [64] or a high-fat diet [65] together with salt excess. In the SGK1 knockout mice, the stimulating effect of mineralocorticoids on salt appetite [66] and the stimulating effect on intestinal glucose uptake [67] is blunted and the uptake of glucose into brain, adipocytes and skeletal muscle following a glucose load is decreased [68].

GSK3

Glycogen synthase kinase 3 (GSK3) was originally identified as one of five protein kinases that phosphorylate the rate-limiting enzyme glycogen synthase (GS) of glycogen synthesis in response to insulin [69]. GSK3 is a serine threonine kinase encoded by two isoforms GSK-3α and GSK-3β [70].

GSK3 is ubiquitously expressed and evolutionarily conserved kinase [71]. In addition to GS,GSK3 phosphorylates a broad range of substrates, including several transcription factors such as c-Myc, c-Jun and c-Myb [72] and the translation factor eIF2B [73]. GSK3 has also been implicated in the regulation of cell fate in *Dictyostelium* [74] and is a component of the Wnt signalling pathway required for *Drosophila* and *Xenopus* development [75]. In mammalian cells, on stimulation with insulin or other growth factors, GSK3 is rapidly phosphorylated at serine 21 in GSK-3α or serine 9 in GSK-3β, resulting in inhibition of GSK3 kinase activity [76]. Protein kinase B (PKB/Akt), a serine-threonine kinase located downstream of phosphatidylinositol 3-kinase (PI3K), has been demonstrated to phosphorylate both of these sites in vitro and in vivo, suggesting that growth factors downregulate GSK3 activity through the PI3K–PKB signalling cascade [56]. Consistent with its position downstream of the PI3K–PKB pathway, GSK3 activity suppresses cell proliferation and survival.

Regulation of GSK3 by the Wnt pathway

In multicellular organisms, GSK3 functions in several distinct signalling pathways. Activation of any of these pathways leads to inactivation of GSK3 kinase activity by one of the three distinct mechanisms: (i) inhibition by the action of Dishevelled, (ii) NH2-terminal domain serine phosphorylation or (iii) tyrosine phosphorylation.

In the absence of a Wnt signal, GSK3 interacts with β-catenin, axin and APC in the cytoplasm and phosphorylates these proteins, leading to the Slimb/βTrCP-mediated ubiquitination and proteolytic degradation of β-catenin. Upon binding of Wnt by a seven-transmembrane domain receptor, dishevelled is activated resulting in the downregulation of GSK3 kinase activity [77](Figure 4).

Regulation of GSK3 by other signalling pathways

GSK3 phosphorylates GS at four distinct sites. Inhibition of GSK3 causes activation of glycogen synthase due to less phosphorylation leading to conversion of glucose into glycogen. Stimulation of glycogen synthesis by insulin also involves the dephosphorylation of serine residues in glycogen synthase.

Several signalling mechanisms independent of Wnt have been proposed to explain inhibition of GSK3. Some studies support the involvement of mitogen-activated protein kinase (MAPK) in GSK3 regulation.

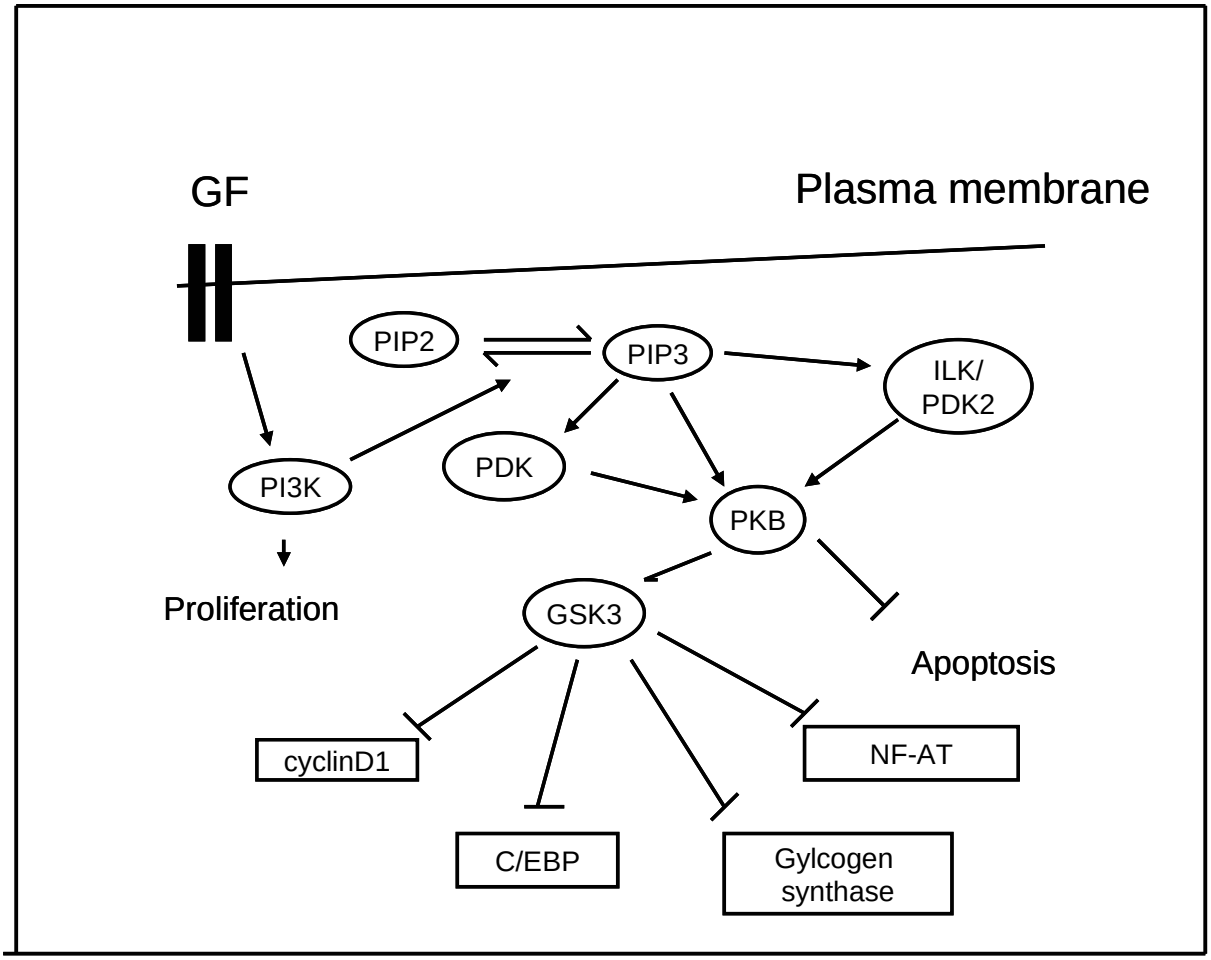

Figure 4: Role of GSK3 in phosphatidylinositol 3-kinase signalling.

Upon receptor tyrosine kinase activation, PI3 kinase is recruited to the plasma membrane and phosphorylates phosphoinositides at the 3‘-position of their inositol ring. This, in turn, recruits PH-domain-containing proteins such as PKB and the PDKs. Once phosphorylated by the PDKs, PKB is activated and phosphorylates GSK-3 leading to its inhibition.

GSK3 inhibition

GSK3 can be inhibited by Akt phosphorylation, which is part of insulin signal transduction. Therefore, Akt is an activator of many of the signalling pathways blocked by GSK3 Experimentally, it has been shown that certain concentrations of lithium chloride (LiCl) and/or 6-bromoindirubin-3'-oxime (BIO) will inhibit GSK3 [78] in the Wnt signalling pathway. This inhibition of GSK3 is currently believed to underlie the therapeutic usefulness of lithium salts for the treatment of mood disorders [79]. GSK3 phosphorylation by PKC kinases in hemopoietic cells regulate growth [80].

Mouse phenotype

Dario R. Alessi generated GSK3 knockin mice in which the codon encoding Ser21 of GSK3alpha and Ser9 of GSK3beta was changed to encode a non-phosphorylatable Alanine residue. The GSK3 knockin mice display no overt phenotype. GSK3 knockin mice develop and grow normally (shown by growth curves from 4-16 weeks of age) and are non-diabetic. These mice can dispose of injected glucose at the same rate as wild type mice. They possess normal fasted glucose and insulin levels [81].

APC

The adenomatous polyposis coli (APC) gene was identified by positional cloning in 1991. APC is a large gene, encompassing 15 exons with an open reading frame of 8,538 base pairs. It encodes a protein of 2,843 amino acids with a molecular weight of 310kD. APC is classified as a tumor suppressor gene, since inactivation of both alleles results in the loss of control of cell growth and proliferation. Patients with FAP have a germ-line mutation in one of the two alleles of the APC gene. These mutations result from point mutations, insertions or deletions that lead to a premature stop codon and a truncated functionally inactive protein [82]. More than 300 different APC mutations have been described [83].

The APC gene product is widely expressed in many tissues, including brain, eye, esophagus, stomach and liver tissues. Its structure is characterized by numerous functional domains which mediate protein-protein interactions in cell adhesion, the formation of epithelial cell-cell contacts, regulation of ß-catenin and maintenance of cytoskeletal microtubules [84].

APC in WNT pathway

WNT acts as a ligand for a 7-pass transmembrane Frizzled receptor. When WNT is absent, ß -catenin is ubiquinated and degraded by the proteosomal pathway, resulting in low levels of ß-catenin. WNT absence allows DSH to activate GSK3, which then phosphorylates ß-catenin, followed by its ubiquination by TrCP and proteosomal degradation. This involves a multiprotein destruction complex composed of APC, AXIN, GSK3, and TrCP. A LEF/TCF transcription factor together with cofactors CBP, CtBP, and Groucho repress target genes. When WNT is present, DSH inhibits phosphorylation of ß-catenin by GSK3. This results in excess ß-catenin which translocates to the nucleus and together with LEF/TCF, upregulates target genes. Regulation of ß-catenin is essential for the tumor suppressor effect of APC. This can be circumvented by mutations in either APC or ß-catenin, resulting in familial adenomatous polyposis.

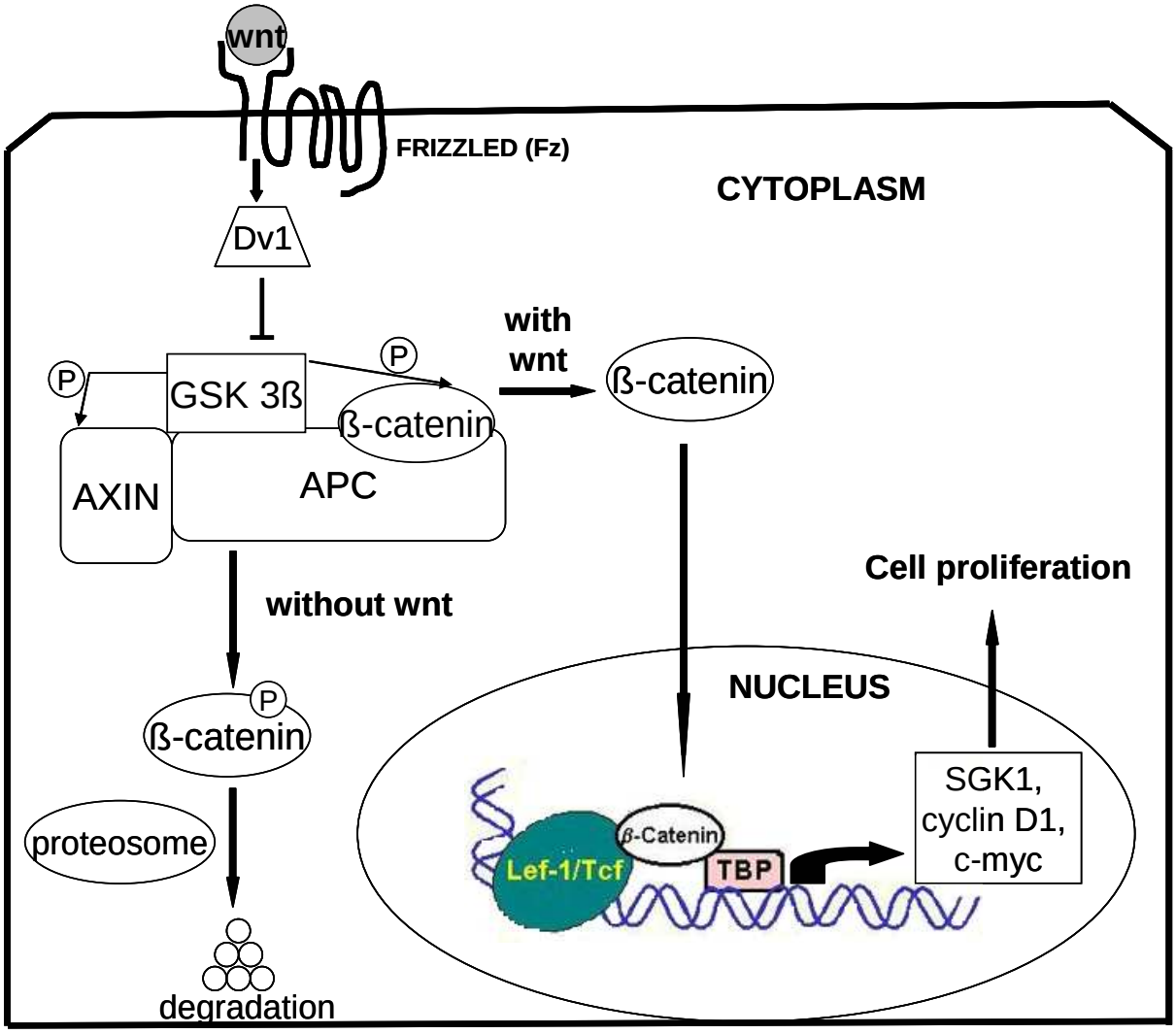

Figure 5: A simplified illustration of canonical WNT pathway.

Mouse Phenotype

Mice homozygous for the Min mutation die as embryos, while heterozygous mice develop a severe phenotype characterized by numerous small intestinal adenomas and mammary tumors [85]. Two different APC mutant mice having mutations at codon 1638 were generated by gene targeting. APC 1638N heterozygous mice that have a much milder phenotype (five to six small intestinal tumors per mouse) and APC1638T heterozygous mice that do not develop intestinal tumors [86;87]. A fourth mouse model, APC d716, develops 200 to 500 intestinal adenomas.

SGK3

Serum and glucocorticoid inducible protein kinase 3 (SGK3) is an isoform of SGK like SGK2. SGK2 and SGK3 share 80% amino acid sequence identity in their catalytic domains with each other and with SGK [88]. For these reasons, SGK has been termed SGK1. SGK3, also termed cytokine-independent survival kinase (CISK) [89], is distinguished by an NH2-terminal PX (Phox) domain. Like SGK1, SGK3 is expressed in all human and murine tissues examined, but expression is particularly high in the mouse heart and spleen and in the embryo. SGK3 appears to be localized to vesicular compartments in transfected 293 cells. This localization is dependent on the PX domain, the removal of which by truncation of the NH2 terminus results in diffuse staining of SGK3 throughout the cell [89].

Regulation of SGK3 kinase activity

SGK3 protein kinase becomes functional after activation by phosphorylation, which is accomplished through a signalling cascade involving PI3-kinase, PDK1 and PDK2 or "hydrophobic motif" (H-motif) kinase [55;90]. Degradation of PIP3 by phosphatase and PTEN [91] leads to the disruption of activation of the SGK3. SGK3 is also activated by oxidation, insulin and IGF-I through the same signalling cascade.

Functions

Ion channels

Like *sgk1*, *sgk3* regulates many ion channels like the Epithelial Na^+ channel-ENaC [1], renal epithelial Ca^{2+} channel-TRPV5 [92] , renal and inner ear Cl^- channel-ClC-Ka [93], ubiquitous Cl^- channel-ClC2 [94], cardiac voltage-gated Na^+ channel-SCN5A [95], cardiac and epithelial K^+ channels-KCNE1/KCNQ1 [96], voltage-gated K^+ channels-Kv1.3, Kv1.5 [97] and Kv4.3, and amino-3-hydroxy-5-methyl-4-isoxazolic acid (AMPA) receptor [98].

