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Mohammed S. Razzaque *Editor*

Phosphate Metabolism

From Physiology to Toxicity

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Preface

I am pleased to present this book, entitled *Phosphate Metabolism: From Physiology to Toxicity*. Phosphate is a widely distributed mineral in the human body. It is an integral component of bone and is also involved in cell signaling, energy metabolism, and nucleic acid synthesis. Phosphate is present in virtually every human system, and its optimal balance is essential for the biological activities of the cells. This book volume contains the works of the authors who are actively involved in research in determining various aspects of phosphate regulation in health and diseases. All chapters present the current state of knowledge, ranging from physiologic regulation of phosphate homeostasis to adverse effects of phosphate toxicity, and discuss future clinical perspectives. The chapters in this book fall broadly into three groups based on their focus: (1) factors regulating phosphate homeostasis, (2) mode of cytotoxic effects of phosphate, and (3) organ damage induced by phosphate toxicity. Despite widening our understanding of in vivo regulation of phosphate, there has not been much in-depth research conducted to determine the underlying mechanisms of organ-specific phosphate toxicity and its long-term cumulative effects on human health. I expect that this book will encourage more interdisciplinary collaboration to enhance our understanding of phosphate toxicity and identify possible interventions to delay or reduce the eventual debilitating health consequences associated with the dietary phosphate burden. My time and efforts of writing, editing, and organizing this book will be worthwhile if the content inspires young physicians and scientists to take on the challenges of finding innovative ways of reducing the amount of phosphate-based preservatives in our processed foods and drinks. As editor, I hope that the broad portfolio of its contents offered to the readership will help foster a more enlightened insight into phosphate regulation in the human body.

I would like to take this opportunity to express my thanks and gratitude to each of the contributors of this book volume for sharing their knowledge and expertise. Finally, I acknowledge my family's kind support and encouragement (Rafi, Yuki, Lisa, Newaz, Zahid, Muhit, Shahed, and my mother, Nilufar Begum) that helped me complete this book.

Erie, PA, USA — Mohammed S. Razzaque

Contents

Phosphate Metabolism: From Physiology to Toxicity

1

Mohammed S. Razzaque

Abstract

Systemic phosphate homeostasis is tightly controlled by the delicate cross-organ talk among intestine, kidney, bone, and parathyroid glands. The endocrine regulation of phosphate homeostasis is primarily mediated by fibroblast growth factor 23 (FGF23), vitamin D, and parathyroid hormone (PTH). Bone-derived FGF23 acts on the proximal tubular epithelial cells of the kidney to partly maintain the homeostatic balance of the phosphate. FGF23, through binding with its cell surface receptors in the presence of klotho, can activate downstream signaling kinases to reduce the functionality of the sodium-phosphate (NaPi) co-transporters of the kidney to influence the systemic phosphate homeostasis. Given the complexity of molecular regulation of phosphate homeostasis, providing information on all aspects of its homeostatic control in a single volume of a book is an overwhelming task. As the Editor, I have organized the chapters that I believe will provide necessary information on the physiologic regulation and pathologic dysregulation of phosphate in health and diseases. Readers will be able to use this volume as a quick reference for updated information on phosphate metabolism without prior acquaintance with the field.

Keywords

FGF23 · Klotho · Vitamin D · PTH · EMT · Cytotoxicity

1.1 Phosphate Homeostasis

Phosphate exerts essential biological functions in humans; around 85% of it is present in the bone [1]. Low phosphate status is linked to musculoskeletal deformities in humans, including rickets or osteomalacia and skeletal myopathy [2–4]. Contrary, high phosphate status is linked to cardiovascular calcification, commonly observed in patients with chronic kidney disease (CKD) on hemodialysis [5–8]. Fibroblast growth factor 23 (FGF23) is the master regulator of systemic phosphate homeostasis [2, 4]. Several other humoral factors, including parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [1,25 $(OH)_2D$], also play crucial roles in fine-tuning phosphate balance [9, 10]. Of relevance, phosphate, $1,25(OH)_2D$, and PTH have feedback regulation on FGF23, while calcium can also induce FGF23 (Fig. 1.1). The osteoblasts and osteocytes mainly produce FGF23; it can specifically bind to the FGF receptors in klotho expressing organs, including kidney and parathyroid glands [11–13]. In the kidney FGF23 suppresses the

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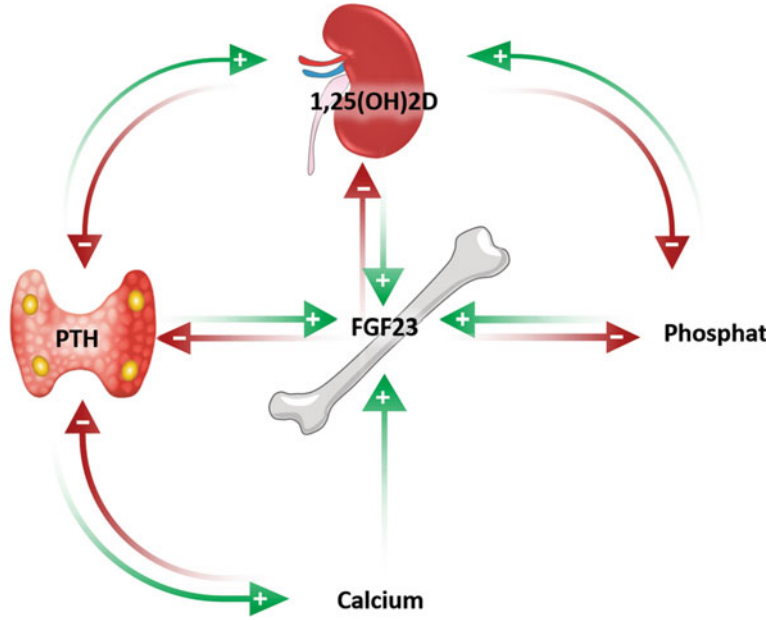

Fig. 1.1 Simplified diagram showing feed-back regulation of FGF23 by phosphate, 1,25(OH)2D, and PTH. Calcium can also positively influence FGF23 synthesis

expression of sodium-phosphate (NaPi) cotransporters (type IIa and type IIc) in the proximal renal tubules to reduce renal phosphate reabsorption [14–19]. FGF23 also reduces the generation of the active form of vitamin D [1,25 $(OH)_2D$] by suppressing the renal expression of 1α-hydroxylase, which converts inactive 25-hydroxyvitamin D [25$(OH)_2D$] to active 1,25 $(OH)_2D$. Multiple steps of vitamin D metabolism are illustrated in our earlier publications [20–25]. Of importance, 1,25$(OH)_2D$ can increase intestinal phosphate absorption. FGF23, therefore, can reduce both intestinal phosphate absorption (indirectly by reducing vitamin D activities) and renal phosphate reabsorption (directly by suppressing NaPi co-transporter activities) to reduce the overall phosphate content of the body, which might reflect as a low serum level of phosphate. Bone cell-derived full-length FGF23 is biologically active [26, 27], while proteases like Furin can cleave the full-length FGF23 into inactive smaller fragments [28]. A phosphate-rich diet and 1,25$(OH)_2D_3$ can increase the serum level of full-length FGF23 in experimental animals [29]. Furthermore, transforming growth factor β (TGFβ), erythropoietin (EPO), inflammation, iron deficiency, and hypoxia are the positive regulator of FGF23, whereas insulin is shown to be a negative regulator [30–33] (Fig. 1.2).

1.2 Book Chapters

The chapters in this book fall broadly into three groups based on their focus: (1) factors regulating phosphate homeostasis, (2) mode of cytotoxic effects of phosphate, and (3) organ damage induced by phosphate toxicity. The organ cross-talk during physiologic and pathologic phosphate regulations by various factors is elaborated in the chapter contributed by Akimbekov et al. [34]. The chapter is adequately illustrated to present simplistic views of the roles and regulation of FGF23, vitamin D, and PTH in controlling phosphate homeostasis [34]. In a follow-up chapter, Nakatani et al. has discussed the effects of FGF23 on vitamin D metabolism and explained how increased serum FGF23 might be an important indicator of adverse clinical outcomes of patients with kidney diseases [35]. Existing evidence suggests that even in individuals with normal renal function, FGF23 plays an important role in vitamin D metabolism; an increased level of FGF23 and a decreased level of vitamin D are

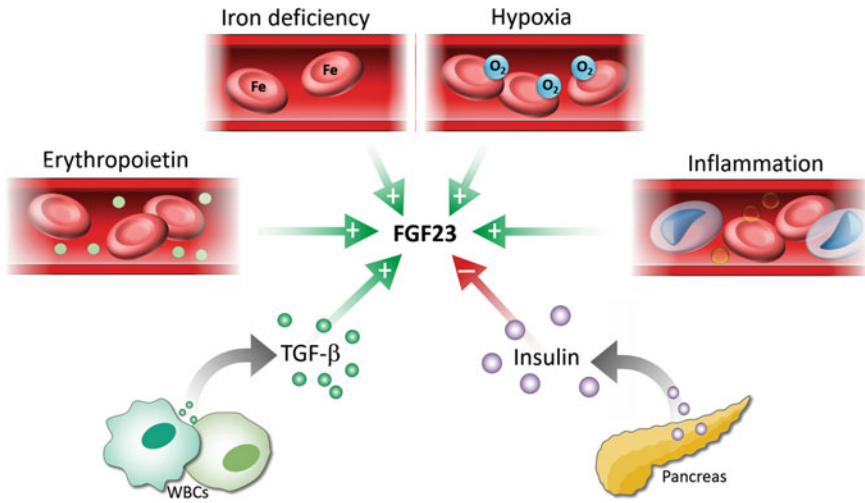

Fig. 1.2 Factors influencing production of FGF23. Transforming growth factor β (TGFβ), erythropoietin (EPO), inflammation, iron deficiency, and hypoxia are the positive regulator of FGF23, whereas insulin is a negative regulator

associated with commonly encountered adverse events in patients with CKD [36].

One of the unresolved areas of phosphate homeostasis is how the body senses the alteration of phosphate status to trigger the release of phosphate-regulatory factors. The chapter contributed by Takashi and Fukumoto detailed about phosphate sensing, and explained how FGFR1c has the potential to be a phosphate sensor [37]. Studies have also proposed PiT1, PiT2, and calcium-sensing receptor (CaSR) as phosphate-sensors, although detail mechanisms are not yet clear [37]. In a separate chapter, Abbasian and colleagues have elucidated how endothelial cells can sense the elevated inorganic phosphate concentration to generate subcellular signals to induce various vascular responses, ranging from angiogenesis to endothelial-mesenchymal transition (EndoMT) [38]. Phosphate toxicity can cause cytotoxicity (cell stress, senescence, apoptosis, and necrosis) and induce epithelial to mesenchymal transition (EMT) [39]. Potential subcellular regulations of phosphate-induced TGF-β-dependent and -independent EMT are elaborated by Lewis and colleagues [40]. Hu and Moe, in their chapter, described the underlying mechanisms of phosphate-induced cellular senescence and detailed the potential roles of klotho and plasminogen activator inhibitor-1 in senescence [41]. The role of phosphate-induced inflammation in tissue /organ damage and tumorigenesis is an evolving area of research and is briefly deliberated in another chapter (Fig. 1.3) [42]. A separate chapter by Michigami et al. discussed how phosphate could generate abnormal cell signaling and oxidative stress to induce cytotoxicity and inflammatory events [43].

Processed foods often contain high phosphate-rich additives that are more readily absorbed into the body than organic phosphate sources. The Food and Drug Administration (FDA) does not mandate food manufacturers or retailers to report per serving phosphate amounts on food labels [44]. A chapter of this book volume is devoted to elaborating the common dietary sources of natural and artificial phosphate-containing foods in the U.S. and Japan [45]. Cardiovascular anomalies are the major consequence of phosphate toxicity [5, 12, 24, 44, 46]. How phosphate can act as one of the cardiovascular toxins, is discussed in the chapter contributed by Leifheit-Nestler et al. [46]. Studies have shown that magnesium-based phosphate binders can reduce phosphate-induced cardiovascular calcification [47–49]. Bruna et al. reviewed how bacteria sense and respond to intracellular changes in phosphate and magnesium concentrations. The

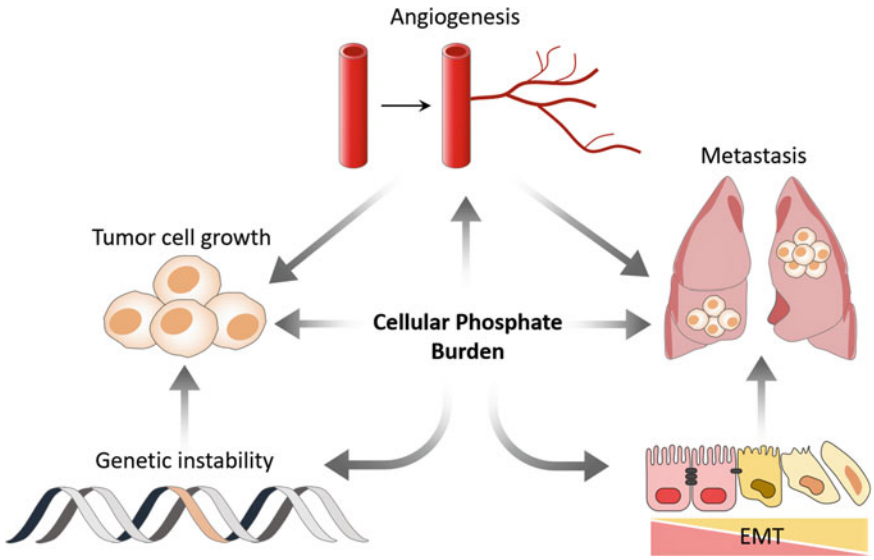

Fig. 1.3 Cellular phosphate burden can stimulate tumorigenesis, possibly by exerting mitogenic effects on tumor cells, promoting angiogenesis, inducing chromosome instability, and facilitating metastasis

authors provided experimental evidence to explain how these two minerals are functionally linked, and that excessive cytoplasmic phosphate microenvironment mimics conditions resulting from insufficient cytoplasmic magnesium and contrariwise [50].

Further understandings into the molecular details of phosphate-medicated cytotoxicity, phenotypic alteration of cell behaviors, and inflammatory events are likely to offer novel therapeutic targets for minimizing phosphate toxicity-induced tissue and organ damages, with far-reaching impacts on tumorigenesis, vasculogenesis, fibrogenesis and neuronal toxicity [51–53].

1.3 Conclusion

All the chapters in this book present the current state of understanding of physiologic and pathologic regulations of phosphate homeostasis. The wide range of topics that are covered will provide the reader with a fundamental understanding of phosphate regulation during health and diseases. The goal is to encourage more interdisciplinary collaboration to enhance our understanding of organ-specific phosphate toxicity and identify possible approaches to reduce the amount of phosphate-based preservatives used in the processed foods and drinks. The sincere expectation will be that among the readers, a few will be encouraged to take up the challenge and reap the rewards for themselves to enhance further understanding of pathomechanisms of phosphate toxicity that will lead to better patient care.

Acknowledgement I want to express my sincere gratitude to Dr. Nuraly Akimbekov (Al-Farabi Kazakh National University, Kazakhstan) for his help in drawing the illustrations. I also wish to thank Dr. Margo Wolfe for reading the manuscript and providing useful suggestions.

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Phosphate Burden and Inflammation

2

Anna Sarah Erem, Satoko Osuka, and Mohammed S. Razzaque

Abstract

Phosphate is an essential macromineral often introduced to the body through dietary intake. The mechanisms for maintaining phosphate levels are tightly controlled via hormonal interactions and excretion via the kidneys. However, western diets consist of high levels of inorganic phosphate, which can overwhelm the regulatory mechanisms in place for maintaining homeostasis. Recent studies have found that phosphate burden can lead to activation of inflammatory signaling in various parts of the body. In addition, individuals with impaired kidney function may also experience exacerbated symptoms of phosphate overload due to decreased filtration and elimination. Many disease states can arise as a result of phosphate burden and subsequent inflammatory signaling, including cardiovascular diseases, tumorigenesis, depression, and neuronal disorders. While the pathophysiological causes of these diseases have been elucidated, there remains a need to address the clinical impacts of excessive dietary phosphate intake and to clarify potential drug candidates that may help alleviate these conditions. This brief chapter looks to explain the overall connection between phosphate burden and inflammation in various diseases.

Keywords

Inflammation · Phosphate burden · FGF23 · Cytokines · IL-1 · Tumorigenesis

2.1 Phosphate in the Human Body

Phosphate was first discovered by Hennig Brand in 1669 using a urine preparation; since then, extensive research has been performed investigating its essential role in living organisms. This mineral is a crucial component in the structure of nucleic acids and phospholipid membranes; in addition, it is involved in several biological processes, including the phosphorylation of proteins, and the formation of cyclic AMP and ATP [19]. Phosphate is a relatively abundant mineral found in the human body, measuring between 500 and 800 grams (g) [13]. The normal plasma concentration of inorganic phosphate in adults is 2.5–4.5 mg/dL; men typically have a

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slightly higher concentration than women [13]. Around 85% of phosphate is found in the bones and teeth, 10–15% in soft tissues, and approximately 1% in blood. Serum phosphate levels, therefore, might not always reflect the total amount and distribution of phosphate. Blood is constantly being filtered by the kidneys, and phosphate is partly reabsorbed there. In individuals with a normal phosphate intake, between 80 and 90% of filtered phosphate is reabsorbed [17].

Fibroblastic growth factor 23 (FGF23), calcitriol, and parathyroid hormone (PTH) levels are responsible for regulating phosphate concentrations in the body by regulating the amount of urinary excretion [31, 32]. Intake of dietary phosphate stimulates PTH secretion which in turn increases bone FGF23 synthesis and release, and is key in triggering the synthesis of calcitriol by the kidneys. FGF23 suppresses both PTH and calcitriol levels, and calcitriol inhibits PTH synthesis and secretion, while stimulating FGF23 release. The regulatory triangle formed between these three substrates creates a fine balance for the maintenance of serum phosphate levels. Since filtration and urinary excretion rely heavily on the kidneys, individuals with reduced kidney function are susceptible to high levels of phosphate in their blood. This disruption in homeostasis can lead to the development of hyperphosphatemia.

Hyperphosphatemia is a condition defined by high plasma concentrations of phosphate (>4.5 mg/dL) [19]. It is often caused by a decrease in renal processing and excretion of phosphate; in other cases, thyroid diseases such as hypoparathyroidism or pseudohypothyroidism may be to blame [19]. While most individuals with hyperphosphatemia are asymptomatic, other conditions, such as hypocalcemia may exacerbate symptoms (e.g., tetany) [13]. Hyperphosphatemia is sometimes associated with concomitant cardiovascular disease in patients with chronic kidney disease (CKD). In those with normal kidney function, elevated phosphate levels have been associated with cardiovascular events and vascular calcification.

2.2 Inflammation Associated with Phosphate Toxicity

Inflammation is a biological process generated in response to pathogens and tissue injury. It is a feed-forward system that, once activated, continues to recruit inflammatory cells and drive inflammation at the cellular level. While it is beneficial in most cases, chronic inflammation can increase the risk of developing diseases such as cancer, CKD, and cardiovascular disease (CVD). Traditionally, identified mediators of inflammation are cytokines and chemokines; however, lipid mediators involving phosphate may also play a role in inflammation, and tumorigenesis [13].

Phosphate is a relatively abundant mineral found in almost all the places in the human body. While it has important physiologic roles in maintaining musculoskeletal functions, it can also become pathogenic when it precipitates with calcium in extra-skeletal systems. This ectopic precipitation can lead to cell damage and initiate inflammatory responses. In healthy individuals, calcium-phosphate crystals are adsorbed by the serum protein fetuin-A; this interaction prevents the crystals from forming large, pathogenic precipitates. However, these formations can also break off into nanoparticles, known as calciprotein particles (CPPs), which circulate in the blood. Over time, serum CPP levels increase due to aging and elevated phosphate. CPPs have been implicated in chronic non-infectious inflammation and vascular stiffness [17].

The kidneys are vital in maintaining phosphate homeostasis, as excess phosphate is excreted from the body in urine. Elevated levels of plasma FGF23 have been linked to expression of inflammatory cytokines (IL-6, TNF-α), C-reactive protein (CRP), fibrinogen, and flares in autoimmune diseases in patients with CKD [39]. Increases in serum phosphate have also been found to promote inflammation in individuals with CKD via induction of inflammatory gene programs in the liver [9]. In rat models of CKD, fluctuations in dietary phosphate intake were found to influence inflammation and vascular calcification [37].

The role of dietary phosphorus in inflammation and oral diseases has also been studied in human diseases. Of relevance, Western diets contain high levels of refined carbohydrates, fat, sodium, and phosphorus, contributing to the growing number of cardiovascular and metabolic diseases. For instance, inorganic phosphate salts are used as a flavor enhancer and preservative [17]. Ingested phosphate is absorbed through the small intestine after it is cleaved by alkaline phosphatases in the cells lining the intestine [17]. Higher phosphorus intake significantly correlated with the presence of gingivitis and elevated levels of pro-inflammatory cytokine IL-1 beta, and inversely correlated with the anti-inflammatory cytokine IL-4 in saliva [12].

As mentioned, phosphate toxicity is associated with the activation of cellular stress response systems and inflammation. Cortisol, which is released by the hypothalamic-pituitary-adrenal axis response to stress and inflammation, is speculated to be associated with phosphate toxicity and depression [6]. In turn, phosphate toxicity may negatively impact adrenal gland function, potentially leading to adrenal insufficiency and increased depression. Furthermore, Alzheimer's disease is associated with hyperphosphorylated tau protein which self-assembles into neurofibrillary tangles, perhaps utilizing excessive amounts of phosphate in the brain and central nervous system (CNS) [6].

2.3 Mechanisms of Phosphate-Induced Inflammation

Exposure to inorganic phosphate activates inflammatory signaling pathways, particularly through activation of the transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NK- κB) [45]. This has been studied extensively in vascular smooth muscle cells (VSMCs) in regard to inflammatory signaling and its effects on calcification in cardiovascular disease. NF-κB affects multiple signaling pathways to mediate inflammatory signaling and calcification. For example, it inhibits IκB kinase (IKK), which is known to have an anti-calcific effect [1, 41]. NF-κB can also be activated by the pro-inflammatory mediator TNF and TNF-related weak inducer of apoptosis (TWEAK) [16]. VSMCs that have been exposed to high levels of inorganic phosphate *in vitro* have been shown to release the inflammatory cytokine IL-6 [44]. IL-6 induces expression of CBFA1 through activation of the transcription factor STAT3 [18]. In addition, IL-6 plays a role in the inorganic phosphate-induced senescence of VSMCs. This is linked to oxidative stress, which activates a DNA damage response and can further induce inflammation [10, 43]. Exposure to inorganic phosphate can also result in upregulation of IL-1β and the NALP3 inflammasome, which can promote senescence [15, 42].

2.4 Phosphate Toxicity and Cardiovascular Disease

Phosphate toxicity is associated with an increased risk of cardiovascular disease [11, 28, 34]. This includes heart failure, chronic obstructive pulmonary disease (COPD), and cardiovascular mortality caused by stroke or noncoronary heart diseases [39]. While it has been shown that patients with CKD are more susceptible to CVD, circulating excess phosphate can also play a role in vascular calcification and cardiovascular events in individuals with normal kidney function [8, 39].

