STIMULATION OF RAT TYPE IIA SODIUM DEPENDENT PHOSPHATE COTRANSPORTER (NAPI-2) BY SERUM AND GLUCOCORTICOID-INDUCED KINASE SGK1 AND NA^+/H^+ EXCHANGE REGULATING FACTOR NHERF2

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ABSTRACT

Inorganic phosphate (Pi) is reabsorbed in the renal proximal convoluted tubules mainly via the electrogenic sodium dependent phosphate cotransporter NaPi Type IIa (NaPi-IIa). The isoforms of NaPi-IIa have been cloned from different species such as rat (NaPi-2). Serum and glucocorticoid-induced kinase 1 (SGK1) and rat sodium dependent phosphate cotransporter (NaPi-2) are highly expressed in the brush border membrane (BBM) of proximal tubule cells. The significance of the kinase in regulation of sodium dependent phosphate cotransporter (NaPi-2) has, however, remained elusive. On the other hand, the carboxyl-terminal tail of NaPi-2 contains information for apical expression, and interacts by means of its three terminal amino acids with several PSD95/DglA/ZO-1-like domains (PDZ)-containing proteins such as Na^+/H^+ exchanger 3 regulatory factors NHERF1 or NHERF2. Both, NHERF1 and NHERF2 modulate the targeting and trafficking of several proteins into the plasma membrane. Trafficking of the Na^+/H^+ exchanger NHE3 is controlled by NHE regulating factor NHERF2 and serum and glucocorticoid-inducible kinase SGK1. To test for a possible involvement in NaPi-2 regulation, cRNA encoding NaPi-2 was injected into *Xenopuslaevis* oocytes with or without additional injection of cRNA encoding SGK1 and/or NHERF2. Using two-electrode voltage-clamp, the transport activity was quantified as the substrate-induced current. Exposure to 1 mM phosphate induced an inward current (I_P) in NaPi-2 expressing oocytes but not in water injected oocytes. Coexpression of SGK1 in NaPi-2 expressing oocytes significantly stimulated the phosphate-induced inward current. Moreover, coexpression of NHERF2 also significantly stimulated the phosphate-induced inward current in NaPi-2 expressing oocytes. The effect of SGK1 on NaPi-2 is mimicked by additional coexpression of NHERF2. The observations suggest that SGK1 and NHERF2 regulate NaPi-2 activity and are thus likely to participate in the stimulatory effect of some hormones, such as growth hormone and insulin, on renal phosphate transport. The present results thus disclose novel signaling mechanisms regulating NaPi-2 activity and renal phosphate transport, which may be important for regulation of phosphate homeostasis.

Keywords: *NaPi-2, Proximal tubule, SGK1, NHERF 2*

INTRODUCTION

The maintenance of Pi homeostasis is a critical event to the appropriate growth and well-being of both young and adult animals (Adedokun and Adeola, 2013), because it is necessary for the development, maintenance, and repair of bone and tissues (Mulroney *et al.*, 2004). The major regulation of Pi homeostasis occurs at the kidney (Tenenhouse and Murer, 2003). The homeostasis of Pi is maintained via renal glomerular filtration followed by tightly controlled tubular reabsorption (Prasad and Bhadauria, 2013). The renal tubular reabsorption of phosphate requires cellular phosphate uptake across the brush border and exit at the basolateral cell membrane (Biber and Murer, 1994). One of the key components involved is sodium dependent phosphate cotransporter system localized in the brush border membrane of the tubular epithelium which mediates the uptake of Pi against the electrochemical gradient from primary urine into the cell (Forster *et al.*, 1997).

The sodium dependent phosphate cotransporter systems mediating the transport have been classified in three different types according to both molecular and functional characteristics (Albano *et al.*, 2015). Type I cotransporter (NaPi-1) induces an anion channel (Yanagawa *et al.*, 1999), type IIa is the renal brush border sodium dependent phosphate

cotransporter and type IIb is found apically in many tissues including the small intestine and the lung, but not the kidney (Murer *et al.,* 2000). Type III cotransporters are being found every where including renal tubules (Werner and Kinne, 2001).

