Phosphate overload induces podocyte injury via type III Na-dependent phosphate transporter

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1Division of Endocrinology and Metabolism, Department of Internal Medicine, 2Education and Research Center of Animal Models for Human Diseases, 3Laboratory of Molecularbiology and Histochemistry, Joint Research Laboratory, and 4Department of Surgical Pathology, Fujita Health University, Aichi; 5Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya; 6Department of Pediatrics, Kyorin University School of Medicine, Tokyo; and 7Department of Structural Pathology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

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Sekiguchi S, Suzuki A, Asano S, Nishiwaki-Yasuda K, Shibata M, Nagao S, Yamamoto N, Matsuyama M, Sato Y, Yan K, Yaoita E, Itoh M. Phosphate overload induces podocyte injury via type III Na-dependent phosphate transporter. Am J Physiol Renal Physiol 300: F848–F856, 2011. First published February 9, 2011; doi:10.1152/ajprenal.00334.2010.—Uptake of Pi at the cellular membrane is essential for the maintenance of cell viability. However, phosphate overload is also stressful for cells and can result in cellular damage. In the present study, we investigated the effects of the transgenic overexpression of type III Pi transporter Pit-1 to explore the role of extracellular Pi, in glomerular sclerosis during chronic renal disease. Pit-1 transgenic (TG) rats showed progressive proteinuria associated with hyperalbininemia and dyslipidemia. Ultrastructural analysis of TG rat kidney by transmission electron microscopy showed a diffuse effacement of the foot processes of podocytes and a thickening of the glomerular basement membrane, which was progressively exhibited since 8 wk after birth. TG rats died at 32 wk of age due to cachexia. At this time, more thickening of the glomerular basement membrane and segmental sclerosis were observed in glomeruli of the TG rats. Immunohistochemical examination using anti-connexin 43 and anti-desmin antibodies suggested the progressive injury of podocytes in TG rats. TG rats showed higher Pi uptake in podocytes than wild-type rats, especially under low Pi concentration. When 8-wk-old wild-type and TG rats were fed a 0.6% normal phosphate (NP) or 1.2% phosphate (HP) diet for 12 wk, HP diet-treated TG rats showed more progressive proteinuria and higher serum creatinine levels than NP diet-treated TG rats. In conclusion, our findings suggest that overexpression of Pit-1 in rats induces phosphate-dependent podocyte injury and damage to the glomerular barrier, which result in the progression of glomerular sclerosis in the kidney.

phosphate; podocyte; proteinuria

Pi IN EXTRACELLULAR FLUID is known to play important roles in maintaining cellular function (34). Pi influx from the extracellular milieu is controlled by an active Pi transport system driven by an inwardly directed Na gradient. There are at least three types of Na-dependent Pi transporters, type I, II, and III, in mammalian cells (34). The plasma Pi concentration is mainly controlled by type IIa and IIc Na-dependent Pi cotransporters that are located in the proximal tubules of the kidney, whereas type IIb is expressed in several tissues including the lung and small intestine. Type III Pi transport systems, namely Pit-1 and Pit-2, are ubiquitously expressed and are considered to be essential for Pi influx in systemic cells. As for bone-forming cells, Pit-1 is also necessary to induce extracellular mineralization by expressing Pit-1 on matrix vesicles, which are released from osteoblasts and chondrocytes (4, 23, 32). In addition to bone formation, arterial calcification is modulated by the transition of arterial smooth muscle cells from contractile to the chondro-osseous form. This program of transdifferentiation is thought to be induced through the expression of Pit-1. Pi uptake via Pit-1 is required for osteochondrogenic phenotypic changes and calcification of vascular smooth muscle cells in vitro (9, 11, 17). These findings suggest that Pit-1 is a key player in both physiological and pathological mineralization.

Pi uptake at the cellular membrane is essential for the maintenance of cell viability because Pi is required for ATP production. However, accumulating evidence suggests that Pi overload from the extracellular milieu causes cell stress (26). We have previously reported that high Pi medium induces cell death of rat A-10 vascular smooth muscle cells (20). However, osteoblast-like cells, which generally use Pi to initiate extracellular calcification, remain viable in a high Pi milieu. Moreover, such conditions actually enhance calcification of osteoblast-like cells in vitro (10, 32). It has been shown that the extracellular signal to stimulate the proliferation of osteoblasts induces the enhancement of Pi uptake (i.e., Pi transport activity) (25, 33, 39). These findings suggest that cell survival under high-Pi milieu conditions depends on the phenotype of the cells.

