Hyperphosphatemia in chronic kidney disease exacerbates atherosclerosis via a mannosidases-mediated complex-type conversion of SCAP N-glycans

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Blood phosphate levels are linked to atherosclerotic cardiovascular disease in patients with chronic kidney disease (CKD), but the molecular mechanisms remain unclear. Emerging studies indicate an involvement of hyperphosphatemia in CKD accelerated atherogenesis through disturbed cholesterol homeostasis. Here, we investigated a potential atherogenic role of high phosphate concentrations acting through aberrant activation of sterol regulatory element-binding protein (SREBP) and cleavage-activating protein (SCAP)-SREBP2 signaling in patients with CKD, hyperphosphatemic apolipoprotein E (ApoE) knockout mice, and cultured vascular smooth muscle cells. Hyperphosphatemia correlated positively with increased atherosclerotic cardiovascular disease risk in Chinese patients with CKD and severe atheromatous lesions in the aortas of ApoE knockout mice. Mice arteries had elevated SCAP levels with aberrantly activated SCAP-SREBP2 signaling. Excess phosphate in vitro raised the activity of α-mannosidase, resulting in delayed SCAP degradation through promoting complex-type conversion of SCAP N-glycans. The retention of SCAP enhanced transactivation of SREBP2 and expression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, boosting intracellular cholesterol synthesis. Elevated α-mannosidase II activity was also observed in the aortas of ApoE knockout mice and the radial arteries of patients with uremia and hyperphosphatemia. High phosphate concentration in vitro elevated α-mannosidase II activity in the Golgi, enhanced complex-type conversion of SCAP N-glycans, thereby upregulating intracellular cholesterol synthesis. Thus, our studies explain how hyperphosphatemia independently accelerates atherosclerosis in CKD.


KEYWORDS: α-mannosidase activity; atherosclerosis; HMGCR; hyperphosphatemia; SCAP N-glycans conversion

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Translational Statement

Hyperphosphatemia accelerates atherosclerotic cardiovascular disease, but the underlying mechanisms are still unclear. This paper describes a new mechanism by which hyperphosphatemia increased the activity of α-mannosidase II in the Golgi, which enhanced sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP)–mediated SREBP2 activation in lipid synthesis by modulating SCAP N-glycans. Our work provides a mechanistic explanation for a high incidence of atherosclerotic cardiovascular disease in patients with chronic kidney disease and the underlying mechanisms by which phosphate directly affects vessel lipid homeostasis without changing blood lipid levels. It may also contribute to the design of effective dual therapeutic strategies targeting both phosphate and lipid metabolisms in the chronic kidney disease population.

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of mortality in patients with chronic kidney disease (CKD).¹ Cholesterol blood levels in patients with CKD are usually normal or even low but are associated with extraordinarily high ASCVD risk.² This clinical phenomenon implies that cholesterol metabolism in patients with CKD differs from that of the general population and that nonclassical risk factors may disrupt lipid...
homeostasis in CKD to significantly increase ASCVD by unknown mechanisms.2–5

Phosphate, whose level is tightly regulated in the human body (physiologic range 0.8–1.5 mmol/l), plays vital roles in the regulation of various physiological processes such as membrane integrity, intracellular signaling, and skeletal mineralization.1–8 Hyperphosphatemia accounts for morbidity in 23% of patients with stage 4 CKD and about 70% of patients on dialysis.9 Compelling clinical evidence suggests that hyperphosphatemia contributes to higher atherosclerotic death risk in Western patients with CKD and the general population.10–12 Moreover, feeding apolipoprotein E (apoE) (apoE/-/-) mice a phosphate-rich diet resulted in more severe atherosclerotic lesions independently of vascular calcification, whereas oral phosphate binder could decrease the progression of atherosclerosis in uremic apoE/-/- mice.13–15 Emerging studies indicate the involvement of hyperphosphatemia in atherogenesis in CKD through disturbed cholesterol homeostasis,16,17 but the underlying mechanisms remain largely unknown.