The isoforms of the type II sodium dependent phosphate cotransporters from several mammalian species have been cloned such as the rat (NaPi-2) and human (NaPi-3) (Magagnin *et al.,* 1993), opossum kidney (NaPi-4) (Sorribas *et al.,* 1994), flounder (NaPi-5) (Forster *et al.,* 1997), rabbit (NaPi-6) (Verri *et al.,* 1995) and murine (NaPi-7) (Hartmann *et al.,* 1995). The type II cotransporters in the renal brush border are rate limiting for renal tubular phosphate reabsorption in the respective species (Murer, 1992).

The type II sodium dependent phosphate cotransporters are tightly regulated by parathyroid hormone (PTH) (Traebert *et al.,* 2000) which inhibits proximal tubular phosphate reabsorption by stimulation of NaPi-internalization and degradation (Pfister *et al.,* 1998). Further hormones regulating renal tubular phosphate transport include insulin (Li *et al.,* 1996) and growth hormone (Costanzo *et al.,* 1974), which mediates its effect through IGF-1 (Hammerman *et al.,* 1980). Insulin (Bandyopadhyay *et al.,* 1999) and insulin like growth factor IGF-1 (Vanhaesbroeckand Alessi, 2000) are both known to stimulate PI3 kinase, which has previously been demonstrated to enhance insertion of NaPi into the cell membrane (Pfister *et al.,* 1999). Downstream targets of PI3 kinase include phosphoinositol dependent kinase 1 (PDK1) (Park *et al.,* 1999), which activates the protein kinase B (PKB) (Vanhaesbroeck and Alessi, 2000) and Serum and glucocorticoid-inducible kinase SGK1 (Kobayashi and Cohen, 1999).

PDZ-binding motifs are found in the C-terminal tails of numerous integral membrane proteins where they mediate specific protein-protein interactions by binding to PDZ-containing proteins (Yun *et al.,* 2002). The C-terminal tail of NaPi-2 contains a PDZ binding motif, which may bind to the PDZ domains of NHE regulating factors NHERF1 or NHERF2. Both, NHERF1 and NHERF2 are expressed in the proximal renal tubule (Shenolikar and Weinman, 2001). Moreover, it has been shown that NHERF1 bind to the NaPi-2 and play a role in the apical expression of these cotransporters (Hernando *et al.,* 2002). Thus, the possibility was considered that NHERF2 also participates in the regulation of NaPi-2 activity.

Recent experiments disclosed a role of serum and glucocorticoid dependent kinase SGK1 in the interaction of NHE3 with NHERF2 (Yun, 2003; Yang *et al.,* 2014). SGK1 has originally been cloned as a glucocorticoid sensitive gene from rat mammary tumor cells (Firestone *et al.,* 2003). The human isoform has been identified as a cell volume regulated gene (Waldegger *et al.,* 1997). Subsequent studies revealed the genomic regulation of SGK1 by aldosterone (Cowling and Birnboim, 2000), 1,25 $(OH)_2D_3$ (Akutsu *et al.,* 2001), TGFß (Waldegger *et al.,* 1999) and a variety of further cytokines (Lang and Cohen, 2001).

SGK1 is expressed in a wide variety of human epithelial tissues including intestine, kidney and placenta (Waldegger *et al.,* 1997; Wallace *et al.,* 2011). Thus, they may participate in the regulation of transport in those tissues. Similarly NaPi-2 and NHERF2, SGK1 is expressed in the rat proximal renal tubule and OK cells (Fuster *et al.,* 2007) and may well participate in the regulation of NaPi-2.

The present experiments have been performed to explore whether rat type IIa sodium dependent phosphate cotransporter (NaPi-2) is regulated by SGK1 and NHERF2. To this end, cRNA encoding wild-type rat NaPi-2 has been injected with or without cRNA encoding NHERF2 and/or wild-type SGK1 into *Xenopusleavis* oocytes.

MATERIALS and METHODS

cRNA synthesis

cRNA encoding the wild-type rat sodium dependent phosphate cotransporter NaPi-2 (Werner *et al.,* 1991), the Serum and glucocorticoid-inducible kinase SGK1 (Kobayashi *et al.,* 1999), and the Na^+/H^+ exchange regulating factor NHERF2 (Yun *et al.,* 2002) were synthesized in vitro as described previously (Busch *et al.,* 1995; Forster *et al.,* 1997; Wagner *et al.,* 2000).