Carriers and Pumps

SGK3 has been shown to upregulate amino acid transporters like EAAT1 and EAAT5 [99;100]. Na^+-dicarboxylate cotransporter NaDC-1 is stimulated by SGK1 and SGK3, an effect requiring the participation of NHERF2 [101]. Both SGK1 and SGK3 stimulate the activity of Creatine transporter-CreaT (SLC6A8) [102]. SGK3 modulates Na^+-K^+-ATPase activity (Henke

et al., 2004). In *Xenopus* oocytes, SGK3 has been shown to stimulate the activity of the Na^+-glucose cotransporter SGLT1 [103], which serves to absorb intestinal glucose.

SGK3 has been shown to confer cell survival [89]. The antiapoptotic effect of SGK3 has been attributed in part to its ability to phosphorylate forkhead transcription factors, such as FKRHL1[89]. Moreover, SGK3 has been shown to phosphorylate and thus inactivate Bad [104]. Phosphorylated Bad binds to the chaperone 14–3-3 and is thus prevented from traveling to the mitochondria, where it triggers apoptosis [105].

Mouse phenotype

The phenotype of SGK3 knockout mice is surprisingly mild. SGK3 null mice do not exhibit any of the phenotypes associated with the various reported PKB knockout mice [106]. Mice lacking SGK3 are viable and fertile and display a defect in post-natal hair follicle development. Starting at post-partum day 4 (PP4), a clear difference in hair follicle progression can be observed which becomes more pronounced in later stages. In addition, the hair follicle of the knockout animals is disorganized, suggesting that SGK3 may also be involved in cell differentiation and migration. SGK3 null mice also show a transient growth defect until 7 weeks of age [106]. This delayed growth may be due to a decrease in intestinal glucose transport through the sodium-dependent glucose transporter SGLT1 [107]. Closer inspection of the SGK3 knockout mice revealed a subtle decrease of locomotion [60].

AIMS OF THE STUDIES

The present studies aimed at identifying the role of three different kinases involved in various renal epithelial transport mechanisms and their regulation in hormone secretion.These include the following

a) The role of PKB/SGK resistant GSK3 in renal water and electrolyte excretion as well as steroid hormone release.
b) SGK1 dependence of renal electrolyte balance and hormone regulation in APC min mice.
c) The role of SGK3 in renal phosphate handling.

Rationale for the present studies

a) The role of PKB/SGK resistant GSK3 in renal water and electrolyte excretion as well as steroid hormone release

GSK3 activity is inhibited by insulin [108;109], an effect mediated by protein kinase B [56;110] and the serum and glucocorticoid inducible kinase SGK [111].

At least in theory, inhibition of GSK3 could contribute to SGK1 dependent effects of insulin on renal electrolyte transport. Insulin stimulates the renal epithelial Na^+ channel ENaC and thus leads to renal retention of NaCl [112-114]. The effect of insulin on ENaC involves the Phosphatidylinositide-3 (PI3)- kinase [115-117] and the serum and glucocorticoid inducible kinase SGK1 [33;34;118]. Whether or not the effects of SGK1 on ENaC [33;34;119] and further renal tubular transport systems [60] involves regulation of GSK3, has never been addressed.

Moreover, GSK3 could participate in the insulin like growth factor (IGF)-dependent regulation of aldosterone and cortisol synthesis [120].

The first study explored, whether phosphorylation and inactivation of GSK3 could participate in the regulation of renal tubular electrolyte transport and steroid hormone release. To this end aldosterone and cortisol release as well as renal electrolyte excretion were analysed in mice carrying mutations of GSK3α,ß (*gsk3*^{KI}), in which the serines of the PKB phosphorylation sites were replaced by alanine [81]. Those mice have previously been shown to lack the known effect [81;121] of insulin on muscle glycogen synthase [81]. Steroid hormone release and renal electrolyte excretion, have, however, not been analysed in those mice.

b) SGK1 dependence of renal electrolyte balance and hormone regulation in APC min mice

Lack of APC leads to accumulation of ß-catenin, which travels into the nucleus and triggers the expression of several genes [122;123] including the serum and glucocorticoid inducible kinase SGK1 [124;125]. As shown in gastric glands [126], SGK1 expression may indeed be enhanced in those mice. Among other targets, SGK1 phosphorylates glycogen synthase kinase 3 (GSK3) [111], which in turn phosphorylates ß-catenin and is thus involved in APC-dependent regulation of cellular functions [127-129]. SGK1 is a stimulator of a wide variety of renal transport proteins [60]. Moreover, lack of SGK1 leads to an increase of plasma mineralocorticoid concentration [61].

The present study was thus performed to elucidate whether adrenal hormone levels and/or electrolyte homeostasis are altered in *apc*$^{Min/+}$ mice and if so whether the difference may be dependent on the presence of SGK1. To this end, metabolic cage experiments were performed in *apc*$^{Min/+}$ mice, in their wild type littermates (*apc*$^{+/+}$) and in *apc*$^{Min/+}$ mice lacking in addition SGK1 (*apc*$^{Min/+}$/*sgk1*$^{-/-}$).

c) The role of SGK3 in renal phosphate handling

Phosphate is a critically important component of bone minerals [130;131]. Accordingly, adequate mineralization of bone depends on the precise tuning of phosphate balance, which is a function of intestinal absorption and renal excretion [130;132;133]. The latter depends on cellular uptake of phosphate across the apical membrane of proximal tubular cells [134], which is accomplished mainly by the Na^+-coupled phosphate transporter NaPiIIa [132;134]. The carrier is downregulated by parathyroid hormone PTH [132], a hormone at least in part effective through cAMP. Renal tubular phosphate reabsorption is stimulated by insulin [135;136] and by insulin-like growth factor IGF-1 [137]. Little is known, however, about the signalling involved in insulin and IGF-1 mediated regulation of phosphate excretion.

The signalling of insulin involves the serum and glucocorticoid-inducible kinase SGK3 [88], which, similar to protein kinase B [138] and SGK1 [55], is activated through phosphatidylinositol (PI)-3 kinase and phosphoinositide-dependent kinase PDK1.

The present study thus explored the possibility that SGK3 may be involved in the regulation of renal tubular transport. To this end, in vitro regulation of NaPiIIa by SGK3 was studied in the *Xenopus* oocyte expression system and in vivo significance of SGK3-sensitive

phosphate transport elucidated by analyzing gene targeted mice lacking functional SGK3 (*sgk3*^{KO}) as well as their wild type littermates (*sgk3*^{WT}) .

MATERIALS

Equipment

Accucheck Sensor Comfort	(Roche Diagnostics, Manheim, Germany)
Balance	(Sartorius, Göttingen, Germany)
BioPhotometer Eppendorf	(Eppendorf, Wesseling-Berzdorf, Germany)
Centrifuge 5417 R	(Eppendorf, Hamburg, Germany)
Dri-chem clinical chemistry analyzer	(FUJI FDC 3500i, Sysmex, Norsted, Germany).
Flame photometry	(AFM 5051, Eppendorf, Hamburg, Germany)
Gamma Counter	(Perkin Elmer, Massachusetts, USA)
Heparinized capillaries	(Hirschmann laborgerate,Eberstadt,Germany)
Hot air oven	(Memert,Schwabach,Germany)
High resolution microCAT-II	(Siemens, Germany)
Magnetstirrer	(IKA, Staufen, Germany)
Metabolic cages	(Techniplast, Hohenpeissenberg, Germany)
Multireactiontubes	(Eppendorf, Hamburg, Germany)
MultiChanelPipet	(Eppendorf, Hamburg, Germany)
Multilevel counter	(Victor 1420, PerkinElmer, Boston, USA)
Osmometer	(Osmomat 030, Gonotec, Berlin, Germany)
Petri dishes	(Greiner Bio-one, Frickenhausen, Germany)
Pipettes	(Eppendorf, Hamburg, Germany)
Pipette tips	(Carl Roth, Karlsruhe, Germany)
Shaker VIBRAX VXR	(IKA, Staufen, Germany)
Spectronic GENESYS 6	(Thermo Fisher Scientific Inc. Massachusets, USA)
SpeedVac SVC 100	(Savant Life Sciences, Bath, UK)
Sterile PS-tube 4.5 ml 12.4/75 MM	(Greiner bio-one, Frickenhausen, Germany)
Sterile filters	(Millipore, Cork, Ireland)
30-gauge insulin syringe	(BD micro-fine, Heidelberg, Germany).
Syringes, Omnifix-H, 1ml	(Braun, Melsungen, Germany)
Ultracentrifuge	(Beckman Coulter, Krefeld, Germany)
UV-cuvettes 8.5mm	(Plastiband, Antwerp, Belgium)
Vortex	(Labnet Abimed, Langenfeld, Germany)
Waterbath	(Labortechnik, Seelbach, Germany)

Kits

Albumin determination kit	(microfluoral, Progen, Heidelberg, Germany)
Aldosterone kit	(Demeditec, Kiel, Germany)
ADH RIA kit	(Immunotech, Marseille, France; IBL, Hamburg, Germany)
Corticosterone ELISA kit	(DRG Instruments, Marburg, Germany)
Creatinine determination kit for Plasma - creatinine PAP	(Lehmann, Berlin, Germany)
Creatinine kit for urine – Jaffe Kreatinin	(Labor technik,Berlin,Germany)
Gamma-B 1.25-Dihydroxy-vitamin D	(IDS, Boldon, UK)
Glucose kit: gluco-quant	(Roche Diagnostics, Mannheim, Germany)
Inorganic Phosphate	(Roche Diagnostics, Mannheim, Germany)
Insulin ELISA kit	(Mercodia, Uppsala, Sweden)
Intact parathormone ELISA kit	(Immunotopics, San Clemente, CA, USA)
Mouse intact PTH Elisa kit	(Immunotopics, California, USA)
Plasma leptin ELISA kit	(Linco, St. Charles, USA)
Urea determination kit	(Lehman,Germany)

Chemicals

Aqua ad injectabili	(Ampuwa, Niefern, Germany)
Calcium chloride	(Sigma-Aldirch, Hannover,Germany)
Dexamethasone phosphate disodium salt	(Sigma, Taufkirchen, Germany)
Diethylether	(Carl Roth, Karlsruhe, Germany)
DOCA pellets	(50 mg, Innovative Research of America, Sarasota, Florida, USA)
Ethanol absolute (99%)	(Carl Roth, Karlsruhe, Germany)

Evans Blue	(Sigma, Taufkirchen, Germany)
Flumazenil	(Roche, Mannheim, Germany)
HEPES	(Sigma, Taufkirchen, Germany)
Ketamine	(Sigma-Aldrich, Hannover, Germany)
Magnesiumchloride	(Sigma-Aldrich; Hannover, Germany)
Methanol absolute (99%)	(Carl Roth, Karlsruhe, Germany)
Midazolam	(Sigma, Taufkirchen, Germany)
Mouse albumin standard	(Sigma, Taufkirchen, Germany)
Natriumchloride	(Sigma-Aldrich, Hannover, Germany)
Nitric acid(HNO_3)-	(Sigma, Taufkirchen, Germany)
Nitrogen liquide	(Linde, Wiesbaden, Germany)
Normal saline 0.9%	(Fresenius Kabi Bad Homburg, Germany).
Phosphate-buffered saline	(PBS tablets, Invitrogen, Karslruhe, Germany).
Potassium chloride	(Carl Roth, Karlsruhe, Germany)
Sterilium	(Carl Roth, Karlsruhe, Germany)

Diets (Altromin, Heidenau, Germany)

Standard diet C1310/1314 [0.24% Na^+, 0.71% K^+, 0.95% Ca^{2+} (wt/wt)]
Control diet C1000 [0.24% Na^+, 0.71% K^+, 0.95% Ca^{2+} (wt/wt)]
Low-salt diet C1036 [0.015% Na^+, 0.71% K^+, 0.95% Ca^{2+} (wt/wt)]

Software

Blood pressure computerized data acquisition	(PowerLab 400 and Chart 4, Colorado Springs, Colorado Springs, USA)
Chart version.4.2	(Axon Instruments ,USA)
Data link version 1.0.0	(Herbert &Scheneider Software &CAM, Siglingen, Germany)
GraphPad Instat version 3.05	(GraphPad Software Inc., San Diego, USA)
Inveon Research Workplace	(Siemens, Germany)
Magellan version 3.11	(Tecan GmbH, Crailsheim, Germany)
Sigma plot version 7.0	(Systat Software Inc., Erkrath, Germany)

Animals

All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals and were approved by local authorities.

gsk3^{KI} mice were kindly provided by Dario Alessi which were bred homozygously. The mice (6 males, age 16 weeks) were fed a control diet (C1000, Altromin), 4 days a low salt diet (C1036, Altromin) or 4 days a high salt diet (1% NaCl solution in drinking water), as indicated. The mice had free access to tap drinking water and/or 1% saline as indicated.

Mice with mutated APC (*apc*$^{Min/+}$) and their wild type littermates (*apc*$^{+/+}$) with a mixed (C57/Bl-6-Sv129J) background were generated by breeding of male *apc*$^{Min/+}$ mice initially obtained from the Jackson Laboratory. Where indicated, additional mice were generated by crossbreeding *apc*$^{Min/+}$ with gene targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) to generate mice carrying the mutant APC and simultaneously lacking SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$). Sex and age matched mice of 3 months age were used for the experiments.

Generation and basic properties of the SGK3 knockout mice (*sgk3*^{KO}) were described previously [106]. The mice were compared to their wild type littermates (*sgk3*^{WT}*)* and genotyped by PCR on tail DNA using SGK3 and neo-R-specific primers as previously described [106]. The mice (n = 10-13 in each group, age 3 months) were fed a control diet (C1314, Altromin, Lage, Germany) with free access to tap drinking water.

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METHODS

Collection of urine and feces

For evaluation of the renal and fecal excretion as well as daily food and fluid intake, mice were placed individually in metabolic cages for 24-hour urine and feces collection with free access to fluid and food. They were allowed a 3-day habituation period when food & water intake, urinary flow, urinary excretion of salt, fecal excretion and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently 24h collection of urine was performed for three consecutive days in order to obtain the urinary parameters. To assure quantitative urine collection, metabolic cages were siliconized and urine was collected under water-saturated oil.

Preparation of feces

To prepare feces for the analysis of the electrolyte content, feces samples were dried at 80°C for about 3 hours and weighed. After addition of 5 ml of 0.75 M HNO_3 to the feces, samples were electrically shaken for 48 hours to yield a homogenous creamy mass. The mass was centrifuged at 3000 g for 10 minutes, 1 ml of the supernatant was centrifuged a second time at 10000 g for 5 minutes, and the resulting second supernatant was used for further analysis. Measured electrolyte concentrations were multiplied with 5 to obtain excreted amount per day (in µmol/24h).

Determination of plasma, urinary and fecal concentrations

To obtain blood specimens, animals were lightly anesthetized with diethylether and approximately 130 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Blood losses were replaced with 400 µl of 0.9 % NaCl subcutaneously.

Plasma, urinary and fecal concentrations of Na^+, K^+ and Ca^{2+} were measured by flame photometry. Plasma and urinary creatinine concentrations were measured using an enzymatic colorimetric method (Creatinine PAP). Urinary creatinine concentrations were measured using the Jaffe method. Plasma, urinary and fecal phosphate were measured photometrically using kits from Roche Diagnostics. Plasma and urinary calcium and plasma phosphate concentrations in SGK3 were determined by a photometric method according to the manufacturer's instructions (dri-chem clinical chemistry analyzer FUJI FDC 3500i).

Urinary and plasma aldosterone concentrations were determined by using a commercial RIA kit (Demeditec), plasma and urine corticosterone concentrations were determined using an ELISA kit (DRG Instruments). Plasma concentrations of insulin were determined using an enzyme immunoassay kit (Mercodia). To estimate ADH release, the urinary ADH excretion has been determined as a surrogate for the plasma ADH concentration. Direct determination of plasma ADH concentration would require large volume, which could not be obtained by decapitation of the mice. Urinary ADH concentrations were determined by radioimmunoassay (Immunotech). In view of the circadian rhythm of hormone release, plasma corticosterone levels were determined prior to the beginning of the dark cycle at 5 p.m

Plasma intact parathormone concentrations were measured using an ELISA-kit (Immunotopics). Radioimmunoassay kits were employed to determine the concentrations of plasma 1,25(OH)-vitamin D_3 (IDS). Plasma leptin levels were determined using an ELISA kit (Linco). All measurements were done according to the manufacturer's instructions.