Many animal models have been used to study proteinuria (7). The glomerular barrier, which protects against proteinuria, consists of the podocyte, glomerular basement membrane (GBM), and glomerular endothelial cells (6). Among them, the terminally differentiated podocyte functions as a critical size and charge barrier to prevent proteinuria, and podocyte injury, with or without loss of renal function, results in proteinuria (27). Podocytes are injured by both immune- and non-immune-mediated diseases such as membranous nephropathy, minimal-change disease, viral infection, and diabetes (13, 14, 22). Serum Pi concentration is mainly regulated by reabsorption at the proximal tubules in the kidney (8). Clinically, hyperphosphatemia occurs most commonly as a result of impaired excretion due to renal failure. At the same time, hyperphos-
phatemia itself might contribute to the progress of renal damage resulting from Pi-induced cellular stress on glomerular cells such as podocytes. Healthy rats have mechanisms to normalize a high-phosphate diet, suggesting that we need to establish an indirect model to explore the effect of high Pi on glomerular function in vivo.

In the present study, we examined the age-dependent change in renal function in the transgenic (TG) rat overexpressing type III Pi transporter Pit-1, which is systemically expressed to handle cellular Pi influx, to explore the effect of high Pi on glomerular function in vivo. In the present study, we examined the age-dependent change in renal function in the transgenic (TG) rat overexpressing type III Pi transporter Pit-1, which is systemically expressed to handle cellular Pi influx, to explore the effect of high Pi, “stress” on kidney function in vivo. Our findings suggest that Pit-1 overexpression in rats induces phosphate-dependent podocyte injury and damage to the glomerular barrier, which result in the progress of glomerular sclerosis in the kidney.

METHODS

Animals. The mouse Pit-1 gene was ubiquitously expressed under the control of a cytomegalovirus early enhancer element and chicken β-actin (CAG) promoter in TG rats, and upon pronuclear DNA microinjection into the rat zygotes (31). The expression of the mouse Pit-1 gene in TG rats was confirmed by both Southern blot analysis and PCR. All rats were housed at 24°C with a 12:12-h light-dark cycle and were allowed free access to tap water and a normal rodent chow. Generally, TG rats died in 24–32 wk because of malnutrition and cachexia, while wild-type (WT) rats survive more than 2 yr (data not shown). Proteinuria was measured by the biuret method on a 24-h urine collection at each time point. Routine serum chemistries were measured with a Hitachi 7180 automatic analyzer (Hitachi High Technologies, Tokyo, Japan). All the rats were anesthetized by ether and were killed by exsanguination from the ventral aorta. When indicated, a 0.6% normal-phosphate diet (NP) and 1.2% high-phosphate diet (HP) started at 8 wk of age, and the animals were housed the same as above. This research adheres to the APS Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training. All animal procedures and a statement of protocol were approved by the institutional animal care and use committee and government authorities.

Quantitative real-time PCR. Total RNA was isolated from primary culture cells of podocyte with TRIZol (Invitrogen, Carlsbad, CA) and was subjected to quantitative real-time PCR (qRT-PCR) using the ABI PRISM7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Mouse Pit-1 and rat Pit-1 amplification was compared with the amplification of a housekeeping gene encoded 18S. The sequences of primers used for the RT-PCR were as follows: mouse Pit-1, 5'-AACTGTGGTCATCGCATCAAAC-3' and 5'-ACAGAGCCCACCTTGCATAATGT-3'; rat Pit-1, 5'-GATTCTTTAAACAGCCAAGCA-3' and 5'-GAAGCTGGTGAGGCCAACAA-3'; eukaryotic 18S rRNA (Hs99999901_s1, Applied Biosystems). PCR was performed at 95°C for 15 s, 60°C for 60 s (40 cycles).