Sterol regulatory element-binding protein 2 (SREBP2), which is synthesized as an immature precursor embedded in the endoplasmic reticulum (ER), principally controls the expression of genes involved in cholesterol uptake and biosynthesis. The stability and maturation of precursor SREBP2 relies on binding to SREBP cleavage-activating protein (SCAP), a sterol sensor.18,19 A small reduction of ER membrane cholesterol alters SCAP conformation, resulting in its detachment from its anchoring protein insulin-induced gene 1 (INSIG1) and translocation of SCAP/SREBP2 complex from ER to Golgi, where SREBP2 is enzymatically cleaved and releases its active NH2 terminal fragment SREBP2-N. SREBP2-N can enter the nucleus to bind with an SRE to activate 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) and low-density lipoprotein receptor (LDlr) gene transcription.20,21 Intracellular cholesterol de novo synthesis is increased via upregulated HMGCR, and native LDL intake is strengthened by upregulated LDLr.

During translocation to the Golgi, asparagine (N)-linked carbohydrates of SCAP are successively modified by α-mannosidase (α-MAN) I, N-acetylglucosamine (GlcNAc) transferase I, and α-MAN II.22 The modifications cause the N-glycans of SCAP to be heterogeneously converted from a format of high-mannose glycans to complex glycans containing multiple branches (defined as “complex-type conversion”). Part of the SCAP protein then recycles back to the ER to form a new complex with another SREBP2, while the remainder is subject to degradation by the ubiquitin proteasome system.23 Glucose deprivation prohibited N-glycosylation of SCAP, which made it more susceptible to degradation and therefore restricted its biofunction in the transactivation of SREBPs.24 Increased biosynthesis of cholesterol mediated by HMGCR contributes to lipid accumulation and foam cell formation in vascular smooth muscle cells (VSMCs).25 SCAP dysfunction has been implicated in atherosclerosis observed in chronic inflammatory status and diabetes.25–29 However, whether increased serum phosphate (P3+ level in patients with CKD affects SCAP posttranslational glycosylation, and whether the modifications contribute to the development of ASCVD remains unclear.

In the present study, we analyzed clinical epidemiology data from a Chinese CKD population, which supported our argument that hyperphosphatemia accelerated ASCVD. We also explored the aberrant activation of SCAP-SREBP2 signaling in the arteries of hyperphosphatemic apoE/-/- mice and uremic patients. We sought to determine how excessive phosphate perturbs SCAP posttranslational glycosylation, causing unregulated intracellular cholesterol synthesis with foam cell formation. These studies provide a novel mechanistic explanation for the accelerated atherosclerosis in the CKD population.

METHODS

A cross-sectional study in Chinese CKD population

A study population including 438 patients with CKD from stage 3 to 5 admitted to the First Affiliated Hospital of Chongqing Medical University from January 1, 2013 to February 28, 2016. Serum P3+ levels were the mean of 3 measurements of fasting morning serum P3+ concentration within 1 month. All the subjects were categorized into 4 serum P3+ ranges as follows: <1.21 mmol/l, 1.22 mmol/l to ~1.46 mmol/l, 1.47 mmol/l to ~1.78 mmol/l, and >1.78 mmol/l, by quartile spacing strategy. Details are described in the Supplementary Methods. The study was approved by the Human Research Ethics Committee at Chongqing Medical University. All subjects were included after providing informed consent.

Study animals

A hyperphosphatemic animal model was established by feeding high-phosphate diet (HPD) as previously published.13 Eight-week-old male apoE/-/- mice were raised with standard chow (n = 15) or HPD (n = 15) (Baitai Hongda Biotec, Jiangsu, China) for 16 weeks. The formulas of the main nutrition gradients were shown in Supplementary Table S1. The post valve aortic root (about 5 mm in length) was used to prepare frozen serial sections. The remaining aortas were stored in −80 °C for mRNA and protein examination. All procedures of the animal study were approved by the Sub委员会 on Animal Care Research at Chongqing Medical University.

Cell culture

The detailed methods of our primary human aortic VSMCs culture were based on our previous work.25 The cells of sixth or seventh passages were used for experiments. Phosphate stocking medium was made of NaH2PO4 and Na2HPO4 (adjusting pH to 7.40).

SCAP protein degradation

The protocols for determining SCAP protein degradation followed previous investigations.25,26

Crude ER isolation and examination of SCAP N-glycans

Treated VSMCs on 4 150-cm2 flasks were harvested to examine the format of SCAP N-glycans following the approach of Nohturfft et al.22
Table 1 | Multivariate logistic regression analysis of the correlation between serum P3⁺ level and ASCVD risk, stratified by serum P3⁺ levels

<table>
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<tr>
<th>Item</th>
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<th>P value</th>
<th>Odds ratio</th>
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<td>Serum P3⁺ levels</td>
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<td>(mmol/l)</td>
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<tr>
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<tr>
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<td>1.057</td>
<td>0.002</td>
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ASCVD, atherosclerotic cardiovascular disease.