Blood pressure

Systolic arterial blood pressure was determined by the tail-cuff method (IITC, Model 179) at baseline and after respective treatments. The tail-cuff approach to determine arterial blood pressure requires certain precautions to reduce the stress of the animals, including appropriate training of the mice over multiple days and adequate pre-warming to dilate the tail artery. The animals were placed in a heated chamber at an ambient temperature of 28-30 °C for 15 min and from each animal 10-20 blood pressure traces were recorded in one session. The readings from 3 days were then averaged to obtain a mean blood pressure under the respective treatment. All recordings and data analysis were done using a computerized data acquisition system and software (PowerLab 400 and Chart 4, both Ad Instruments, Colorado, USA). All measurements were done by one person during a defined time (between 2-4 p.m).

Mineralocorticoid Treatment

To induce mineralocorticoid excess, $gsk3^{KI}$ and $gsk3^{WT}$ mice (8 months old, n = 6 each) were implanted with 21-day-release 50 mg DOCA pellet (Innovative Research of America) in the neck area during superficial general anesthesia (intraperitoneal midazolam 5 mg/kg + ketamin 50 mg/kg), which was partially antagonized by flumazenil (0.5 mg/kg i.p.) afterwards. Prior to the pellet implantation (control period) the mice had free access to plain tap water. After

the implantation, the tap water was replaced by 1% NaCl (high salt). Throughout the entire study, mice had free access to a standard mouse diet (C1310, Altromin). Renal excretion was determined before and after 18 days of DOCA+1%NaCl treatment.

For analysis of dexamethasone (DEXA) effects, $gsk3^{KI}$ and $gsk3^{WT}$ mice were injected with dexamethasone phosphate disodium salt dissolved in 0.9% saline at a concentration of 1 mg/ml) at a dosage of 10 µg/g body weight (BW) for four consecutive days at 5 p.m. Mice had free access to a standard mouse diet (C1310, Altromin) and tap water. Renal excretion was evaluated before and after 4 days of dexamethasone treatment.

Determination of plasma volume

Plasma volume was assessed by dye dilution using Evans Blue. Mice were anesthetized with diethylether and 30-50 µl of an Evans Blue stock solution (3 mg/ml in 0.9% NaCl) was injected intravenously into the left retroorbital plexus using a 30-gauge insulin syringe (BD micro-fine). The exact applied volume was determined by weighing the syringe before and after injection. Blood samples (20-25 µl) were drawn from the right retroorbital plexus during superficial diethylether anesthesia after 10, 30, 60 and 120 mins which each time yielded a volume of 10 µl plasma after centrifugation. Absorbance was measured at 620 nm against blank mouse serum after recovery in 90 µl phosphate-buffered saline. Plasma concentrations of Evans Blue were calculated using the stock solution dissolved in mouse serum as a standard. Division of the applied dose of Evans Blue (in mg) by the y-intercept (in mg/ml) resulted in the distribution volume of Evans Blue which was normalized for body weight.

Determination of Bone Density

For the analysis of bone density, animals were sacrificed and legs were amputated and fixated in formalin. The samples were scanned with a high resolution microCAT-II (Siemens, Germany) small animal computed tomography (CT) scanner using a field of view of 3.1 x 3.1 x 4.8 cm^3. The X-ray tube parameters were set at 80kVp and 400 µA. The images were acquired with 720 angular projections (exposure time 1200 ms per projection) over 360° and binned with a factor of two, yielding a spatial resolution of ~38 µm. The total scan time was 24 minutes. Reconstructed CT images were analyzed with the Inveon Research Workplace software (Siemens, Germany) by drawing a standard-sized container around the femur and applying a region growth routine to segment the trabecular bone structure. For all samples, the

same upper and lower density threshold were applied and compared the relative numbers of trabecular bone density.

In vitro experiments in SGK3

For generation of cRNA, constructs were used encoding wild type NaPiIIa [139] and SGK3 [88] The cRNA was generated as described previously [98]. SGK3 cDNA was kindly provided by Sir Philip Cohen, the cDNA encoding NaPiIIa by Heini Murer. For electrophysiology, *Xenopus* oocytes were prepared as previously described [140;141]. 7.5 ng of wild type SGK3 and 15 ng of NaPiIIa cRNA were injected (one day) after preparation of *Xenopus* oocytes. All experiments were performed at room temperature 4-5 days after injection. Two electrode voltage-clamp recordings were performed at a holding potential of -50 mV. The data were filtered at 10 Hz, and recorded with Digidata A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments). The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. 3 mM phosphate was added to induce NaPiIIa dependent currents. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA and results with $p < 0.05$ were considered statistically significant

Statistical analysis

Data are provided as means ± SEM, n represents the number of independent experiments.. All data were tested for significance using paired or unpaired Student t-test or ANOVA where ever applicable and only results with $p < 0.05$ were considered statistically significant. GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, USA) was used.

RESULTS

The role of PKB/SGK resistant GSK3 in renal water and electrolyte excretion as well as steroid hormone release

Food (Figure 6A) and water (Figure 6B) intakes were slightly but significantly larger in *gsk3*^{KI} than in *gsk3*^{WT} mice. In parallel to the enhanced food intake, fecal excretion was again significantly larger in *gsk3*^{KI} than in *gsk3*^{WT} mice (Figure 6C). Despite the increased food and water intake, body weight was similar in *gsk3*^{KI} and in *gsk3*^{WT} mice (Figure 6D).

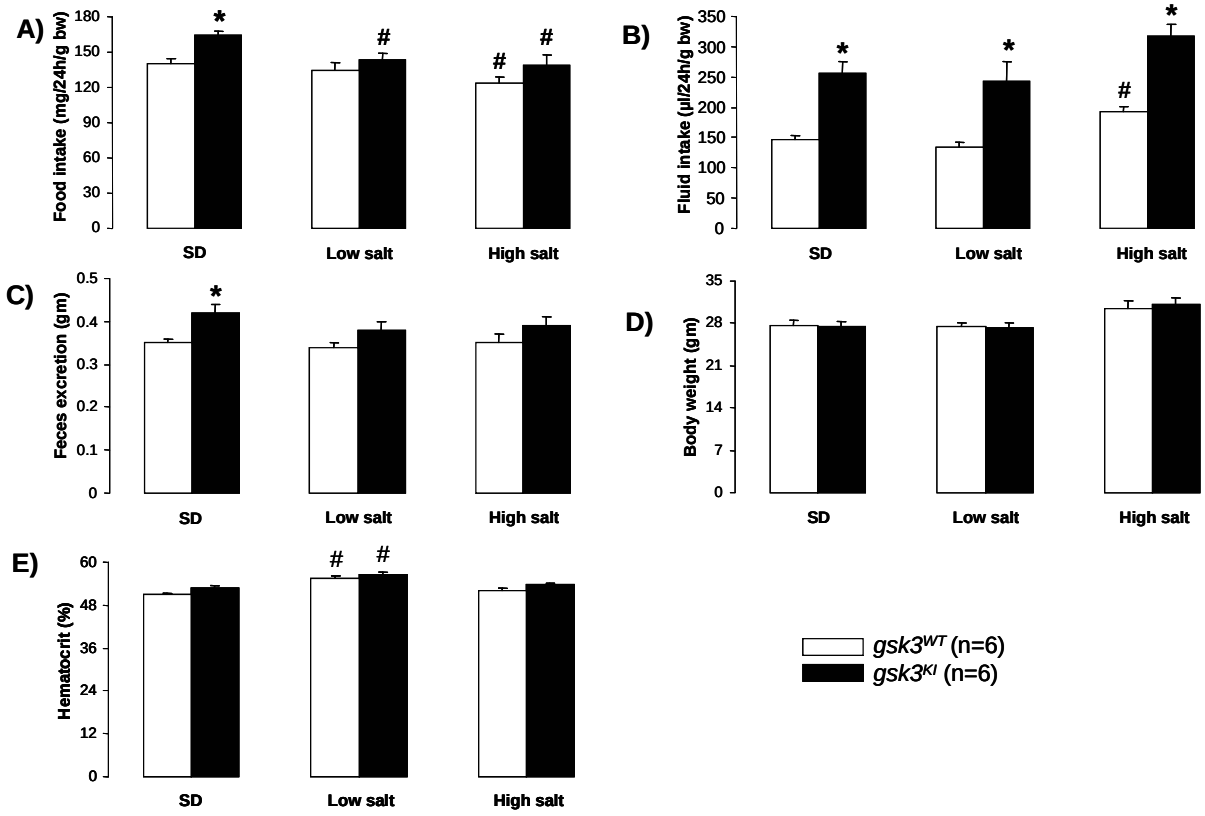

Figure 6: Food and fluid intake, fecal excretion, body weight and packed cell volume in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet, low salt and high-salt intake.

Arithmetic means ± SEM (n=6 each group) of food intake (A), fluid intake (B), fecal excretion (C), body weight (D) and packed cell volume (hematocrit, (E) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) under standard diet (SD), under low-salt diet (Low salt) and under high-salt diet (High salt). # $p < 0.05$ vs respective value under SD,* $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Packed cell volume (hematocrit) tended to be higher in *gsk3*^{KI} than in *gsk3*^{WT} mice, a difference, however, not reaching statistical significance (Figure 6E). Further experiments were performed to explore the food and fluid intake in normal cages. Food (211.2 ± 8.7 mg/24h/g bw, n = 8 vs 149.6 ± 7.42 µg/24h/g bw) and fluid inake (311.4 ± 19.5 µl /24h/g bw, n = 8 vs 192.8 ± 12.4 µl /24h/g bw) were significantly larger in *gsk3*^{KI} than in *gsk3*^{WT} mice.

Plasma insulin concentrations were similar in *gsk3*^{KI} mice (0.5 ± 0.1 ng/ml) and in *gsk3*^{WT} mice (0.4 ± 0.1 ng/ml). In contrast, plasma corticosterone concentrations were significantly lower in *gsk3*^{KI} mice than in *gsk3*^{WT} mice (Figure 7B). Moreover, aldosterone concentration was significantly lower in *gsk3*^{KI} than in *gsk3*^{WT} mice (Figure. 7A). Low salt diet increased and high salt diet tended to decrease plasma aldosterone levels. Neither diet abrogated the difference between the genotypes (Figure 7A). In contrast to plasma hormone concentrations, the urinary excretion of aldosterone was significantly higher and corticosterone excretion tended to be higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. Urinary excretion of vasopressin (ADH) was similar in *gsk3*^{KI} and *gsk3*^{WT} mice during control diet and low salt diet but increased to significantly higher levels during high salt intake (Figure 7C).

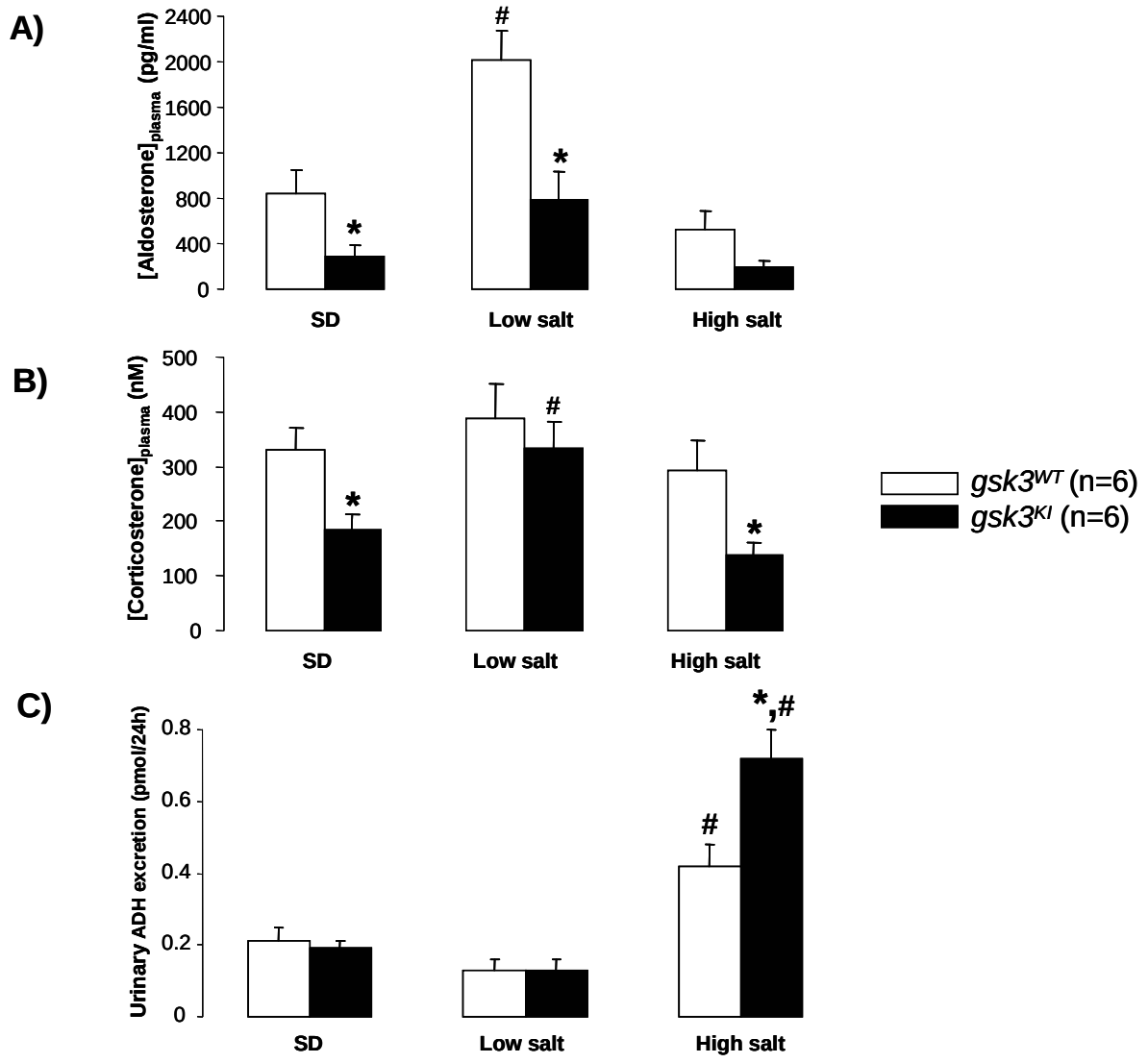

Figure 7: Plasma aldosterone and corticosterone concentrations and urinary vasopressin excretion in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet, low-salt and high-salt intake.

Arithmetic means ± SEM (n=6 each group) of aldosterone plasma concentrations (A), Corticosterone concentrations (B) and urinary vasopressin excretion (C) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT} , open bars) under standard diet (SD), under low-salt diet (Low salt) and under high-salt diet (High salt). # $p < 0.05$ vs respective value under SD,* $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Plasma Na^+ concentration was slightly but significantly lower in *gsk3*^{KI} than in *gsk3*^{WT} mice under control diet (Figure 8A). Low salt diet significantly decreased plasma Na^+ concentration in *gsk3*^{WT} mice but not in *gsk3*^{KI} mice and thus dissipated the difference between *gsk3*^{KI} and *gsk3*^{WT} mice. High salt diet was followed by a significant increase of plasma Na^+ concentration in both, *gsk3*^{KI} and *gsk3*^{WT} mice, and again dissipated the difference

between genotypes (Figure 8A). Plasma K^+ concentration was similar in *gsk3*^{KI} and *gsk3*^{WT} mice under control, low and high salt diet (Figure 8B).