Research now shows that the connection between hyperphosphatemia and cardiovascular events is linked to pro-inflammatory signaling and effects [3, 38, 40]. Circulating phosphate levels can be associated with inflammatory mediators such as IL-6 and CRP [39]. Phosphate exposure activates pro-inflammatory cellular signaling via the expression of the transcription factor NF-κB in VSMCs. In VMSCs, exposure to phosphate can initiate oxidative stress; however, when phosphate burden is pharmacologically blocked, VSMC-calcification is inhibited

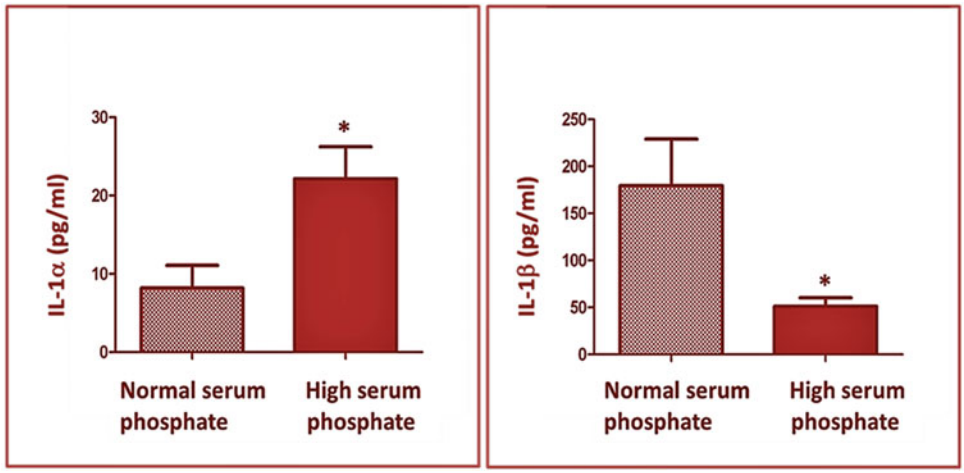

Fig. 2.1 Serum IL-1α and IL-1β levels in hyperphosphatemic klotho knockout mice (n = 5) and age-matched (6-weeks-old) normophosphatemic wild-type mice (n = 4). Note that compared to control wild-type mice (with normal serum phosphate), klotho knockout mice (with high serum phosphate) show elevated IL-1α and reduced IL-1β levels (*: $p < 0.05$)

[2, 20]. Oxidative stress causes DNA damage, which then activates a DNA damage response. This process can lead to more inflammation and vascular calcification [36].

In CKD patients, high phosphate levels are associated with cardiovascular disease. Multiple *in vitro* studies have investigated the effects of phosphate concentrations on human aortic smooth muscle cells. Exposure to phosphate induces the expression of pro-inflammatory mediators such as interleukins (IL-1β, IL-6, IL-8), and TNF-α [21, 44, 46]. In addition, an increase in reactive oxygen/nitrogen species (ROS/RNS) production, can be detected, further indicating an inflammatory response [21]. Animal models of CKD have also demonstrated that high-phosphate diets induce inflammation both locally in the arteries and systemically, which may also play a role in the pathogenesis of the vascular calcification associated with hyperphosphatemia [29]. It is worth noting, klotho knockout mice are a well-studied model for phosphate toxicity that can lead to cardiovascular calcification and premature aging [22–26, 33, 35]. Dysregulated inflammatory cytokines were detected in serum collected from hyperphosphatemic klotho knockout mice compared to their age-matched normophosphatemic wild-type mice (Fig. 2.1). Such molecular signature of inflammation in klotho knockout mice with phosphate toxicity was associated with significantly reduced longevity. More importantly, reducing phosphate toxicity in klotho knockout mice could reduce cardiovascular calcification and increase longevity [23, 27].

2.5 Phosphate Toxicity and Tumorigenesis

Phosphate toxicity leading to cellular phosphate burden has been identified as a cause of cancer cell growth. Tumor cells are unable to regulate phosphate homeostasis due to their higher expression of phosphate cotransporters and ability to store more phosphate than healthy cells. Animal models have demonstrated that high dietary intake of phosphate leads to the growth of skin and lung tumors [39].

A potential interaction between the excess of blood vessels supplying a tumor (inflammatory hyperemia) and hyperphosphatemia has been speculated in the formation and progress of cancer [5]. Hyperemia increases the blood flow rate and volume to tumors. This combined with the

inflammatory nature of hyperphosphatemia leads to an increase in inorganic phosphate circulating within the tumor microenvironment [5]. Elevated intracellular phosphorus levels may trigger the synthesis of ribosomal RNA (rRNA), which leads to increased protein synthesis, driving tumor growth [5]. Research has also been performed investigating the role of sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) in inflammation and cancer [7, 14]. These pro-inflammatory metabolites have been found to participate in and promote tumorigenesis [4, 30].

2.6 Conclusions

Phosphate toxicity can induce a wide range of tissue injuries, including cardiovascular damage and premature aging (Fig. 2.2) [26, 27]. Recent investigations have highlighted the inflammatory effects of excess phosphate and various disease states. An elevated level of circulating phosphate is partly the result of an increase in dietary phosphate intake, insufficient kidney function, and abnormal FGF23 and PTH levels. Combined, these factors can lead to tumorigenesis, cardiovascular events, and mental health conditions. These facts raise the point that phosphate intake should be monitored, particularly in susceptible groups such as those with chronic kidney disease. Further work is needed in elucidating the exact mechanisms and treatments available to prevent inflammation from worsening any of these conditions.

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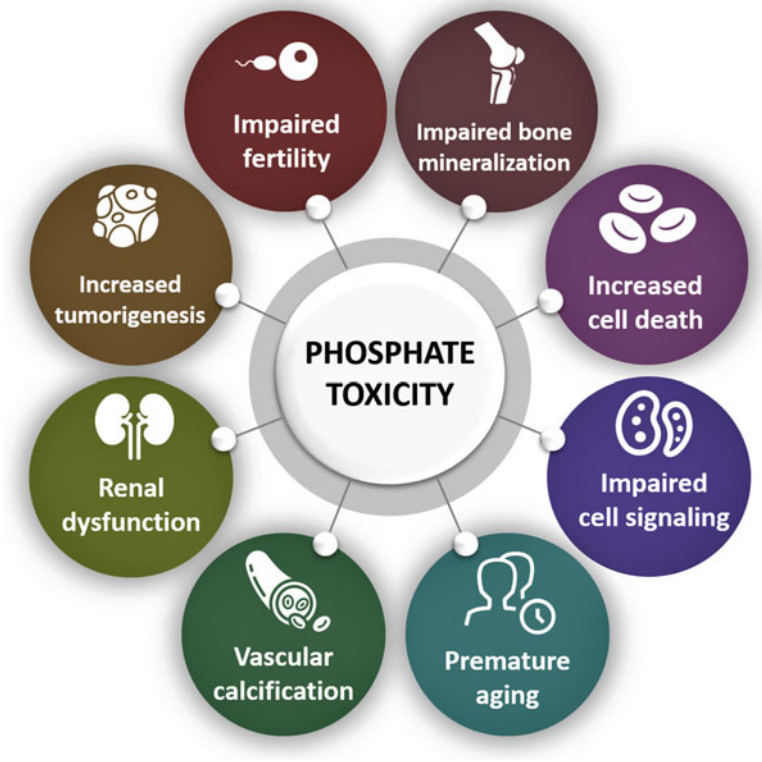

Fig. 2.2 Partial list of various pathologies induced by phosphate toxicity [34]

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Extracellular Phosphate, Inflammation and Cytotoxicity

3

Toshimi Michigami, Miwa Yamazaki, and Mohammed S. Razzaque

Abstract

Phosphorus is an essential nutrient that plays a crucial role in various biological processes, including cell membrane integrity, synthesis of nucleic acids, energy metabolism, intracellular signaling, and hard tissue mineralization. Therefore, the control of phosphorus balance is critical in all living organisms, and the fibroblast growth factor 23 (FGF23)-αKlotho system is central to maintain phosphate homeostasis in mammals. Although phosphate is indispensable for basic cellular functions, its excessive retention is toxic and can affect almost all organ systems' functionality. In human patients, hyperphosphatemia has been implicated in an increase in morbidity and mortality. Also, mouse models with hyperphosphatemia generated by disruption of the FGF23-αKlotho system exhibit extensive tissue damage, premature aging, and a short lifespan. Experimental studies using cell and animal models suggest that cytotoxic and inflammatory effects of elevated phosphate are partly mediated by abnormal cell signaling and oxidative stress. This review provides an overview of our current understanding regarding the toxicity of phosphate.

Keywords

Phosphate toxicity · Fibroblast growth factor 23 (FGF23) · αKlotho · Phosphate-induced signaling · Oxidative stress

3.1 Introduction

Phosphorus is an essential nutrient for all organisms, being involved in various biological processes that include cellular membrane composition, synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), intracellular signaling and energy metabolism, as well as skeletal mineralization in vertebrates. Phosphorus ingested through food usually exists as both organic and inorganic forms of phosphate in the body. In human adults, ~90% of the total phosphorus is distributed in the bone as hydroxyapatite (calcium-phosphate) crystals, and the remainder is mostly present in soft tissues. The extracellular fluid contains <1% of phosphorus [54, 55]. Phosphorus in serum exists mostly as inorganic phosphate (Pi) in the form of free ions HPO_4^{2-} and $H_2PO_4^{-}$, and HPO_4^{2-} is dominant in physiological pH. Intracellular phosphate mostly exists as a bound form or inorganic

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phosphate esters, phospholipids in the cell membrane, or phosphorylated intermediate molecules involved in various biochemical processes, which include the generation, storage, and transport of cellular energy through the formation of adenosine 5′-triphosphate (ATP) by oxidative phosphorylation [54, 55].

Mammalian cells take up Pi from extracellular fluid mainly through membrane transporters that function dependently on sodium (Na^+) gradient across the plasma membrane. These Na^+-dependent Pi transporters (Na^+/Pi cotransporters) have been classified into three families in mammals [87]. Type I transporter that belongs to the solute carrier family 17 (SLC17) is involved in the transport of organic ions in addition to Pi, and human type 1 transporter NPT1 has been suggested to function as a urate transporter [16]. Among type II transporters, type IIa and IIc encoded by the *SLC34A1* and *SLC34A3* genes in human, respectively, are predominantly expressed in brush border membrane of proximal tubules in the kidney and responsible for renal reabsorption of Pi, while type IIb transporter encoded by *SLC34A2* is expressed in various tissues and accountable for active transcellular absorption of Pi in the small intestine. Type III Na^+/Pi cotransporters PiT1 and PiT2 encoded by *SLC20A1* and SLC20A2, respectively, are widely expressed with different expression patterns, and their main function is likely to supply Pi to individual cells [32, 87].

Renal reabsorption and intestinal absorption of Pi are critical for Pi homeostasis in mammals. Renal Pi reabsorption by type IIa and IIc Na^+/Pi cotransporters (NaPi2a and NaPi2c) is suppressed by several humoral factors, including parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), which increases urinary Pi excretion [66, 67]. Intestinal Pi absorption by type IIb Na^+/Pi cotransporter (NaPi2b) is increased by both 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] and low dietary phosphate [30]. It was reported that mice deficient for NaPi2b absorbed approximately 50% less phosphate than wild-type animals, confirming the major contribution of this transporter in Pi homeostasis [76]. However, in humans, the daily need for phosphate is covered by intestinal absorption from ingested food, so serum Pi level is virtually maintained by renal phosphate excretion.

3.2 FGF23-αKlotho System and Phosphate Metabolism

Mounting evidence has established the central roles of FGF23 in Pi metabolism. FGF23 is a secreted protein of 32 kDa produced by bone and exerts its effects on the distant target organs, including the kidney, in an endocrine fashion. In the kidney, FGF23 increases Pi excretion by suppressing the expression of NaPi2a and NaPi2c. Also, FGF23 decreases the production of 1,25$(OH)_2$D by suppressing the expression of 25-hydroxyvitamin D-1α-hydroxylase and inducing that of 25-hydroxyvitamin D-24-hydroxylase [55, 66, 67]. To evoke its signal through FGF receptor (FGFR), FGF23 requires αKlotho [46, 86]. αKlotho is a 130-kDa type I membrane protein, which was initially identified as an aging-related factor [45]. In addition to the transmembrane form, soluble form of αKlotho is also detectable in serum and CSF, although its physiological roles are not clarified yet [37, 93, 94]. Because of the predominant expression of a transmembrane form of αKlotho in the kidney, parathyroid gland, and the choroid plexus, these organs have been considered to be the physiological targets for FGF23 action [45]. In the parathyroid gland, it has been shown that FGF23 suppresses the gene expression and secretion of PTH [8]. In addition, we previously reported that the placenta also expresses αKlotho and that a high level of maternal FGF23 up-regulates the placental expression of the *Cyp24a1* gene encoding 25-hydroxyvitamin D-24-hydroxylase and affects fetal vitamin D metabolism in a mouse model of human X-linked hypophosphatemic rickets [61, 62].

Disruption of FGF23-αKlotho system by loss-of-function mutations in FGF23, αKlotho, or GalNAc-transferase 3 that is an enzyme responsible for *O*-glycosylation of FGF23, causes hyperphosphatemic familial tumoral calcinosis [3, 35, 36]. Similarly, mice deficient for *Fgf23*

or *αKlotho* also exhibit hyperphosphatemia and increased level of $1,25(OH)_2D$ [70, 77]. Thus, FGF23-αKlotho system is central in Pi and vitamin D metabolism, and its impairment leads to excessive retention of Pi in the body.

On the other hand, the excess action of FGF23 has been implicated in the pathogenesis of various hypophosphatemic diseases, including some heritable rickets/osteomalacia [26]. Mutations in FGF23 that make the protein resistant to cleavage are responsible for autosomal dominant hypophosphatemic rickets (ADHR), which is characterized by renal Pi wasting, hypophosphatemia, and inappropriately low levels of serum $1,25(OH)_2D$ [2]. In addition, loss-of-function mutations in several genes such as the p*hosphate-regulating gene homologous to endopeptidases on X chromosome* (*PHEX*), *dentin matrix protein 1* (*DMP1*) and *family with sequence similarity 20, member C* (*FAM20C*) cause increased levels of FGF23 and hypophosphatemic rickets [25, 34, 49, 84], and overproduction of FGF23 by tumors also causes hypophosphatemic osteomalacia [78]. Recently, Burosumab, a monoclonal antibody against FGF23, has been developed as a new drug to treat FGF23-related hypophosphatemic rickets/osteomalacia [13, 26].

Although the physiological actions of FGF23 require αKlotho, recent studies have suggested that massively elevated levels of circulating FGF23 as found in patients with chronic kidney disease (CKD) might result in pathological changes in cells and tissues lacking αKlotho [74].

3.3 Phosphate and Morbidity and Mortality

Although phosphorus is an essential nutrient for all living organisms, excessive retention of Pi in the body is toxic and causes various cellular and tissue injuries [24, 38, 68, 69]. Mice deficient for *Fgf23* or *αKlotho*, which exhibit markedly elevated levels of serum Pi and $1,25(OH)_2D$, suffer from premature aging, vascular calcification, and early mortality. They also manifest hypogonadism, infertility, emphysema, and generalized tissue atrophy [45, 56]. Interestingly, it was shown that a low-phosphate diet corrected hyperphosphatemia, prevented vascular calcification, and prolonged survival in *Fgf23*-deficient mice, despite the persistent elevation in $1,25(OH)_2D$ levels [82]. Moreover, genetic inactivation of NaPi2a in *αKlotho*-deficient mice restored severe hyperphosphatemia and reduced vascular and soft tissue calcification, even in the presence of extremely high levels of serum calcium and $1,25(OH)_2D$. These mice lacking both *NaPi2a* and *αKlotho* also recovered body weight, regained reproductive ability, reduced their tissue atrophy, and exhibited the longer survival, compared to the mice deficient for *αKlotho* alone [63, 64]. However, when fed with a high-phosphate diet, the *NaPi2a/αKlotho* double-deficient mice again suffered from premature aging and shortened lifespan [64]. These observations in mouse models clearly indicate that excessive Pi can be toxic to multiple organs and accelerates the aging process.

Human studies also have suggested the toxic effects of Pi. In CKD (CKD), elevated serum Pi has been implicated as a risk factor for cardiovascular diseases and higher mortality in both dialysis and non-dialysis patients [9, 23, 27, 43, 65, 81]. Hyperphosphatemia and elevated calcium-phosphate product levels are associated with calcification of soft tissues and blood vessel walls in CKD patients and are predictive of high morbidity and mortality [20, 27]. In addition to the passive precipitation of calcium-phosphate in soft tissues, substantial evidence suggests that high extracellular Pi induces the expression of osteoblastic genes in vascular smooth muscle cells, which contributes to calcification [40]. It has also been reported that higher serum Pi is associated with high mortality even in individuals with preserved renal function. Tonelli, et al. performed a post hoc analysis of the data from the Cholesterol And Recurrent Events (CARE) study and found a graded independent relation between higher serum Pi and the risk of death and cardiovascular events in 4127 subjects, most of whom had serum Pi levels within the normal range [85]. Li, et al. performed a meta-analysis study using 24 clinical trials with a total of

147,634 patients without CKD and revealed a positive association between serum Pi level and mortality [47]. A prospective study demonstrated that a higher serum Pi predicted mortality in renal transplantation recipients [18].

Phosphate overload can also be associated with a diet rich in phosphate additives and treatment with phosphate-containing laxatives or enema [7]. It was reported that the high-phosphate diet containing much food additives increased the serum and urinary Pi levels as well as the excretion of hydroxyproline and cyclic adenosine monophosphate (cAMP), while decreased serum and urinary calcium levels [7]. A randomized controlled trial demonstrated that avoiding phosphate-containing food additives resulted in a modest improvement in hyperphosphatemia among patients with end-stage renal disease [83]. The administration of phosphate-containing enemas also caused serum Pi elevation and decreased serum calcium [29]. Several case reports describe the complications such as tetany, hypocalcemic coma, and brain damage, induced by administration of phosphate-containing enema [22, 50, 80].

3.4 Phosphate and Pathological Calcification

Pathological calcification is one of the primary mechanisms for harmful effects induced by phosphate overload. Hyperphosphatemia and the increased calcium-phosphate products may facilitate the ectopic calcification of soft tissue and blood vessels [72, 73]. In addition to the accelerated formation of calcium-phosphate crystals, *in vitro* studies using cultured vascular smooth muscle cells (VSMCs) demonstrated that elevated extracellular Pi caused osteoblastic transdifferentiation [17, 40]. The treatment of VSMCs with high Pi induced the expression of *RUNX2*, a master transcription factor required for osteoblastic differentiation, and *BGLAP* encoding osteocalcin, an osteoblast-specific matrix protein [40]. Since it was reported that ablation of Runx2 prevented vascular calcification in mice [48], the Pi-induced up-regulation of Runx2 might substantially contribute to the ectopic calcification caused by phosphate overload.

3.5 Phosphate and Inflammation

Inflammation is a common feature of advanced renal disease [60]. It was reported that serum Pi and calcium-phosphate product levels directly correlated with serum levels of C-reactive protein (CRP) and interleukin-6 (IL-6) in 133 patients with CKD not on dialysis and not receiving calcium supplements, phosphate binders, or vitamin D, whereas HDL-cholesterol and estimated glomerular filtration rate (eGFR) inversely correlated with the levels of inflammatory state [57]. The authors of this study suggested that serum Pi was an independent risk factor for the presence of an inflammatory state, based on their logistic regression analysis. In an animal study, Yamada, et al. reported that dietary phosphate overload resulted in an increase in serum and tissue levels of TNF-α and developed malnutrition in adenine-induced CKD rat model [91]. Emerging evidence has suggested that pro-inflammatory response might mediate the development of phosphate overload-related vascular calcification [88].

Duchenne muscular dystrophy is a lethal inherited disease caused by dystrophin deficiency and characterized by progressive muscle degeneration, increased macrophage infiltration, and ectopic calcification. It was reported that dietary phosphorus overload dramatically aggravated the dystrophic phenotype in the dystrophin-deficient *mdx* mouse, a model of Duchenne muscular dystrophy, by increasing the number of necrotic muscle fibers and the degree of inflammation associated with infiltration of M1 macrophages [89]. More recently, a human study has demonstrated that dietary phosphorus enhances inflammatory response in gingivitis [28]. Thus, phosphate overload may induce inflammation in various conditions.

3.6 Phosphate-Induced Signaling and Cytotoxicity

Although the underlying mechanisms are still not fully understood, the direct inflammatory and cytotoxic effects of extracellular Pi are likely to be involved. It has been shown that elevated extracellular Pi itself triggers signaling to regulate gene expression and cellular functions in some cell types [6, 41, 44, 55, 93, 94]. Especially, Pi-induced signaling has been extensively investigated in bone cells. In an osteoblastic cell line MC3T3-E1, elevated extracellular Pi induced the expression of several genes, including that for osteopontin [4–6, 19, 59, 71]. In early chondrocytes of proliferating stage, extracellular Pi up-regulated the *cyclin D1* expression [44]. In mature chondrocytes, elevated Pi induced the expression of matrix Gla protein (MGP) that is related to mineralization [41]. These studies revealed that gene regulation by the elevated extracellular Pi involved the type III Na^+/Pi cotransporter Pit-1 and the activation of MEK/ERK pathway [4, 41, 44, 55]. It is also reported that signaling pathways activated by elevated Pi include FGF receptor signaling, G-protein signaling, N-ras signaling [11] and Akt/mTORC signaling [31, 42]. In addition to the effects on gene expression, elevated extracellular Pi facilitates apoptosis in terminally differentiated chondrocytes [51, 52, 75]. Thus, extracellular Pi is physiologically an important regulator of proliferation, differentiation and function of bone cells. However, as mentioned above, a pathologically elevated concentration of extracellular Pi induced the expression of several osteoblast-specific genes such as *Runx2* and *osteocalcin* in vascular smooth muscle cells in a Pit-1-dependent manner, leading to a phenotypical change to predispose calcification [40]. By expressing wild-type or various mutant Pit-1 proteins in Pit-1-deficient vascular smooth muscle cells, Chavkin, et al. demonstrated that both Pi-uptake-dependent and –independent functions of Pit-1 are involved in vascular calcification [14].

As described above, an elevation in extracellular Pi also triggers a signal for apoptosis in hypertrophic mature chondrocytes [52, 75]. In pathological conditions, elevated levels of extracellular Pi may accelerate apoptosis in other cell types as well. Indeed, the apoptosis rate was increased in various organs in the hyperphosphatemic *αKlotho*-deficient mice, which was restored in the *NaPi2a/αKlotho* double-deficient mice with lowered serum Pi [64]. ISO-HAS is an endothelial cell line originally established from human hemangiosarcoma [53]. Using this cell line (obtained from Cell Resource Center for Biomedical Research Institute for Development, Aging and Cancer, Tohoku University, Japan), elevated Pi-induced apoptosis was associated with the cleavage of caspase-3 (Fig. 3.1).