Statistics

Statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL). All data were analyzed by 2-tailed Student t test as well as by 1-way analysis of variance as appropriate. P < 0.05 was considered statistically significant.

RESULTS

Hyperphosphatemia correlated with increased ASCVD risk in Chinese patients with CKD

In this study, 438 patients with CKD were divided into atherosclerosis and nonatherosclerosis groups according to the diagnosis of ASCVD; their basic characteristics are described in Supplementary Table S2. Hyperphosphatemia and several other parameters such as maintenance dialysis showed positive correlations with the incidence of ASCVD (Supplementary Table S3). Multivariate logistic stepwise regression analysis suggested that serum P3⁺ levels between 1.47 mmol/l to ~1.78 mmol/l (P = 0.010) and >1.78 mmol/l (P = 0.002) were positively correlated with the incidence of ASCVD. ASCVD risk increased with rising serum P3⁺ level (serum P3⁺ level, 1.47 to ~1.78 mmol/l; odds ratio, 2.348; serum P3⁺ level, >1.78 mmol/l, odds ratio, 2.878) (Table 1).

Hyperphosphatemia disrupted SCAP mediated cholesterol homeostasis in the atherogenesis of apoE⁻/⁻ mice

Four weeks after feeding an HPD, the serum P3⁺ levels of the mice were significantly elevated and sustained until sacrificed (HPD 2.68 mmol/l vs. standard chow 2.14 mmol/l). There were no alterations of other indexes such as total cholesterol (TC) and triglyceride when sacrificed, except for parathyroid hormone, the secondary change of elevated serum P3⁺ levels (Supplementary Table S4). HPD feeding resulted in a more severe atheroma burden relative to standard chow (Figure 1a and b). The serum P3⁺ level correlated positively with the atheroma burden in the aortic root (Figure 1c).

We observed transcription changes of cholesterol metabolism-related genes in aortas but not in livers in HPD-fed mice (liver data not shown; primers are shown in Supplementary Table S5). mRNA levels of HMGCR, LDLr, and SREBP2, but not of INSIG1, SCAP, liver X receptor α (LXRα) or adenosine triphosphate–binding cassette transporter A1 (ABCA1), were increased in HPD-fed mice (Figure 1d). The protein levels of SCAP, HMGCR, and SREBP2-N, but not INSIG1, LDLr, LXRα, and ABCA1 were dramatically elevated in HPD-fed mice relative to the control (Figure 1e and f). Moreover, immunohistochemistry staining further confirmed SCAP protein increment in the aortas of HPD-fed mice (Figure 1g).

Excess phosphate induced translocation of SCAP and cholesterol accumulation in vitro

VSMCs and macrophages are the main sources of atheromatous foam cells. Neutral lipid deposition was increased when VSMCs and THP-1 macrophages were treated with excess phosphate (3.0 mmol/l) (Figure 2a and Supplementary Figure S1A). The TC and cholesterol ester (CE) levels, but not free cholesterol in excess phosphate-treated VSMCs were significantly higher than in control cells (Figure 2b). Excess phosphate increased intracellular TC but not CE or free cholesterol in THP-1 macrophages (Supplementary Figure S1B).

Excess phosphate increased the mRNA levels of HMGCR, LDLr, and SREBP2, but did not increase levels of INSIG1, LXRα, or ABCA1 (Figure 2c and Supplementary Figure S1C; primers are shown in Supplementary Table S6). It did not affect the protein levels of INSIG1, LDLr, LXRα, and ABCA1, but it did increase that of HMGCR and SREBP2-N (Figure 2d and e and Supplementary Figure S1D and E). Excess phosphate did not affect SCAP mRNA expression (Figure 2f and Supplementary Figure S1H) but increased its protein level (Figure 2f and g and Supplementary Figure S1F and G). Excess phosphate enhanced colocalization of SCAP within the Golgi (Figure 2i and Supplementary Figure S1I). VSMCs were more sensitive to excess phosphate than THP-1 macrophages were, so VSMCs were used alone in subsequent experiments. Levels of LDLr mRNA increased 1.35-fold in excess phosphate-treated VSMCs, but its protein expression reached only 1.16-fold of control. This small variation in LDLr protein level did not increase Dil-LDL uptake as shown by fluorescence photography (Figure 2j) and fluorospectrophotometry (Figure 2k). In line with our in vivo findings, we found that excess phosphate increased SCAP protein at the posttranscriptional stage, enhancing SCAP escort for SREBP2 transactivation to upregulate the expression of HMGCR, thus facilitating cholesterol de novo synthesis and intracellular cholesterol accumulation.