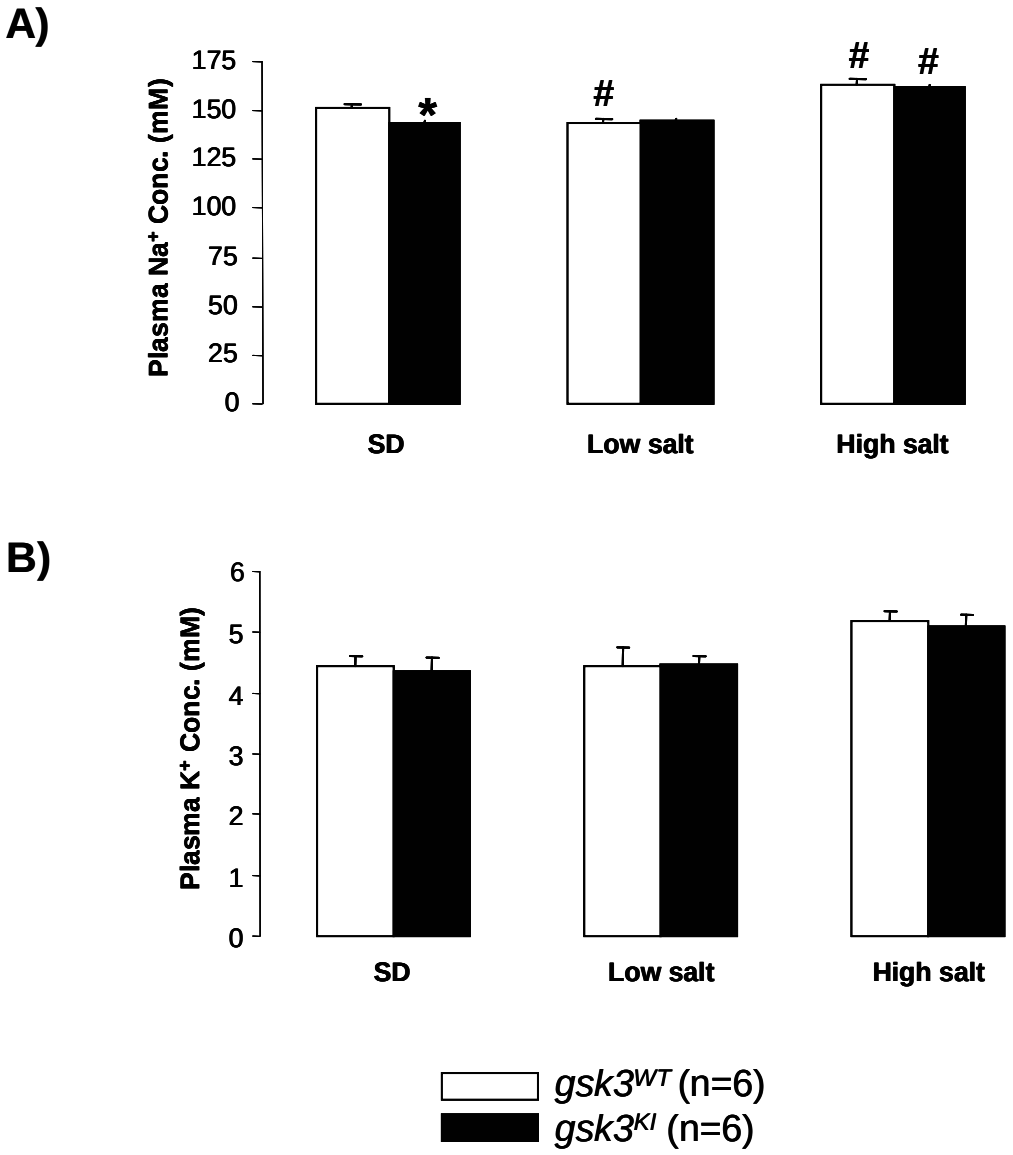

Figure 8: Plasma Na^+ and K^+ concentrations in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet, low-salt and high-salt intake.

Arithmetic means ± SEM (n=6 each group) of plasma Na^+ (A) and K^+ (B) concentrations in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) under standard diet (SD), under low salt diet (Low salt) and under high-salt diet (High salt). # $p < 0.05$ vs respective value under SD,* $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Urinary flow rate was significantly larger in *gsk3*^{KI} than in *gsk3*^{WT} mice, an effect persisting under both, low and high salt diet (Figure 9A). Urinary Na^+ excretion was similarly

larger in *gsk3*^{KI} than in *gsk3*^{WT} mice. Low salt diet significantly decreased urinary Na^+ output to similarly low levels in both genotypes (Figure 9B). High salt diet significantly increased renal Na^+ excretion in both genotypes. Urinary Na^+ excretion remained, however, significantly larger in *gsk3*^{KI} than in *gsk3*^{WT} mice during high salt diet (Figure 9B). Under control diet, urinary K^+ excretion was again significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice (Figure 9C). The difference was, however, dissipated by low and high salt diet (Figure 9C).

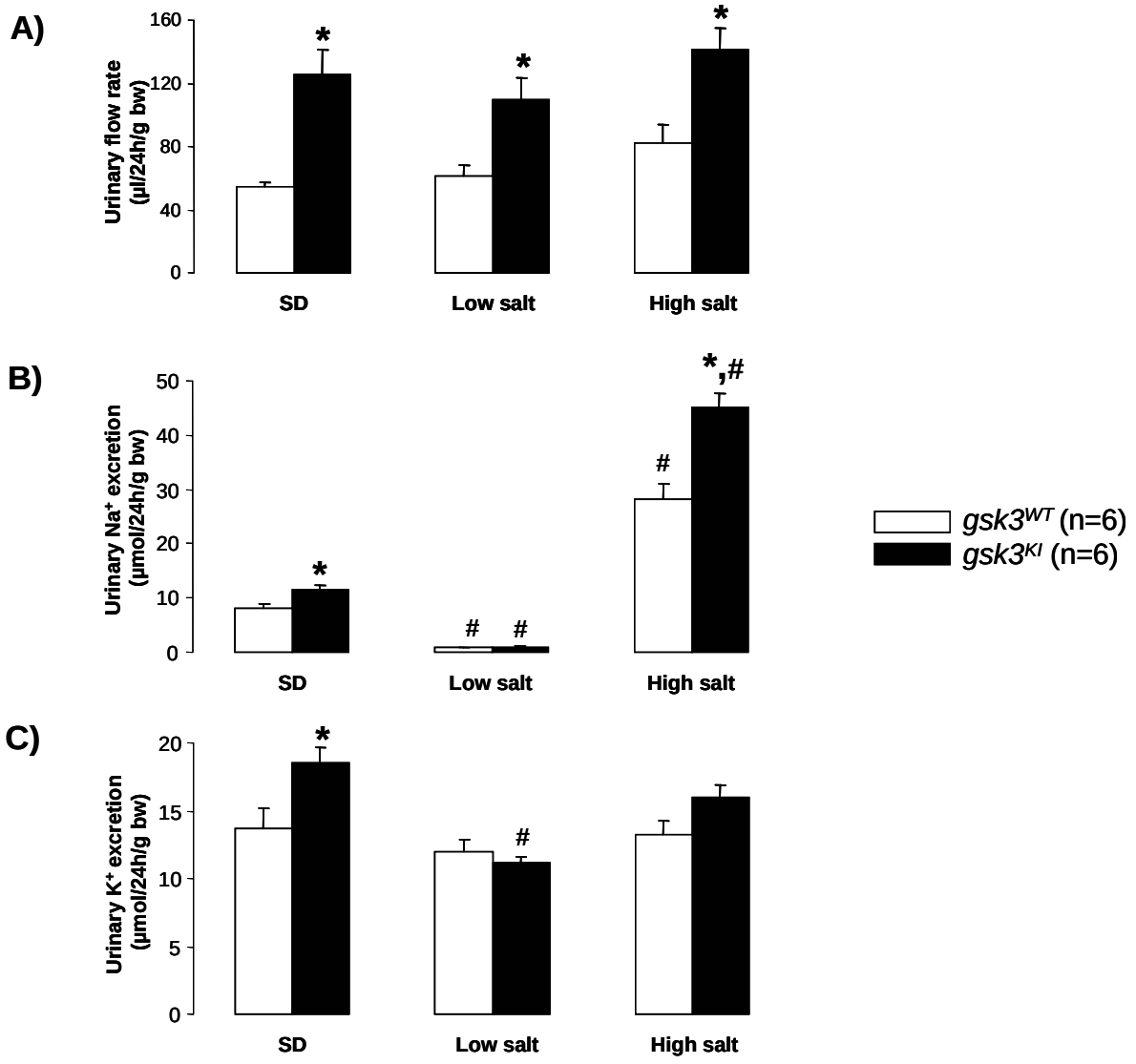

Figure 9: Urinary flow rate and urinary excretion of Na^+ and K^+ in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet, low-salt and high-salt intake.

Arithmetic means ± SEM (n=6 each group) of urinary flow rate (A), and urinary excretion of Na^+ (B), and K^+ (C) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) under standard diet (SD), under low-salt diet (Low salt) and under high-salt diet (High salt). #p<0.05 vs respective value under SD, *p<0.05 vs respective value of *gsk3*^{WT} mice.

Under control diet the creatinine clearance as well as urinary urea, creatinine, glucose and aldosterone excretions were significantly larger in *gsk3*^{KI} mice than in *gsk3*^{WT} mice (Table 1). Plasma creatinine and urea concentrations were slightly but significantly lower in *gsk3*^{KI} than in *gsk3*^{WT} mice under control diet (Table 1). Plasma glucose concentration was not significantly different between *gsk3*^{KI} and *gsk3*^{WT} mice (Table 1).

As shown in Figure 10, urine osmolarity was significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. Water deprivation further increased urine osmolarity, an effect, reaching statistical significance only in *gsk3*^{KI} mice (Figure 10B). Plasma osmolarity was not significantly different between *gsk3*^{KI} and *gsk3*^{WT} mice and was not significantly altered by water deprivation (Figure 10C). Body weight decreased during water deprivation (Figure 10E), an effect significantly larger in *gsk3*^{KI} mice (Figure 10D).

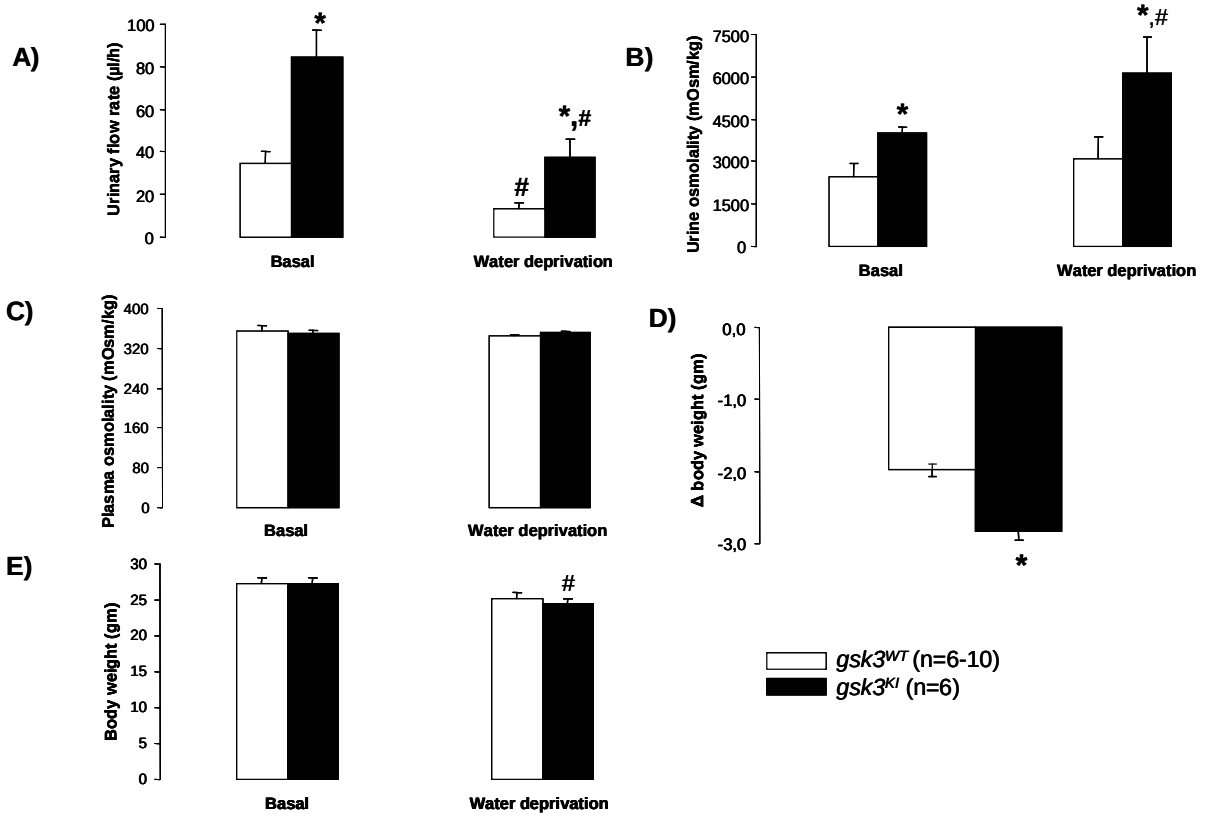

Figure 10: Urinary flow rate, plasma and urinary osmolarity as well as body weight in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet before and after water deprivation. Arithmetic means ± SEM (n=6 each group) of urinary flow rate (A), urinary (B), and plasma (C) osmolarity, change of (Δ) body weight (D) as well as body weight (E) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars). # $p < 0.05$ vs respective value under SD, * $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Additional experiments were performed to elucidate, whether the decreased aldosterone levels in *gsk3*^{KI} mice resulted in a reduced salt appetite. To this end, the mice were offered two drinking bottles, one containing plain tap water and the other 1 % saline. As illustrated in Figure 11, the *gsk3*^{WT} mice significantly preferred saline. The *gsk3*^{KI} mice drank similar volumes of saline but larger volumes of tap water than *gsk3*^{WT} mice. As a result, *gsk3*^{KI} mice drank similar volumes of saline and tap water (Figure 11).

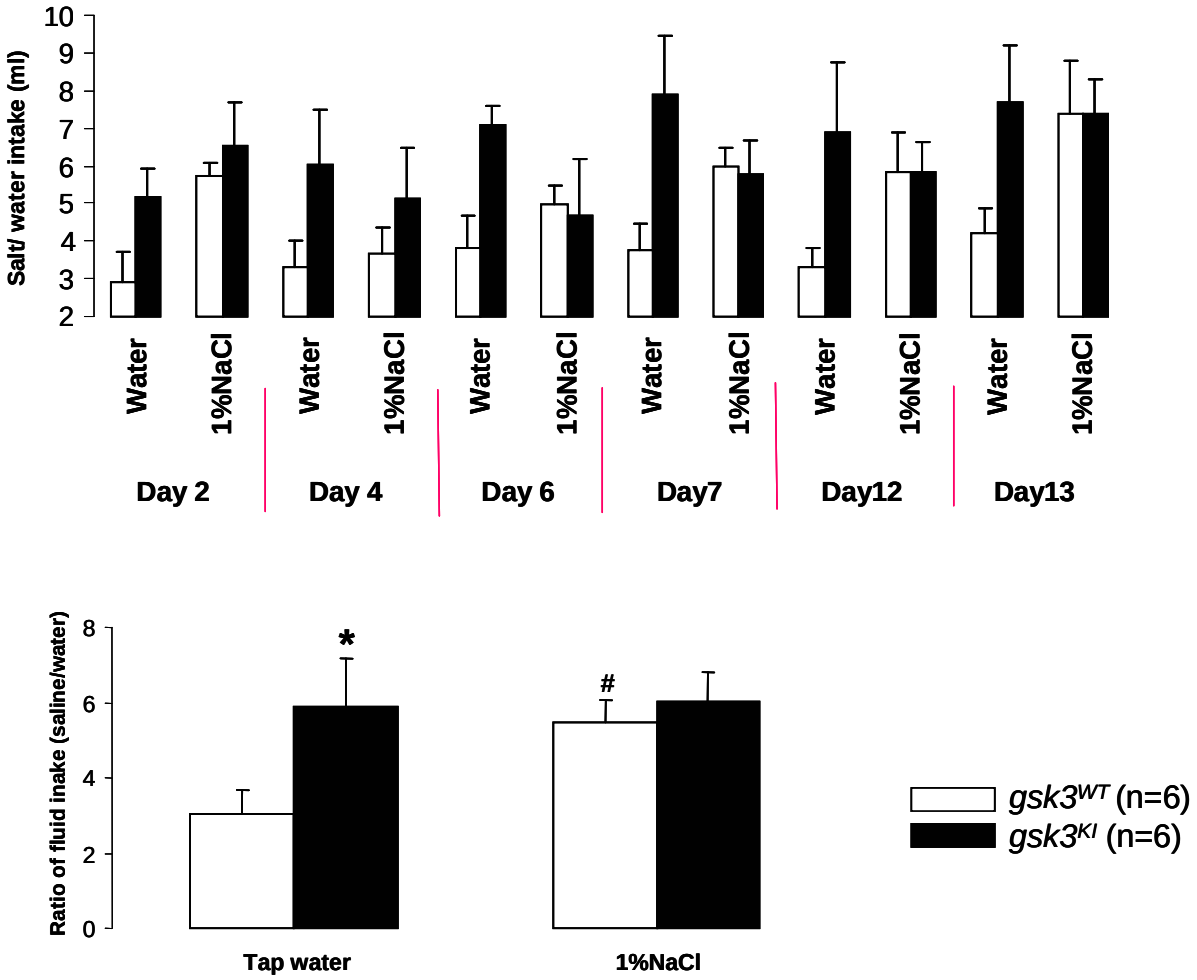

Figure 11: Fluid intake from tap water or saline by *gsk3*^{KI} and *gsk3*^{WT} mice.