Expression in Xenopus laevis oocytes

Dissections of *Xenopuslaevis* ovaries, collection, and handling of the oocytes were done as described by Wagner *et al.,* 2000. Oocytes were injected with 7.5 ng SGK1 and/or 5ng NHERF2 cRNA on the first day after preparation of the oocytes. On the second day after preparation 1ng wild-type rat NaPi-2 was injected. Control oocytes were injected with H_2O. All steps were performed at room temperature 3-8 days after injection of the respective cRNAs.

Voltage-clamp analysis

As shown in Fig. 1, two electrode voltage clamp recordings were performed at a holding potential of -50 mV. The data were filtered at 10 Hz and recorded with MacLab digital to analog converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). The control bath solution (ND96) contained 96 mMNaCl, 2 mMKCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. Where indicated, phosphate was added at 1 mmol/l. The final solutions were titrated to the pH 7.4 using HCl or KOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s. In two electrode voltage clamp experiments substrate induced currents were recorded following the application of phosphate. The magnitude of the induced currents varied two- to fivefold, depending on the time period after cRNA

injection and on the batch of oocytes (from different animals). Thus, comparisons were made only within the same batch of oocytes.

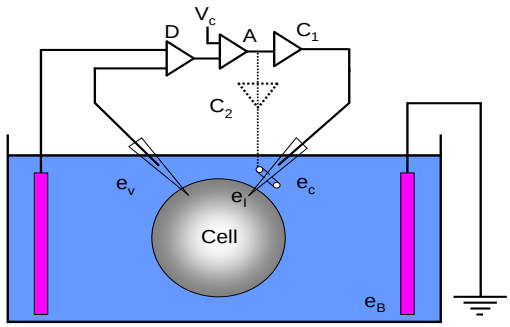

Fig. 1: The two-electrode voltage-clamp. The voltage recording electrode e_V monitors the membrane potential; this is compared with a command voltage V_c, and the magnified difference is applied to a current injection electrode, e_I. A bath electrode e_B serves as the return path for the injected current.

Statistical analysis

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. All data were tested for significance using the Student *t*-test, and only results with $P < 0.05$ were considered as statistically significant.

RESULTS

NaPi-2 induced inward currents

Addition of phosphate (1 mM) to *Xenopusleavis* oocytes expressing NaPi-2 led to an inward current (I_P) approaching -4.7 ± 0.5 nA (n = 14) at a holding potential of -50 mV (Fig. 2). Water injected oocytes, not injected with NaPi-2, did not show any electrogenic phosphate transport and the phosphate induced inward currents were negligible (-0.3 ± 0.1 nA, n = 8).

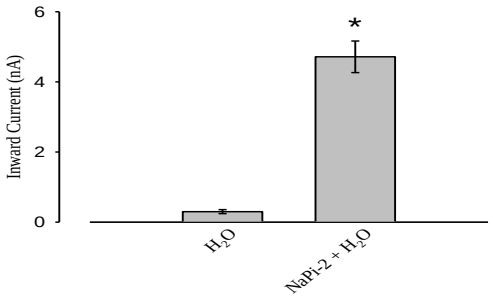

Fig. 2: Phosphate induced inward current (IP) in *Xenopus* oocytes expressing NaPi-2. *Xenopuslaevis* oocytes were injected with either water (H_2O), or cRNA encoding wild-type rat type IIa sodium dependent phosphate cotransporter (NaPi-2) alone. Addition of 1 mM phosphate into ND96 solution induced inward current in *Xenopus* oocytes expressing NaPi-2. In contrast, very small phosphate-induced inward current was observed in H_2O-injected oocytes may be due to expression of endogenous proteins. * indicates statistically significant difference to current in H_2O-injected oocytes.