Excess phosphate promoted intracellular cholesterol accumulation via SCAP-SREBP2-HMGCR pathway in VSMCs

The mRNA levels of HMGCR and SREBP2 were stably increased when the concentration of phosphate rose from 1.0...
to 5.0 mmol/l without affecting SCAP mRNA levels (Supplementary Figure S2A). HMGCR and SCAP protein increased as the phosphate concentration elevated (Supplementary Figure S2B), suggesting a dose-dependent SCAP response to phosphate signaling. A physiological phosphate concentration (1.0 mmol/l) changed neither SCAP nor HMGCR protein levels over the 72-hour time course, but excess phosphate increased HMGCR and SCAP protein levels dramatically and they remained high after 24 hours (Supplementary Figure S2C).

Sodium-dependent phosphate cotransporter is the main channel for phosphate uptake in VSMCs, to which phosphofructate sodium (PFA) is a specific competitive inhibitor that can dose-dependently block sodium-dependent phosphate
cotransporter. PFA did not reduce HMGCR and SREBP2 mRNA levels with a physiological concentration of phosphate, but PFA inhibited their excess phosphate elevated expressions (Figure 3a and b). PFA did not affect SCAP mRNA levels (Figure 3a), but it overrode the increased SCAP, HMGCR, and SREBP2-N protein levels observed after excess phosphate.
treatment (Figure 3b). Meanwhile, PFA inhibited intracellular TC and CE accumulation induced by excess phosphate, but it did not change their levels within the physiological concentration of phosphate (Figure 3c). These results imply that blocking VSMC phosphate uptake can abrogate the activation of SCAP signaling by excess phosphate.

When knocking down SCAP by short hairpin RNA (shRNA), the mRNA and protein levels of HMGCR and SCAP decreased, even in the presence of excess phosphate (Figure 3g). Excess phosphate increased SCAP protein level but knocking down SREBP2 decreased HMGCR and SREBP2-N in the nucleus (Figure 3h). Moreover, knocking down SREBP2 reduced intracellular TC and CE content and offset the accumulating effects on them of excess phosphate (Figure 3i). These results indicated that excess phosphate specifically promoted cholesterol accumulation in VSMCs via SCAP-SREBP2-HMGCR signaling.

Excess phosphate delayed SCAP degradation by promoting complex-type conversion of its N-glycans

Cycloheximide is an inhibitor for intracellular protein synthesis, which can be used to determine the half-life of

Figure 3 | Excess phosphate (Pi) promoted intracellular cholesterol accumulation via sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP)–SREBP2–3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) pathway in vascular smooth muscle cells (VSMCs). The VSMCs were cultured in experimental medium with or without Pi and indicated treatments for 24 hours. (a,d,g) The gene expression determined by real-time polymerase chain reaction. β-Actin served as the reference gene. Values are means ± SD from 3 experimental repeats (n = 12). (b,e,h) The protein levels examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats. β-Actin served as the reference for cytoplasmic proteins, and lamin A served as the reference for proteins in the nucleus. (c,f,i) Quantification of intracellular free cholesterol (FC) and cholesterol ester (CE). Values are means ± SD of duplicate wells from 3 experimental repeats (n = 12). Statistical significance was assessed using 1-way analysis of variance with Bonferroni or Tamhane T2 as appropriate (a,d,f,g,i,j,l). *P < 0.01. AU, arbitrary unit; Ctrl, control; PFA, phosphoformate sodium; sh, short hairpin; SREBP2-N, NH2 terminal fragment of SREBP2; TC, total cholesterol.
proteins in molecular biology. SCAP protein levels significantly decreased at the 8th hour after cycloheximide treatment and reduced to 22% at the 24th hour compared to that of hour 0 within a physiological phosphate concentration. However, with excess phosphate, the SCAP protein at the 24th hour remained at 48.9% of that at hour 0 (Figure 4a and b), indicating that excess phosphate increased SCAP protein retention by delaying its degradation. GlcNAc (20 mmol/l), which facilitates N-glycosylation, significantly increased mRNA levels of HMGCR and SREBP2, while tunicamycin (Tuni; 1 μg/ml, an inhibitor of N-glycosylation) decreased them even in the presence of excess phosphate (Figure 4c). Neither GlcNAc nor Tuni altered the mRNA level of SCAP (Figure 4d). However, GlcNAc increased SCAP, HMGCR, and SREBP2-N protein levels equivalent to those of excess phosphate, and Tuni decreased them in the same condition (Figure 4e). These data implied that the effects of excess phosphate on SCAP signaling were probably mediated by altering the N-glycans of SCAP.