Arithmetic means ± SEM (n=6 each group) of volumes tap water intake (water) or 1% saline (1% NaCl) drunk per day (upper panel)as well as the preference of saline, i.e., the ratio of saline/water drunk (lower panel) by GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars). * $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Systolic blood pressure was slightly but significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice during control diet and during low salt diet (Figure 12), a difference abrogated by high salt diet. Low salt diet significantly decreased the blood pressure in *gsk3*^{WT} mice. To test, whether the difference between *gsk3*^{KI} and *gsk3*^{WT} mice was due to differences in circadian rhythm, systolic blood pressure was measured at both, 1 p.m and 10 p.m. Systolic blood pressure was significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice at both, 1 p.m. (101.26 ± 1.8 vs 90.3 ± 1.8 mmHg) and 10 p.m. (102.46 ± 2.6 vs 89.4 ± 1.2 mmHg, n=6-7).

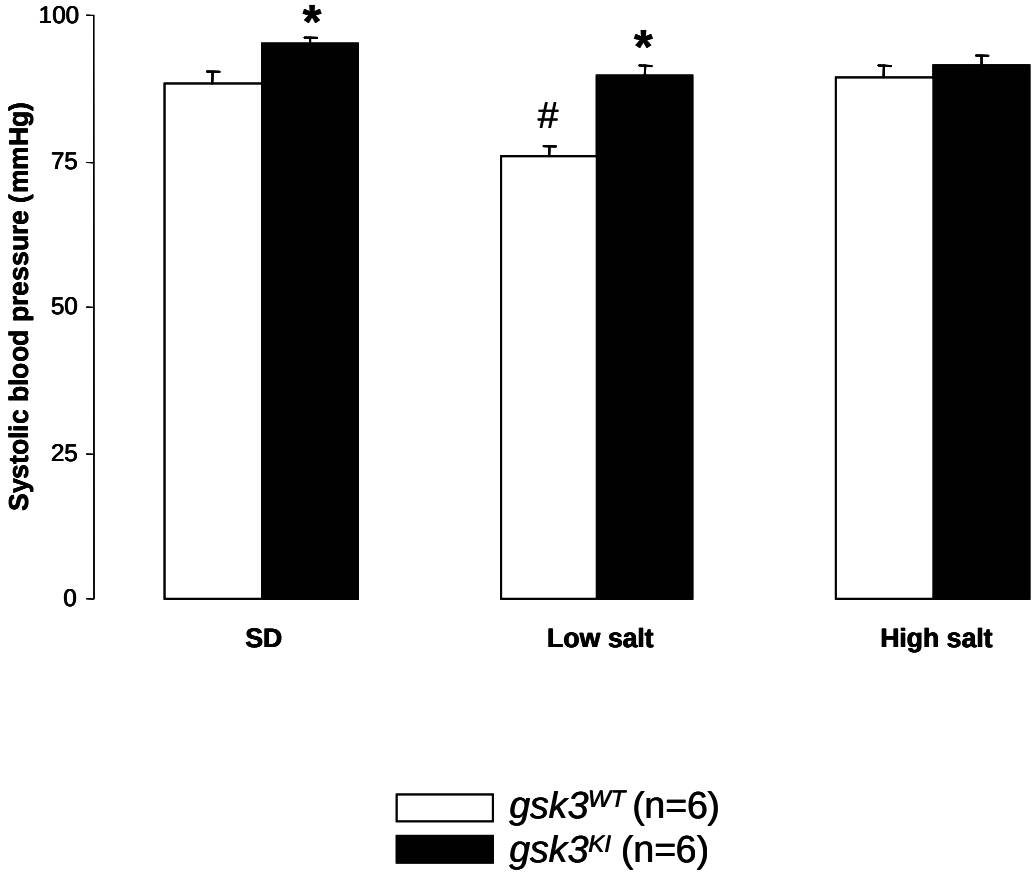

Figure 12: Systolic blood pressure in *gsk3*^{KI} and *gsk3*^{WT} mice on standard diet, low-salt and high-salt intake.

Arithmetic means±SEM (n=6 each group) of systolic blood pressure (BP) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) under standard diet (SD), under low-salt diet (Low salt) and under high salt diet (High salt). # $p < 0.05$ vs respective value under SD, * $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Body temperature was significantly higher in *gsk3*^{KI} mice (38.8 ± 0.2 ^{0}C, n = 9) than in *gsk3*^{WT} mice (36.8 ± 0.2 °C, n = 9).

Further experiments were performed to explore urinary Na^+ and K^+ excretion during mineralocorticoid or glucocorticoid excess. Prior to hormone application urinary Na^+ and K^+ excretion was significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. Following treatment with DEXA (Figure 13B) or DOCA (Figure 14B) the urinary Na^+ excretion was still significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. Urinary K^+ excretion, however, was not significantly different between the genotypes after the DEXA or DOCA treatment (Figure 13C and Figure 14C).

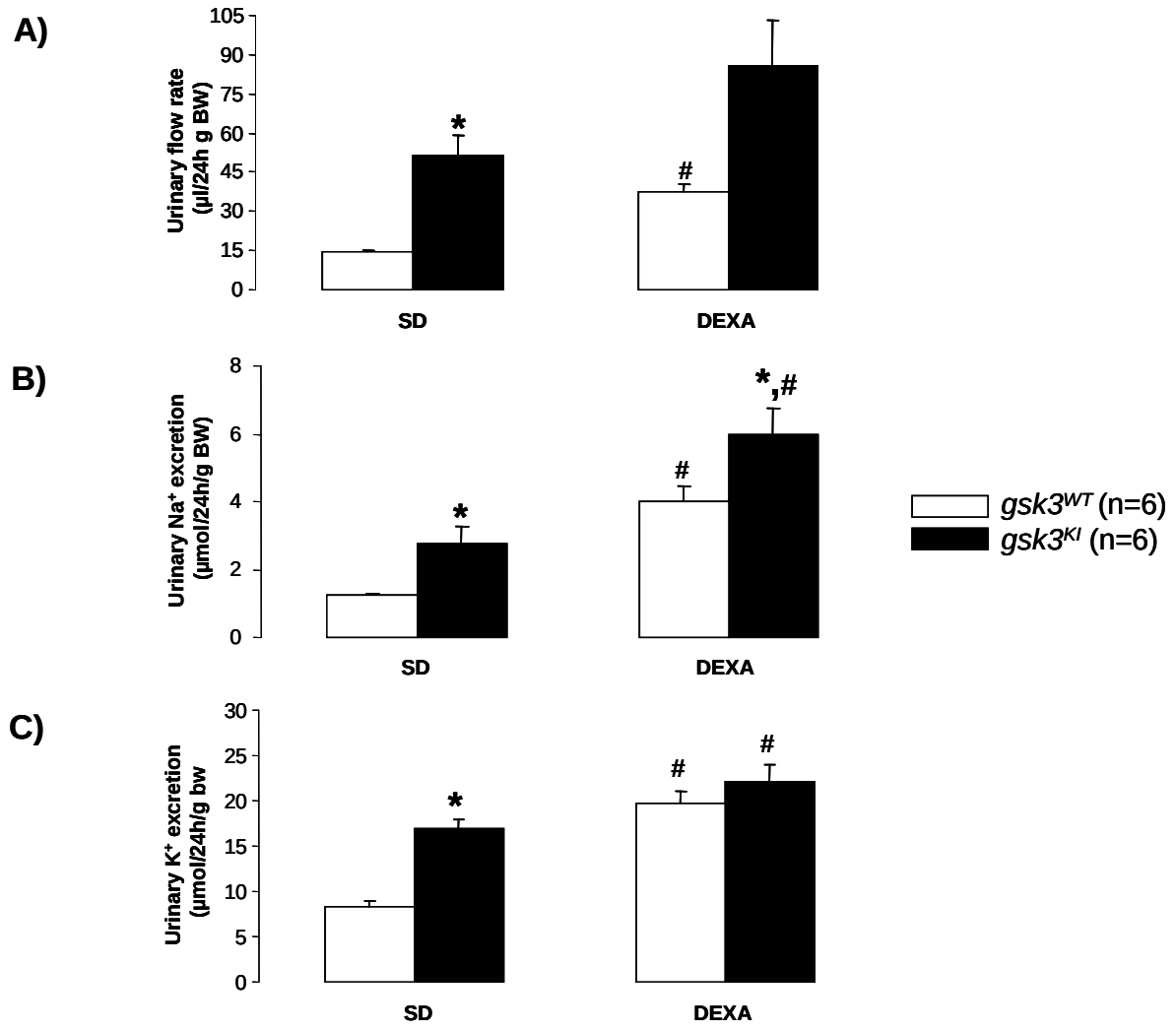

Figure 13: Urinary flow rate and urinary excretion of Na^+ and K^+ in *gsk3*^{KI} and *gsk3*^{WT} mice before and after DEXA treatment.

Arithmetic means ± SEM (n=6 each group) of urinary flow rate (A) and urinary excretion of Na^+ (B) and K^+ (C) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) before (left panels) and after (right panels) 4-days DEXA treatment. # $p < 0.05$ vs respective value under SD, * $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

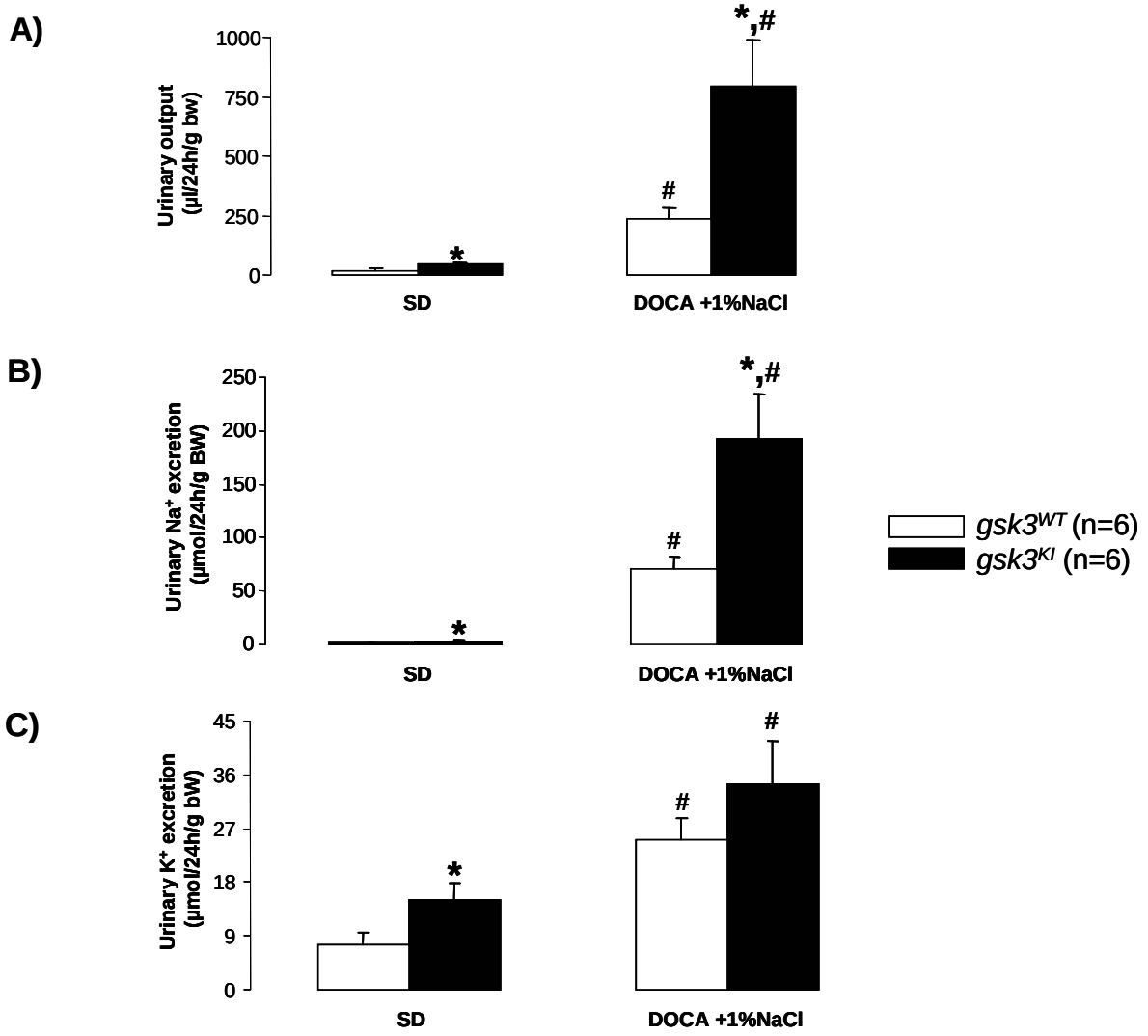

Figure 14: Urinary flow rate and urinary excretion of Na^+ and K^+ in *gsk3*^{KI} and *gsk3*^{WT} mice before and after DOCA treatment.

Arithmetic means ± SEM (n=5–6 each group) of urinary flow rate (A) and urinary excretion of Na^+ (B) and K^+ (C) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and their wild-type littermates (*gsk3*^{WT}, open bars) before (left panels) and after (right panels) an 18-day DOCA + 1% NaCl treatment. # $p < 0.05$ vs respective value under SD, * $p < 0.05$ vs respective value of *gsk3*^{WT} mice.

Table 1: Effect of standard diet, low salt and high salt diet on plasma concentrations and urinary excretion of electrolytes and hormones.

Creatinine, urea concentrations in plasma, renal excretions of glucose, urea, creatinine clearance, aldosterone, corticosterone and fractional renal excretions of Na^+ and K^+ in both *gsk3*^{KI} and in *gsk3*^{WT} mice.

	Standard diet (n=6)		**Low salt diet (n=6)**		**High salt diet (n=6)**	
	***gsk3*^{WT}**	***gsk3*^{KI}**	***gsk3*^{WT}**	***gsk3*^{KI}**	***gsk3*^{WT}**	***gsk3*^{KI}**
[Creatinine]$_{plasma}$ (mg/dl)	0.13 ± 0.01	0.09 ± 0.01*	0.18 ± 0.03	0.21 ± 0.02$^{\#}$	0.30 ± 0.05$^{\#}$	0.25 ± 0.05
[Urea]$_{plasma}$ (mg/dl)	54.9±2	47.0±2*	47.2±5	45.6±3	51.5±3	44.0±2*
[Glucose] $_{plasma}$ (mg/dl)	137.6±6.7	126.6±9.0	ND	ND	ND	ND
Urinary creatinine (µg/24h/g BW)	13.9±1.2	17.0±0.7*	22.6±1.5$^{\#}$	22.4±1.1$^{\#}$	16.5±1.3	16.5±1.3
Creatinine clearance (µl/min/g BW)	7.4±0.6	14.4±1.7*	9.9±1.7	8.0±0.8$^{\#}$	4.3±0.7$^{\#}$	4.9±1.3$^{\#}$
Urine urea (mg/24h)	150.2±14	207.2±11*	161.5±11	174.6±7.3$^{\#}$	189.2±21	226.1±15
Urine glucose (mg/24h)	0.4±0.05	1.7±0.5*	0.5±0.1	1.8±0.3	0.5±0.04	0.86±0.04
FE Na^+ (%)	0.49±0.06	0.42±0.07	0.04±0.01$^{\#}$	0.04±0.01$^{\#}$	3.14±0.48$^{\#}$	4.02±0.81$^{'\#}$
FE K^+ (%)	30.6±3.38	21.8±2.35	17.0±1.95	14.3±2.37	54.9±6.16$^{\#}$	59.7±13.27$^{\#}$
Urine aldosterone (ng/24h)	8.3±0.8	12.7±1.1*	19.7±4.8$^{\#}$	22.1±4.1	7.4±0.7	10.8±1.1
Urine corticosterone (nmol/24h)	0.11±0.02	0.23±0.07	0.20 ± 0.03$^{\#}$	0.42±0.14$^{\#}$	0.15±0.02$^{\#}$	0.29±0.11

Arithmetic means ± SEM; $^{\#}$ $p<0.05$ vs. respective value under SD, * $p<0.05$ vs. respective value of *gsk3*^{WT} mice, ND- Not determined.