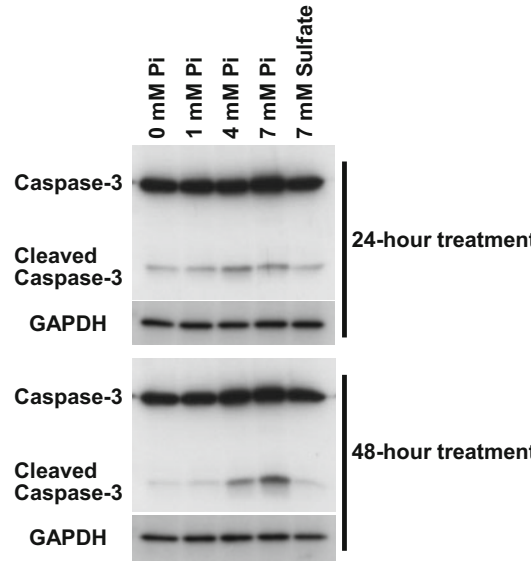

Fig. 3.1 Increased extracellular phosphate (Pi) accelerated the activation of caspase-3, a crucial mediator of apoptosis, in an endothelial cell line, ISO-HAS. The cells were starved in the Pi-free medium for 24 h and then treated for 24 or 48 h with the indicated concentration of Pi or sulfate as a negative control. Media containing Pi or sulfate were prepared by addition of sodium salts to Pi-free medium. The experiments were performed in the presence of 0.1% fetal bovine serum, which provided additional 0.01 mM Pi. Whole cell lysates were harvested and subjected to Western blotting using antibodies against caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Treatment with high Pi caused an increase in the amount of cleaved caspase-3, suggesting apoptosis

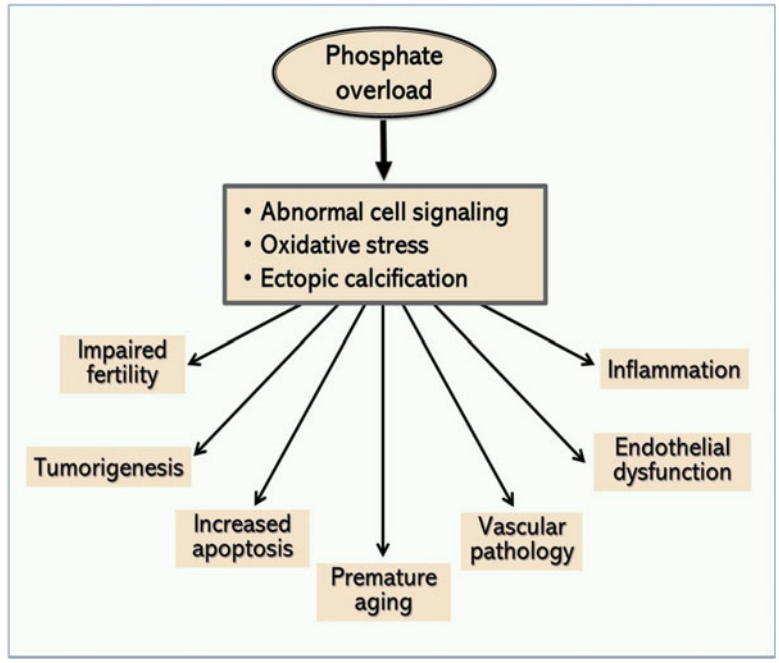

Fig. 3.2 Simplified diagram illustrating various toxic effects of the elevated extracellular phosphate

A recent study using HEK293 cells and HeLa cells suggested that abnormally elevated extracellular Pi might rewire interwoven network of signaling pathways such as Akt pathway, ERK pathway and JNK pathway, leading to a wide variety of cytotoxic effects including abberant proliferation, endoplasmic reticulum (ER) stress, epithelial-mesenchymal transition (EMT) and cell death [31] (Fig. 3.2).

3.7 Phosphate and Oxidative Stress

A line of evidence has revealed the involvement of oxidative stress in the harmful effects by elevated extracellular Pi. Zhao, et al. reported that high extracellular Pi induced the generation of mitochondrial reactive oxygen species (ROS), which promoted the nuclear translocation of nuclear factor κB p65 and accelerated the osteogenic transdifferentiation of the cultured smooth muscle cells [95]. Nguyen, et al. demonstrated that high extracellular Pi induced defective insulin secretion and cytotoxicity in pancreatic β-cells, which was also mediated by oxidative stress and hyperpolarization of mitochondria [58]. It has been demonstrated that oxidative-stress induces DNA damage and senescence in human fibroblasts [15]. In *αklotho*-deficient mice characterized by tissue atrophy and premature aging, urinary 8-hydeoxy-2′-deoxyguanosine (8-OHdG), a marker for oxidative stress-induced DNA damage, is increased [42, 92]. In brown adipose tissue of *αklotho*-KO mice, we demonstrated that hyperphosphatemia-induced activation of Akt/mTORC1 signaling pathway might be involved in suppressing antioxidant genes and oxidative damage [42], thus, the cytotoxic effects of Pi overload appear to be partly mediated by increased oxidative stress. It has also been reported that elevated Pi induces autophagy, which counteracts vascular calcification by reducing matrix vesicle release [21].

It is reported that hyperphosphatemia impairs endothelial cell function [79]. Dialysis patients have an increased concentration of circulating procoagulant endothelial microparticles, contributing to cardiovascular occlusive events [10]. Abbasian, et al. reported that treatment of cultured human endothelial cells with elevated extracellular Pi led to a rise in intracellular Pi

concentration and a marked increase in cellular tropomyosin-3, plasma membrane blebbing, and release of microparticles. This effect of Pi was suggested to be independent of oxidative stress or apoptosis [1].

3.8 Effects of Phosphate on Tumorigenesis

The toxicity of phosphate is also implicated in tumorigenesis. It was shown that a high Pi diet increased the development of lung and skin cancers in animal models [12, 39]. In a mouse model of lung cancer, high dietary Pi stimulated pulmonary Akt activity and increased lung tumorigenesis [39], and knockdown of type IIb Na^+/Pi co-transporter in the lung suppressed the lung tumorigenesis [33]. In humans, a population-based prospective study investigated the relationship between serum Pi and risk of cancer in human, and showed a positive link between Pi quartiles and the risk of cancer of the pancreas, lung, thyroid gland, and bone in men, and cancer of the esophagus, lung, and nonmelanoma skin cancer in women [90]. Interestingly, the risks of breast, endometrial and other endocrine cancers were lower in the population of higher Pi levels in both men and women, probably due to the effects of hormonal factors [90].

3.9 Conclusions

Although phosphate is an essential nutrient involved in various biological processes, its overload in the body can be toxic and cause damage to multiple organs. The pathological effects of Pi include premature aging, calcification and blood vessels and soft tissues, impaired fertility, increased inflammation, accelerated cell death, and tumorigenesis, leading to a short lifespan. Abnormal cell signaling and oxidative stress have been implicated in the direct cytotoxicity of the elevated extracellular Pi, although the underlying molecular mechanisms are still not fully understood. Excess phosphate retention can be associated with various conditions, including renal dysfunction, administration of phosphate-containing laxatives and enemas, and ingestion of diet rich in phosphate food additives. Since phosphate overload can induce severe medical complications, phosphate balance in the diet should be considered for health promotion.

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Phosphate-Sensing

4

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Abstract

The blood level of phosphate is tightly regulated in a narrow range. Hyperphosphatemia and hypophosphatemia both lead to the development of diseases, such as hyperphosphatemic tumoral calcinosis and rickets/osteomalacia, respectively. Although several humoral factors have been known to affect blood phosphate levels, fibroblast growth factor 23 (FGF23) is the principal hormone involved in the regulation of blood phosphate. This hormone is produced by bone, particularly by osteocytes and osteoblasts, and has the effect of lowering the blood level of phosphate in the renal proximal tubules. Therefore, some phosphate-sensing mechanism should exist, at least in the bone. However, the mechanisms through which bone senses changes in the blood level of phosphate, and through which the bone regulates FGF23 production remain to be fully elucidated. Our recent findings demonstrate that high extracellular phosphate phosphorylates FGF receptor 1c (FGFR1c). Its downstream extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling pathway regulates the expression of several transcription factors and the *GALNT3* gene, which encodes GalNAc-T3, which plays a role in the regulation of post-translational modification of FGF23 protein, which in turn enhances FGF23 production. The FGFR1c-*GALNT3* gene axis is considered to be the most important mechanism for regulating the production of FGF23 in bone in the response to a high phosphate diet. Thus—in the regulation of FGF23 production and blood phosphate levels—FGFR1c may be considered to function as a phosphate-sensing molecule. A feedback mechanism, in which FGFR1c and FGF23 are involved, is present in blood phosphate regulation. In addition, other reports indicate that PiT1 and PiT2 (type III sodium-phosphate cotransporters), and calcium-sensing receptor are also involved in the phosphate-sensing mechanism. In the present chapter, we summarize new insights on phosphate-sensing mechanisms.

Keywords

FGF23 · Phosphate-sensing · PiT1, PiT2 · *GALNT3* · $1,25(OH)_2D$ · FGFR1c

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4.1 General

Phosphate is an essential mineral for humans and plays many functions in the body. Approximately 85% of phosphate is stored in the bone as a hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] in humans [24]. The skeleton, which is composed of hydroxyapatite crystals and matrix proteins, is physically hard enough to support the weight of the body. Thus, chronic hypophosphatemia induces rickets/osteomalacia and skeletal muscle myopathy [26]. On the other hand, hyperphosphatemia is also harmful to the body. Hyperphosphatemia leads to ectopic calcification, which is typically seen in patients with hyperphosphatemic tumoral calcinosis [26]. Hyperphosphatemia is also well known to induce vascular calcification, resulting in ischemic heart disease and stroke in patients with end-stage renal disease (ESRD) on hemodialysis [45]. Therefore, the blood phosphate level needs to be regulated within a narrow range. It is well known that there are several humoral factors that help to maintain blood phosphate level in an appropriate range [16]. Fibroblast growth factor 23 (FGF23), a bone derived hormone, is the principal hormone in the regulation of blood phosphate [14, 31, 42]. Therefore, it could be considered that a phosphate-sensing mechanism has the function of regulating FGF23 production in bone. However, phosphate-sensing mechanism has been largely unknown.

4.2 Fibroblast Growth Factor 23: FGF23

Several humoral factors maintain the blood level of phosphate in humans, FGF23, parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] [16]. Among these humoral factors, FGF23 is the principal hormone in the regulation of blood phosphate levels [14, 31, 42]. This hormone is produced by bone (particularly osteocytes and osteoblasts). The main action of FGF23 is observed in the renal proximal tubules [14, 31, 42]. In the presence of α-Klotho (the expression of which is limited to several tissues, including the kidney and parathyroid glands), FGF23 can bind to the FGF receptor 1c (FGFR1c) [27, 28, 50]. FGF23 suppresses the type IIa and IIc sodium-phosphate cotransporters expression in the renal proximal tubules and inhibits proximal tubular phosphate reabsorption. In addition, FGF23 reduces the synthesis of 1,25$(OH)_2$D by suppressing the expression of *CYP27B1*, which produces 25-hydroxyvitamin D-1α-hydroxylase, and by enhancing the expression of *CYP24A1*, which encodes 25-hydroxyvitamin D-24-hydroxylase. Because 1,25$(OH)_2$D enhances intestinal phosphate absorption, FGF23 reduces the blood phosphate level by inhibiting both proximal tubular phosphate reabsorption and intestinal phosphate absorption via the reduction of 1,25$(OH)_2$D [42].

4.2.1 The Structure of FGF23 Protein

FGF23 protein (a peptide with 251 amino acids) is produced by bone. After cleavage of a signal peptide (24 amino acids), FGF23 protein (227 amino acids) is secreted into circulation [40]. Before or during its secretion, part of this protein is proteolytically cleaved into inactive fragments. FGF23 can be cleaved between arginine (Arg) 179 and serine (Ser) 180 by enzymes, such as a Furin, which recognize the Arg176-X177-X178-Arg179 motif [42]. The important thing is that only full-length FGF23 protein is biologically active [18, 41, 53]. This cleavage was previously demonstrated to be inhibited by *O*-glycosylation of threonine (Thr) 178 in FGF23 protein initiated by UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransderase 3 (GalNAc-T3)—a gene product of *UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransderase 3* (*GALNT3*)— [17]. Thus, *O*-glycosylation of FGF23 protein—which is initiated by GalNAc-T3—works to increase active full-length FGF23 protein. On the other hand, the phosphorylation of Ser180 in FGF23 protein by family with sequence similarity 20, member C (FAM20C) was reported to inhibit *O*-glycosylation of FGF23 protein by GalNAc-T3 and enhance the processing of FGF23 protein (Fig. 4.1) [44].

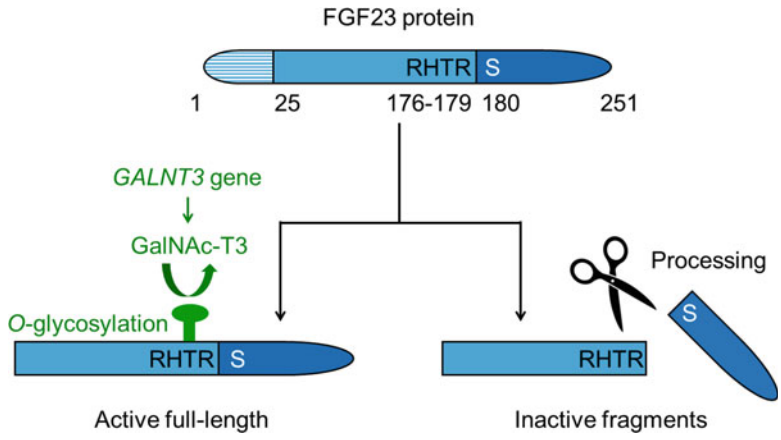

Fig. 4.1 The structure of FGF23 protein and posttranslational modification via a gene product of *GALNT3*

4.2.2 The Posttranslational Modification of FGF23 Protein Via a Phosphate Responsive Gene: *GALNT3*

Inactivating mutations of *GALNT3* cause hyperphosphatemic familial tumoral calcinosis (HFTC) in humans [48]. These patients show that hyperphosphatemia and high blood 1,25 $(OH)_2D$ levels like *Fgf23* knockout mice [48]. It could be considered that FGF23 protein, in which *O*-glycosylation is impaired, is susceptible to this processing, which results in low active full-length FGF23 and impairs the actions of FGF23. *GALNT3* is one of 20 *GALNT* gene families in humans; however, the gene product of *GALNT3* is necessary for the initiation of *O*-glycosylation of Thr178 in FGF23. No other *GALNT* gene products can initiate *O*-glycosylation [5]. According to these facts, it could be considered that the activity of FGF23, namely the level of full-length FGF23 in the blood, is regulated by the transcription and translation of *FGF23*, as well as by the posttranslational modification of FGF23 by the gene product of *GALNT3*. Previously, it was reported that a high phosphate diet increased blood levels of full-length FGF23 in both mice and humans [15, 37]. Recent data demonstrated that a high phosphate diet did not enhance the expression of *Fgf23* in the bone, while a high phosphate diet increased blood full-length FGF23 level in mice [46]. On the contrary, a high phosphate diet increases *Galnt3* levels in bone [46]. Taken together, a high phosphate diet is considered to increase the blood level of full-length FGF23 by increasing the expression of the *Galnt3* gene. Furthermore, when we performed Western blotting in *in vitro* experiments to determine the ratio of full-length FGF23 to cleaved fragments of FGF23 in culture media of the osteoblastic UMR106 cell line, the ratio of full-length FGF23 protein under a high extracellular phosphate condition was higher in comparison to that under a low phosphate condition [46]. *In vitro* experiments demonstrated that high extracellular phosphate levels increased the *Galnt3* gene expression in a dose dependent manner, and enhanced the GalNAc-T3 protein (encoded by *Galnt3*) expression [46]. Taken together, we consider *Galnt3* to be a phosphate responsive gene.

As mentioned above, the posttranslational modification of FGF23 protein was reported to be regulated not only by GalNAc-T3 but also by FAM20C. Both a high phosphate diet and high levels of extracellular phosphate also enhanced the expression of *Fam20c* [46]. From the observation that the blood full-length FGF23 level was increased by a high phosphate diet, the role of GalNAc-T3 in response to high phosphate levels seemed to be dominant over that of FAM20C. In addition, the processing of FGF23 protein is mediated by proteases like Furin [44]. Neither a high phosphate diet nor a high level of extracellular phosphate changed the expression of *Furin* [46]. It was already reported that $1,25(OH)_2D_3$ increases the blood level of full-length FGF23 in murine models [38]. $1,25(OH)_2D_3$ enhanced the expression of *Fgf23* in osteoblastic UMR106

cells, however, $1,25(OH)_2D_3$ had no effect on the expression of *Galnt3* [46]. Furthermore, a high phosphate diet increased the blood level of PTH, while PTH was reported to suppress the expression of *Galnt3* [23]. It is therefore considered unlikely that $1,25(OH)_2D_3$ and PTH are involved in the increase in the expression of *Galnt3* or in the increase in the blood level of full-length FGF23 that is caused by high phosphate levels.

Taken together, posttranslational modification of FGF23 by the gene product of *Galnt3* is considered to be the main mechanism of the response to high dietary phosphate and *Galnt3* is considered to be responsive to phosphate.

4.3 Phosphate-Sensing

4.3.1 Intracellular Signaling Induced by Phosphate: MEK/ERK Pathway

Alternations of extracellular phosphate have been shown transduce signals into the cells to regulate the expression of gene and the behavior of cells [33]. High levels of extracellular phosphate were shown to stimulate the extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway [4]. Furthermore, high levels of extracellular phosphate were demonstrated to induce a number of genes, including *osteopontin* (*Opn*), *dentin matrix protein 1* (*Dmp1*), *cyclin D1*, and *early growth response 1* (*Egr1*) through this same pathway [3, 25, 35, 54].

It was recently reported—based on a DNA microarray analysis using osteoblastic UMR106 cells—that high levels of extracellular phosphate induced the expression of several transcription factors downstream of the MEK/ERK pathway [46]. High extracellular phosphate induced the phosphorylation of ERK1/2 in a dose-dependent manner [46]. In addition, inhibition of the MEK/ERK pathway suppressed the enhancement of *Galnt3* gene, a phosphate responsive gene as mentioned above, by high extracellular phosphate [46]. MEK/ERK pathway activation by high levels of extracellular phosphate is therefore considered necessary for the induction of *Galnt3*. The same study also revealed the necessity of a number of transcription factors (e.g., *Egr1* and *ETS variant 5* [*Etv5*]) for high levels of extracellular phosphate to induce the expression of *Galnt3* [46]. However, *Egr1* and *Etv5* were not sufficient to enhance the *Galnt3* gene expression; thus, other transcriptional activators are needed.

4.3.2 A Potential Candidate Molecule for Phosphate-Sensing in the Bone: FGFR1c

As mentioned above, high extracellular phosphate was reported to activate the MEK/ERK pathway by several research groups [3, 4, 25, 35, 46, 54]. Until today, however, the upstream molecules of the MEK/ERK pathway have been unknown. The MEK/ERK pathway is activated by a number of receptor tyrosine kinases (RTKs), including—but not limited to—epidermal growth factor receptor (EGFR) and FGFRs [32]. An experiment was performed in order to investigate whether high extracellular phosphate levels can activate RTKs, and if so, to determine the RTKs that are activated using proteomics. Osteoblastic UMR106 cells were treated with high levels of extracellular phosphate and an LC-MS/MS analysis was performed after digestion of the cell extract with trypsin and immunoprecipitation with an anti-phosphotyrosine antibody [1]. The proteomic analysis demonstrated that FGFR1 was the only RTK to be phosphorylated by high levels of extracellular phosphate [46].

Alternative splicing produces two types of FGFR1 (FGFR1b and FGFR1c) [36]. Osteoblastic UMR106 cells only express FGFR1c endogenously [46]. Furthermore, this analysis identified, two types of FGFR1 peptides—one with phosphotyrosine 583 and 585, the other with phosphotyrosine 653 and 654—in which high extracellular phosphate levels induced tyrosine (Tyr) phosphorylation [46]. Six Tyr residues, which are located in the cytoplasmic region of FGFR1, Tyr653, 583, 463, 766, 585, and 654, are sequentially phosphorylated in this order following the activation of FGFR1 [19, 29]. Among them, the phosphorylation of the two Tyr residues (Tyr653 and 654) is known to dramatically increase the tyrosine kinase activity of FGFR1

[36]. Parallel-reaction monitoring (PRM) was applied to quantify the amount of this peptide with phosphotyrosine 653 and 654, which revealed that high extracellular phosphate levels increased the amount of this peptide approximately threefold [46]. Moreover, treatment with an FGFR inhibitor and *Fgfr1* silencing aborted both the enhanced *Galnt3* expression and the ERK1/2 phosphorylation induced by high levels of extracellular phosphate [46]. These findings were in line with previous reports that suggested that FGFR1 is involved in the cellular responses to high levels of extracellular phosphate [35, 54]. In addition, these findings are supported by the findings of a previous study reporting that— in patients with osteoglophonic dysplasia—some activating mutations in *FGFR1* gene induce high blood levels of FGF23 as well as hypophosphatemia [52].

4.3.2.1 The Significance of FGFR1c as a Phosphate-Sensing Molecule *In Vivo*

In addition to these *in vitro* data, some *in vivo* experiments to examine the significance of FGFR1c have been reported. First, enhanced phosphorylation of ERK1/2 in the bone by a high phosphate diet was visible in the whole tissue extracts by immunoblotting in intact animals [46]. Second, an FGFR inhibitor, NVP-BGJ398 [22], was administered to mice fed a high phosphate diet. Although the blood phosphate levels of mice fed a high phosphate diet were significantly higher than those of mice fed a control diet, under NVP-BGJ398 treatment, neither the blood level of full-length FGF23 nor *Galnt3* gene expression in the bone showed a clear increase, confirming the proposed function of FGFR in *Galnt3* gene induction [46]. Finally, a selective ablation of *Fgfr1* in the bone by crossing *Osteocalcin-Cre* [55] mice with floxed *Fgfr1* [49] mice abolished the increase of the blood level of full-length FGF23 and the *Galnt3* gene upregulation in bone by a high phosphate diet [46]. These *in vivo* data again support the function of FGFR1c in the regulation of the *Galnt3* gene expression by high phosphate.

4.3.2.2 The Specific FGFR1c Signal Transduction Mediated by Phosphate

Although high levels of extracellular phosphate induced the expression of the *Galnt3* gene through activated FGFR1c, it was unclear whether canonical FGFR ligands (e.g., FGF2) also enhance the expression of *Galnt3*. However, FGF2 did not enhance the expression of *Galnt3* gene [46]. It is therefore possible that a molecular mechanism is involved in the differences of FGFR1c activation caused by high levels of extracellular phosphate and the canonical FGFR ligands. Given that FGFR1 activates the MEK/ERK pathway through FGFR substrate 2α (FRS2α)—a phosphorylation substrate of activated FGFR—and that FRS2α is necessary to activate the downstream MEK/ERK pathway [20, 21], we investigated the phosphorylation of FRS2α. FGF2 was observed to phosphorylate Tyr196 and 436 of FRS2α; however, only Tyr196 was phosphorylated by high levels of extracellular phosphate. Monitoring of the time course of ERK1/2 activation revealed that ERK1/2 activation by high levels of extracellular phosphate was transient; in contrast, activation by FGF2 was sustained [46]. Thus, there are differences in signal transduction by high extracellular phosphate and the canonical FGFR ligands.