Figure 4 | Excess phosphate (Pi) delayed sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) degradation by promoting complex-type conversion of its N-glycans. (a,b) The vascular smooth muscle cells (VSMCs) were cultured in experimental medium with 50 μmol/l cycloheximide (CHX) in the absence or presence of Pi for the indicated times. (a) The protein levels were examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats. (b) The densitometric scans of protein bands were processed by ImageJ software (National Institutes of Health, Bethesda, MD). Values are means ± SD from 3 experiments (n = 6). (c–g) The VSMCs were cultured in experimental medium with or without Pi and indicated treatments for 24 hours. (c,d) The gene expression was determined by real-time polymerase chain reaction. β-Actin served as the reference gene. Values are means ± SD from 3 experiments (n = 12). (e) The protein levels examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats. Lamin A served as the reference for proteins in the nucleus. β-Actin served as the reference for cytoplasmic proteins. (f) N-glycans of SCAP were analyzed as described in the Methods section. (g) The colocalization of SCAP with Golgi apparatus was observed by confocal microscopy (arrows). The results are typical of those observed in 4 separate experiments. Bars = 50 μm. Statistical significance was assessed using 1-way analysis of variance with Bonferroni or Tamhane T2 as appropriate (b–d). *P < 0.01; **P < 0.05. AU, arbitrary unit; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; GlcNAc, N-acetylglucosamine; HMGCR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; PNGase, peptide:N-glycosidase; SREBP2-N, NH2 terminal fragment of SREBP2; Tuni, tunicamycin. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 5 | Excess phosphate (Pi) enhanced the activity of \( \alpha \)-mannosidase II isoform A1 (\( \alpha \)-MAN 2A1) and \( \alpha \)-MAN 2A2 in vitro. (a–h,k) The vascular smooth muscle cells (VSMCs) were cultured in experimental medium with or without Pi and indicated treatments for 24 hours. (a,b,c,h) The activity of \( \alpha \)-MAN II determined by the enzymatic assay as described in the Methods section. Values are means ± SD of duplicate wells from 3 experiments (n = 9). (d) Intracellular free cholesterol (FC) and cholesterol ester (CE) assayed by a quantitative method. Values are means ± SD of duplicate wells from 3 experiments (n = 12). (e,g) The gene expression was determined by real-time polymerase chain reaction. \( \beta \)-Actin served as the reference gene. Values are means ± SD of duplicate wells from 3 experiments (n = 12). (f) The protein levels were examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats. \( \beta \)-Actin served as the reference for cytoplasmic proteins. (k) The colocalization of sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) with Golgi apparatus observed by confocal microscopy (arrows). The results are typical of those observed in 4 separate experiments. Bars = 50 μm. (j) The VSMCs were cultured in experimental medium with 50 μmol/l cycloheximide (CHX) in the absence or presence of Pi and transfection of both short hairpin RNA (shRNA) for \( \alpha \)-MAN 2A1 and 2A2 for 16 hours. (i) The SCAP protein levels were examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats. (j) The densitometric scans of SCAP protein bands were processed by ImageJ software (National Institutes of Health, Bethesda, MD). Values are means ± SD of duplicate wells from 3 experiments (n = 6). Statistical significance was assessed using 1-way analysis of variance with Bonferroni or Tamhane T2 as appropriate (a–d,g,j) and a 2-tailed Student t test (e). *P < 0.01; **P < 0.05. AU, arbitrary unit; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; HMGCR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; PFA, phosphoformate sodium; Swain, swainsonine; TC, total cholesterol. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
SCAP contains a glycosylated peptide of 170 amino acids that is protected from proteolysis by trypsin. This trypsin-protected fragment acquires 2 N-linked high-mannose-type glycans that are sensitive to hydrolysis by endoglycosidase H (endo H) when the nascent SCAP was synthesized. The 2 glycans will become endo H–resistant when further modified by α-MAN II in the Golgi, converting to the complex-type glycans. Within the physiological phosphate concentration, N-glycans of SCAP largely remained high-mannose-type, as shown that the apparent mass of the most trypsin-protected fragments became smaller (without glycans) after endo-H digestion, which hydrolyzed their high-mannose-type glycans (Figure 4f, lane 5 vs. lane 4). Excess phosphate converted >90% of SCAP N-glycans to the complex type, because most trypsin-protected fragments are still with glycans after endo H digestion (endo H resistant) (Figure 4f, lane 4 vs. lane 1). Excess phosphate was more efficient than GlcNAc in facilitating the complex-type conversion of SCAP N-glycans (Figure 4f, lane 4 vs. lane 2). Tuni nearly abolished phosphate-induced conversion of SCAP N-glycans from high-mannose-type to the complex-type, as shown by half of the trypsin-protected fragments remaining sensitive to endo H (Figure 4f, lane 5 vs. lane 4), which hydrolyzed them to make the apparent mass of the fragments as small as if N-glycans had been removed, whether or not they have been modified in the Golgi, using endoglycosidase peptide:N-glycosidase F (as a positive control) (Figure 4f, lane 6). Interestingly, we found that both GlcNAc and excess phosphate increased SCAP colocalization with Golgi apparatus (Figure 4g, GlcNAc and excess phosphate vs. control), which was attenuated by Tuni (Figure 4g, Tuni vs. control), even in the presence of excess phosphate (Figure 4g, Tuni vs. excess phosphate + Tuni).