SGK1 dependence of renal electrolyte balance and hormone regulation in APC min mice

The body weight was similar in *apc*$^{Min/+}$ mice, *apc*$^{+/+}$ mice and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice (Table 2). Similarly, food intake and fluid intake were not significantly different between the genotypes. Urine flow tended to be lower in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice, a difference, however, not reaching statistical significance. Mean fractional urinary excretion of K^+ was significantly higher in *apc*$^{Min/+}$ and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice than in *apc*$^{+/+}$ mice. In contrast, absolute urinary excretion of Na^+ was significantly lower in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice. The absolute Na^+ excretion tended to be higher in *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice than in *apc*$^{Min/+}$ mice and was not significantly different from the respective value in *apc*$^{+/+}$ mice (Table 2). Thus, lack of SGK1 appeared to abrogate the decrease of renal Na^+ excretion in mice carrying the mutant APC.

Fecal excretion was studied to further clarify the electrolyte homeostasis and as shown in Table 2, feacal dry weight was not different between the genotypes. Fecal sodium and potassium excretion was significantly higher in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice. The additional lack of SGK1 could reverse the difference in fecal sodium excretion but did not reverse the increase of fecal potassium excretion (Table 2).

Serum creatinine concentration was significantly higher in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice, a difference, which was abrogated by additional lack of SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice). The glomerular filtration rate was also significantly lower in *apc*$^{Min/+}$ mice and the additional lack of SGK1 tended to partially reverse the difference (Table 2).

Plasma corticosterone and aldosterone concentrations were significantly higher in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice (Figure 15). While plasma corticosterone concentration was similar in *apc*$^{+/+}$ mice and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice, plasma aldosterone was even higher in *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice than in *apc*$^{Min/+}$ mice (Figure 15).

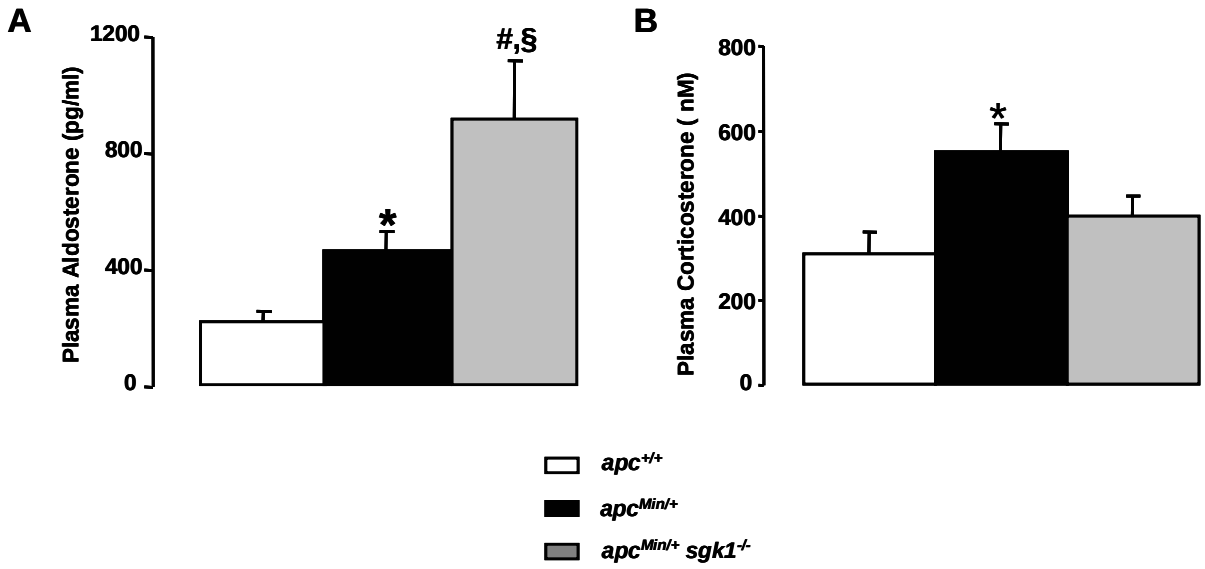

Figure 15: Plasma aldosterone and corticosterone concentrations in *apc*$^{Min/+}$ mice, *apc*$^{+/+}$ mice and *apc*$^{Min/+}$/*sgk1*$^{-/-}$ mice

Arithmetic means ± SEM of plasma aldosterone concentrations(n = 7-9) (A) and corticosterone concentrations (n = 11-13) (B) in mice carrying a defective APC (*apc*$^{Min/+}$, black bar), their wild type littermates (*apc*$^{+/+}$, white bar) and mice with defective APC and in addition lacking SGK1 (*apc*$^{Min/+}$/*sgk1*$^{-/-}$, grey bar). * indicates statistically significant ($p<0.05$) difference between *apc*$^{+/+}$ and *apc*$^{Min/+}$ mice. # indicates statistically significant ($p<0.05$) difference between *apc*$^{Min/+}$ and *apc*$^{Min/+}$/*sgk1*$^{-/-}$ mice. § indicates statistically significant ($p<0.05$) difference between *apc*$^{+/+}$ and *apc*$^{Min/+}$/*sgk1*$^{-/-}$ mice.

To test whether enhanced corticosterone and aldosterone concentrations were the result of volume depletion, plasma volume was determined utilizing Evans blue distribution. As illustrated in Figure 16, plasma volume was significantly higher in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice, a difference partially reversed by additional lack of SGK1 (*apc*$^{Min/+}$/*sgk1*$^{-/-}$ mice).

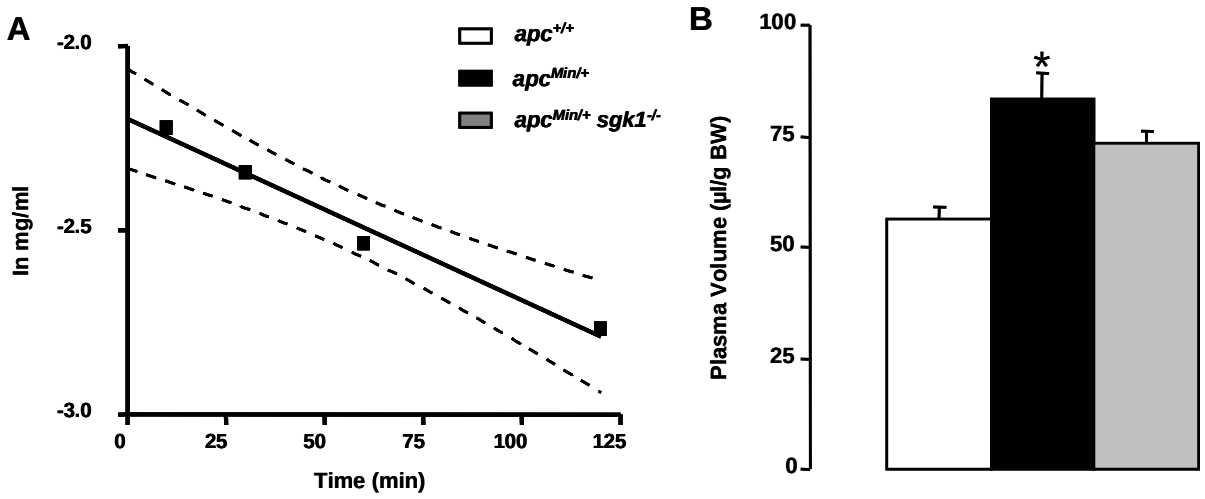

Figure 16: Plasma volume in *apc*$^{Min/+}$mice, *apc*$^{+/+}$mice and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice

A. Representative experiment demonstrating the time-dependent decay of Evans Blue plasma concentration.

B. Arithmetic means ± SEM (n = 8-14) of relative plasma volume (per gram body weight) of mice carrying a defective APC (*apc*$^{Min/+}$, black bar), their wild type littermates (*apc*$^{+/+}$, white bar) and mice with defective APC and in addition lacking SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$, grey bar). Plasma volume has been determined utilizing Evans Blue.* indicates statistically significant ($p<0.05$) difference between *apc*$^{+/+}$ and *apc*$^{Min/+}$ mice.

As body weight was similar in the three genotypes despite the differences in plasma volume, the increased extracellular volume of APC deficient mice may have been paralleled by a decrease of body fat. Thus, serum leptin concentrations were measured in order to depict differences in body fat. Serum leptin concentrations were significantly lower in both *apc*$^{Min/+}$ and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice than in *apc*$^{+/+}$ mice (Table 2). Thus, APC deficiency decreased the body fat, an effect not sensitive to additional lack of SGK1.

Additional experiments were performed to elucidate whether the altered renal Na^+ output was paralleled by enhanced salt appetite. To this end, animals were offered two bottles, one with tap water and the other with saline. As shown in Figure 17, neither *apc*$^{Min/+}$ mice nor *apc*$^{+/+}$ mice preferred water or saline and there were no differences in salt appetite between the two genotypes.

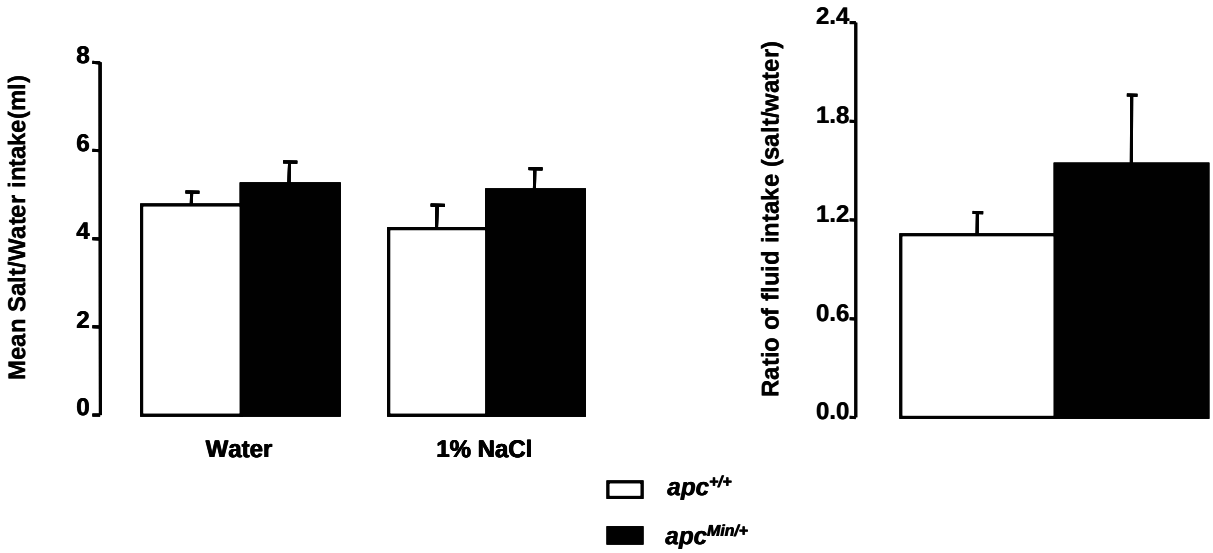

Figure 17: Plain tap water and saline consumption of in *apc*$^{Min/+}$ mice and *apc*$^{+/+}$ mice

Arithmetic means ± SEM (n = 12 each group) of daily drinking volumes of plain tap water (water) and 1% saline (NaCl) in mice carrying a defective APC (*apc*$^{Min/+}$, black bar) and their wild type littermates (*apc*$^{+/+}$, white bar).

Hyperaldosteronism is known to elevate blood pressure. Thus, blood pressure was determined in an additional series of experiments. As illustrated in Figure 18, blood pressure was indeed significantly higher in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice. The difference was partially reversed by additional lack of SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$).

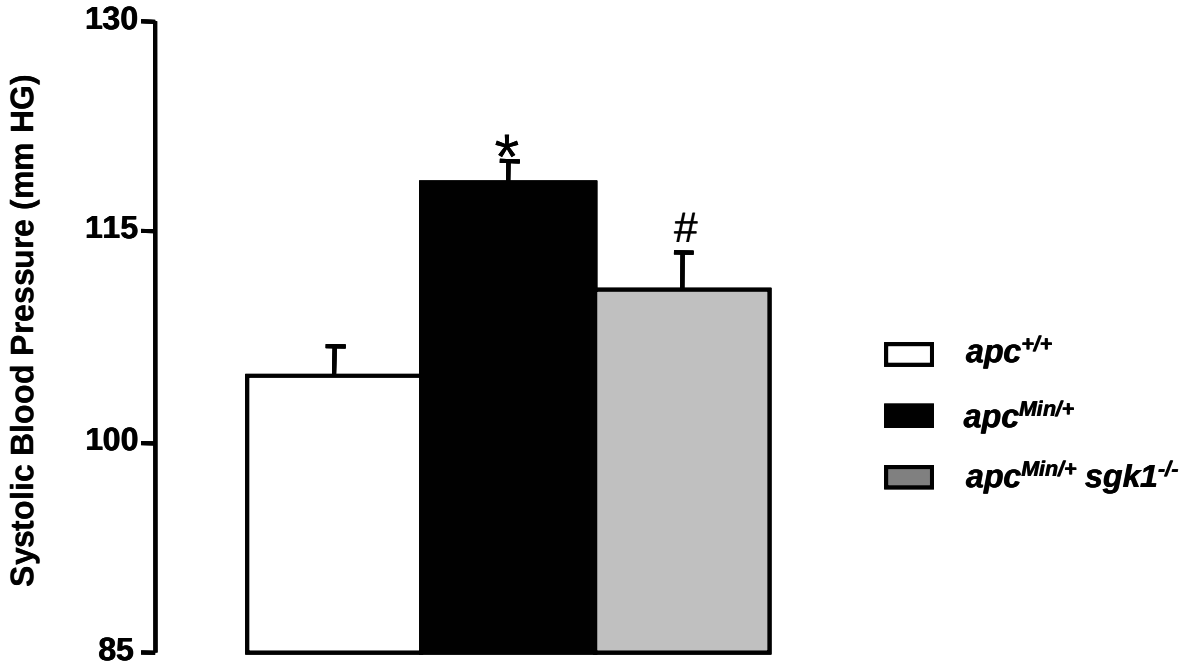

Figure 18: Systolic blood pressure in *apc*$^{Min/+}$ mice, *apc*$^{+/+}$ mice and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice

Arithmetic means ± SEM of systolic blood pressure (BP) in mice carrying a defective APC (*apc*$^{Min/+}$, n = 7, black bar), their wild type littermates (*apc*$^{+/+}$, n = 6, white bar) and mice with defective APC and in addition lacking SGK1 (*apc*$^{Min/+}$*/sgk1*$^{-/-}$, n = 5, grey bar). * indicates statistically significant ($p<0.05$) difference between apc+/+ and *apc*$^{Min/+}$ mice. # indicates statistically significant ($p<0.05$) difference between *apc*$^{Min/+}$and *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice.