Light microscopy. Kidneys were fixed in formalin and embedded in paraffin. Sections of 2- to 3-μm thickness were stained with hematoxylin and cosin or periodic acid-Schiff (PAS) reagent. Using light microscopy at low-power magnification, a plan of suitable sections was drawn with the position of every glomerulus marked, which generally covered the whole section. At high-power magnification, glomeruli were examined for lesions.

Electron microscopy. Small pieces of tissue, 2 × 4 mm, were fixed in 2.5% glutaraldehyde (GA)/0.05 M sodium phosphate, pH 7.4 (PB), for 2 h. After washing with PB, samples were dehydrated in a graded ethanol series and treated with n-butyl glycidyl ether before being
embedded in Epon 812 (Taab Laboratories, Aldermaston, UK). Ultrathin sections (0.1 μm) were doubly stained with 2% uranyl acetate and 1% lead citrate and then observed using a JEM-1010 (JEOL, Tokyo, Japan) for transmission electron microscopy (TEM) at an accelerating voltage of 80 kV.

**Immunofluorescence microscopy.** The indirect immunofluorescence technique was applied to frozen kidney sections and outgrowths from glomeruli as described previously (38). In brief, the rat kidneys were snap-frozen at −70°C, sectioned at a thickness of 3 μm in a cryostat, and fixed in 2% paraformaldehyde in PBS for 5 min. The following antibodies were used as primary antibodies: monoclonal anti-nephrin antibody (5-1-6; courtesy of Dr. H. Kawachi, Niigata University, Niigata, Japan) (36); murine monoclonal anti-desmin antibody (clone D-33; DakoCytomation, Glostrup, Denmark); rabbit anti-connexin 43 antibody (Sigma, St. Louis, MO); rabbit polyclonal anti-podocin antibody (kindly provided by Dr. H. Tsukaguchi, Kansai Medical University, Moriguchi, Japan) (28); murine monoclonal anti-synaptopodin antibody (clone G1D4; Progen, Heidelberg, Germany) (19); murine monoclonal anti-zonula occludens-1 (ZO-1) antibody (Zymed Laboratories, South San Francisco, CA); and rabbit anti-laminin antibody (DakoCytomation). For double-label immunofluorescence microscopy, murine monoclonal anti-nephrin antibody and rabbit anti-laminin antibody, murine monoclonal anti-desmin antibody and rabbit anti-laminin antibody, and rabbit anti-connexin 43 antibody and murine monoclonal anti-ZO-1 antibody were mixed and applied as primary antibodies simultaneously. After washing with PBS, the sections were stained with FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG, rewashed with PBS, and subsequently reacted with FITC- or TRITC-conjugated anti-mouse IgG. PBS, normal rabbit serum, or murine IgG monoclonal antibody (against rotavirus), shown not to react with rat glomeruli, was used as a negative control for the primary antibodies. Immunofluorescence of the sections was observed with a laser-scanning confocal microscope (A1Rsi; Nikon, Tokyo, Japan).

**Primary culture of podocytes.** Glomerular isolation from rat kidney was performed as previously reported (12). Briefly, male rats at ages of 7–9 wk were anesthetized by inhalation of diethyl ether. Kidneys were removed and perfused with a mixture of Dynabeads (4.5 μm) and iron powder (6 μm), removed, and minced into 1-mm³ pieces, and digested in collagenase solution containing 1 mg/ml collagenase. The collagenase-digested tissues were gently pressed through a 100-μm cell strainer. Glomeruli containing Dynabeads and iron powder in the cell suspension were gathered by a magnetic particle concentrator and washed with PBS. Collected glomeruli were seeded onto type I collagen-coated culture dishes in DMEM/F-12 (1:1) containing 5% FBS supplemented with 0.5% Insulin-Transferrin-

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![Image](http://ajprenal.physiology.org.org)
Fig. 4. Ultrastructural analysis of glomerulus of WT (A–E) and Pit-1-overexpressing TG rats (F–J) by transmission electron microscopy at 20 days (A and F) and 8 (B and G), 24 (C and H), and 32 wk of age (D, E, I, and J). Original magnification: ×30,000 (A–D, F–I); ×3,000 (E and J).
Selenium-A liquid media supplement, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated in a 37°C humidified incubator with 5% CO2. Subculture of primary cultured podocytes was performed after 4 days of culture of isolated glomeruli. Cellular outgrowths were detached with trypsin-EDTA and passed through a 25-μm sieve to remove the remaining glomerular cores. The filtered cells were cultured on type I collagen-coated dishes for 1 more day. Phagocytic cells were removed from the culture by incubation of primary cultures with immune complex-coated magnetic beads followed by attachment to a magnetic particle concentrator. We confirmed that the podocyte markers such as nephrin, podocin, synaptopodin, and podocalyxin were expressed in our primary cultured podocytes from both WT and TG rats (data not shown).