**Excess phosphate enhanced the activity of α-MAN 2A1 and 2A2 in vitro**

With the increase of phosphate concentrations, α-MAN II activity increased (Figure 5a). Interestingly, PFA did not affect α-MAN II activity within physiological phosphate concentrations, but PFA could reverse the elevated enzyme activity by excess phosphate (Figure 5b). Swainsonine is a potent and selective inhibitor of α-MAN II. Excess phosphate significantly increased the activity of α-MAN II, which can be inhibited by swainsonine (2.5 μg/ml) (Figure 5c). This inhibition also reduced TC and CE levels, even in the presence of excess phosphate (Figure 5d).

α-MAN 2A1 and 2A2 are the 2 isoforms of α-MAN II, which are located mainly in the Golgi and manipulate complex-type conversion of SCAP N-glycans. Excess phosphate did not affect either the gene (Figure 5e) or protein (Figure 5f) levels of α-MAN 2A1 and α-MAN 2A2. However, knocking down either α-MAN 2A1 or α-MAN 2A2 with shRNA suppressed both HMGCR and SREBP2 mRNA expression, which can be reversed by excess phosphate (Figure 5g). In the same manner, knocking down either α-MAN 2A1 or α-MAN 2A2 with an shRNA significantly decreased α-MAN II activity, and the effects can be alleviated by excess phosphate (Figure 5h). Knocking down the 2 isoforms simultaneously with both shRNA resulted in accelerated SCAP degradation after cycloheximide treatment, and this effect can be disrupted by excess phosphate (Figure 5i and j). Knocking down the 2 isoforms simultaneously also reduced SCAP colocalization with Golgi, which was reversed by excess phosphate (Figure 5k).

**Hyperphosphatemia enhanced the activity of arterial α-MAN II**

Hyperphosphatemia did not affect the levels of the genes (Figure 6a) and proteins (Figure 6b) of α-MAN 2A1 and α-MAN 2A2, but it significantly increased α-MAN II activity in the aorta of apoE<sup>−/−</sup> mice (Figure 6c). We also detected elevated SCAP protein levels in radial arteries of uremic patients with hyperphosphatemia (with serum phosphate level of 2.43 ± 0.57 mmol/l) by immunohistochemistry staining (Figure 6d). There was increased activity of α-MAN II, when compared with the control group (with serum phosphate level of 1.27 ± 0.15 mmol/l) (Figure 6e).