Table 2: Body weight, food and fluid intake, urinary flow, fecal dry weight, creatinine clearance, absolute and fractional urinary Na^+, K^+ excretion, plasma and fecal Na^+, K^+ concentrations and plasma leptin concentration in *apc*$^{+/+}$ mice, *apc*$^{Min/+}$ mice and *apc*$^{Min/+}$/*sgk1*$^{-/-}$ mice (n = 13-16, except leptin [n = 6]).

	apc$^{+/+}$	*apc*$^{Min/+}$	*apc*$^{Min/+}$/*sgk1*$^{-/-}$
Body weight (g)	25.6 ± 1.0	25.0 ± 0.9	24.3 ± 0.7
Food intake (mg/24h)	4.0 ± 0.2	3.9 ± 0.2	3.8 ± 0.2
Fluid intake (ml/24h)	6.1 ± 0.4	5.5 ± 0.2	5.5 ± 0.3
Urinary flow (µl/24h/g BW)	38.9 ± 3.9	28.2 ± 4.7	34.2 ± 4.3
Fecal Dry Weight (mg/24h/g BW)	48 ± 5	54 ± 3	52 ± 3
$[Creatinine]_{plasma}$ (mg/dl)	0.30 ± 0.02	0.43 ± 0.04*	0.36 ± 0.03
Urinary creatinine (µg/24h/g BW)	27 ± 2	22 ± 2	21 ± 2
Creatinine clearance (µl/min/g BW)	6.6 ± 0.6	4.0 ± 0.5*	4.4 ± 0.6*
Urinary Na^+ excretion (µmol/24h)	134 ± 14	79± 14*	123 ± 17
Urinary K^+ excretion (µmol/24h)	509 ± 26	431 ± 45	448 ± 50
Plasma Na^+ concentration (mM)	150 ± 2	151 ± 2	152 ± 2
Plasma K^+ concentration (mM)	3.82 ± 0.10	3.76 ± 0.09	3.73 ± 0.08
Plasma Leptin (ng/ml)	2.38 ± 0.57	0.77 ± 0.13*	0.67 ± 0.16*
FE Na^+ (%)	0.42± 0.03	0.43 ±0.07	0.61 ± 0.06
FE K^+ (%)	66.± 6	98 ± 1*	96 ± 10*
Fecal Na^+ (µmol/24h)	148 ± 17	206 ± 14*	120 ± 108#
Fecal K^+ (µmol/24h)	229 ± 15	286 ± 20*	286 ± 19*

* indicates statistically significant ($p<0.05$) difference with respect to *apc*$^{+/+}$ mice.

# indicates statistically significant ($p<0.05$) difference with respect to *apc*$^{Min/+}$ mice.

The role of SGK3 in renal phosphate handling

A first series of experiments analysed the in vitro influence of the serum and glucocorticoid-inducible kinase isoform SGK3 on NaPiIIa, the major renal tubular phosphate transporter. Exposure of noninjected *Xenopus* oocytes to phosphate (3 mM) in the bath solution did not induce a significant current, indicating that those oocytes do not express significant endogenous electrogenic phosphate transport (Figure 19). In oocytes injected with cRNA encoding NaPiIIa, however, the addition of phosphate (3 mM) induced an inward current (Ipi). Coexpression of SGK3 significantly increased Ipi in NaPiIIa-expressing oocytes. Expression of SGK3 alone did not induce Ipi, indicating that SGK3 was indeed effective by stimulating NaPiIIa.

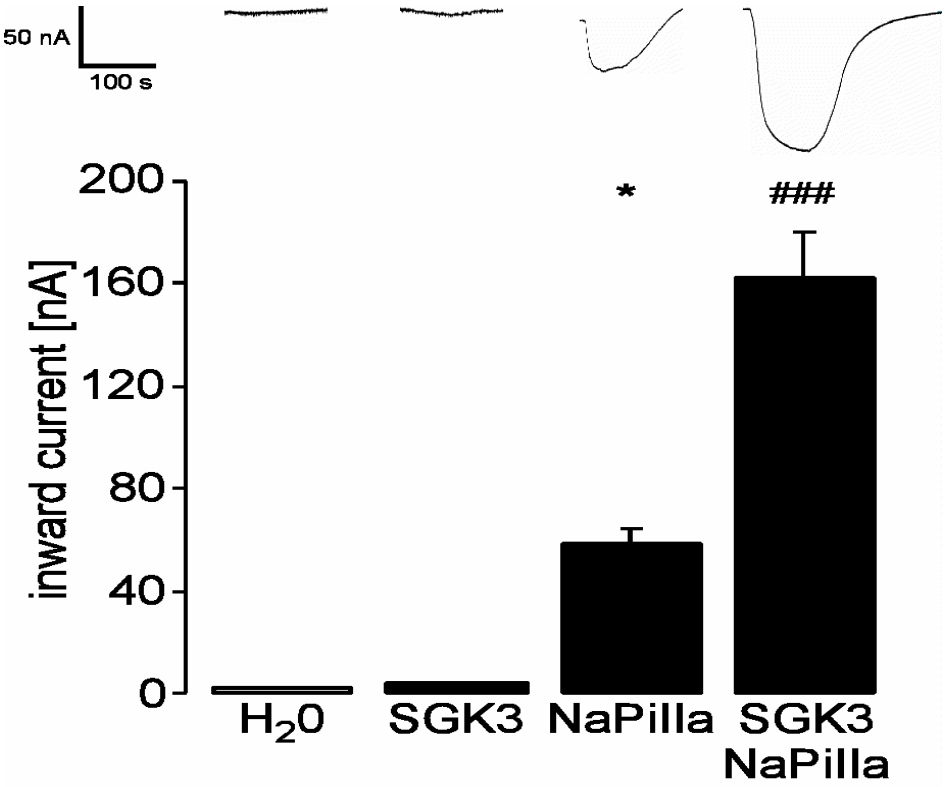

Figure 19: Coexpression of SGK3 stimulates electrogenic phosphate transport in NaPiIIa-expressing Xenopus oocytes

Arithmetic means ± SEM of phosphate (3 mM)-induced currents (IPi) in Xenopus oocytes injected with water (H_20) or SGK3 or NaPiIIa, or SGK3 and NaPiIIa cRNA. *indicates significant difference from absence of NaPiIIa cRNA. ### indicates significant absence from SGK3 cRNA ($p<0.001$).

A second series of experiments explored whether SGK3 participates in the regulation of renal phosphate excretion in vivo. To this end, metabolic cage experiments were performed in gene targeted mice lacking functional SGK3 (*sgk3*^{KO}) and their wild-type littermates (*sgk3*^{WT}). As shown in Table 3, the body weight was similar in *sgk3*^{KO} mice and *sgk3*^{WT} mice. Fluid intake tended to be slightly higher in *sgk3*^{KO} than in *sgk3*^{WT} mice, a difference, however, not reaching statistical significance (Table 3). Food intake was slightly but significantly higher in *sgk3*^{KO} than in *sgk3*^{WT} mice (Table 3).

No significant differences were observed between the genotypes in plasma Ca^{2+} and phosphate concentrations (Table 3).

Urinary flow rate and creatinine clearance each tended to be slightly higher in *sgk3*^{KO} mice than in *sgk3*^{WT} mice, a difference, however, not reaching statistical significance (Table 3). Absolute (Table 3) and fractional (Figure 20) excretion of Ca^{2+} were not significantly different between the two genotypes. In contrast, absolute (Table 3) and fractional (Figure 20) excretion of phosphate were significantly higher in *sgk3*^{KO} mice than in *sgk3*^{WT} mice. Neither in the *sgk3*^{WT} nor in the *sgk3*^{KO} mice glucosuria was observed.

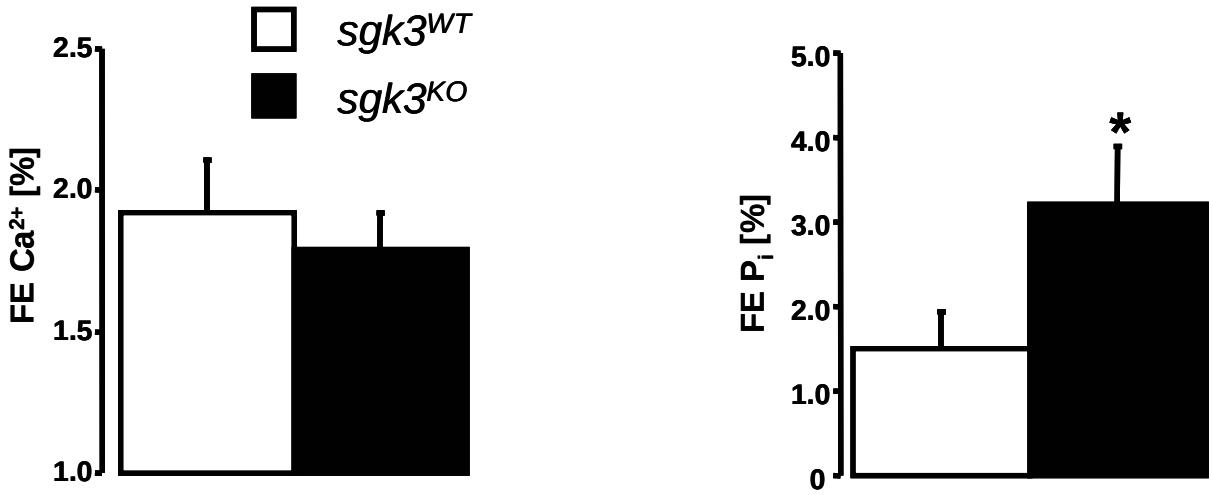

Figure 20: Fractional excretion of calcium and phosphate in *sgk3*^{KO} and *sgk3*^{WT} mice

Arithmetic means ± SEM (n = 10-12 each group) of fractional urinary calcium (left panel) and phosphate (right panel) excretion in SGK3 knockout mice (*sgk3*^{KO}, closed bars) and their wild type littermates (*sgk3*^{WT}, open bars).* $p<0.05$ vs. respective value of *sgk3*^{WT} mice.

The phosphaturia could have been secondary to increased parathyroid hormone release. Thus, plasma PTH levels were determined. As illustrated in Figure 21C, PTH plasma

concentration was not significantly different between genotypes. However, plasma $1,25(OH)_2D_3$ concentration was significantly lower in *sgk3*^{KO} than in *sgk3*^{WT} mice(Figure 21D).

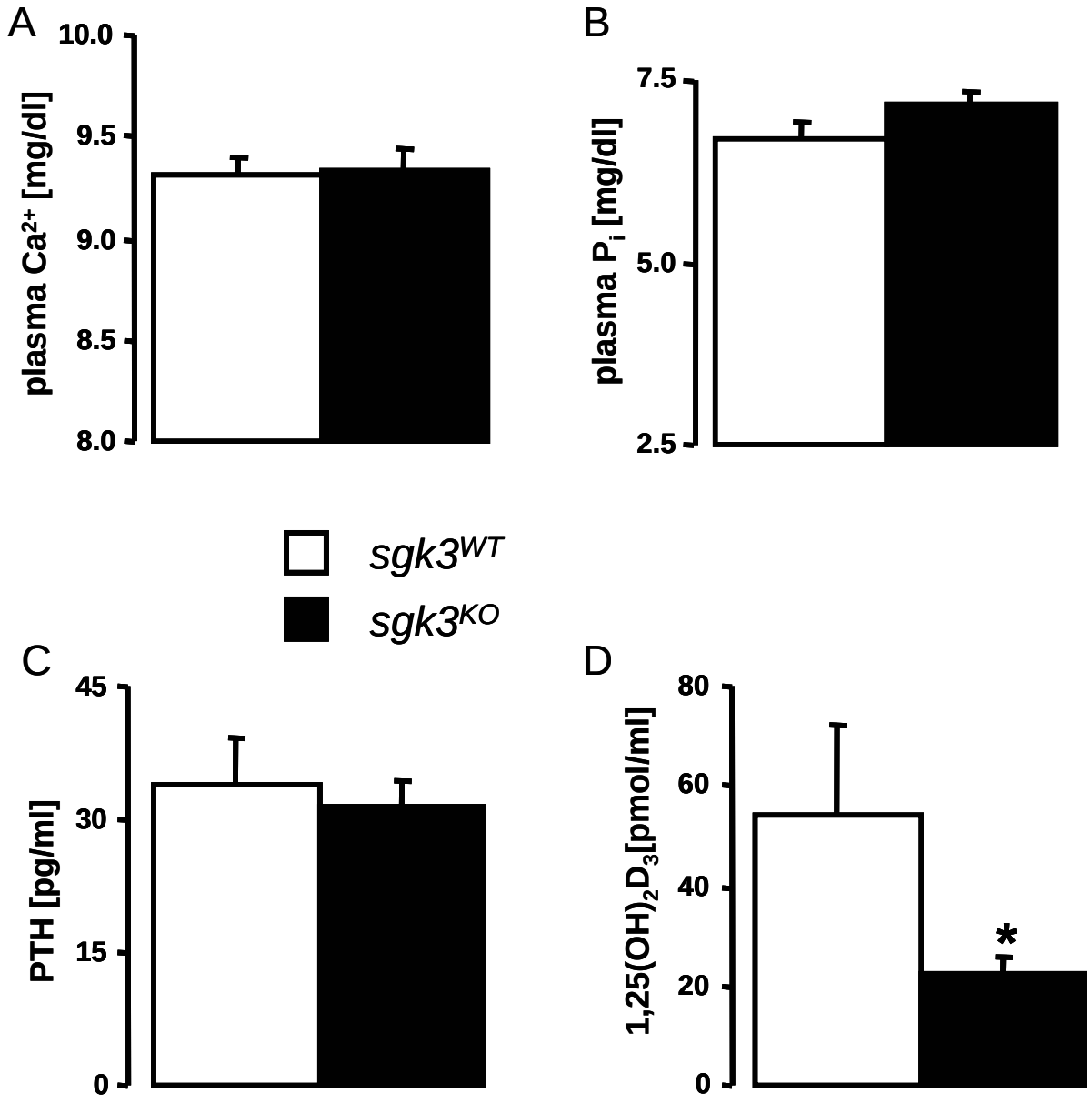

Figure 21: Plasma calcium, phosphate, PTH and $1,25(OH)_2D_3$ concentrations in *sgk3*^{KO} and *sgk3*^{WT} mice

Arithmetic means ± SEM of plasma calcium (A), phosphate (B) (n = 10 each group), PTH (C) and $1,25(OH)_2D_3$ (D) (n = 10-12 each group) concentration in SGK3 knockout mice (*sgk3*^{KO}, closed bars) and their wild type littermates (*sgk3*^{WT}, open bars).* $p<0.05$ vs. respective value of *sgk3*^{WT} mice.

Renal loss of phosphate was expected to foster demineralization of bone. Thus, bone density was determined in *sgk3*^{KO} and *sgk3*^{WT} mice. As shown in Figure 22, the bone density was indeed lower in *sgk3*^{KO} mice than in *sgk3*^{WT} mice.

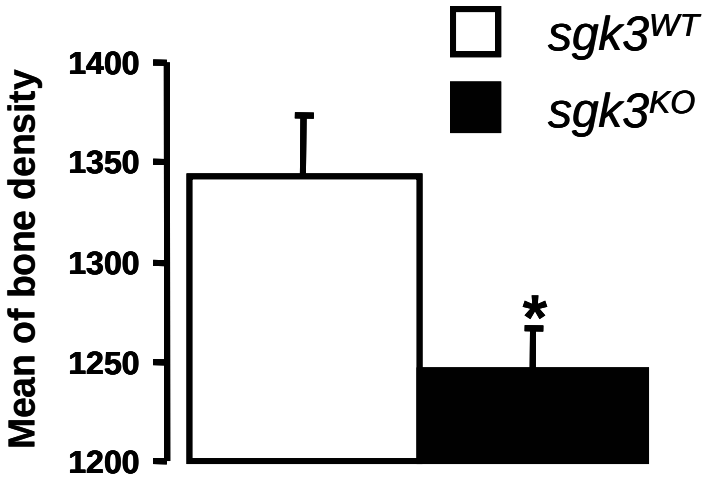

Figure 22: Bone density of *sgk3*^{KO} and *sgk3*^{WT} mice

Arithmetic means ± SEM (n = 6 each group) of bone density in SGK3 knockout mice (*sgk3*^{KO}, closed bars) and their wild type littermates (*sgk3*^{WT}, open bars).* $p<0.05$ vs. respective value of *sgk3*^{WT} mice.

Table 3: Analysis of blood and urine of *sgk3*^{KO} and *sgk3*^{WT} mice

Body weight, food and fluid intake, plasma concentrations and renal excretions of Ca^{2+} and phosphate, urinary flow rate, creatinine clearance and fractional renal excretions of Ca^{2+} and phosphate in SGK3 knockout mice (*sgk3*^{KO}) and their wild type littermates (*sgk3*^{WT}).