Three typical intracellular signaling pathways are known to exist downstream of the FGFRs; these are the MEK/ERK pathway, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, and the phospholipase C-γ (PLCγ)/calcineurin/Nuclear Factor of Activated T cells (NFAT) pathway [20]. Among these, FRS2α activates the MEK/ERK and PI3K/Akt pathways. In contrast, PLCγ/calcineurin/NFAT activation occurs independently of the phosphorylation of FRS2α [20]. PRM demonstrated that the protein level of ERK2 with phosphorylated Tyr183 and 185 was increased by both high extracellular phosphate and FGF2. However, while FGF2 effectively increased the protein level of phosphorylated PLCγ, high extracellular phosphate did not. While Akt was also phosphorylated by FGF2, Akt was not phosphorylated by high

extracellular phosphate. Furthermore, a PI3K inhibitor and a calcineurin inhibitor did not influence the induction of *Galnt3* gene expression by high levels of extracellular phosphate [46]. Taken together, these findings indicate that the PI3K/Akt and PLCγ/calcineurin/NFAT pathways are not involved in the induction of the expression of *Galnt3* by high levels of extracellular phosphate.

4.3.2.3 The Activation Model of Unliganded FGFR1c by Phosphate

FGFR1c is necessary for the induction of the *Galnt3* gene expression by high levels of phosphate. Thus, phosphate is considered to function as the first messenger to regulate the level of full-length FGF23 in the blood and a feedback system—which involves FGFR1c and FGF23—exists in regulation of the blood level of phosphate. However, the exact mechanism through which FGFR1c is activated by high phosphate levels remains to be elucidated. FGFRs can form either homodimers to facilitate the binding of paracrine FGFs (e.g., FGF1 and FGF2) or heterodimers with coreceptors (e.g., Klothos) to bind endocrine FGFs including FGF23 [20]. The phosphorylation and dimerization of FGFRs are known to be coupled with the activation of FGFR by the binding of FGFR ligands, while the strength of FGFR activation differs according to the type of FGFR ligand [39]. Several reports have proposed a dimerization of unliganded FGFR model [12, 30, 39]. For the dimerization of unliganded FGFRs on the cell surface, the transmembrane domain is pivotal, while the extracellular and intracellular tyrosine kinase domains are not [12]. Activating mutations in *FGFR1* that change amino acids in the FGFR1 transmembrane domain have been identified in patients with osteoglophonic dysplasia [52]. Furthermore, FGFR1 with these mutations has been reported to be more prone to induce FGFR1 dimerization in comparison to intact FGFR1 in the absence of canonical FGFR ligands [12]. We hypothesize that the activation of unliganded FGFR1c by high extracellular phosphate levels is mediated by an alternation in the protein structure of FGFR1c (e.g., stabilization of the interaction between the transmembrane domains).

4.3.3 The Involvement of Type III Sodium-Phosphate Cotransporters: PiT1 and PiT2

Several studies indicate the involvement of sodium-phosphate cotransporters in the process of phosphate-sensing [4, 25, 35, 54]. Bacteria and yeast use some types of phosphate transporters as phosphate-sensors [33]. However, such a phosphate "transceptor" model has not been established in mammals. Mammalian sodium-phosphate cotransporters are classified into two distinct families: type II and III sodium-phosphate cotransporters [6]. *SLC20A1* and *SLC20A2* encode the two types of type III sodium-phosphate cotransporters, PiT1 and PiT2, respectively [51]. Treatment with phosphonoformic acid (foscarnet: PFA), which is a sodium-phosphate cotransporter inhibitor, was shown to block the uptake of phosphate by cells as well as the effects of phosphate on the expression of *Opn* [4]. Studies in several types of cultured cells suggested the involvement of PiT1 in the signal transduction and gene regulation triggered by high extracellular phosphate [25, 54]. In addition, several reports have indicated that silencing *Slc20a1*, which encodes PiT1, reduced the activation of FGFR1 and MEK/ERK pathway by high levels of extracellular phosphate [25, 54]. Conversely, PiT2—rather than PiT1—was recently reported to be involved in the regulation of FGF23 secretion high extracellular phosphate [8]. Moreover, these authors reported that extracellular phosphate both induced PiT1-PiT2 heterodimerization and mediated the MEK/ERK pathway activation independently of the uptake of phosphate. They concluded that phosphate binding to Ser128 (in PiT1) and Ser113 (in PiT2) is the key factor in mediating phosphate signaling through the PiT proteins [7]. In contrast, our data showed that a high phosphate diet did not induce the expression of *Slc20a2*, which encodes PiT2, in the bone, and the induction of *Galnt3* gene by high extracellular phosphate was not suppressed by the silencing on *Slc20a2* in osteoblastic UMR106 cells [46]. It remains to be clarified how the transporters solely can activate the MEK/ERK signaling in mammalian cells. Furthermore, whether or not PiT1 and

PiT2 (type III sodium-phosphate cotransporters) play a role in the activation of unliganded FGFR1c by high phosphate remains unclear.

4.3.4 A Molecule for Phosphate-Sensing in the Parathyroid Glands: Calcium-Sensing Receptor

Calcium-sensing receptor (CaSR) is the key molecule for the regulated secretion of PTH, which is the principal hormone to maintain the blood calcium (Ca) level [10]. PTH not only works to increase blood Ca level but also inhibit phosphate reabsorption in the renal proximal tubules [9, 13]. Although increased blood phosphate level stimulates PTH secretion from parathyroid glands, phosphate-sensing mechanism on the parathyroid cells also remained unclear [2, 34, 43, 47]. It was recently reported that extracellular phosphate stimulates PTH secretion through CaSR in parathyroid cells. Phosphate was found to function as a noncompetitive antagonist for CaSR and phosphate-binding sites in the extracellular domain of CaSR [11]. Thus, several molecules may have phosphate-sensing mechanisms that work in a cell- or tissue-specific manner.

4.4 Perspectives

The identification of FGF23 has advanced the understanding of not only the regulatory mechanism of phosphate metabolism but also the pathogenesis of hypophosphatemic and hyperphosphatemic diseases. Recent findings have uncovered an unreported function of FGFR1c and demonstrated a novel molecular basis for phosphate-sensing in regulated production of FGF23 in bone (Fig. 4.2). However, the precise

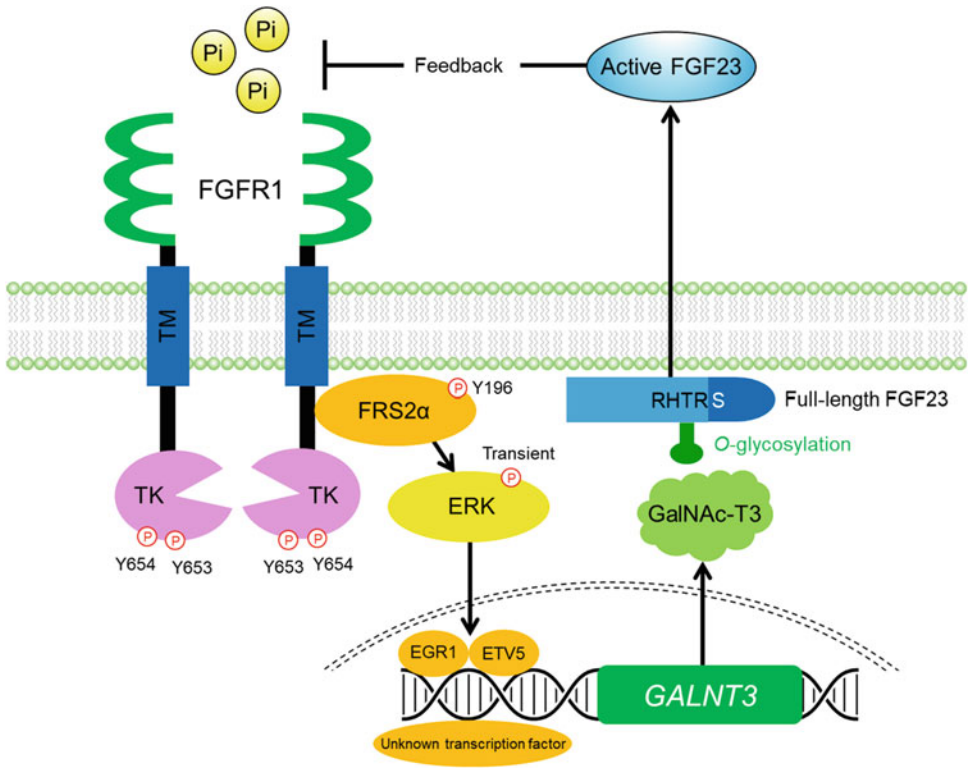

Fig. 4.2 The model of phosphate-sensing in the bone to regulate the production of FGF23 and the blood level of phosphate

mechanism through which FGFR1c is activated by high phosphate remains to be fully elucidated. Furthermore, while PiT1 and PiT2 are also reported to play a role in phosphate-sensing, the precise mechanism of their involvement has not been determined.

As the identification of CaSR has led to the discovery of new drugs for the treatment of primary and secondary hyperparathyroidism, elucidating the mechanism of phosphate-sensing may facilitate the optimization of treatment strategies for abnormal phosphate metabolism.

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Vitamin D and Phosphate Interactions in Health and Disease

5

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Abstract

Vitamin D plays an essential role in calcium and inorganic phosphate (Pi) homeostasis, maintaining their optimal levels to assure adequate bone mineralization. Vitamin D, as calcitriol ($1,25(OH)_2D$), not only increases intestinal calcium and phosphate absorption but also facilitates their renal reabsorption, leading to elevated serum calcium and phosphate levels. The interaction of $1,25(OH)_2D$ with its receptor (VDR) increases the efficiency of intestinal absorption of calcium to 30–40% and phosphate to nearly 80%. Serum phosphate levels can also influence $1,25(OH)_2D$ and fibroblast growth factor 23 (FGF23) levels, i.e., higher phosphate concentrations suppress vitamin D activation and stimulate parathyroid hormone (PTH) release, while a high FGF23 serum level leads to reduced vitamin D synthesis. In the vitamin D-deficient state, the intestinal calcium absorption decreases and the secretion of PTH increases, which in turn causes the stimulation of $1,25(OH)_2D$ production, resulting in excessive urinary phosphate loss. Maintenance of phosphate homeostasis is essential as hyperphosphatemia is a risk factor of cardiovascular calcification, chronic kidney diseases (CKD), and premature aging, while hypophosphatemia is usually associated with rickets and osteomalacia. This chapter elaborates on the possible interactions between vitamin D and phosphate in health and disease.

Keywords

Vitamin D · PTH · FGF23 · Klotho · Phosphate · Kidney · Intestine · Bone

5.1 Introduction

Vitamin D research has more than 100 years of history since McCollum and Davis's discovered the "growth-promoting fat-soluble vitamin" that was found in cod liver oil [20]. The effect of this growth-promoting factor in the treatment of rickets was so effective that cod liver oil was regarded as a panacea and gave a powerful impetus to further research on vitamin D throughout the world [71]. In the last 20 years, it has been shown that vitamin D's biological activities extend far beyond its involvement in calcium

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metabolism. Along with proven efficacy in pathological conditions and diseases such as rickets, bone loss, and osteomalacia, some novel effects of vitamin D on very diverse physiological processes have been well established [8, 39]. Vitamin D deficiency remains a critical health issue worldwide, and it has been estimated that around one billion people suffer from various vitamin D-related disorders [35].

The biological effects of $1,25(OH)_2D$ can be divided into two types: skeletal (primarily related to calcemic and phosphatemic activities) and non-skeletal, typically not associated with mineral metabolism [15]. The homeostasis of serum phosphate mediated by vitamin D is of paramount importance for adequate bone mineralization, muscle contraction, nerve conduction, and many other vital functions [26]. This brief chapter reviews our understanding of vitamin D-mediated regulation of phosphate homeostasis in health and diseases.

5.2 Physiological Regulation of Phosphate Homeostasis

Phosphorus is the sixth most abundant chemical element in the body [34]. In nature it mainly exists as phosphates, the form most suitable for living organisms [14]. In mammals, the phosphate group is primarily concentrated (~85%) in bones and teeth as hydroxyapatite. The remaining ~15% are distributed in the other tissues as intracellular ortho- and pyrophosphate groups, either free ("inorganic") or as a part of nucleotides, coenzymes, and high-energy phosphate compounds. (referred to as "organophosphates"). Inorganic phosphates exist in two forms: monovalent dihydrogen phosphate ($H_2PO_4^-$) and divalent hydrogen phosphate (HPO_4^{2-}). In the cytosol dihydrogen phosphate is contributing bulk amounts (62% of all cytosolic phosphates).

The extracellular fluid contains only <1% of the whole pool of body's inorganic phosphates [27, 33]. Interestingly, compared to the cytosol, the proportion $H_2PO_4^-$/ HPO_4^{2-} is inverted, so that the major component is now hydrogen phosphate (61% of all phosphates). In general, a 70-kilogram adult with 25% body fat content would have total body phosphorus of approximately 630 g (~21 mol) [34].

Due to its unique chemical structure, various phosphate groups (especially as nucleoside triphosphates) are key players in cellular energy metabolism, in genetic information storage, in signaling pathways, and as phospholipid components of the cell membranes [37]. Inorganic phosphates, together with bicarbonate and protein buffer systems, constitute the basis of the acid-base homeostasis of the body [42].

A healthy adult consumes 1000 mg on average of dietary phosphate per day (Fig. 5.1). Of this amount 700 mg. is absorbed in the small intestine through passive and active pathways [97]. The unabsorbed phosphate is excreted in the feces. Approximately 150 mg. phosphate is secreted into the gut in the saliva, intestinal and pancreatic secretions, while some of it is reabsorbed [47]. Although dietary phosphate intake differs from day to day, principally, phosphate homeostasis is adjusted by intestinal absorption, renal reabsorption, and skeletal resorption. The average serum phosphate concentration in healthy adults is 2.5–4.9 mg/dl [67].

The kidneys filter about 9000 mg. of phosphate daily, 80–90% of which is reabsorbed mainly in the proximal tubule [68]. At least three distinct cotransporters are involved for phosphate transcellular reabsorption in the proximal tubule, namely NaPi-IIa (SLC34A1), NaPi-IIc (SLC34A3), and PiT-2 (SLC20A2) [7] (Fig. 5.2). Phosphate reabsorption is coupled with sodium-dependent (Na^+) transport. Type NaPi II cotransporters are capable of transporting both $H_2PO_4^-$ and HPO_4^{2-} across brush border membrane (BBM) of the proximal tubules [90]. In contrast, in the small intestine, phosphate is absorbed by both transcellular (active) and paracellular (passive) processes, with the active transport being mainly mediated by NaPi-IIb [55].

Given the generally acknowledged role of phosphate in almost every molecular and cellular function, altered phosphate balance can lead to untoward effects. The serum phosphate homeostasis is firmly regulated by endocrine

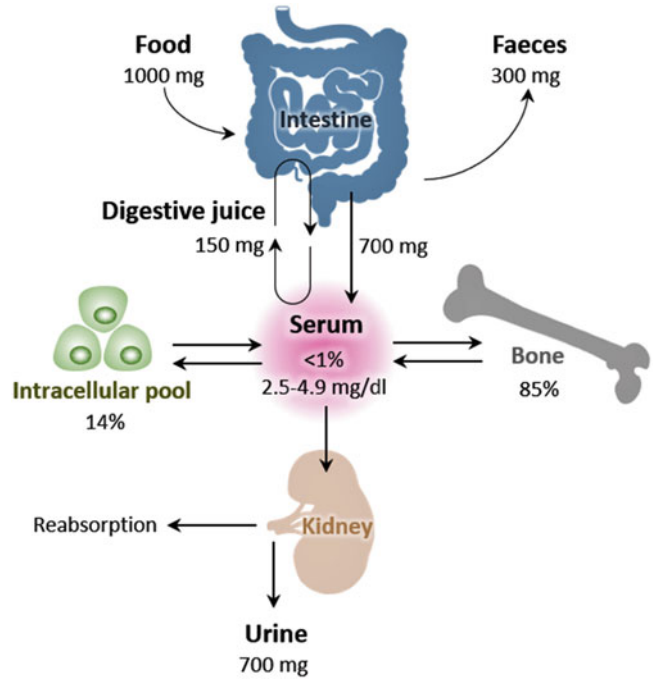

Fig. 5.1 Phosphate flows and balances in the human body [66, 83]

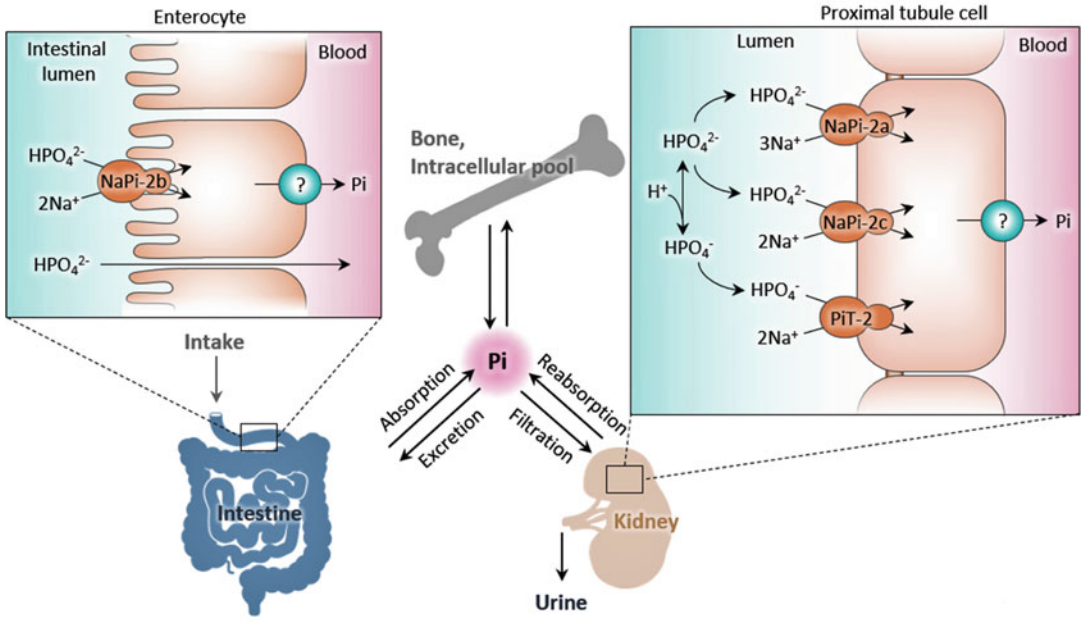

Fig. 5.2 Main transcellular phosphate traffic mechanisms

communication among parathyroid hormone (PTH), calcitriol ($1,25(OH)_2D$), and fibroblast growth factor 23 (FGF-23) [5, 11].

5.2.1 Parathyroid Hormone (PTH)

PTH, a polypeptide containing 84 amino acids with MW 9500 Da, is secreted by chief cells of parathyroid glands [92]. Extracellular calcium concentration is the main modulator of PTH secretion [60]. PTH stimulates calcium resorption from bone tissue, increases calcium reabsorption in the renal tubules, facilitates hydroxylation of 25(OH)D to $1,25(OH)_2D$ in the kidneys, and induces renal excretion of phosphate [50, 69].

In bone tissue, PTH at a permissive level of $1,25(OH)_2D$ promotes calcium resorption by activating osteoclasts [93]. In the intestine, PTH increases the reabsorption of calcium and phosphate by enhancing $1,25(OH)_2D$ synthesis [69]. High serum PTH levels and hypophosphatemia lead to activation of vitamin D-activating enzyme 1α-hydroxylase [57]. $1,25(OH)_2D$ facilitates absorption of calcium and phosphate for bone mineralization and homeostatic metabolism, preventing low serum levels of these elements [43]. PTH also stimulates the synthesis of vitamin D in the kidneys [52].

The effect of PTH on the renal tubules leads to decreased phosphate reabsorption and its increased renal excretion due to the lowered NaPi cotransporters. In general increased PTH secretion results in a decrease in serum phosphate levels [30]. The main role of $1,25(OH)_2D$ is to determine the availability of calcium and phosphate to form new bone and prevent the development of hypocalcemia and hypophosphatemia [3, 30]. This hormone increases intestinal phosphate absorption elevating its serum concentration.

Secretion PTH by the parathyroid glands is mainly triggered by low extracellular calcium by acting on Ca-sensing receptors (CaSR) [85]. Stimulation of CaSR (they belong to the class of G-protein-coupled receptors) activates multiple heterotrimeric G-proteins, in turn passing the signal to mitogen-activated protein kinase (MAPK) pathways. This cascade of reactions ultimately leads to the suppression of PTH secretion by a negative feedback loop. It has been shown that $1,25(OH)_2D$ upregulates the transcription of the gene encoding the CaSR in the parathyroid gland [13]. Additionally, a low level of calcium indirectly induces parathyroid hyperplasia [23]. However, there is also evidence of the opposite effect of stimulation of parathyroid cell proliferation in response to a high calcium concentration [81].

Interestingly, high serum phosphate levels (hyperphosphatemia) also increase PTH secretion independently of shifts in extracellular calcium [41, 86]. The further secretion of PTH is directly suppressed by $1,25(OH)_2D$, acting on VDR of parathyroid glands [79].

5.2.2 Vitamin D (Calcitriol)

From a biological point of view, vitamin D is a steroid hormone, as it is synthesized in the body and has a highly specific receptor (VDR). Most vitamin D (90–95%) is formed in the skin under the influence of UVB light, and only a minor fraction of it is obtained from dietary sources [8].

Vitamin D is stored mainly in the liver with a half-life of approximately 14 days. When a larger amount of vitamin D is absorbed, its excess is stored mainly in adipose tissue [1]. Furthermore, vitamin D in association with the vitamin D-binding protein (VBP) is transferred to the liver, where it is hydroxylated to form 25 (OH)D, which subsequently undergoes 1-α-hydroxylation in the renal tubules, turning into $1,25(OH)_2D$. This biologically active form of vitamin D is under control by serum PTH, phosphate, and FGF23 concentrations. The synthesis of $1,25(OH)_2D$ is stimulated by low serum phosphate levels and high PTH concentrations [78].