**DISCUSSION**

In this study, we report for the first time that hyperphosphatemia in Chinese patients with CKD is an independent risk factor for ASCVD and that higher serum P<sup>3+</sup> levels are significantly associated with a higher ASCVD risk, which accords with clinical observations in Western countries. Our present work using a mouse model also revealed evidence that the severity of the aortic atheroma burden paralleled serum P<sup>3+</sup> levels. These results demonstrated a clear link between hyperphosphatemia and ASCVD.

The known mechanisms involved in the pathophysiology of phosphate-induced cardiovascular risk are vascular calcification and endothelial dysfunction, but the potential link between hyperphosphatemia and atherosclerosis is reportedly uncertain. Previous studies by Phan et al. and Ellam et al. both showed that hyperphosphatemia accelerates atherogenesis in apoE<sup>−/−</sup> mice, but the molecular mechanisms remain unclear. Our data obtained in the present study confirmed the proatherosclerotic effect of hyperphosphatemia and showed for the first time that phosphate has a direct proatherogenic role by perturbing VSMC local cholesterol metabolism. We did not detect significant changes in blood lipid levels in our hyperphosphatemic mouse model, but aortic atheroma lesions increased significantly. In line with this observation, the lipid profile was not affected by a HDP in the study from Ellam et al., and sevelamer benefited atherogenesis in uremic apoE<sup>−/−</sup> mice with hyperphosphatemia without impacting circulating cholesterol as in the study from Phan et al. and Nikolov et al. These studies suggest that atherosclerosis induced by hyperphosphatemia is not necessarily dependent on hyperlipidemia and that disturbed cholesterol homeostasis (e.g., increased intracellular cholesterol synthesis) of local cells on the vessel wall could lead to an accumulation of cholesterol in the arteries. In fact, local vascular lipid homeostasis could be
affected by phosphate or other factors (e.g., inflammation) without changes of overall lipid profiles. Modified LDL uptake via CD36 and scavenger receptor A was thought to be the main pathway for atherogenic cholesterol accumulation in artery cells. However, intracellular cholesterol homeostasis is coordinately regulated by cholesterol influx, cholesterol efflux, and de novo cholesterol synthesis. Emerging evidence has highlighted the importance of HMGR-mediated cholesterol biosynthesis in atherogenesis. Excess phosphate significantly increased both mRNA and protein expression of HMGR in VSMCs and macrophages. The elevation of HMGR level was proven to affect LDL uptake by phosphate or other factors (e.g., inflammation) without changes of overall lipid profiles. Modified LDL uptake via CD36 and scavenger receptor A was thought to be the main pathway for atherogenic cholesterol accumulation in artery cells. However, intracellular cholesterol homeostasis is coordinately regulated by cholesterol influx, cholesterol efflux, and de novo cholesterol synthesis. Emerging evidence has highlighted the importance of HMGR-mediated cholesterol biosynthesis in atherogenesis. Excess phosphate significantly increased both mRNA and protein expression of HMGR in VSMCs and macrophages. The elevation of HMGR level was proven to affect LDL uptake by phospholipid transcytosis in the Golgi, its glycan structure is cleaved by α-MAN I in the ER. These high-mannose-type glycans are sensitive to endo H digestion. The glycans can then be catalyzed by GlcNAc transferase I in the medial Golgi, which allows for the generation of glycans branches. Successively, the glycans are converted to multibranched, complex-type glycans by decorations of sialic acid, galactose, and fucose in the trans-Golgi, which is catalyzed by α-MAN II (2A1 and 2A2). They then became resistant to endo H digestion, suggesting that α-MAN II is the key enzyme for the formation of SCAP complex-type glycans.

Excess phosphate raised the activity of α-MAN II, leading to the conversion of most SCAP glycans from the high-mannose-type to the complex-type (Figure 4f, lane 4). Gene silencing of α-MAN 2A1 and α-MAN 2A2 blocked this conversion, which further differentiated the detrimental role of SCAP in atherosclerotic foam cell formation in vivo. Here, blocking phosphate transport by PFA and gene silencing of SCAP or SREBP2 dramatically interrupted the effect of excess phosphate on SCAP/SREBP2 signaling and cholesterol accumulation, indicating that SCAP may act as a key phosphate-responsive protein, which orchestrates cholesterol homeostasis by controlling SREBP2 activation.