P<sub>i</sub> transport activity. Membranous P<sub>i</sub> transport activity was analyzed in primary-cultured podocytes cultured on day 5. P<sub>i</sub> transport activity was determined in Earle’s buffered salt solution (EBSS) containing 0.01–2.0 mM H<sub>3</sub>PO<sub>4</sub> as previously described (33). Before the transport assay, the cell layer was rinsed three times with EBSS without radioactive or cold substrate. The transport measurement was started after adding 0.3 ml of EBSS containing the labeled substrate (1 μCi/ml). After a 10-min incubation, the uptake solution was aspirated, and the cell layer was rinsed three times with 0.3 ml of ice-cold substrate free EBSS. At the end of the experiment, cells were solubilized with 0.25 ml of 0.2 N sodium hydroxide and the radioactivity contained in a 200-μl aliquot was counted by using a standard liquid scintillation technique.

Statistical analysis. Results are expressed as means ± SE. A two-sided unpaired Student’s t-test or ANOVA for multiple comparisons was used for statistical analysis. A difference between experimental groups was considered to be significant when the P value was <0.05.

RESULTS

The overexpression of mouse Pit-1 mRNA in podocyte of Pit-1 TG rat was confirmed by qRT-PCR (Fig. 1). Pit-1 TG rats began to display a lower serum albumin concentration in 8-wk-old rats compared with their WT littermates. After 8 wk, the serum albumin level of TG rats progressively decreased until their death at ~30 wk old (Fig. 2A). In accordance with the decrease in the level of serum albumin, total serum cholesterol concentrations in TG rats increased (Fig. 2B). In addition, severe proteinuria was found in TG rats from 12 wk, which increased further according to their aging (Fig. 2C). By contrast, the concentration of serum creatinine in TG rats was not different from that of WT rats throughout their lives (Fig. 2D).

TG and WT kidney sections with PAS staining were histologically examined at 10 days and 8, 12, and 32 wk of age. No difference was observed between WT and TG rats at 10 days (data not shown) and 8 wk (Fig. 3, A and B). In TG rats aged 12 wk, hyaline and vacuolar degeneration was frequently observed in podocytes (Fig. 3D). Hyaline droplet-positive podocytes were observed in >70% of glomeruli. Some of the podocytes exhibited distinct cellular hypertrophy. Tubular atrophy and interstitial cell infiltration were minor and focal. In 32-wk-old TG rats, most glomeruli showed sclerotic lesions, including an increase in mesangial matrix, collapse of the basement membrane, and adhesion of the capillary loop to Bowman’s capsule (Fig. 3F). Tubular atrophy and interstitial cell infiltration were correspondingly conspicuous. These changes were not observed in the age-matched WT rats (Fig. 3, C and D).

Morphometry coupled with TEM was used to observe differences in the ultrastructure of podocyte processes and GBM in the kidneys of both TG and WT rats (Figs. 4 and 5). Analysis of the morphometry data revealed that, although there was little difference between WT and TG from day 10 until day 20 after birth (Fig. 4, A and F), the widths of the GBM in TG rat kidneys was greater than those of their WT littermates, even at 8 wk old (Fig. 4, B and G). The difference in the thickness of the GBM in kidneys from TG and WT rats became more pronounced with age (Figs. 4, C–E and H–J, and 5). In addition to the thickening of the GBM, the podocytes gradually lost their foot processes and underwent effacement (Fig. 4, F–J).