Vitamin D promotes the intestinal absorption of calcium and phosphate, significantly increases their renal reabsorption, and also inhibits the PTH secretion [40] (Fig. 5.3). Thus the major effects of $1,25(OH)_2D$ are to augment the intestinal absorption of both calcium and phosphate for proper bone mineral matrix formation [40]. In the intestine and kidneys, $1,25(OH)_2D$ increases the

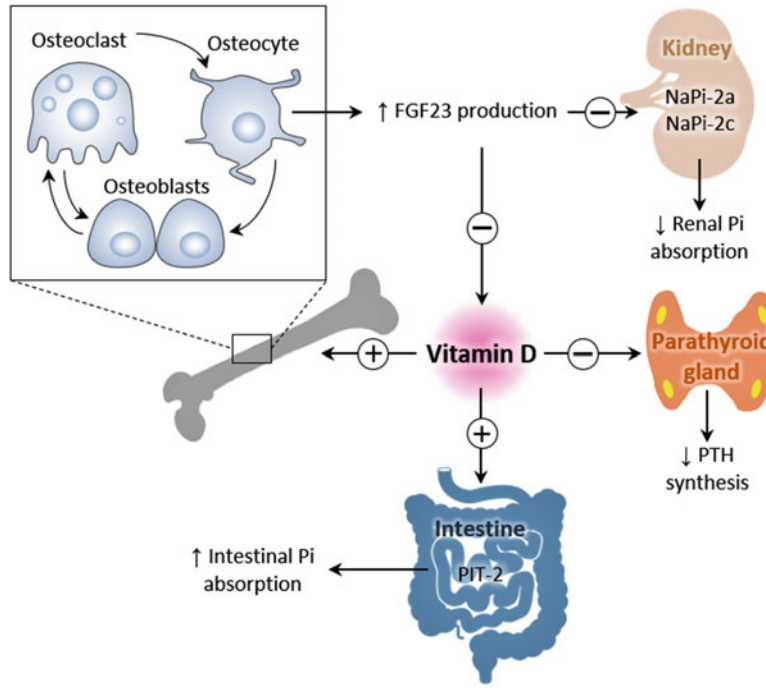

Fig. 5.3 Possible regulation of phosphate homeostasis by vitamin D

formation of calcium-binding proteins (calbidins), which promote transmembrane calcium transport to control homeostasis [2]. In bone, 1,25$(OH)_2$D potentiates the effects of PTH, stimulates bone resorption by osteoclasts, and promotes maturation of monocytes into osteoclasts [70, 84]. In parathyroid glands, 1,25 $(OH)_2$D binds to the VDR, resulting in the suppression of PTH production [96]. The optimal level of serum phosphate is maintained by the interaction of hormones; lowering serum phosphate level by PTH and FGF23, while, increasing serum phosphate level by elevating its absorption in the intestine (1,25$(OH)_2$D) and its resorption from bones (PTH, 1.25$(OH)_2$D) [37]. PTH directly activates osteoclasts and causes phosphate resorption, and indirectly enhances intestinal phosphate absorption by stimulating 1,25 $(OH)_2$D production [44].

Activation of the VDR is a potent and rapid modulator of FGF23 expression, thus forming a "classical" endocrine negative feedback loop between FGF23 and vitamin D [17]. In addition, 1,25$(OH)_2$D is a potent suppressor of PTH gene expression [9].

5.2.3 Fibroblast Growth Factor 23 (FGF23)

FGF23, secreted in bone (osteocytes, osteoblasts, and odontoblasts), is an around 32 kDa glycoprotein, which can be converted in its inactive form through cleavage by a proconvertase-type enzyme into two smaller fragments: 18 kDa (amino fragment) and 12 kDa (carboxy fragment) [32].

FGF23, like PTH, reduces renal phosphate reabsorption, which leads to a drop-in plasma phosphate levels [18]. This hormone also suppresses the secretion of PTH and inhibits the 1α-hydroxylase activity of the kidneys, thus reducing the synthesis of 1,25$(OH)_2$D [46, 51]. FGF23 acts by stimulating its receptors, for the normal function of which a cofactor is needed, i.e. the Klotho protein, synthesized, mostly in the kidneys [87]. The transmembrane Klotho protein is essential for FGF23 to exert its phosphaturic effects in the kidney [72–74, 89].

A decrease in serum phosphate under the FGF23 is achieved by inhibiting phosphate reabsorption in the renal tubules, as well as by

stimulating PTH secretion and suppressing 1,25 $(OH)_2D$ synthesis [12, 51, 56, 72, 91]. In contrast, calcitonin, is another hormone produced by the thyroid gland, slightly lowers serum calcium due to inhibition of renal and intestinal calcium reabsorption, reducing calcium and phosphate resorption from bones [36]. Plasma calcium is regulated by a complex system involving PTH and $1,25(OH)_2D$ on the intestine, bones, and kidneys. As mentioned, parathyroid gland cells respond to serum calcium concentration via CaSR. A high level of calcium in extracellular fluid stimulates CaSR receptors and activates cellular mechanisms, which ultimately leads to inhibition of PTH release [6].

Imbalance of calcium and phosphate is manifested as a shift in the calcium, phosphate levels in serum and the levels of serum hormones [PTH and 25(?H)D], as well as the development of bone pathology and cardiovascular calcification with soft anomalies [76, 88]. The exact etiology and pathogenesis of serum phosphate derangements (hyperphosphatemia and hypophosphatemia) will need further studies.

5.3 Hyperphosphatemia

Renal failure is the most common cause of hyperphosphatemia [80]. The decline in estimated glomerular filtration rate disrupts phosphate homeostasis: when it falls below 30 mL/min/1.73 m^2, the reabsorption of phosphate is maximally suppressed and fractional excretion markedly reduced. As a result, the serum level of phosphate increases [16, 21]. A primary increase in tubular reabsorption of phosphate is less common and can be observed in hypoparathyroidism, acromegaly, and tumoral calcification [38].

Excessive phosphate can be released from the intracellular compartment, which is observed in acute tumor lysis syndrome, rhabdomyolysis, hemolysis, hyperthermia, profound catabolic stress, and acute leukemia. Tumor lysis syndrome is commonly observed in malignant hematological patients, particularly non-Hodgkin's lymphoma and acute leukemia, following chemotherapy [4]. Risk factors for developing the syndrome include impaired renal function, increased levels of lactate dehydrogenase, and hyperuricemia [95]. The latter is caused by the disturbances in FGF23-mediated phosphate regulation in the proximal tubule of the kidney [10]. Increased intestinal phosphate absorption is mainly caused either by the use of phosphate-containing oral laxative, or by vitamin D overdoses [59].

5.4 Hypophosphatemia

Hypophosphatemia may be a consequence of the decreased intestinal absorption, internal redistribution, and increased urinary loss of phosphate [31]. The acute shift of phosphate from the extracellular to the intracellular compartment is most often caused by respiratory alkalosis and refeeding syndrome in hospitalized patients [19, 54]. Respiratory alkalosis causes an increase in intracellular pH, which stimulates phosphofructokinase, leading to severe hypophosphatemia with plasma phosphate of >0.32 mmol/L [82]. The intracellular shift of phosphate is also observed in the treatment of diabetic ketoacidosis and hungry bone syndrome, which occurs after parathyroidectomy performed for patients with long-standing hyperparathyroidism [31]. At the same time, in the postoperative period, serum calcium and phosphate concentrations significantly decrease.

Low phosphate intake rarely causes hypophosphatemia, probably because the phosphate content in the diet almost always exceeds the phosphate loss through the gastrointestinal tract, and the kidneys can reabsorb nearly all of the filtered phosphate [24]. Excessive urinary loss of phosphate is observed in both primary and secondary hyperparathyroidism caused by impaired vitamin D metabolism, Fanconi syndrome, diuretics, and tumor-induced osteomalacia (TIO) [31, 48]. TIO is a rare paraneoplastic syndrome characterized by hypophosphatemia, phosphaturia, decreased $1,25(OH)_2D$ level, normal 25(OH)D levels, and osteomalacia [29]. Overproduction of FGF23 caused by TIO reduces tubular phosphate reabsorption and $1,25(OH)_2D$ production [58].

5.5 Genetic Disorders Associated with Hypophosphatemia

Several inherited abnormalities are characterized by phosphate-wasting syndromes, commonly mediated by FGF23. These diseases, resulted by impaired FGF23 metabolism, include autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemic rickets (XLHR), and autosomal recessive hypophosphatemic rickets (ARHR) [94].

ADHR (OMIM 193100) is produced by *FGF23* gain-of-function mutation, which causes the resistance of the mutant FGF23 to proteolytic degradation [22]. ADHR manifests as a defect in renal phosphate transport, associated with decreased 1,25(OH)2D levels, while the PTH levels remain normal. ADHR is characterized by hypophosphatemia, renal phosphate loss, short stature, and bone disorders [25].

ARHR (OMIM 241520) is caused by mutations in the *DMP1* gene (located on chromosome locus 4q21). Patients with ARHR suffer from decreased renal phosphate reabsorption and typically display hyperphosphaturia, hypophosphatemia, reduced $1,25(OH)_2D$ concentration, with PTH values remaining normal [28, 49].

XLHR (OMIM 307800) appears as a result of mutations inactivating *PHEX* (phosphate-regulating gene with homologies to endopeptidases located on the X-chromosome). The *PHEX* gene encodes a zinc-dependent metalloproteinase, and is strongly expressed in osteoblasts, osteocytes, and odontoblasts [53]. The XLHR symptoms include growth retardation, hypophosphatemia, osteomalacia, and defective renal phosphate reabsorption. The diseased state is resistant to phosphate and vitamin D therapy [63].

5.6 Conclusions

Serum phosphate levels are tightly regulated by hormonal and metabolic factors mainly related to the triad "vitamin D-PTH-FGF23" as well as dietary phosphate. Experimental studies have convincingly shown that disorders and disturbances in phosphate regulation can lead to serious systemic complications [45, 61, 62, 64, 65, 75, 77]. Particular attention should be placed on the central activity of vitamin D in phosphate metabolism, as 1,25(OH)2D both, directly and indirectly, impact serum phosphate levels. However, despite the well-studied pivotal roles of vitamin D in phosphate homeostasis, many aspects remain unclear. For instance, what are the underlying mechanisms by which vitamin D acts on renal phosphate reabsorption, and how exactly do calcium and vitamin D modulate FGF23 production? A better understanding of these processes and interactions would help to develop more efficient strategies for the treatment of phosphate-related disorders.

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Fibroblast Growth Factor 23 as Regulator of Vitamin D Metabolism

6

Shinya Nakatani, Ayumi Nakatani, Katsuhito Mori, Masanori Emoto, Masaaki Inaba, and Mohammed S. Razzaque

Abstract

Fibroblast growth factor 23 (FGF23) is a hormone produced by osteocytes in bone that acts on the kidneys to regulate phosphate and vitamin D metabolism.FGF23 levels were shown to be increased in the early stage of chronic kidney disease (CKD), with a slight decline in estimated glomerular filtration rate (eGFR) even when the range was restricted to above 60 mL/min/1.73 m^2, indicating that subtle phosphate load is a stimulator of FGF23 in serum. FGF23 is also known to inhibit vitamin D activation from 25-hydroxyvitamin D (25-OH-D) to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], while it stimulates its degradation from 25-OH-D to 24,25-dihydroxyvitamin D [24,25$(OH)_2$D]. Previously, we demonstrated a significant and negative association of serum FGF23 with serum 1,25$(OH)_2$D and 1,25$(OH)_2$D/25-OH-D ratio, a putative parameter for CYP27B1, and confirmed the physiological effects of FGF23 on phosphate and vitamin D metabolism in non-CKD subjects. Elevated FGF23 by itself is reported to be associated with various adverse outcomes, including left ventricular hypertrophy, endothelial dysfunction, and activation of the renin-angiotensin-aldosterone system, leading to increased mortality even in non-CKD individuals. On the other hand, our previous study showed that the impaired incremental response of serum FGF23 in response to oral phosphate load in diabetic patients can help to significantly increase serum phosphate (Yoda et al., J Clin Endocrinol Metab 97:E2036–43, 2012) and thus may contribute to progression of vascular calcification in those patients (personal observation). It is suggested that increased serum FGF23 might be an important indicator of adverse outcomes in non-CKD as well as CKD patients.

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Keywords

FGF23 · Vitamin D · Phosphate · CKD-MBD · Klotho

6.1 Physiology of FGF23

Fibroblast growth factor 23 (FGF23) is a hormone produced by osteocytes in bone that acts on the kidneys to regulate phosphate and vitamin D metabolism through activation of the FGF receptor (FGFR)/α-klotho co-receptor complex [31]. This hormone induces phosphaturia by decreasing phosphate reabsorption in the proximal tubule through down-regulation of luminal sodium-phosphate co-transporters [48, 53]. Furthermore, it inhibits the activation step of vitamin D from 25-hydroxyvitamin D (25-OH-D) to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] [6, 52], while it stimulates the degradation step of 25-OH-D to 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] [53, 54, 62], thus inhibiting intestinal phosphate absorption (Fig. 6.1). Consequently, FGF23 has been proposed as a major regulator of phosphate and vitamin D metabolism.

6.2 Direct and Indirect Effects of FGF23 on Various Factors

Chronic kidney disease (CKD) is known to be complicated with CKD-mineral and bone disorder (CKD-MBD), which is mainly comprised of

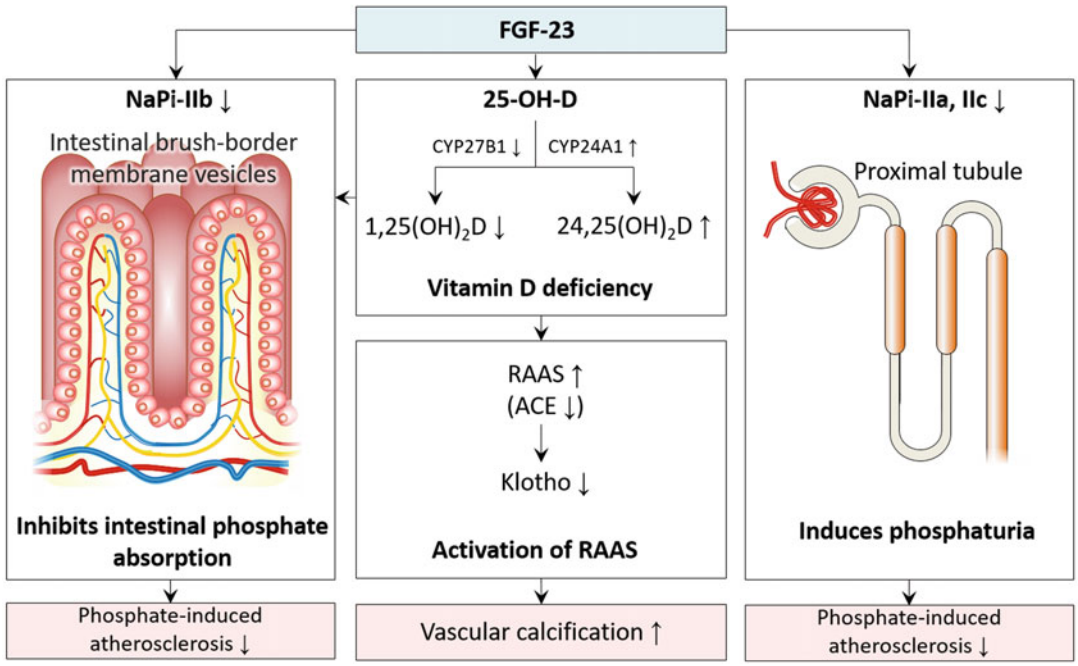

Fig. 6.1 Putative mechanisms of the effect of elevated FGF23 in non-CKD subjects

Even in non-CKD subjects, FGF23 inhibits CYP27B1, leading to decreased serum 1,25$(OH)_2$D. Elevated FGF23 and decreased serum 1,25$(OH)_2$D induces activation of RAAS, while RAAS decreases klotho expression, causing vascular calcification

On the other hand, FGF23 decreases phosphate reabsorption in the kidneys and intestines via down-regulation of the cotransporters NaPi-IIa and NaPi-IIc in the proximal tubule, and NaPi-IIb in the intestinal brush-border membrane vesicles. This protects against development of phosphate-induced atherosclerosis and particularly vascular calcification

ACE angiotensin-converting enzyme, *FGF23*, fibroblast growth factor 23, *NaPi* sodium dependent inorganic phosphate, *RAAS* renin-angiotensin-aldosterone system, *25-OH-D* 25-hydroxyvitamin D, *1,25$(OH)_2$D* 1,25-dihydroxyvitamin D, *24,25$(OH)_2$D* 24,25-dihydroxyvitamin D

renal bone disease and vascular calcification, leading to increased cardiovascular morbidity and mortality [29, 34, 39]. Among the various abnormalities related to CKD-MBD, phosphate load has been hypothesized as an initial causative factor [10, 27]. Since FGF23 regulates phosphate metabolism, it is considered to be a predictor of cardiovascular outcome in CKD patients [27]. Findings supportive of that view include the association of elevated FGF23 level with vascular calcification [28, 57], left ventricular hypertrophy (LVH) [11, 16], and increased arterial stiffness and endothelial dysfunction [21, 37], leading to increased mortality in patients with CKD and end-stage kidney disease (ESKD) [12, 15, 24, 26]. Of importance, even in non-CKD subjects, elevated FGF23 levels have been shown to be associated with LVH [51], endothelial function [37], and increased mortality [2, 45, 56] (Fig. 6.2).

In addition, animal experiment results suggest that FGF23 directly stimulates the renin-angiotensin-aldosterone system (RAAS) by suppressing angiotensin-converting enzyme (ACE) expression in the kidneys, independent of other abnormalities related to mineral and bone disorders [8] (Fig. 6.1). Activation of RAAS has been linked with numerous adverse consequences, such as hypertension, endothelial dysfunction, progression of atherosclerosis, and diabetic nephropathy [46] (Fig. 6.2). Indeed, FGF23-mediated activation of local RASS in the heart was reported to promote cardiac hypertrophy and fibrosis [3], while activation of RAAS has also been found to reduce the expression of klotho in the kidneys [9]. Thus, the effect of FGF23 on RAAS may be a potential cause of various adverse outcomes.

Controversy remains regarding whether FGF23 is a contributor to vascular calcification. This hormone helps to maintain phosphate levels within a normal range in response to phosphate overload, and we previously reported that oral phosphate loading significantly increases serum

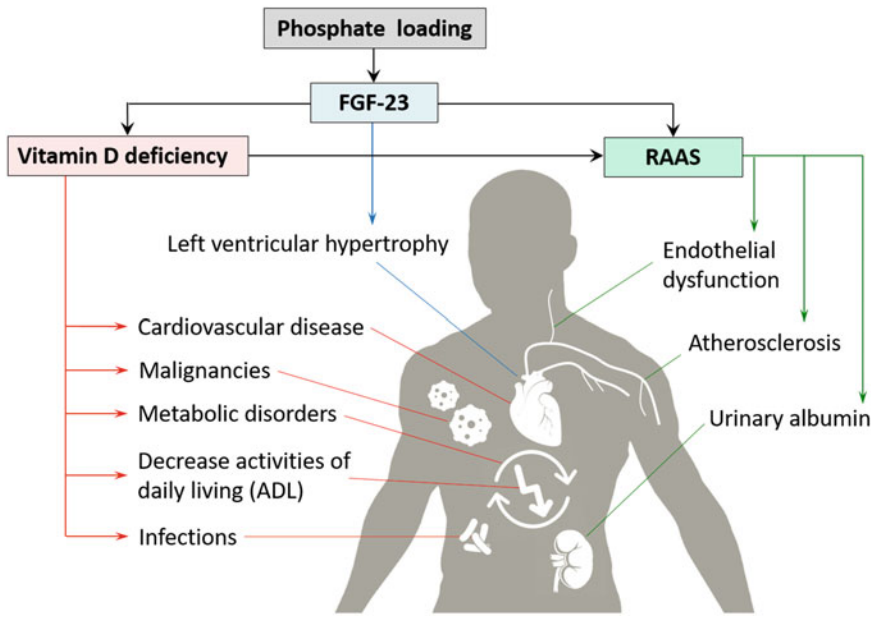

Fig. 6.2 Putative mechanisms of effects of elevated FGF23 in non-CKD subjects
Elevated FGF23 alone, decreased $1,25(OH)_2D$, and activated RAAS are each associated with numerous adverse outcomes, leading to high mortality

ACE angiotensin-converting enzyme, *ADL* activities of daily living, *CVD* cardiovascular disease, *FGF23* fibroblast growth factor 23, *P* phosphate, *RAAS* renin-angiotensin-aldosterone system

FGF23 and parathyroid hormone (PTH) to attenuate an increased serum phosphate level [60]. In patients with type 2 diabetes mellitus (DM), impaired bone formation resulting from osteoblast/osteocyte deficit is the main feature of DM bone abnormalities. Our prior study found impaired incremental responses of serum FGF23 and PTH in DM patients, which was in contrast to their increases in non-DM subjects. As a result, a significant rise in serum phosphate is observed in type 2 DM but not non-DM patients, clearly indicating the protective effect of FGF23 against the increase of serum phosphate after oral phosphate load in non-DM individuals, which protects against development of phosphate-induced atherosclerosis and particularly vascular calcification [7] (Fig. 6.1). Furthermore, a cohort study that included 1501 patients revealed that baseline plasma FGF23 level was not associated with the severity of calcium (Ca) content in the coronary artery [49]. Serum FGF23 level by itself may be a surrogate marker for vascular calcification in CKD patients [23], because it reflects phosphate load. It is therefore considered that increased serum FGF23 might serve as an important indicator of adverse outcomes in CKD as well as non-CKD patients.

6.3 Significance of Inhibition of Vitamin D Activation by FGF23 in Non-CKD Individuals

Vitamin D, specifically biologically active 1,25 $(OH)_2D$, plays a key role in bone and mineral metabolism. Vitamin D deficiency, defined by serum 25-OH-D < 20 ng/mL, is estimated to occur in 36–57% of the general population [18] and 50–86% of CKD patients [33]. It is increasingly recognized that vitamin D insufficiency (25-OH-D:20-29 ng/mL), in addition to vitamin D deficiency, is a risk factor for various diseases, such as diabetes, infections, cardiovascular disease, and cancer [19, 20]. Furthermore, harmful effects of vitamin D deficiency have recently been recognized even in the general population, based on an association with increased risk of all-cause mortality and reduced activities of daily living [36, 47, 55, 64] (Fig. 6.2). Together with the notion that vitamin D might be a primitive steroid-like hormone, because the nuclear vitamin D receptor belongs to the steroid receptor superfamily, these observations support the importance of vitamin D.

6.4 Potential Role of FGF23 in Regulation of Vitamin D Metabolism in Non-CKD Individuals

Serum FGF23 level has been reported to be increased even at an early stage of CKD in patients with an eGFR $\geq$60 mL/min/1.73 m^2 [24]. While both FGF23 and PTH have potent phosphaturic activities, they have opposite effects in terms of vitamin D metabolism, as FGF23 inhibits [6, 52] and PTH stimulates [5, 14, 17] vitamin D activation from 25-OH-D to 1,25 $(OH)_2D$. To examine the physiological role of FGF23 in regulation of vitamin D metabolism and thus phosphate metabolism in non-CKD patients (eGFR$\geq$60 mL/min/1.73 m^2), we measured serum FGF23 along with three vitamin D metabolites, 25-OH-D, 1,25$(OH)_2D$, and 24,25 $(OH)_2D$, in subjects with eGFR $\geq$60 mL/min/1.73 m^2 [40]. To avoid confounding factors, subjects with DM as well as those taking corticosteroids, vitamin D, phosphate-binder, supplementary Ca, estrogen, or thyroid hormone were excluded from the study. The results showed that serum FGF23 was significantly and inversely correlated with serum 1,25$(OH)_2D$ and 1,25 $(OH)_2D$/25-OH-D ratio, putative parameters for CYP27B1. Additionally, in multiple regression analysis that included FGF23, PTH, and eGFR as independent variables, FGF23 emerged as a significant factor showing an independent negative association with 1,25$(OH)_2D$ and 1,25 $(OH)_2D$/25-OH-D ratio. These results suggested that FGF23 inhibits CYP27B1, leading to decreased serum 1,25$(OH)_2D$ even in non-CKD individuals. In addition, FGF23 was found to be significantly and independently associated in a positive manner with 24,25$(OH)_2D$ /1,25 $(OH)_2D$ ratio. Thus, based on the findings

showing that FGF23 plays an important role in vitamin D catabolism in non-CKD individuals, it was speculated that phosphate overload exists even in non-CKD patients, and that an increase in serum FGF23 acts directly to attenuate increased serum phosphate, and indirectly to stimulate phosphaturia and inhibit activation of vitamin D, resulting in suppression of intestinal phosphate absorption, based on the effect of 1,25 $(OH)_2D$ to stimulate Na-Pi co-transport in intestinal brush-border membrane vesicles [38, 50, 59].