Stimulation of NaPi-mediated currents by coexpression of SGK1

As shown in Fig. 3, coexpression of SGK1 led to a marked increase of phosphate induced inward currents in NaPi-2 expressing oocytes. Significantly ($p < 0.05$) higher phosphate induced inward currents were observed in oocytes injected with SGK1 and NaPi-2 (-7.4 ± 0.9 nA, n = 14) than the respective value in oocytes expressing NaPi-2 alone.

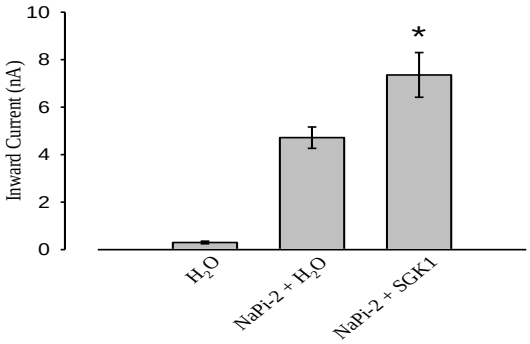

Fig. 3: Phosphate induced inward current (I_P) in *Xenopus* oocytes expressing NaPi-2 with SGK1. *Xenopuslaevis* oocytes were injected with either cRNA of encoding wild-type rat type IIa sodium dependent phosphate cotransporter (NaPi-2) alone or together with SGK1. Coexpression of SGK1 with NaPi-2 significantly stimulated the phosphate induced inward current (I_P) in contrast to coexpression of NaPi-2 + H_2O (*$p < 0.05$). * indicates statistically significant difference to current in *Xenopus* oocytes expressing NaPi-2 alone.

Stimulation of NaPi-mediated currents by coexpression of NHERF2

Coexpression of NHERF2 together with NaPi-2 increased significantly phosphate-induced inward currents in NaPi-2 expressing oocytes (Fig. 4). Significantly ($p < 0.05$) higher phosphate induced inward currents were observed in oocytes injected with NHERF2 and NaPi-2 (-8.4 ± 0.9 nA, n = 14) than phosphate induced inward currents in oocytes expressing the cotransporter alone.

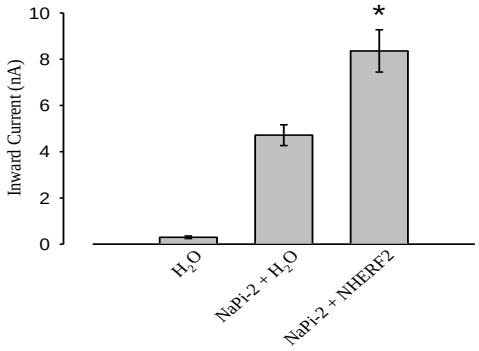

Fig. 4: Stimulation of NaPi-2 transport activity by NHERF2. *Xenopuslaevis* oocytes were injected with cRNA of wild-type rat type IIa sodium dependent

phosphate cotransporter (NaPi-2) alone or together with NHERF2. Coexpression of NHERF2 with NaPi-2 significantly increased phosphate induced inward currents in contrast to coexpression of NaPi-2 + H_2O (*p < 0.05). * indicates statistically significant difference to current in *Xenopus* oocytes expressing NaPi-2 alone.

NHERF2 further increase the effect of SGK1on the NaPi-mediated currents

As shown in Fig. 5, upon coexpression of NaPi-2 with both, SGK1 and NHERF2, phosphate induced inward currents were significantly larger than phosphate induced inward currents in oocytes expressing NaPi-2 with SGK1 or NaPi-2 with NHERF2 alone. In *Xenopus* oocytes coexpressing NaPi-2 together with both, NHERF2 and SGK1, phosphate induced inward currents approached -12.9 ± 1.5 nA, n = 14).