By immunofluorescence microscopy, we next examined podocyte constituent proteins such as nephrin, podocin, synaptopodin, and podocalyxin and podocyte injury markers such as desmin (37) and connexin 43 (38) in the early stage of the pathological proteinuria in TG rats. In 8 wk- and 9-wk-old TG rats, before they developed distinct proteinuria, we could not find any significant change in localization or staining intensity of nephrin compared with WT rats of the same age (Fig. 6, A and B). Other constituent proteins such as podocin, synaptopodin, and podocalyxin also showed same staining intensity between WT and TG rats (data not shown). In contrast, immunostaining for podocyte injury markers showed dramatic changes in these TG rats. Podocytes exhibited focally and segmentally conspicuous staining for desmin and connexin 43 (Fig. 6, C–F). The connexin 43-enhanced area was more widely distributed than the desmin-enhanced area. These findings suggest that podocytes may be injured in the early stage of pathological proteinuria in TG rats.

To explore the involvement of P<sub>i</sub> overload in the mechanism of podocyte injury, at first we evaluated the mouse Pit-1 gene by using primary cultured podocytes from both TG and WT rats. The overexpression of mouse Pit-1 mRNA in podocytes of Pit-1 TG rats was confirmed by qRT-PCR (Fig. 1). We next examined the P<sub>i</sub> uptake in primary cultured podocytes of the glomerulus in kidneys from both TG and WT rats. Na-depend-
dent P, transport activity was determined by measuring $^{32}$P uptake from the extracellular milieu using primary cultured podocytes from TG and WT rats. TG rats showed higher P uptake in the podocytes than WT rats, especially under low P concentration (Fig. 7). To investigate the effect of augmentation of P uptake, the effect of dietary phosphate on the progress of proteinuria in Pit-1 TG rats by feeding the rats with an HP diet for the long term. Since they were 8 wk old, we fed them with NP and HP diets for 12 wk. After 4 wk, the serum albumin level of TG rats taking HP (TG-HP) decreased more than TG rats taking NP (TG-NP) (Fig. 8A). Although there was little difference in serum albumin or proteinuria between NP-fed and HP-fed WT rats, TG-HP had severe proteinuria earlier than TG-NP in accordance with the decrease in the level of serum albumin (Fig. 8, A and B). Afterward, urinary protein secretion in TG-HP caught up with that in TG-NP in 12 wk. Furthermore, TG-HP rats showed an elevation of serum creatinine levels, while TG-NP rats had same concentration as WT rats (Fig. 8C).

**DISCUSSION**

In the present study, we have demonstrated that the overexpression of type III Na-dependent P transporter Pit-1 in rats induced the overt proteinuria associated with hypoalbuminemia and dyslipidemia. Pathological proteinuria is associated with both effacement of foot processes and the loss of slit diaphragms (1, 21). Podocytes cover the GBM, and their interdigitated foot processes interlink with slit diaphragms. Both GBM and the slit diaphragms are essential components of the glomerular filtration barrier, which prevents protein loss into the urine. The GBM itself is acellular, and the GBM is thought to be thickened by an increase in the accumulation of new and abnormal matrix proteins, which are produced by the overlying injured podocytes. Light microscopic examination of kidney sections stained with PAS revealed that the pathological changes in the glomeruli of TG rat kidneys occurred after birth and became more apparent with age. Furthermore, ultrastructural analysis of both TG and WT rat kidneys showed that there...
was normal development of glomerulus until 10 days after birth, when glomerulogenesis is complete. Afterward, the effacement of podocyte foot processes and the thickening of the GBM progressively occurred in TG rats in accordance with massive proteinuria. Thus it is likely that proteinuria in TG rats is caused by podocyte injury in accordance with GBM thickening occurring in the adult but not during glomerulogenesis. Although severe proteinuria progressively increased in TG rats, their serum creatinine levels were unaffected. Diabetic nephropathy is known to be one of the most common cause of glomerulosclerosis, but the plasma glucose level in TG rats was not affected in this study (data not shown). Furthermore, glomerular damage apparently proceeded to renal tubular atrophy and intercellular cell infiltration in TG rats. These results suggest that Pit-1 overexpression mainly damaged glomeruli of TG rat kidneys.