	***sgk3*^{WT}**	***sgk3*^{KO}**
Body weight (g)	24.01±0.69	23.48±0.75
Food intake (mg/g BW)	180.9±8.9	210.4±8.9*
Fluid intake (mg/g BW)	299.9±16.7	318.7±16.6
[Ca^{2+}]plasma (mg/dl)	9.31±0.08	9.33±0.11
[P_i]plasma (mg/dl)	6.70±0.22	7.15±0.19
Urine Ca^{2+} (µmol/24h/g BW)	0.30±0.02	0.31±0.03
Urine P_i (µmol/24h/g BW)	0.18±0.03	0.62±0.12*
Urinary flow (µl/24h/g BW)	51.47±8.77	63.93±5.47
Creatinine clearance (µl/min/g BW)	4.78±0.55	6.05±0.95
Fractional excretion of Ca^{2+} (%)	1.92±0.19	1.79±0.13
Fractional excretion of P_i (%)	1.50±0.42	3.20±0.69*

Arithmetic means ± SEM (n = 10 - 13); * indicates significant difference between genotypes (p<0.05).

DISCUSSION

The role of PKB/SGK resistant GSK3 in renal water and electrolyte excretion as well as steroid hormone release

The present study reveals several subtle differences between *gsk3*^{KI} and *gsk3*^{WT} mice. Food intake was significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice even though the body weight was similar in both genotypes. In search for a possible explanation for this seeming discrepancy, we hypothesized that *gsk3*^{KI} mice had enhanced metabolic turnover and thus determined body temperature. Rectal temperature was indeed significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. The elucidation of enhanced body temperature is beyond the scope of this study and future studies shall be directed to elucidate the underlying cause. Enhanced metabolic turnover is further reflected by enhanced urinary urea excretion, an indicator for enhanced protein degradation [142]. Increased urinary urea excretion could have contributed to the higher urine osmolarity in *gsk3*^{KI} mice [143]. Notably, plasma urea concentration is rather lower and thus, renal urea clearance enhanced.

Enhanced food intake is paralleled by increased electrolyte intake. Enhanced renal Na^+ excretion may at least partially be due to reduced plasma aldosterone levels. Hypoaldosteronism should, however, not increase but decrease urinary K^+ excretion. Plasma K^+ concentration was not different between *gsk3*^{KI} and *gsk3*^{WT} mice at any of the diets offered to the mice and differences in plasma K^+ concentration cannot account for the kaliuresis of the *gsk3*^{KI} mice. Thus, a renal mechanism presumably causes enhanced renal K^+ excretion. Lithium, an inhibitor of GSK, downregulates distal nephron ENaC expression [144], which is expected to decrease distal tubular K^+ secretion. Conversely, overactivity of GSK3 in *gsk3*^{KI} mice may enhance ENaC activity, thus enhancing the driving for K^+ secretion.

Fluid intake was again significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. The enhanced fluid intake parallels the enhanced urinary flow rate of *gsk3*^{KI} mice. The enhanced urinary flow rate in *gsk3*^{KI} mice does not reflect decreased ability of urinary concentration, as urinary osmolarity is increased. Moreover, water deprivation did not abrogate the differences in urinary output between *gsk3*^{KI} and *gsk3*^{WT} mice. At high salt diet, the increased urinary osmolarity could be explained by increased ADH excretion. Beyond that, GSK3 may participate in the regulation of renal tubular water transport. Along those lines lithium, an inhibitor of GSK3, causes nephrogenic diabetes insipidus [145]. Lithium increases

cycloxygenase expression, leading to enhanced formation of prostaglandins and subsequent polyuria [145].

The increased fluid intake of *gsk3*^{KI} mice was not dependent on salt intake. Moreover, when offered the choice between tap water and saline, the *gsk3*^{KI} mice drank similar volumes of saline as the *gsk3*^{WT} mice, but continued to drink larger volumes of tap water than their wild-type littermates. Accordingly, unlike the *gsk3*^{WT} mice, *gsk3*^{KI} mice did not drink significantly more saline than tap water. The enhanced fluid intake of *gsk3*^{KI} mice cannot be explained by enhanced plasma Na^+ concentration or osmolarity. Notably, inhibition of GSK3 by lithium has been reported to upregulate eNOS [146] and inhibition of NO-synthase by L-NAME enhances thirst following Lipopolysaccharide (LPS) injection [147]. Taken together, one may speculate that unrestrained GSK3 could downregulate NO-synthase and that the lowering of NO induces thirst. Clearly, additional experimental effort is needed to determine the role of GSK3 in the regulation of thirst.

The role of GSK3 in the regulation of eNOS [146] may further contribute to or even account for the differences of blood pressure between *gsk3*^{KI} and *gsk3*^{WT} mice. Blood pressure was higher in *gsk3*^{KI} than in *gsk3*^{WT} mice both, at 1 p.m. and 10 p.m.

In both, *gsk3*^{KI} and *gsk3*^{WT} mice, hematocrit increased significantly following a low Na^+ diet. The reduced salt intake led to a decrease of plasma Na^+ concentration, a difference reaching statistical significance in *gsk3*^{WT} mice. The decreased extracellular Na^+ concentration is expected to favour water movement from extracellular space into cells and thus increase the erythrocyte volume at the expense of plasma volume.

Under control diet the creatinine clearance was significantly higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. The present study did not attempt to define the underlying mechanisms. It is noteworthy, however, that GSK3ß expressed in the glomerula inactivates Snail, which in turn decreases the transcription of nephrin [148]. Thus, GSK3ß presumably participates in the regulation of glomerular function.

Plasma aldosterone at 10 a.m. and plasma corticosterone levels were at 5 p.m. indeed significantly lower in *gsk3*^{KI} than in *gsk3*^{WT} mice. However, the urinary excretion of aldosterone was significantly higher and corticosterone excretion tended to be higher in *gsk3*^{KI} than in *gsk3*^{WT} mice. The discrepancy may result from an influence of GSK on circadian rhythm [79;149]. Moreover, according to unpublished observations, plasma protein concentration is decreased in *gsk3*^{KI} mice. Decreased plasma protein binding could contribute to the reduced plasma steroid levels in those mice.

gsk3^{KI} mice are devoid of SGK1-dependent regulation of GSK3. Accordingly, some parallel findings were expected in *gsk3*^{KI} mice and in gene targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$). However, the properties of *gsk3*^{KI} and *sgk1*$^{-/-}$ differ in a variety of aspects. As compared to their wild type littermates, *sgk1*$^{-/-}$ mice have enhanced plasma aldosterone levels and are unable to adequately decrease urinary Na^+ output following exposure to salt-deficient diet [61]. In contrast, *gsk3*^{KI} have lower plasma aldosterone concentrations than *gsk3*^{WT} mice and adequately decrease urinary Na^+ excretion during salt depletion. Moreover, *sgk1*$^{-/-}$ mice are unable to adequately increase renal K^+ excretion during acute and chronic K^+ loading [62]. In contrast, renal K^+ excretion is rather enhanced in *gsk3*^{KI} mice despite normal plasma K^+ concentration. Thus, SGK1-dependent regulation of renal Na^+ and K^+ excretion is obviously not due to phosphorylation and inhibition of GSK3. Instead, SGK1 is partially effective through stimulation of ENaC expression [114], phosphorylation of the ENaC alpha-subunit [150] and phosphorylation of Nedd4-2 [151]. Apparently, GSK3 influences renal water and electrolyte excretion via SGK1 independent mechanisms.

In conclusion, insensitivity of GSK3 to the inhibitory action of PKB and SGK1 leads to a decrease of plasma corticosterone and aldosterone levels, decreased salt appetite, enhanced renal Na^+ excretion, hyponatremia, enhanced susceptibility to water deprivation and increased blood pressure. The present observations disclose a completely novel element in the regulation of water and electrolyte metabolism.

SGK1 dependence of renal electrolyte balance and hormone regulation in APC min mice

The present observations reveal several subtle differences between mice carrying a mutation in the APC gene ($apc^{Min/+}$) and their wild type littermates ($apc^{+/+}$). Most importantly, the $apc^{Min/+}$ mice had higher plasma aldosterone and plasma corticosterone concentrations than the $apc^{+/+}$ mice.

Lack of APC could, at least in theory, modify electrolyte homoestasis by decreased degradation of ß-catenin with subsequent increase of ß-catenin levels and stimulation of SGK1 expression. As demonstrated earlier, genes upregulated by ß-catenin include SGK1 [125;126], which in turn phosphorylates glycogen synthase kinase 3 (GSK3) [152] and thus blunts the downregulation of ß-catenin by GSK3 dependent phosphorylation [127;129]. SGK1 further stimulates the expression and/or activity of a variety of channels and carriers [33;60;122;123;140;141;152-154] and is thus important for renal Na^+ retention [61;155]. Enhanced expression of SGK1 would be expected to foster renal Na^+ retention and thus increase plasma volume. Accordingly, urinary Na^+ excretion was indeed significantly decreased in $apc^{Min/+}$ mice, a difference reversed by additional lack of SGK1. Mirror-like changes were observed in fecal Na^+ excretion. Unlike renal tubular Na^+ reabsorption [61;155], colonic Na^+ reabsorption appears not to depend on SGK1 [156]. The fractional urinary Na^+ excretion was not significantly different between $apc^{Min/+}$ mice and $apc^{+/+}$ mice, as glomerular filtration rate was similarly lower in $apc^{Min/+}$ mice than in $apc^{+/+}$ mice, a difference, however, not reversed by additional lack of SGK1. The observations point to a role of APC in the maintenance of glomerular function.

According to the experiments with Evans blue, plasma volume was significantly larger in $apc^{Min/+}$ mice, an effect partially blunted in $apc^{Min/+}/sgk1^{-/-}$ mice. Thus, SGK1 dependent Na^+ retention could have led to hypervolemia. Increased SGK1 expression following treatment with a PPARgamma agonist was similarly shown to enhance plasma volume [157], an effect in part dependent on SGK1.

Hypervolemia would in turn be expected to decrease aldosterone release. The opposite is observed, i.e. plasma aldosterone levels are significantly higher in $apc^{Min/+}$ mice than in $apc^{+/+}$ mice. Moreover, the hyperaldosteronism was not reversed but augmented by additional knockout of SGK1. The further increase of plasma aldosterone concentration is in line with the enhanced plasma aldosterone levels observed in SGK1 deficient mice, a result of impaired

renal Na^+ retention in those animals [61;155]. Clearly, the hyperaldosteronism of *apc*$^{Min/+}$ mice is not due to enhanced SGK1 expression and is neither due to volume depletion nor due to hyperkalemia. Instead, APC-dependent signalling may influence aldosterone release more directly. Corticosterone levels were not significantly different between *apc*$^{Min/+}$*/sgk1*$^{-/-}$ and *apc*$^{Min/+}$ mice. Thus, unlike the increase of aldosterone levels, the increase of corticosterone levels may be dependent on the presence of SGK1.

Even though plasma aldosterone levels were even higher in *apc*$^{Min/+}$*/sgk1*$^{-/-}$ mice, urinary Na^+ excretion tended to be higher in those mice than in *apc*$^{Min/+}$ mice, an expected finding in view of the known effect of SGK1 on renal tubular Na^+ transport [61;155]. The enhanced plasma aldosterone levels could further explain the increased fractional K^+ excretion and the elevated blood pressure in *apc*$^{Min/+}$ mice than in *apc*$^{+/+}$ mice.

In contrast to plasma volume, body weight was not different between *apc*$^{Min/+}$ mice and *apc*$^{+/+}$ mice. Thus, the possibility was considered that the abundance of body fat was decreased in *apc*$^{Min/+}$ mice. As body fat mass is correlated with leptin plasma concentrations[158], plasma leptin levels were determined. As a result, APC deficiency was paralleled by marked decrease of plasma leptin concentrations, pointing to reduced body fat mass. Recent observations indeed point to an inhibitory effect of ß-catenin signalling on adipocyte differentiation [159-161].

In conclusion, plasma aldosterone and corticosterone levels, plasma volume and blood pressure were higher in mice carrying the defective APC gene. The effects are partially reversed by lack of SGK1 pointing to a role of this kinase in APC-dependent regulation of electrolyte homoestasis. However, SGK1 deficiency augments the effect of defective APC on plasma aldosterone levels, pointing to a SGK1 independent role of APC in the regulation of adrenal hormone release. Clearly, the signalling cascade of APC and SGK1 is more complicated than a simple serial chain and the present data shed some light but still do not clarify the final interactions.

The role of SGK3 in renal phosphate handling

The present observations disclose a novel function of SGK3, i.e. the stimulation of renal tubular phosphate transport. According to the experiments on *Xenopus* oocytes, coexpression of SGK3 leads to a marked increase in NaPiIIa activity. The in vivo relevance of SGK3 sensitive NaPiIIa regulation is underscored by the phosphaturia of *sgk3*^{KO} mice.

The phosphaturia of *sgk3*^{KO} mice was not due to increased plasma phosphate concentrations and occurs without significant alterations of PTH plasma concentrations. The hormone is well known to downregulate renal phosphate transport [132], and its release is inhibited by enhanced plasma phosphate concentration [162]. The plasma levels of $1,25(OH)_2D_3$ are significantly decreased in *sgk3*^{KO} mice. The rate-limiting enzyme in the generation of $1,25(OH)_2D_3$ is the renal 1α-hydroxylase [163;164], which is stimulated by PTH [164] and cellular phosphate depletion [165].

As $1,25(OH)_2D_3$ is a powerful stimulator of intestinal phosphate transport [166], the decreased plasma $1,25(OH)_2D_3$ concentrations may have contributed to the renal phosphate loss.

The *sgk3*^{KO} mice suffer from a subtle but significant impairment of bone mass, which may again be partially due to decreased formation of $1,25(OH)_2D_3$. The hormone is known to counteract apoptosis of osteoblasts [167] and is thus known to enhance bone mineralization [168]. The effect of $1,25(OH)_2D_3$ is mediated by the phosphatidylinositol 3-kinase pathway [169], and may thus at least partially involve SGK3. The demineralization of bone may further be due to phosphate depletion, as phosphate inhibits the formation of new osteoclasts and stimulates apoptosis of mature osteoclasts [170]. The present observations do not rule out the participation of further mechanisms. For instance, SGK3 shares several functions with Akt2/PKBß [171], which may confer survival of osteoblasts [172] and osteoclasts [173;174].

In conclusion, the present observations reveal a novel function of SGK3, i.e. its involvement in the regulation of $1,25(OH)_2D_3$ plasma concentration, renal phosphate excretion and mineralization of bone.

ABBREVIATIONS

ACE	Angiotensin converting enzyme
ADH	Anti Diuretic hormone
ADP	Adenosine diphosphate
APC	Adenomatous polyposis coli
ASDN	Aldosterone sensitive distal nephron
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BBM	Brush border membrane
BIO	6-bromoindirubin-3´-oxime
BW	Body weight
$CaCl_2$	Calcium chloride
CCD	Cortical collecting duct
CISK	Cytokine independent survival kinase
Dexa	Dexamethasone
dL	decilitre
DOCA	Deoxycorticosterone acetate
ECF	Extracellular fluid
ENaC	Epithelial sodium channel
FAP	Familial adenomatous polyposis
FE	Fractional excretion
GFR	Glomerular filtration rate
GS	Glycogen synthase
GSK3	Glycogen synthase kinase-3
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IGF-1	Insulin like growth factor-1
Ins	Insulin
KCl	Potassium Chloride
MAPK	Mitogen-activated protein kinase
MCD	Medullary collecting duct
mg	milligram
$MgCl_2$	Magnesium Chloride
Min	minute
MR	Mineralocorticoid receptor
NaCl	Sodium Chloride
NaPi	Sodium-phosphate cotranspoter
Ng	nanogram
NHERF2	Sodium-hydrogen exchanger regulatory factor 2
Nmol	nanomoles
PBS	Phosphate buffered saline
PDK1	Phosphoinositide dependent kinase 1
pg	Picogram
Pi	Phosphate
PI3K	Phosphatidylinositide-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PTH	Parathyroid hormone
ROMK	Renal outer medullary potassium channel

SD	Standard diet
SGK	Serum and glucocorticoid inducible kinase
µg	microgram
µL	microlitre

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