6.5 Potential Role of FGF23 in Regulation of Phosphate Metabolism in Non-CKD Individuals

A recent cohort study found that serum FGF23, but not phosphate, was negatively correlated with eGFR in non-CKD postmenopausal female subjects with eGFR $\geq$60 mL/min/1.73 m^2 [43]. Serum phosphate levels begin to increase at a later stage of CKD [24], thus it is probable that an increase in FGF23 acts to protect against development of hyperphosphatemia, as noted in our previous study [60]. Serum FGF23 levels have been shown to be not correlated with serum phosphate in subjects with normal kidney function [35, 40]. Furthermore, we found a significant and independent association of serum FGF23 with urinary albumin-to-creatinine excretion in non-CKD postmenopausal female subjects with eGFR $\geq$60 mL/min/1.73 m^2 [43]. In CKD mice, transcriptome analysis revealed that FGF23 regulates several genes associated with kidney injuries, including Neutrophil Gelatinase-Associated Lipocalin (NGAL), and carbonic anhydrase 14 (Car 14) [8]. Together, these observations suggest that FGF23 exerts a direct effect to cause kidney damage. Alternatively, phosphate overload, as reflected by increased serum FGF23, might induce kidney injury, as noted in previous reports [1, 41]. Therefore, it is considered that FGF23 may play an important role in regulation of phosphate metabolism, based on its effect on vitamin D metabolism as well as the established role of PTH in individuals with normal kidney function.

6.6 Vitamin D Metabolism and FGF23-Klotho Axis in Non-CKD Individuals

Klotho, a 130-kDa transmembrane β-glucuronidase that catalyzes the hydrolysis of steroid β-glucuronides [32], is required for FGF23 to activate FGFRs and their downstream molecules [58]. Since the klotho/FGFR complex binds to FGF23 with higher affinity than either alone, FGF23 exerts its biological effects via activation of FGFR in a klotho-dependent manner [58]. Interestingly, it has been demonstrated that klotho deficiency causes vascular calcification in CKD patients [22] and that vitamin D deficiency is associated with RAAS activation [44]. In addition, angiotensin II has been found to negatively regulate renal expression of klotho [61, 63] (Fig. 6.1). Thus, activation of RAAS and klotho deficiency by the effects of FGF23 on vitamin D metabolism may lead to vascular calcification in CKD patients as well as non-CKD individuals.

6.7 Future Prospects

Therapeutic strategies to control serum FGF23 level and improve adverse outcomes in non-CKD patients remain to be clarified. Clinical trials will be needed to examine the effects of therapeutic intervention, such as vitamin D, phosphate binders, or dietary phosphate restriction, on serumFGF23 levels, and also to determine how such changes impact adverse outcomes in CKD as well as non-CKD cases. Restriction of dietary phosphate, particularly phosphate additives, may be important, because a large amount of rapidly absorbable inorganic phosphate can be found in various food additives and preservatives [42]. A

small-scale short-term pilot study of CKD stage 3–4 patients showed a significant decrease in FGF23 in those who consumed a diet that included the combination of lanthanum carbonate and 900 mg phosphate, though that level was not significantly decreased in those with a lanthanum carbonate and *ad libitum* diet [25]. Another report summarized the results of several interventional clinical trials that used phosphate binders and/or a phosphate restriction diet for examination of FGF23 levels, though most of those studies had a relatively short duration [4]. Long-term large-scale clinical trials focused on phosphate restriction in healthy and non-CKD subjects are needed. Administration of phosphate binders, such as lanthanum carbonate and sevelamer hydrochloride, has been reported to lower serum FGF23 levels in patients with CKD stage 3 and ESKD, respectively [13, 30]. Thus, it may be possible to decrease the FGF23 level in serum and improve adverse outcomes with phosphate binders in patients without CKD.

FGF23 has direct and/or indirect effects outside of the field of MBD, which suggests additional possibilities for treatment of adverse outcomes associated with an elevated level. Since FGF23 stimulates RAAS, administration of ACE inhibitors, angiotensin-receptor blockers, and aldosterone receptor antagonists may be justified.

6.8 Conclusion

Even in individuals with normal kidney function, FGF23 plays an important role in regard to vitamin D metabolism. An elevated FGF23 level and vitamin D deficiency are associated with various adverse events. Therefore, it is possible that an increase in vitamin D and decrease in FGF23 could be instrumental in engendering adverse outcomes, even in non-CKD individuals.

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Phosphate and Cellular Senescence

7

Ming Chang Hu and Orson W. Moe

Abstract

Cellular senescence is one type of permeant arrest of cell growth and one of increasingly recognized contributor to aging and age-associated disease. High phosphate and low Klotho individually and synergistically lead to age-related degeneration in multiple organs. Substantial evidence supports the causality of high phosphate in cellular senescence, and potential contribution to human aging, cancer, cardiovascular, kidney, neurodegenerative, and musculoskeletal diseases. Phosphate can induce cellular senescence both by direct phosphotoxicity, and indirectly through downregulation of Klotho and upregulation of plasminogen activator inhibitor-1. Restriction of dietary phosphate intake and blockage of intestinal absorption of phosphate help suppress cellular senescence. Supplementation of Klotho protein, cellular senescence inhibitor, and removal of senescent cells with senolytic agents are potential novel strategies to attenuate phosphate-induced cellular senescence, retard aging, and ameliorate age-associated, and phosphate-induced disorders.

Keywords

Aging · Age-associated disease · Cellular senescence · Fibrosis · Klotho · Phosphate · Phosphorus · Phosphotoxicity · Plasminogen activator inhibitor-1 · p16 · p21

7.1 Introduction

Phosphorus, the element of phosphate biologic moiety, is the sixth most abundant element after hydrogen, oxygen, carbon, nitrogen, and calcium in the human body. While the term "phosphorus" is used in conventional clinical laboratory reporting, there is no elemental phosphorus in mammalian biology, the term phosphate (inorganic or organic) will be used despite the accepted clinical parlance. In the human body, 85% of phosphate is in bone and teeth as hydroxyapatite, 14% is located intracellularly as various organic phosphate compounds and some inorganic phosphate; and only 1% extracellularly [72, 123, 168]. Of the 1% located in extracellular

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space, 20% is protein bound [72, 123, 168] and the inorganic phosphate is distributed as pyrophosphate (minor) and orthophosphate (major) in various valences determined by the plasma pH.

Serum phosphate serves as an exchange pool among various phosphate-containing and phosphate-regulating organs [72, 123, 168]. Phosphate homeostasis is principally maintained by gut absorption and urine phosphate excretion which is controlled by a complex but tightly and efficiently regulated network consisting of several calciophosphotropic hormones including parathyroid hormone, 1,25-$(OH)_2$ vitamin D, fibroblast growth factor (FGF)-23, and Klotho [65, 72].

The terminology in this field can be confusing and necessitates some front-end clarification. *Hyperphosphatemia* refers to elevated serum or plasma phosphate level (measured as the mass of phosphorus constituent per volume) (Fig. 7.1). It is a "state" and may or may not be associated with disease. *Phosphate loading* refers to an amount of phosphate intake into a system, with overload being a flux that is higher than what is necessary for health. This may or may not be associated with hyperphosphatemia or ill effects. *Positive balance* occurs when intake exceed excretion at the organism level. *Phosphotoxicity* refers to a cellular, organ, or whole organism condition where excess phosphate (extracellular or intracellular) begets an undesirable phenotype- one that predisposes to or actually constitute disease.

A positive imbalance can result from insufficient renal phosphate excretion and/or excessive phosphate intake, and is emerging as a novel detrimental contributor to aging and age-associated disease [25, 94–96, 123, 131, 132]. As stated above, the ill effects of excessive phosphate is collectively termed "phosphotoxicity" with no designation of the cause or specific resultant phenotypes. Aging is a progressive and inevitable process with multi-organ deterioration. Aging is triggered and exacerbated by numerous factors including genetic, and epigenetic factors. Among those identified factors such as diabetes, hypertension and others, the role of phosphate in cellular senescence is incompletely elucidated [147].

Cellular senescence is a type of permanent arrest of cell growth, which was initially identified as a defense mechanism to inhibit tumorigenesis and metastasis [17, 26, 37, 109, 124, 154]. Similar to autophagy and apoptosis, an appropriate cellular senescence activity is therefore required to maintain tissue function and regeneration after tissue damage [180]. But chronic and severe cellular senescence also contributes to aging and age-associated diseases.

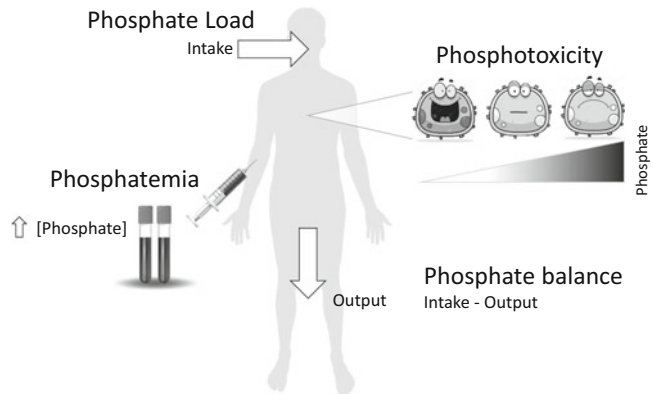


Fig. 7.1 Concepts about phosphate metabolism and balance
Hyperphosphatemia refers to elevated levels of serum or plasma phosphate, which may or may not be associated with disease. External phosphate balance is the amount of phosphate intake minus urinary phosphate excretion. The increased flux of phosphate through the organism may or may not be associated with hyperphosphatemia or ill effects. Phosphotoxicity refers to a state that excess phosphate (extracellular or intracellular) causes an undesirable phenotype at a cellular, organ, or whole organism level

Aging is triggered, driven, and promoted by multiple genetic and acquired detrimental factors.

The cellular and molecular mechanisms whereby phosphate accelerates aging, and excerbates age-associated diseases are complex and multifactorial [68, 122, 123, 135]. High phosphate can reduce autophagy [147] and Klotho [68, 176], induce cell apoptosis [36, 101], and activate cellular senescence [106]. Klotho was discovered in 1997 by Kuro-o and colleagues. Klotho was originally identified as an anti-aging protein and later on was found to directly inhibit cellular senescence, reduce serum phosphate, and prevent phosphate-induced cellular senescence in a tripartite relationship [106]. Therefore, abnormal cellular senescence is attributable in part to the deleterious actions of Klotho deficiency from phosphotoxicity [44, 139] and not necessarily phosphate *per se*.

In this monograph, we will first update current understanding of cellular senescence in aging and age-associated disease, review the effect of phosphate on the activation of cellular senescence and to aging and human disease, and finally discuss the potential of targeting cellular senescence in prevention and treatment of phosphotoxicity.

7.2 Cellular Senescence

7.2.1 Cellular Senescence and Its Signaling Pathways

Cellular senescence was first described by Hayflick and Moorhead when they established an immortal cell line [61]. They found that the cells assume a flattened and enlarged morphology, ceased to proliferate without any responses to growth factors, but were still alive in cultured dishes. Those cells are called senescent cells because they have irreversible growth arrest, which is different from quiescent cells, because quiescent cells are still able to re-enter the cell cycle.

Senescent cells have remarkable morphological and metabolic changes. Those changes include reorganizing chromatin, reprogramming gene expression, and endlessly producing many pro-inflammatory and pro-fibrotic growth factors and cytokines, a phenotype called the senescence-associated secretory phenotype (SASP) [29, 45, 162]. Cellular senescence conferred both protective and deleterious effects depending on the physiological and pathophysiologic scenarios. Although cellular senescence likely functions as a defense mechanism to inhibit malignant transformation of damaged cells, persistent and chronic senescence may promote aging and age-associated pathologies including tissue degeneration, cell dysfunction, and chronic inflammation in the tissues [17, 26, 29, 37, 38, 45, 66, 82, 92, 108, 109, 124, 143, 151, 154, 162].

Cellular senescence is activated and maintained by at least 2 cell signaling pathway: p53/p21 and $p16^{Ink4a}$/retinoblastoma-1 (RB-1). Both are tumor suppressive proteins. These two pathways are activated by DNA damage, reactive metabolites, oncogenic mutations, high mitogen signals, proteotoxic stress, and other yet-to-be-identified factors [17, 18, 26, 66, 82, 143, 154]. Furthermore, cellular senescence is also induced by the perturbation of metabolism including abnormal phosphate metabolism, which may accelerate age-related phenotypes [17, 26, 66, 106, 143, 154].

7.2.2 Senescence-Associated Secretory Phenotype

Senescent cells have a profound phenotype of endless production and secretion of proinflammatory and profibrotic growth factors referred as SASP [29, 45]. The number of senescent cells is widely variable from <1% to >15% depending on physiological and pathophysiologic context, species, and tissue origins, and cell origins [18]. However, a few senescent cells are able to initiate a detrimental positive circle through spreading senescence signal to neighbor cells [45] and to propagate pathologic actions in whole tissue and organ through SASP and even exert systemic effects [125].

Chronic inflammation induced by cellular senescence is the result of unrestrained SASP in the tissue and organ [79]. Obesity-induced

senescent cells can maintain chronic and low-grade inflammation in the pancreas [125]. Therefore, the persistence of senescent cells can promote inflammation and tissue disruption present in chronic disease [124]. Cellular senescence occurs in many types of cells including endocrine cells, endothelial cells, epithelial cells, inflammatory cells, and even stem cells in almost every tissue and organ [18, 26, 66, 154, 157].

7.2.3 Cellular Senescence Effect on Aging

Aging is a continuous and progressive sequence of changes in any organisms that leads to biological dysfunctions and morphological tissue destruction, and causes degenerative pathology [17, 26, 38]. Cellular senescence is present throughout lifetime, occurring in embryogenesis and activated to protect cells against a variety of insults throughout life [17]. With time, the number of senescent cells increases and when coupled with depletion of stem cells and progenitor cells in tissue, causes a decline in tissue regeneration [170].

7.2.4 Cellular Senescence in Human Disease

Cellular senescence has been implicated in many age-associated degenerative phenotypes (Fig. 7.2). In most cases, senescent cells drive chronic degeneration mainly through the secretion of proinflammatory and profibrotic cytokines, growth factors, and proteinases via SASP, inducing inflammation, disrupting tissue structures, and leading to fibrosis. High cellular senescence also causes stem cell depletion [28, 55, 81] and impairs tissue regeneration [13].

7.2.4.1 Tumorigenesis

Cellular senescence was initially proposed to serve as a tumor suppressive mechanism since its discovery in the 1960s [45], but it is now demonstrated that highly active cellular senescence actually drives tumorigenesis.

Cells carrying activated oncogenes such as p53 [58, 90], p21 [1, 91, 174] and p16 [140, 185, 186] were shown to either die through apoptosis or enter stable cell cycle arrest that defines cellular senescence; either way, they may be prohibited from exerting further harm. More importantly, the implication of those oncogenes in activation of cellular senescence and in tumor development have been confirmed both in animal models and in human samples [45, 169]. In addition to tumor initiation, SASP can induce extracellular matrix remodeling through release of numerous inflammatory and growth factors [45] to promote metastasis and induce resistance to therapy [98]. Therefore, cellular senescence is a novel therapeutic target for cancer treatment.

7.2.4.2 Chronic Non-neoplastic Diseases

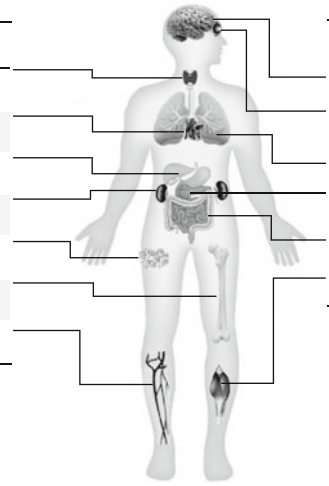
The morbidity and mortality of many chronic degenerative diseases increases with age, including Alzheimer's disease, Parkinson's diseases, Down Syndrome [9], macular degeneration [104], pulmonary disease [156], cardiovascular disease [27], kidney disease [154], liver and digestive disease [6, 12, 46, 50, 87, 88, 164, 193], saropenia [119, 155, 191], osteoporosis and osteoarthritis [75], metabolic disorders such as diabetes [87, 88, 125], autoimmune disease and rheumatic disease [75], and other age-related pathologies, even infectious disease [7, 56, 73, 184] (Fig. 7.2).

7.3 Phosphotoxicity in Aging and Age-Associated Disease

7.3.1 Phosphate Effect on Aging and Klotho

In patients after myocardial infarction, high baseline serum phosphate was associated with high all-cause death after 60 months of follow-up [166]. Since this paper, there are many studies describing the link between serum phosphate and cardiovascular disease in healthy human beings in

Cellular senescence	Phosphotoxicity	Organ
	√	Parathyroid
√	√	Heart
√	√	Liver
√	√	Kidney
		Tumor
		Bone
		Vessel



Organ	Cellular senescence	Phosphotoxicity
CNS	√	√
Eye	√	
Lung	√	√
Pancreas	√	√
Intestines	√	
Muscle	√	√

Fig. 7.2 Cellular senescence and phosphate toxicity in human disease
A summary of human acute and chronic diseases which are confirmed or proposed to be associated with high cellular senescence and phosphotoxicity

the Framingham Offspring Study [35], the Coronary Artery Risk Development Study [48], as well as intermediate phenotypes of vascular and endothelial dysfunction [74, 150], vascular and valvular calcification [3], and greater left ventricular mass [189]. The serum levels of phosphate negatively correlate with anticipated lifespan in animals and human beings [93, 96]. The observational studies in human populations are very compelling but do not provide proof of causality of high phosphate and aging.

Direct evidence to support the detrimental effect of high phosphate on aging comes from animal experiments. The correction of high serum phosphate with low phosphate diet or genetic deletion of sodium-dependent phosphate co-transporter-2a (NaP-2a) in kidney tubules leading to urinary phosphate leak effectively prolongs lifespan and rescues almost all phenotypes in Klotho deficient mice [122, 133]. More importantly, feeding high phosphate food abolishes that benefit conferred by NaPi-2a deletion and brings the premature aging phenotypes back. The longer lifespan associated with high autophagy in mice is at least in part mediated by higher urinary phosphate excretion due to reduced sodium-dependent phosphate cotransporters type II in the kidney [147]. The long-term challenge with high dietary phosphate dramatically abolished the beneficial effects of high autophagy activity [147], further supporting the role of high phosphate in promoting aging.

Klotho is also known as an inhibitor of cellular senescence [16, 20, 102, 105, 106, 126, 129]. High phosphate significantly decreases kidney and circulating Klotho [67–69, 71, 106, 117, 147]. However, this finding has not been reproduced in humans consuming a high phosphate diet [114, 141]. It is conceivable that high phosphate-induced short lifespan is multi-factorial and results at least in part from the reduction of Klotho production. Klotho deficiency may be one of molecular mechanisms behind high phosphate effect on cellular senescence. Moreover, higher levels of baseline serum phosphate are also associated senescence and aging.

7.3.2 Phosphotoxicity and Human Disease

Although phosphate is essential for body structure and function, excessive accumulation of phosphate in the body due to impaired phosphate homeostasis can cause functional and morphologic changes in almost every organ/tissue and

system (Fig. 7.2). All of the chronic diseases associated with high phosphate discussed below are more prominent in the senior population. Interestingly, these diseases are important components in aging which are also triggered and driven by over active cellular senescence.

7.3.2.1 Cancer

Phosphotoxicity has been proposed to promote tumorigenesis (Fig. 7.2). Epidemiologic findings show association between high incidence of some types of cancers and abnormal phosphate metabolism [15, 181], and between high levels of serum phosphate and low survival rate of several types of cancer [167, 190]. Tumor cells store more intracellular phosphate through higher activity of sodium-dependent phosphate transport han in normal cells [41]. High phosphate can act as a mitogenic factor to induce tumor cell proliferation and activate tumor cell growth through induction of tumorigenic signaling [15]. As cellular senescence is one contributor to tumorigenesis, the direct effect of high phosphate on induction of cancer development and metastasis needs to be explored. Furthermore, Klotho is a tumor suppressor through multiple cellular signaling pathways [2, 24, 39, 136, 160, 182], and phosphate-indued reduction in Klotho can also contribute to tumorigenesis.

7.3.2.2 Cardiovascular Disease

Clinical observational studies showed that high serum phosphate is identified as an independent contributor to cardiovascular morbidity and mortality in both chronic kidney disease (CKD) patients and in non-kidney disease subjects [40, 47, 80, 86, 95, 115, 135, 142, 152, 166] (Fig. 7.2) There is also a strong correlation between high serum phosphate and high morbidity and mortality of cardiovascular events such as cardiomyopathy, vascular calcification, arterial stiffness, and hypertension, in dialysis and non-dialysis CKD and experimental animals as well [5, 32, 68, 71, 80, 83, 107, 111, 115, 175]. Furthermore, higher serum phosphate at baseline is related to an increased risk of *de novel* onset of heart failure, myocardial infarction, and other cardiovascular events [166]. Therefore, high phosphate is an indicator and detrimental contributor of cardiovascular disease.

7.3.2.3 Kidney Disease

It has been known for long time that high phosphate contributes to acute kidney damage after large acute phosphate loading in short-term, and leads to kidney fibrosis in normal animals, and promotes progression of CKD animals after long-term phosphate loading [68, 69, 86, 106, 115, 137, 144]. High phosphate is closely associated with complications including cardiovascular disease and metabolic bone disease in CKD. Phosphate binders effectively decrease cellular senescence in vascular smooth muscle cells induced by high phosphate and vascular calcification in uremic rodent model [188]. Phosphotoxicity in the kidney is attributable to high phosphate-induced cell apoptosis, decreased autophagy, and activated cellular senescence [106].

7.3.2.4 Metabolism

High dietary phosphate loading causes abnormal mineral metabolism [22, 67, 94, 114, 141, 152] and disturbed fatty acid metabolism [128] in humans and experimental animals. Disturbed metabolism can be one of underlying mechanisms for phosphotoxicity in some tissues and organs.