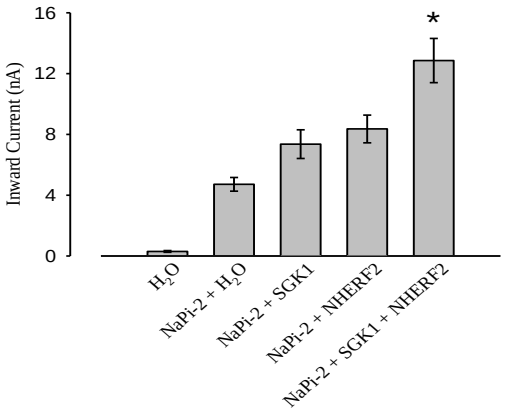

Fig. 5: Phosphate induced inward current (I_P) in *Xenopus* oocytes expressing NaPi-2 with or without coexpression of SGK1. and/or NHERF2. *Xenopuslaevis* oocytes were injected with cRNA encoding wild-type rat IIa sodium dependent phosphate cotransporter (NaPi-2) alone or NaPi-2 together with cRNA encoding SGK1 and/or NHERF2. Coexpression of SGK1 enhances Phosphate induced inward current (I_P), an effect potentiated by additional expression of NHERF2 (*p < 0.05). * indicates significant difference between expression of NaPi-2 alone, or with SGK1, or NHERF2 and coexpression of NaPi-2 together with both, SGK1 and NHERF2.

DISCUSSION

The present experiments disclose two completely novel mechanisms involved in the regulation of renal tubular phosphate transport, i.e., the regulation by NHERF2 and by the protein kinase SGK1. The kinase increases the NaPi-2 activity and stimulates NaPi-2 mediated phosphate transport. As shown for SGK1, the effect is potentiated by additional coexpression of NHERF2. The effect is at least partially due to stimulation of the insertion of the carrier into the cell membrane and delaying the endocytotic retrieval of NaPi-2 cotransporter (Fig. 6).

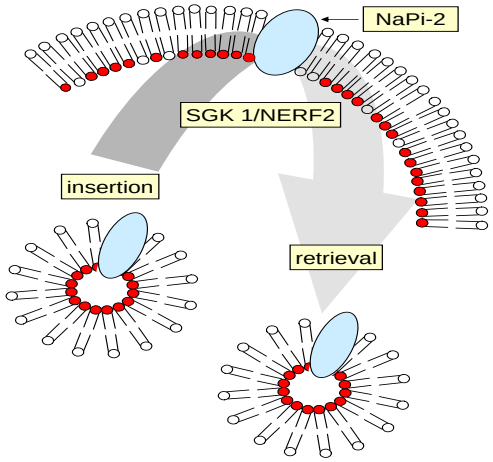

Fig. 6: Proposed model of NaPi-2 cotransporter regulated by SGK1 and NHERF2. The SGK1/NHERF2 stimulates the insertion of new NaPi-2 cotransporters into the plasma membrane and delay the endocytoticretrival of NaPi-2 cotransporters.

In this respect the action of the kinase mimicks that of PI3 kinase (Pfister *et al.*, 1999). In analogy, the serum and glucocorticoid dependent kinase (SGK1) (Webster *et al.*, 1993; Waldegger *et al.*, 1997), another downstream target of PI3 kinase (Kobayashi and Cohen, 1999) stimulates Na^+ channel activity similarly by fostering of channel insertion into the cell membrane (De la Rosa *et al.*, 1999). The SGK1 thus presumably mediates the stimulating effect of IGF-1 on epithelial Na^+ channel activity (Blazer-Yost and Cox, 1988; Blazer-Yost *et al.*, 1992; Blazer-Yost *et al.*, 1996).

SGK1 is not constitutively active but requires activation by phosphorylation. The upstream kinase is the phosphoinositol dependent kinase PDK1 which is in turn activated by IGF-1 through PI3 kinase (Kobayashi and Cohen, 1999; Park *et al.*, 1999). IGF-1 has indeed been shown to stimulate phosphate transport (Caverzasio *et al.*, 1985; Caverzasio and Bonjour, 1988) and inhibition of PI3 kinase leads to internalization and subsequent lysosomal degradation of NaPi (Pfister *et al.*, 1999).

CONCLUSIONS

Rat type IIa sodium dependent phosphate cotransporter (NaPi-2), Serum and glucocorticoid-inducible kinase 1 (SGK1), and Na^+/H^+ exchange regulating factor 2 (NHERF2) are expressed in rat proximal renal tubules. Coexpression of SGK1 enhances NaPi-2 activity and stimulates phosphate transport through NaPi-2, an effect potentiated by coexpression of NHERF2. SGK1 may mediate the effect of IGF-1 and insulin on renal tubular phosphate transport which stimulates SGK1 through PI3 kinase and PDK1. Thus, the present observations unravel a novel signaling pathway in renal tubular transport regulation.