The biosynthesis of N-glycans begins when nascent SCAP is translocated into the lumen of the ER. By generating the heterozygote SM22α-Cre:SCAPflox/+; apoE−/− mice, we have previously reported the importance of SCAP in atherosclerotic foam cell formation in vivo. Here, blocking phosphate transport by PFA and gene silencing of SCAP or SREBP2 dramatically interrupted the effect of excess phosphate on SCAP/SREBP2 signaling and cholesterol accumulation, indicating that SCAP may act as a key phosphate-responsive protein, which orchestrates cholesterol homeostasis by controlling SREBP2 activation.

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Figure 6 | Hyperphosphatemia enhanced the activity of α-mannosidase (α-MAN) II in the artery. (a) The mRNA was extracted from the aorta tissue of the hyperphosphatemia mice and control (Ctrl). The gene expression was analyzed by real-time polymerase chain reaction method. β-Actin served as the reference gene. Values are means ± SD from 7 mice of each group. (b) The protein was extracted from the aorta, and the protein levels were examined by Western blotting. The demonstrated bands were typical from 3 experimental repeats (n = 6 mice). β-Actin served as the loading control. (c) The activity of α-MAN II in the aorta tissue of apolipoprotein E−/− mice. Values are means ± SD from 7 mice of each group. (d) Representative photomicrographs of sterol regulatory element-binding protein cleavage-activating protein (SCAP) antibody-stained immunohistochemistry (ICH) sections from the radial artery of uremic patients. Bars = 100 μm. The values of semiquantitative analysis for the positive areas are expressed as the means ± SD from 8 patients at each group. (e) The activity of α-MAN II in the radial artery of uremia patients. Values are means ± SD from 8 to 9 patients of each group. Statistical significance was assessed using a 2-tailed Student t test (a, c, e). *P < 0.01. α-MAN 2A1, α-mannosidase II isoform A1; AU, arbitrary unit; HPD, high-phosphate diet. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
of α-MAN II in raised phosphate conditions, indicating that α-MAN 2A1 and α-MAN 2A2 controlled complex-type conversion of SCAP N-glycans could be key factors for the pathogenesis of foam cell formation. Our findings further revealed that SCAP with complex-type glycans is more resistant to degradation than SCAP with high-mannose-type glycans (combining the results from Figure 4a, b, and f). A previous study demonstrated in glioblastoma cells that pre-venting N-glycosylation by glucose depletion accelerated the proteasome-dependent degradation of SCAP.24 Our study suggested that not only N-glycosylation but the complex-type conversion of N-glycans by the α-MAN II could be decisive for the stability of SCAP. The best model to test the specific role of phosphate on lipid homeostasis is SCAP VSMCs knockout mice. However, SCAP is an essential molecule for the survival of eukaryotes. Both global and VSMC-specific SCAP knockout are lethal for mice. This is one limitation of this study.

Overall, our study demonstrated that excess phosphate enters VSMCs via sodium-dependent phosphate cotransporter and promotes the complex-type conversion of SCAP N-glycans by augmenting the activity of α-MAN II, which increases SCAP half-life and protein levels. As a result, SCAP with complex-type glycans recycle around the ER and Golgi and overactivate SREBP2, leading to robust de novo cholesterol synthesis and robust cholesterol de novo synthesis, and thereby intracellular cholesterol accumulation and foam cell formation (Figure 7).

DISCLOSURE
All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS
CZ, NOY, ZXR, and QH contributed to the design of the study. CZ, NOY, TTZ, and QL conducted the experiments. HG, TTZ, and NOY collected the clinical data. CZ, TTZ, and NOY analyzed data. CZ, ZXR, JFM, and ZV wrote the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary File (Word)

Figure S1. Excess phosphate induced translocation of SCAP and cholesterol accumulation in THP-1 macrophages.

Figure S2. The dose response and time-course effect of excess phosphate on SCAP-SREBP2 signaling.

Table S1. The formula of the main nutrition gradients in normal chow and high- phosphate diet.

Table S2. Comparison of the basic characteristics between AS and non-AS groups.

Table S3. The univariate logistic regression analysis of vascular risk factors between AS and non-AS groups.

Table S4. Basic characteristics of normal chow- and high-phosphate diet-fed apoE−/− mice when sacrificed.

Table S5. Mouse TaqMan primers for real-time PCR.

Table S6. Human TaqMan primers for real-time PCR.

Supplementary References.

REFERENCES