7.3.2.5 Other Diseases

Hyperphosphatemia is also thought to lead to damage in other organs and tissues (Fig. 7.2) including the lung [23, 76, 78], bone [22, 60, 152], skeletal muscle [15, 128], diabetes [14], liver injury [97], and impaired brain development [77]. Patients with inflammatory disease and tumor in central nervous system have significantly higher levels of phosphate in cerebrospinal fluid; and ones with an intracranial hemorrhage also have elevation of phosphate in cerebrospinal fluid [63].

It is estimated that nearly 1% of total body phosphate is stored within cells as well as in extracellular fluid. Currently, measuring serum phosphate level only estimates the overall status of phosphate in the body, but the levels of serum phosphate may not be a good indicator to always reflect total amount of phosphate in the body.

Sometimes, the early or mild phosphate toxicity might not be associated with detectably high levels of serum phosphate [123]. In fact, several clinical observational studies showed that a very small increase in serum phosphate within normal range of clinical laboratories (3.4–4.5 mg/dl or1.12–1.45 mmol/L), is significantly associated with higher cardiovascular events in non-kidney disease subjects [35, 48, 166]. Therefore, the measurement of tissue or intracellular phosphate may provide more precise and timely status of phosphate metabolism.

7.4 Stimulation of Cellular Senescence by Phosphate

Phosphate-induced cellular senescence is well conserved across species from plants to vertebrates. This effect is found in plant leaf [31, 153]; prokaryotes [33], and cells and tissues in mammals [106, 188]. But the cellular and molecular mechanisms whereby phosphate activates cellular senescence are largely incompletely illustrated. The experiments performed in authors' laboratories showed both direct and indirect effects of high phosphate on activation of cellular senescence in the kidney [106, 188].

7.4.1 Direct Stimulation of Cellular Senescence

After one week of high dietary phosphate treatment (2.0% phosphate w:w), the mice had modest increase in plasma phosphate and nearly normal plasma Klotho. However, the mice had higher p16/p21 expression in the kidneys. Those results imply that high phosphate induces senescence in the kidney which is independent of plasma Klotho (Fig. 7.3). High phosphate (2.0 mM) media induce cellular senescence in cultured kidney cell line without endogenous Klotho expression, supporting that in the absence of Klotho, high phosphate can directly stimulate cellular senescence through p16/p21 signaling pathway [106]. However, whether the phosphate effect on cellular senescence is mediated through an elevation of intracellular phosphate via sodium-dependent phosphate co-transport [100, 112, 173, 177, 187] and/or through other phosphate transport-independent signaling pathway [11] needs to be explored.

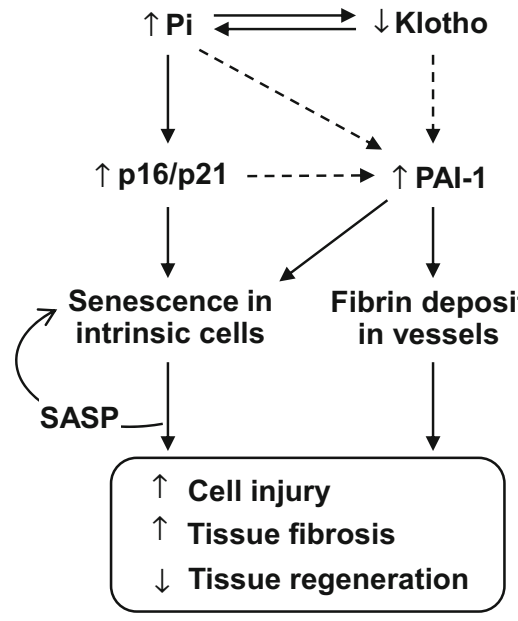


Fig. 7.3 Potential cellular mechanisms of phosphate-induced cellular senescence
High phosphate upregulates p16 and p21 and induces cellular senescence (early phase). High phosphate also increases PAI-1 expression and reduces Klotho protein production (late phase). It is unclear whether high phosphate upregulates PAI-1 in a dependent or independent manner of higher p16/p21 and/or lower Klotho (dash line). Moreover, high PAI-1 activity initiates and accelerates fibrin deposits in vessels, activates cellular senescence, and induces tissue injury and fibrosis. High phosphate-induced Klotho deficiency increases serum phosphate which further reduces Klotho production. Cellular senescence increases senescent cells through activation of a vicious cycle of secreted pro-inflammatory and pro-fibrotic growth factors, and proteases (SASP). Chronic senescence activation in intrinsic cells and vessels, and fibrin deposits in vessels initiate and/or promote fibrosis, hence destructing tissue structure. Senescence in stem cells and progenitor cells impair tissue regeneration

7.4.2 Indirect Stimulation of Cellular Senescence

7.4.2.1 Downregulation of Klotho

It is documented in rodents that high dietary phosphate loading reduces kidney and circulating Klotho [51, 67, 68, 117, 122, 130, 147, 148, 179, 192] although the cellular and molecular mechanism of how phosphate inhibits Klotho is still elusive.

Klotho protein was been shown to suppress cellular senescence (Fig. 7.3) in the kidney of several rodent models including CKD, glomerulonephritis [59], genetic Klotho deficiency [43], and in several cultured cell lines. Cellular senescence induced Klotho deficiency is associated with high Wnt signaling activity [103]. Wnt is a potent inducer of cellular senescence [103]. It has been shown that intracellular, but not extracellular Klotho protein interplays with retinoic-acid-inducible gene-I to suppress SASP and consequently inhibit inflammation [102]. But, other studies confirmed that extracellular domain of Klotho protein is still able to inhibit cellular senescence [24, 92, 106], which provides therapeutic potential to inhibit phosphate-induced cellular senescence with soluble Klotho protein.

7.4.2.2 Upregulation of Plasminogen Activator Inhibitor Type-1

Plasminogen activator inhibitor type-1 (PAI-1) belongs to the superfamily of serine-protease inhibitors, and inhibits both tissue-type and urinary-type plasminogen activators whose primary function is to activate plasminogen. So PAI-1 participates in control of fibrinolysis [21, 64]. The *in vivo* experiments showed that high PAI-1 expression suppresses fibrinolysis, and results in the massive fibrin deposition in the tissue [21, 106]. PAI-1 also regulates endothelial cell replication and angiogenesis [34, 62]. Emerging evidences showed that PAI-1 also makes major contribution to other pathologic processes independently from modulation of fibrinolysis process. PAI-1 is now considered as an inducer of cellular senescence through unclarified mechanism [43, 44, 159, 172].

High dietary phosphate upregulates PAI-1 expression in the kidney of mice *in vivo* and high phosphate media increases PAI-1 in cultured kidney cells *in vitro* [106]. In addition, Klotho deficiency also induces PAI-1 over-expression in the kidney [44, 159]. Therefore, high phosphate works synergistically with Klotho and PAI-1 to amplify cellular senescence.

7.4.2.3 Activation of SASP

High phosphate induces the secretion of pro-inflammatory growth factors such as TNFα and TGFβ, and promotes oxidative stress in experimental animals *in vivo* and in cultured cells *in vitro* [68, 106, 110, 116, 165, 194]. These factors released from damaged tissues or cells would reduce Klotho expression in the kidney in a paracrine or endocrine manner. The experiments conducted in the authors' laboratories showed that senescence markers are first elevated, followed by higher levels of oxidation, SASP, and lower Klotho protein expression. It is conceivable that high phosphate induces cellular senescence, activates oxidative stress, stimulates SASP, and decreases Klotho protein expression [106]. This hypothesis is about to be confirmed.

Taken together, high phosphate activates cellular senescence through upregulating p16/p21 and/or downregulating Klotho production. Klotho deficiency may secondarily induce PAI-1 signaling, which induces fibrin deposition in the vessel, causes tissue fibrosis, and enhances cellular senescence [43, 89] (Fig. 7.3).

7.5 Cellular Senescence: A Novel Downstream Target for the Treatment of Phosphotoxicity

Conventional prevention or treatment of phosphotoxicity consists of tilting the balance between phosphate intake and excretion towards the negative side. Equipped with the knowledge that cellular senescence is one downstream effector of phosphotoxicity, one opens up a novel array of therapeutic options.

7.5.1 Control of Dietary Phosphate Intake and Reduction of Serum Phosphate

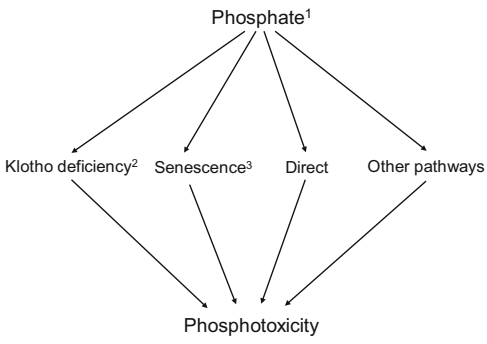
Controlling phosphate absorption is usually prescribed for patients with CKD by restriction of phosphate intake and administration of phosphate binders (Fig. 7.4).

Reducing dietary phosphate intake is a simple and easy strategy to keep normal phosphate homeostasis. While reduction in total phosphate ingestion can be challenging, the form of digested phosphate in food can be modified because bioavailability of different kinds of ingestible phosphate differ tremendously [10, 145]. Inorganic phosphate, which is rare in nature, is much more bioavailable than organic phosphate present in living matter. The most common storage form of phosphorus in plant is phytate, which is very poorly absorbed as humans lack phytases to release the phosphate from the inositol ring [134, 171, 178]. A plant-based diet is able to provide enough dietary protein requirement without increasing risk of high phosphate intake for CKD subjects [53] and lower risk of severity of CKD-mineral bone disorder compared to animal-based protein [113]. Most importantly, one needs to account for the added inorganic phosphate into food mainly from food processing. Almost all processed foods contain phosphate additives [10, 42]. Inorganic phosphate in the preservatives is very readily absorbed across the digestive tract to enter into blood circulation.

Phosphate binders reduce bioavailability of dietary phosphate and minimize phosphate absorption from the intestine [10, 19, 30, 49, 142]. The inhibition of sodium-dependent phosphate co-transporter type 2b, which controls transcellular phosphate absorption and secondarily reduces paracellular transport from gut [85] can complement low phosphate diet and phosphate binders [30, 57] to enhance the control of phosphate balance. It has been shown that phosphate binder effectively blocks cellular senescence activation by high phosphate in vascular smooth muscle cells and vascular calcification in CKD rats [188].

7.5.2 Augmentation of Phosphate Excretion from the Kidney

Klotho is shown to induce phosphaturia by inhibiting sodium-dependent phosphate co-transporters in the kidney tubules through FGF23 dependent pathway via FGF23-FGFR-



1. Reduce phosphate load	Decrease intestinal absorption • Decrease total dietary phosphate • Decrease inorganic phosphate in diet • Phosphate binders • Inhibition of transcellular phosphate transport • Inhibition of paracellular phosphate transport
2. Correct Klotho deficiency	Increase endogenous production • Treat kidney disease • Vitamin D • Off-label use of approved drugs - PPARγ agonists - Antioxidants - RAAS blockade
	Exogenous Klotho • Recombinant protein • Various forms of gene therapy
3. Target cellular senescence	Suppression of senescence induction Removal of senescent cells Interruption of SASP

Fig. 7.4 Potential strategies to treat phosphotoxicity Attenuation of phosphotoxicity can be achieved through reducing phosphate load to the body (Strategy 1), correction of Klotho deficiency (Strategy 2), and direct target of cellular senescence (Strategy 3). Since phosphotoxicity is induced and mediated through multiple cellular and molecular mechanisms, direct inhibition of phosphate-associated intracellular signaling pathways, and modulation of other downstream biologic effectors such as autophagy, mitochondria, and oxidative stress would also participate in alleviation of phosphotoxicity

Klotho complex formation [54] and FGF23-independent pathway via Klotho's enzymatic function [70]. However, clinical utility remains to be confirmed.

7.5.3 Restoration of Plasma Klotho

Elevation of plasma Klotho (Fig. 7.4) to counteract Klotho deficiency that occurs in kidney disease and after high dietary phosphate loading represents a feasible strategy to reduce blood phosphate through induction of phosphaturia, protect kidney disease from chronic progression, and also directly suppress cellular senescence [69, 70, 106, 147].

7.5.3.1 Delivery of Klotho cDNA

One strategy which has been repeatedly approved to be successful to replete Klotho in the research animals is the use of virus, plasmid or minicircle-based vectors to deliver Klotho cDNA. Delivery of Klotho cDNA in rodent can improve kidney function, ameliorate endothelial function, and attenuate uremic cardiomyopathy in acute kidney injury, CKD, and hypertensive models [138, 149, 183, 195].

7.5.3.2 Administration of Recombinant Klotho Protein

Exogenous recombinant Klotho protein has been shown to have at least two effects: direct restoration of serum Klotho levels, and stimulation of endogenous Klotho production in the kidney [69, 70, 146, 148]. Administration of Klotho protein is the only practical and feasible method to elevate serum Klotho to precise and safe levels to date. While Klotho replacement therapy is successful in correction of Klotho deficiency and improvement of renal, cardiovascular, and other diseases in many experimental animals, its clinical application still faces certain hurdles. Obviously ample animal studies have provided solid and convincing evident to support the concept that Klotho protein replacement is far more effective than virus-based Klotho [120].

7.5.3.3 Disinhibition of Endogenous Klotho Production

Another potential strategy to increase serum Klotho is to re-activate or stimulate endogenous Klotho production in the kidney by reversing mechanisms which inhibit Klotho production and/or release of extracellular domain of membrane Klotho protein in diseased kidney or after exposure to dietary phosphate overloading. In experimental CKD rodents, vitamin D receptor agonist augmented serum Klotho levels and reduced the levels of serum phosphate, but the origin of increased soluble Klotho in serum and urine was not identified, indicating that an increase in shedding membrane Klotho may be the source [99]. Experimental study showed that 1,25-$(OH)_2$-vitamin D_3 increased Klotho expression in the kidney [118, 158]. Other medications that can potentially stimulate endogenous Klotho protein production in the kidney include androgen, angiotensin II receptor antagonists, PPAR-γ agonists, and statins [120, 127]. Off-label use of existing medications to pharmacologically activate or de-suppress endogenous Klotho protein production is a viable and safe option. However, to date, there is no clinical trials to test the efficacy of pharmacological interventions in correction of Klotho deficiency. It is also unknown whether these FDA-approved medication can increase endogenous Klotho production or stimulate release of extracellular domain of membrane Klotho in high phosphate-fed animals whose kidney has low Klotho expression is not known.

Another strategy to increase endogenous Klotho expression is epigenetic approach. It has been shown that DNA demethylating agents increase Klotho expression in non-Klotho expressing cells [8]. While theoretically feasible, the effect of modulation of methylation and acetylation of the Klotho gene promoter on upregulation of Klotho in the kidney needs further confirmation.

7.5.4 Direct Suppression of Senescence Signaling Pathway and Removal of Senescent Cells to Ameliorate Phosphotoxicity

Phosphotoxicity exerts its ill effects along multiple pathways, many of which are still not defined. However, among which is the promotion of cellular senescence. Because cellular senescence is characterized by replicative arrest, suppressed apoptosis, and typical secretory phenotype, blockage of cellular senescence activation, induction of apoptosis in senescent cells and interruption of SASP are three targets for intervention of cellular senescence to treat phosphate-induced diseases (Fig. 7.4).

7.5.4.1 Removal of Senescence Stimuli

The inhibition of senescent cells formation needs to remove any senescence stimuli. The control of blood phosphate, treatment of chronic inflammation and use of Klotho protein were shown to inhibit cellular senescence as we discussed above.

7.5.4.2 Removal of Senescent Cells

Because senescent cells are typically considered cells with irreversible fates that cannot re-enter cell cycle, there is increasing interest in targeting and cleaning senescent cells from diseased organs and damaged tissues. The most efforts that scientist have made are largely to repurpose FDA-approved medications or to explore pharmacological agents that can induce senescent cell death. These compounds are together called senolytics [124].

Ideally, selectively stimulating apoptosis with senolytic drugs which block pro-survival pathways including Bcl2, p53/21, is able to remove senescent cells [124]. Quercetin can block Bcl2 activity and probably also p53 signaling to induce apoptosis and remove senescent cells. Some compounds (ABT-737, ABT-262 and A1331852) also induce apoptosis through inhibiting Bcl2 although most of them are only relatively specific.

Other options to clear senescent cells include: (1) Potentiate an immune response with monoclonal antibodies such as anti-PD1, anti-DPP4, and anti-vimentin. These immune-based interventions are most used in cancer therapy [124]; (2) Use silica nanoparticles coated with galacto-oligosaccharides [4]. Cellular uptake and digestion of coated particles are in senescent cells with expression of senescence-associated β-galactosidase. Therefore, the content will be released from the nanoparticle within cells and apoptosis induced in senescent cells [4]. Senolytic application based on senescence-associated β-galactosidase has not been tested *in vivo* [124]. Whether the above agents can ameliorate phosphotoxicity still remains to be determined.

7.5.4.3 Interruption of SASP

If one cannot remove senescent cells from diseased tissue or organ, prevention of release of proinflammatory cytokines, pro-fibrotic growth factors, or matrix-remodeling proteases and/or blockage of their actions can serve as an alternative strategy to interrupt SASP, to reduce senescence amplification, and to limit detrimental and non-cell-autonomous effects of senescent cells [124]. Rapamycin, a well-known inhibitor of the mammalian target of rapamycin signaling pathway [52, 147, 163] and metformin also functioning as mTOR inhibitor [84, 121] effectively suppress inflammation, reduce NF-κB signaling activity, prolong lifespan, and improve health in aged animals [124]. In fact, rapamycin also reduces serum phosphate and increases Klothoexpression [147]. Those two events should consequently suppress cellular senescence [161].

Overall, cellular senescence activation is one of mechanisms mediatin phosphotoxicity. Thus, targeting cellular senescence is one of strategies to reduce phosphotoxicity and treat phosphate-associated diseases, which complements the current therapy consisting of restriction of dietary phosphate intake and administration of phosphate binders to enhance the efficacy (Fig. 7.4).

7.6 Conclusion

With aging, chronic non-communicable diseases are strongly associated with shortened lifespan and diminished life quality in senior population. The detrimental effect of chronically excessive phosphate intake (e.g. processed food) and/or deficiency in phosphate excretion (e.g. CKD) on aging and age-associated diseases draw more and more attention of nephrologists, nutritionists, and basic researchers. Phosphotoxicity has been shown to induce oxidative stress, DNA damage, chronic inflammation, disturbed mineral hormones homeostasis, abnormal autophagy, and cellular senescence.

Cellular senescence is evolutionarily conserved across plant to animal kingdom as a defense mechanism to respond to cellular insults and to prevent the cells from necrosis. However persistent and over active cellular senescence can impair tissue regeneration and amplify tissue damage through SASP. The discovery of phosphate effect on cellular senescence leads to develop novel strategies to prevent or treat phosphotoxicity and phosphate-associated chronic diseases. In addition to the control of phosphate, the successful development of senolytics and inhibition of the SASP of senescent cells called senostatics would render possible to target senescent cells or senescence-related downstream signal pathways for the treatment of many age and phosphate-related diseases that are approximately involved in every organ/tissue/system in humans (Fig. 7.2).

Because phosphate-induced cellular senescence is a chronic physiologic and pathophysiologic process driven by multiple signaling pathways, better understanding of cellular senescence in phosphotoxicity would open a novel horizon to treat phosphate-associated diseases by directly targeting downstream cellular senescence which will be adjoined with effective control of serum phosphate to optimize the efficacy in attenuating phosphotoxicity, extending life span and improving health span (Fig. 7.4). Moreover, every intervention aimed at improving the quantity and quality of human life through targeting phosphate-induced cellular senescence should be thoroughly explored and validated clinically.

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Phosphate Toxicity and Epithelial to Mesenchymal Transition

8

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Abstract

The underlying role of inadequate or excess intake of phosphate is evident in disease states, including metabolic, skeletal, cardiac, kidney and various cancers. Elevated phosphate levels can induce epithelial to mesenchymal transition (EMT) and cell death. EMT and associated lethal, metastatic or fibrinogenic responses are known to be underlying disease processes in fibrotic diseases and various solid tumors. Studies have shown EMT is regulated by induction of different signaling pathways, including TGF-β, RTK, SRC, Wnt and Notch signal transduction. However, cross-talk amongst these signaling pathways is less understood. We have shown that elevated phosphate levels enhanced EMT partially through activating ERK1/2 pathway, resulting in massive cell death. We thus proposed excess phosphate-mediated lethal EMT as one of the underlying mechanisms of phosphate-induced cytotoxicity, which could explain high phosphate-associated renal fibrosis and cancer metastasis in preclinical and clinical studies. This chapter provides the overview of EMT with the highlights of its regulation by various signaling pathways induced by phosphate toxicity. We further put lately reported lethal EMT in the context of phosphate toxicity with the intent to explain it to excessive phosphate-associated pathologies.

Keywords

Phosphate · Cytotoxicity · EMT · Apoptosis

8.1 Phosphate Toxicity

Phosphate (PO_4) is one of the most abundant nutrients in the body and is undeniably essential for the survival of all living organisms. In vivo, inorganic phosphate (Pi), in the form of *di*hydrogen phosphate (H_2PO_4) and *mono*hydrogen phosphate (HPO_4), is synthesized from organic PO_4 metabolism. Pi is involved in numerous essential biological processes, including the synthesis of DNA and RNA, the storage and transfer of energy in the form of ATP, regulation of cell metabolism and cell signaling via protein phosphorylation, and maintenance of cell membrane integrity as phospholipids [53]. Pi also plays a crucial role in skeletogenesis by forming, developing, and maintaining skeleton and dentin in vertebrates [9]. In the body, sufficient phosphorus intake is

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essential for maintaining musculoskeletal functions and beyond. Insufficient intake of dietary phosphorus results in malnutrition, leading to a deficiency in skeletal mineralization and subsequent development of rickets. In contrast, excess Pi may have harmful consequences by inducing pathological calcification [25], oxidative stress secondary to mitochondrial dysfunction [21], and dysregulated signal transduction [34]. These consequences may present as various disorders, including gingivitis [28], dental decay [27], heart disease [10], impaired fertility [64], diabetes [50], kidney disease [51], premature aging [61], and cancer [7].

Recent studies have demonstrated the ability of extracellular Pi to manipulate skeletal and extraskeletal cells. In skeletal cells, elevated Pi levels can alter osteoblast [5] and osteoclast [42] differentiation, as well as vascular calcification [25]. Extraskeletally, Pi has an essential role in cell proliferation [66] and growth [11, 16]. Several studies have provided evidence to suggest that high levels of Pi may play a role in promoting tumorigenesis by altering various metabolic pathways. For instance, high levels of serum phosphate have been found to positively correlate with the development of lung, thyroid, pancreas, and bone cancer in men, and esophageal, non-melanoma skin, and lung cancer in women [82]. In animal models, mice fed with high phosphate diets showed an increased risk of cancer growth than those with low-phosphate diets [7]. A human study found that high phosphate intake in men resulted in not only an increased overall risk of prostate cancer, but an increased risk of advanced-stage or lethal prostate cancer [81]. A possible explanation for this may be due to the excessive expression and activity of phosphate cotransporters on tumor cells and their ability to store more inorganic phosphate in comparison to normal cells [46]. In fact, tumor cells of cancer patients have been found to store nearly double the amount of phosphorus seen in normal cells [6, 23]. This excess intracellular phosphorus leads to increased metabolic activity in tumor cells, the induction of growth-promoting cell signaling, chromosome instability, and create a potential microenvironment for metastasis [7].