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تحفيز الناقل المشترك للصوديوم والفوسفات-٢ في الفئران بواسطة انزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ والعامل المنظم-٢

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يتم إعادة امتصاص الفوسفات الغير عضوي أساساً في الأنابيب الكلوية الملتوية القريبة عبر الناقل المشترك للصوديوم والفوسفات-٢أيه في الفئران. لقد تم استنساخ أشكال مماثلة من الناقل المشترك للصوديوم والفوسفات-٢أيه من كائنات حية مختلفة مثل الناقل المشترك للصوديوم والفوسفات-٢ في الفئران. الانزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ متزامن بشكل كبير في التعبير في نفس المكان مع الناقل المشترك للصوديوم والفوسفات-٢ في الفئران في خلايا الأنابيب الكلوية الملتوية القريبة. أهمية انزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ في تنظيم الناقل المشترك للصوديوم والفوسفات-٢ ما زالت حتى الآن بعيد المنال والدراسة. ومن ناحية أخري يحتوي الطرف الكربوكسيلي للناقل المشترك للصوديوم والفوسفات-٢ على معلومات عن التعبير الطرفي ويتفاعل عن طريق الأحماض الأمنية الثلاثة الطرفية للناقل مع عدة بروتينات التي تحتوي على نطاق الـ بي دي زد مثل العامل المنظم-١ والعامل المنظم-٢ .كل من العامل المنظم-١ والعامل المنظم-٢ تعدل استهداف ونقل العديد من البروتينات إلى غشاء الخلية. يتم التحكم في نقل مبادل الصوديوم والهيدروجين-٣ بواسطة كل من العامل المنظم-٢ وانزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١. لاختبار احتمال تورطهم في تحفيز وتنشيط هذا الناقل تم حقن الحمض النووي الربي الخاص بالناقل المشترك للصوديوم والفوسفات-٢ في البويضات مع أو بدون حقن الحمض النووي الربي الخاص بإنزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ أو العامل المنظم-٢ أو كليهما معاً. باستخدام طريقة ثنائي الإلكترود ذات الجهد الكهربائي المشبك، يمكن حساب كمية الكهرباء كنشاط لهذا الناقل باعتبارها الركيزة الحالية التي يسببها.التعرض للفوسفات ١ ملم يحدث تيارا كهربائي في البويضات المعبرة للناقل المشترك للصوديوم والفوسفات-٢ ولكن ليس في البويضات المحقونة بالماء بدون الناقل .إضافية انزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ في البويضات المعبرة للناقل المشترك للصوديوم والفوسفات-٢ أدى إلى تحفيز بشكل كبير كمية الكهرباء التي يسببها نقل الفوسفات بوساطة الناقل المشترك للصوديوم والفوسفات-٢ في البويضات. وعلاوة على ذلك، أيضا إضافية العامل المنظم-٢ قد حفز بشكل كبير كمية الكهرباء التي يسببها نقل الفوسفات بوساطة الناقل المشترك للصوديوم والفوسفات-٢ في البويضات. تأثير انزيم الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ على الناقل المشترك للصوديوم والفوسفات-٢ في الفئران يزداد بواسطة إضافية العامل المنظم-٢. هذه الملاحظات تشير إلى أن الكيناز المحدث بالسيرم والجلوكوكورتيكويد-١ والعامل المنظم-٢ ينظمان نشاط الناقل المشترك للصوديوم والفوسفات-٢ وهكذا فإن من المرجح أنهم يشاركان معاً في تنشيط تأثير بعض الهرمونات مثل هرمون النمو والأنسولين على نقل الفوسفات الكلوي. هذه النتائج الحالية تكشف عن آليات مبتكرة وجديدة لتنظيم نشاط الناقل المشترك للصوديوم والفوسفات-٢ ونقل الفوسفات الكلوي التي قد تكون ذات أهمية بالنسبة لتنظيم توازن الفوسفات فى جسم الكائن الحي.