Phosphorus is a critical element in bone mineralization, membrane composition, nucleotide structure, and cellular signaling (24). During the development of the organs and tissues including skeletal tissues, cellular Pi uptake is essential for the synthesis of ATP, which is the source of energy in ubiquitous cells (26). Pit-1 knockout mice, generated by homologous recombination in embryonic stem cells, died around midgestation (Caverzasio J, personal communication). Thus Pit-1 appears to play an essential role in cell survival and tissue development. On the contrary, continuous Pi overload of non-skeletal tissues could create cellular stress through, for example, the formation of apoptosome with excess amount of ATP (26). In addition, extracellular and intracellular phosphate concentrations could affect glucose metabolism, insulin sensitivity, and oxidative stress in vivo and in vitro, which potentially affect aging processes (15). In the kidney, several reports suggest that proteinuria increases in accordance with the loss of podocytes under pathological conditions (16, 22, 30). We found that type III Na-dependent Pi transporter Pit-1 was expressed on podocytes in WT rats, and its expression on podocytes was enhanced in Pit-1 TG rats. We also showed that primary cultured podocytes from TG rats had higher Pi uptake activity than those from their WT littermates especially under low Pi concentration in the extracellular milieu. These findings suggest the association between podocyte injury and Pi overload on podocytes even under the normal serum Pi level in Pit-1 TG rats. To detect the podocyte injury in TG rats, we next performed immunohistochemical examinations. It has been reported that podocytes upregulate desmin and connexin 43 in response to injury (37, 38). In this study, we showed that desmin-specific staining of the glomerulus from TG rats was already enhanced at 8 wk after birth, as shown in Fig. 6, and advanced at 32 wk (data not shown). In addition, significant...
increases in immunofluorescent dots for connexin 43 were detected along the glomerular capillary wall in TG rats, indicating increased expression of connexin 43 in podocytes (38). Although the exact mechanism of thickening of the GBM is still to be explored, these findings suggest that podocyte injury plays an important role in the development of glomerular damage in Pit-1 TG rats, resulting in the progress of proteinuria. As high phosphate induced apoptosis of other types of cells such as vascular smooth muscle cells (20, 29), we at first hypothesized that Pi overload from the extracellular milieu might induce apoptosis of podocytes, resulting in the damage to the glomerular barrier. However, we could not find an increase in apoptosis in podocytes from TG rats compared with WT rats (data not shown). Another mechanistic hypothesis might be an interaction between Pi and the cytoskeleton, as with the PDZ (postsynaptic density 95, discs large, ZO-1)-domain containing protein, which has been reported to interact with Na-dependent transporter type IIa and IIc (2). However, there is so far no report about a direct inhibitory effect of Pi on the cytoskeleton itself. Further examination should be required to explore the precise mechanism of Pi-induced podocyte injury.

The amount of phosphorus contained in food as a food additive is currently increasing, and a high intake of phosphorus can cause various diseases (3). Systemic Pi concentration is regulated by diet, hormones, pH, and organ function (24). Above all, the kidney plays a major role in maintaining the physiological Pi level of extracellular fluid through the reabsorption of Pi by type IIa and IIc Na-dependent Pi transporters in the proximal tubules (5, 24), and hyperphosphatemia is a common manifestation of end-stage renal diseases (ESRD). Although hyperphosphatemia and the progress of renal failure occur simultaneously, it seems difficult to clarify whether extracellular Pi itself damages renal function under clinical settings. To explore the role of Pi in the damage to the glomerular filtration barrier in the kidney, we next examined the effect of an HP diet on both WT and Pit-1 TG rats. It has been reported that a prolonged HP diet causes downregulation of the type IIa Na-dependent Pi transporter, and urinary and fecal phosphorus excretions were significantly increased in rats fed with an HP diet (35). In this study, we found that there was little difference in renal function between NP diet-fed and HP diet-fed WT rats, suggesting the quick abolishment of Pi overload under physiological condition. The serum Pi concentration level was not different between NP diet- and HP diet-fed TG rats, while serum creatinine levels were higher than those in NP diet-fed TG. These results as a whole suggest that Pi overload of podocytes could induce and/or progress podocyte injury, resulting in damage to the glomerular barrier in the kidney. Further examination, in both basal and clinical studies, would be required to fully clarify the long-term effect of Pi overload on the glomerular barrier system in the kidney, especially in ESRD.

In conclusion, Pit-1 overexpression in rats induces phosphate-dependent podocyte injury and damage to the glomerular barrier, which result in the progress of glomerular sclerosis in the kidney.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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