Studies have also shown the response of cells to elevated cytotoxic levels of Pi. Mitochondrial oxidative stress in endothelial cells resulted in cell death, possibly by apoptosis and tissue damage [21]. Experimental studies have highlighted the role of Pi-induced endothelial injury as the possible initial event in hyperphosphatemia-associated diseases, including chronic kidney disease, cardiovascular disease, and various cancers. Recent studies have convincingly shown excess Pi-induced ER stress, epithelial-mesenchymal transition (EMT), and cell death [34]. High Pi stimulates Transforming growth factor-β (TGF-β) signaling via the Raf/Mitogen-activated protein kinase (MEK)/Extracellular signal-regulated kinases (ERK) pathways by decreasing E-Cadherin expression in HEK293 cells and increased phosphorylated Smad2 and Snail in both HEK293 and HeLa cells; the resulting effects being EMT to lethal EMT. We believe that lethal EMT exerts a pathologic role in phosphate toxicity-mediated tissue injuries and organ damages. One of the goals of this chapter is to explain how elevated Pi may induce different pathways to initiate EMT and related cellular pathologies.

8.2 EMT Overview

EMT is defined as the loss of epithelial cell polarity and adhesion, gain of invasive and migratory properties, and resulting phenotype conversion to mesenchymal cells. The expression of N-cadherin and vimentin (mesenchymal markers), and loss of E-cadherin (epithelial markers) are the hallmarks of EMT. Classification falls into 3 groups, which is based upon the biological process it plays a role in. Type 1 EMT includes embryogenesis and gastrulation, while type 2 EMT is associated with wound healing and fibrosis. Type 3 EMT is involved with cancer cells invasiveness and metastasis. Figure 8.1 demonstrates a few of the pathways that induce EMT, including TGF-β, Wnt/β-catenin, Notch, proto-oncogene c-Src (Src) and Ras-Mitogen-activated Protein Kinase (MAPK) [26]. These mechanisms then lead to the

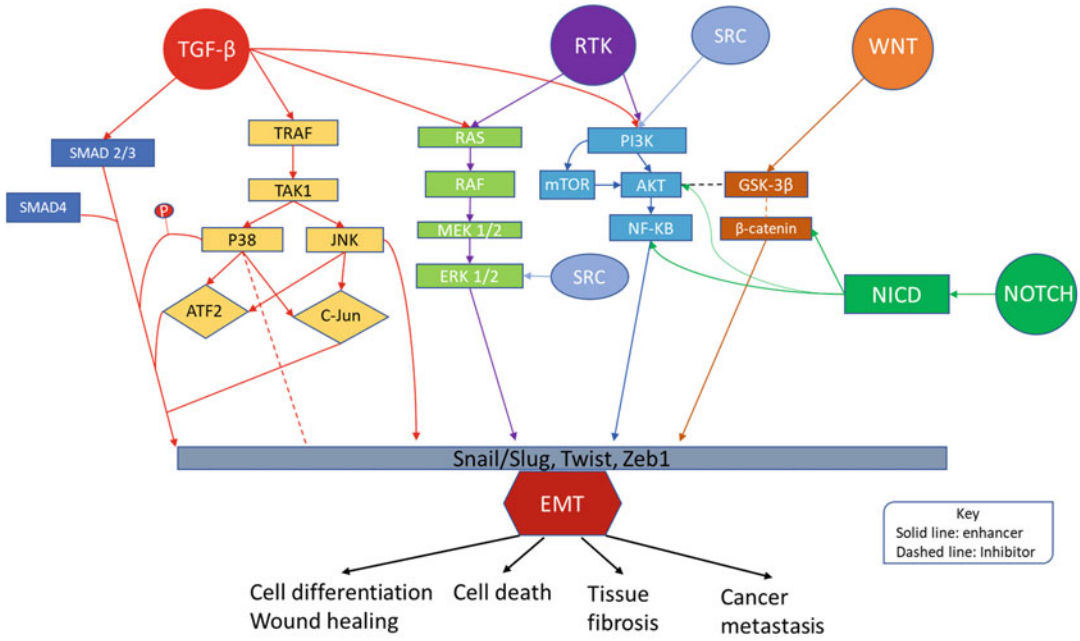

Fig. 8.1 Regulation of EMT

synthesis of transcription factors, including zinc finger proteins Snail 1 and 2 (Slug), basic helix-loop-helix factors Zinc finger homeodomain proteins 1 and 2 (ZEB1/2), and Twist 1, 2 and 3. While EMT has been indicated as a normal process occurring in embryonic differentiation of cells and wound healing, aberrant stimulation of EMT has been identified in metastasis of cancer, as well as tissue fibrosis. The considerable cross-talk that occurs between the different pathways remains less explained. Thus, we will firstly review the pathways involved, downstream effects and major regulators of these pathways.

8.2.1 TGFβ-Induced EMT

TGF-β plays roles in cell proliferation, differentiation and apoptosis [35]. TGF-β has been identified as one of the master regulators of the EMT process [45, 73]. The activation of the TGF-β pathway occurs through several different proteases and integrins, as well as situational activators such as reactive oxygen species or pH-dependent environments. One of the resulting downstream effects of TGF-β signaling is EMT. TGF-β-mediated EMT is either a Smad-dependent or a Smad-independent phenomenon [20, 26, 45].

8.2.1.1 Smad-Dependent EMT

Activation of TGF-β and subsequently TGF-β type I receptor (TBRI) results in Smad2 and Smad3 activation via the phosphorylation of serine residues on Smad. The activated Smad2/3 then forms a trimeric complex with Smad4, which is translocated to the nucleus and binds transcription factors [41, 85]. The transcription of Snail1/2, and Twist1 proteins is then upregulated, leading to the repression of epithelial markers, such as E-cadherin, claudins and occludin. Snail and Twist1 are also involved in the activation of mesenchymal gene expressions such as fibronectin, vitronectin and N-cadherin. Activation of ZEB proteins occurs through an alternative mechanism, resulting from the interaction with Smad-3. However, the downstream effects of ZEB1 and ZEB2 activation can repress epithelial markers and the expression of mesenchymal markers [83]. TGF-β activated Smad was also identified as an enhancer of the MAPK pathway [3, 52] (Fig. 8.1). Specifically, the Smad2/

3-smad4 complex induced by TGF-β upregulates transcription of LEF-1, which can form a complex with β-catenin, and allow the gene transcription that promotes EMT [52]. The significance of Smad-dependent, TGF-β induced EMT was demonstrated in the study performed by Thacker et al., in which cervical cancer cells were treated with curcumin and Emodin. The treatment caused the down regulation of TGF-β signaling via the decreased expression of TGF-β receptor II, Smad3 and Smad 4 and also counterbalanced the tumorigenic effects of TGF- β via the induction of G_2/M phase arrest of cell cycle [75].

8.2.1.2 Smad-Independent EMT

While Smad-dependent TGF-β signaling has been identified as a central mechanism in EMT, Smad-independent pathways have also been shown to play a role in the genesis of EMT. Studies have identified Smad-independent roles in TGF-β-induced EMT for MAP kinases and Phosphoinositide 3- kinase (PI3K)-Protein kinase B (AKT) pathway, as well as other signaling pathways [31, 41].

MAP kinases represent a family of proteins that play a role in different aspects of cell signaling, including gene expression, cell proliferation and cell cycle regulation. Within this family, TGF-β signals ERK, p38 MAPK and c-Jun N-terminal kinases (JNK). ERK 1/2 proteins have been identified as a potent activator of EMT. Stimulation of the TBRI leads to the activation of Raf-Ras, which then activates MEK and is followed by MEK activation of ERK. The effects of activated ERK leads to downregulation of E-cadherin, resulting in a gain of motility and invasiveness. Studies have shown that the ERK-mediated step is important in TGF-β induced EMT [86]. Another study looking at this relationship showed the inhibition of MEK-ERK signaling activities prevented the induction of TGF-β- mediated EMT [8]. Together, these findings highlight the importance of the MAPK-ERK pathways in the evolvement of EMT.

JNK and p38 are modulated by TGF-β signaling through a common mediator, TRAF 4/6. The interaction of TRAF with TGF-βR1 leads to TGF-β activated kinase 1 (TAK1) [79, 84]. The downstream effects of p38 and JNK activation signaling were associated with proliferation, differentiation, and apoptosis. Thus, multiple studies have focused on the role of activated p38 and JNK in EMT. The role of activated JNK is linked to increased cell invasion and motility of cells, though its role in apoptosis is not yet conclusive. Studies have proposed both pro-apoptotic and pro-survival roles of JNK [79], which can be determined by the duration of JNK activation. Persistent JNK stimulation led to apoptosis [13], while short-term JNK activation by growth factors promotes cell survival and proliferation [71]. Activated JNK signaling was also shown to result in the stabilization of Twist1 and enhancement of Smad-mediated effects [20]. The downstream effects of p38 MAPK have been identified in both downregulation of E-cadherin and induction of EMT. The phosphorylation of Smad proteins by p38 can cause downregulation of E-cadherin. Conversely, p38 repression of Snail1 and upregulation of Twist1, was also reported. The study claimed the role of p38 in maintaining the expression of E-cadherin [48]. TAK1 is a necessary step regarding the activity of the Smad pathway. Activated JNK and p38, downstream of effects of TAK1, are required for the activation of c-JUN and Activating transcription factor 2 (ATF2). The JNK-c-JUN was identified as a mediator of TGF-β-induced Smad transcription, while p38-ATF2 complex cooperates with Smads in EMT [24]. The interactions of TGF-β1, MAPK, and Smads promoted EMT in human malignant keratinocyte cell lines, with ERK and p38 enhancing Smad 2/3 transcription [18]. However, in a study on the role of Galectin-1 (Gal-1) in human ovarian cancer cells, it was shown that Gal-1 enhanced EMT through the activation of the MAPK JNK/p38 pathway [89]. These findings show cooperative and isolated signaling mechanisms involvling TGF-β, Smads, and MAPKs, and suggest that further studies are needed to identify the molecular mechanisms of EMT in various pathological states.

The role of *PI3K/AKT* has also been identified as another key regulator of EMT, but the specifics behind this pathway continue to be further

investigated. The study showed PI3K-AKT was a necessary pathway for EMT [3]. Further studies looked at the specific role AKT plays in the signaling process. Long-term activation of AKT resulted in cells with enhanced motility and decreased adhesion, features typical to EMT. These features associated with activated Akt have been linked to the downstream effects of increased expression of Snail1 and repression of E-cadherin [30]. It is thought that TGF-β signaling activates the PI3K pathway by its own receptors or indirectly through Epidermal growth factor (EGF) and Platelet-derived growth factor (PDGF) receptors [26]. Qian et al., found that TGF-β1 stimulation of PI3K/AKT-induced phosphorylation of FOXO3a to be an underlying mechanism of Bleomycin-induced pulmonary fibrosis and potential therapeutic target [63].

8.2.2 Non-TGFβ-Induced EMT Pathways

While the role of TGF-β in EMT is well defined and considered a major part, studies have shown that TGF- β independent mechanisms can also lead to EMT.

The *Src family* of proteins (non-receptor tyrosine kinase) serve as a regulating point of different pathways. Specifically, an increase in Src kinase activity has been shown to promote EMT activity. The studies have highlighted such possibilities by demonstrating that Src regulates E-cadherin, and by increasing or suppressing Src activity resulted in a corresponding induction or suppression of EMT [55].

Wnt signaling is actively involved in tumorigenesis. With a number of those Wnt-mediated tumors displaying activated EMT factors. Wnt acts on GSK-3β, which acts as an inhibitor of β-catenin destruction complex. This inhibition prevents degradation of β-catenin, which accumulates and is translocated to the nucleus. β-catenin then acts as transcriptional inducer of EMT, increasing the expression of Snail/Slug, Twist1/2, and ZEB1/2 [39]. The combined treatment of curcumin and emodin showed effects on the Wnt/β-catenin in cervical cancer, HeLa cells; TGF-β activated the Wnt/β-catenin pathway in HeLa cells but combination therapy with curcumin and emodin downregulated Wnt signaling via the inhibition of β-catenin. [75].

Notch signaling has been identified to play a direct and/or indirect role in EMT signaling. Notch protein acts as a paracrine substance, regulating the actions of cells locally. The role of Notch involves the downstream effects of Notch Intracellular domain (NICD), which is released by Notch receptor interaction with Delta-like 1/3/4 (Dll) or Jagged 1/2 (JAG) interaction. NICD then binds C protein binding factor 1/Suppressor of Lag 1(CSL) and acts to regulate the expression of proteins such as Nuclear factor kappa-B (NF-kB), p21/27, AKT or the β-catenin destruction complex [20, 80]. The binding of JAG2 was also indicated as an indirect inducer of EMT by inhibiting miRNAs. Of note, Notch signaling also plays a role in TGF-β induced EMT. Snail-Notch interaction was identified as a β-catenin activator and E-cadherin repressor [80].

8.2.3 EMT and Diseases

The knowledge gained to this point has implicated the pathological role of EMT in different disease states. Studies conducted thus far have looked explicitly at the role EMT plays in cancer and fibrosis.

8.2.3.1 Cancer Metastasis

As mentioned, EMT has been found to have a significant role in cancer development and metastasis, which is predominantly driven by TGF-β induced EMT. During the late stages of tumor development, cancer cells become resistant to TGF- β induced cytostasis and TGF- β subsequently functions as a tumor promoter by inducing EMT [33]. Activation of EMT can result in decreased cellular adhesion and tight junctions, allowing for cancer cell dissemination, which is pivotal to early metastasis [33]. Mouse models have demonstrated that activation of EMT through the TGF- β signaling pathway with hyperactivity of the Raf/MAPK pathway results in the invasion of cancer cells to distant organs

[38]. The TGF- β pathway has been found to induce metastasis in various cancers, including breast, gastric, lung, and prostate [58]. Interestingly, new evidence also suggests that EMT may play a role in resistance against cancer therapy in a process driven by EMT transcription factors (EMT-TFs) [77]. *In vitro* and xenograft mouse studies have been able to identify increased expression of EMT-TFs on chemoresistant cells [77]. For example, a study performed by Zhu et al. was able to demonstrate a significant positive correlation between expression of TWIST1 (a helix-loophelix EMT-TF), and poor post-operative, and post-chemotherapeutic survival in colorectal cancer patients [88].

8.2.3.2 Fibrosis

One of the main functions of EMT includes wound healing, or type II EMT. The physiological role of EMT was indicated in the re-epithelialization of cutaneous wounds by kertinocytes and post-ovulation in ovarian surface epithelium [1, 2]. Tissue is further repaired through the generation of scar tissue, mostly via myofibroblasts. Studies to this point have identified EMT as one of the driving mechanisms behind myofibroblastic activities [72]. However, over activities of myofibroblasts results in pathologic tissue scarring or fibrosis through EMT and subsequent accumulation of collagen proteins. With that in mind, studies have further looked at the potential association of EMT and fibrosis of different organs. Studies have shown that TGF-β induced loss of epithelial morphology in epicardial cells [15]. EMT is also an important event in the development of pulmonary fibrosis, as the alterations of epithelial to mesenchymal phenotype in alveolar epithelial cells have been documented in the genesis of pulmonary fibrosis [43]. In a study looking at hepatic fibrosis, TGF-β-dependent activation of Smad 2/3 was indicated as the mechanism that mediated phenotype change in hepatocytes [40]. Numerous other studies have also highlighted the role of EMT in cardiac, pulmonary and hepatic fibrosis, as well as renal fibrosis [12, 29, 32, 49, 70]. A partial-EMT model was also identified as the driver of keratinocyte phenotype switch and resulting fibrotic skin lesions in scleroderma [59].

8.2.3.3 Therapeutics by Targeting EMT

Using the information known on EMT signaling pathways, studies have been performed to find potential therapeutic targets for EMT-associated changes. One such study by Zhou et al. looked at the effects of Thalidomide (THL) on EMT in alveolar cells during pulmonary fibrosis. Their results showed that THL decreased expression of the mesenchymal phenotype by inhibiting several signal transducers in TGF-β signaling pathways, which included p38, JNK, ERK, AKT, GSK3β and smads 2/3 [87]. The role of EMT in cancer was indicated to induce not only metastasis (as reviewed at Sect. 8.2.3.1), but also aid in escape of immune response through immunosuppression [44]. The role of Programmed death-1 (PD-1) on EMT cells in non-small cell lung cancer (NSCLC) was investigated in regards to immune evasion, showing the use of AKT pathway inhibitor (LY294002), ERK pathway inhibitor (PD98059) and TAK1 pathway inhibitor (5Z-7) could effectively inhibit the characteristic phenotype expressed in EMT cells [47]. These studies highlight the prospects of reducing EMT by therapeutic manipulation of various signaling pathways.

8.3 Lethal EMT

Studies have described the overexpression of Snail/slug, zinc finger E-box-binding homeobox (Zeb), and Twist1, as well as the decrease in E-cadherin and regulation of β-catenin as major factors in EMT. As shown by Padmanaban et al. E-cadherin loss has been shown to result in increased apoptosis of cells. Cells displaying loss of E-cadherin exhibited nuclear localization of Smad 2/3 [62]. This is consistent with other findings indicating the importance of both TGF-β

and E-cadherin in EMT process. Their study further defined the relationship by showing E-cadherin loss triggers TGF-β signaling dependent ROS, that resulted in in apoptosis of the cells. Recent studies have shown that TGF-β signaling induced fibrosis and cell injury are partly regulated through lethal EMT [17, 73]. Studies have demonstrated the role of EMT in tumor suppression by remodeling the transcription factor landscape and converting SOX4 into a promoter of EMT-mediated cell death [17]. Another study found a potential role of RREB1 in TGF-β induced lethal EMT, by coordinated activation of RAS and TGF-β signaling cascade [73]. Studies have also shown KRAS mutation combined with TGF-β stimulation had a 30-fold increase in Snail1 and Zeb1 expression, decreased E-cadherin, and increased organoid dissociation and apoptosis.

8.4 High Pi-Mediated EMT

Our recent studies showed that abnormally high Pi could markedly enhance EMT by up-regulating the expression of Snail and Vimentin, and repression of E-Cadherin (Fig. 8.2) [34].

Pharmacologically (by the chemical inhibitor of Pi transporters) or genetically (by siRNA knockdown of Pi transporters) blocking cellular Pi transport resulted in prevention of high Pi-mediated EMT, indicating excess Pi's primary effect on the initiation and progression of EMT. Interestingly, elevated Pi-induced EMT was not predominantly mediated by TGF-β signaling but partially regulated by ERK1/2 signaling (Fig. 8.3). Elevated Pi also triggered massive cell death via activating extrinsic and intrinsic apoptotic pathways. Our cell-based studies have also shown that high Pi-mediated lethal EMT was partly mediated by reducing Snail mitigated Pi-triggered apoptosis. This finding is based on an acute high Pi treatment (24–48 h) model. In a chronic high-Pi stress model, where cells are exposed to moderately high levels (above 1–10 mM) of Pi for a longer duration (>3 days), high Pi-mediated EMT may have differential effects, such as increased cell mobility and fibrosis. This speculation is further echoed by high-Pi-related cancer metastasis and renal fibrosis in human and experiment studies.

It is believed that Pi-driven metastases could be the consequence of an adaptation of cancer cells to meet their phosphorus needs for rapid growth [19]. A higher concentration of interstitial Pi was detected in metastatic tumors compared to the non-invasive ones [6], suggesting a requirement of elevated Pi for the rapid cell growth, invasion and migration. Alpha-klotho functions in pair with FGF23 and reduces renal reabsorption of phosphate [76]. Elevated phosphate levels have been linked to decreased klotho expression in mouse genetic studies [56, 65]. Of relevance, klotho has been considered a tumor suppressor with universally depressed in breast cancer, pancreatic cancer, ovarian cancer, lung cancer, colorectal cancer, and melanoma [67]. In mice, secreted klotho protein administration could suppress renal fibrosis and cancer metastasis by inhibiting TGF-β, Wnt and IGF-1 signaling and delaying EMT process [22]. The causal links between Pi overload, hyperphosphatemia and the progression of chronic kidney disease (CKD) have been reported in human [60, 69, 78,

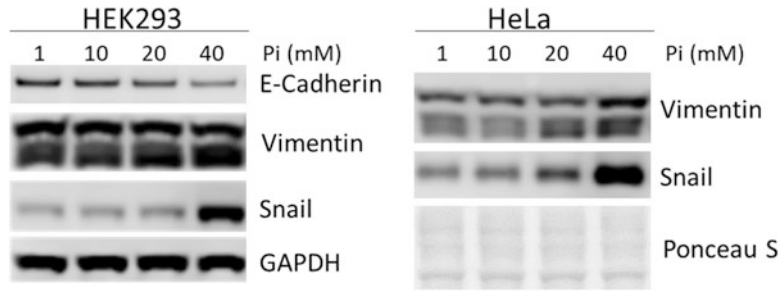

Fig. 8.2 High Pi (24 h treatment) enhanced EMT in HEK293 and HeLa cells by Westen blot analysis

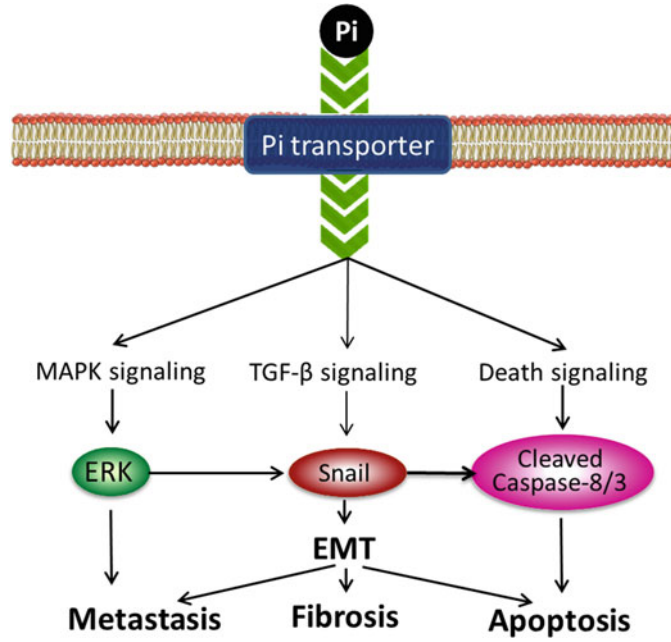

Fig. 8.3 High Pi-mediated EMT

90] and animal [37, 54, 57, 68] studies. Interstitial fibrosis is the main morphological alteration that eventually progresses to CKD. *In vitro*, elevated Pi could induce myofibroblast activation, promote cell proliferation and augment the synthesis of interstitial matrix protein, such as type I collagen [74] and fibronectin [14]. As mentioned, EMT is one of the causal events for fibrosis. Hence, high Pi-related renal fibrosis is partly mediated by the induction of EMT [90].

8.5 Open Questions and Future Directions

Excess Pi-mediated lethal EMT is dispensable of TGF-β signaling. Beyond the MAPK pathway, it will be clinically rewarding to discover other Pi-mediated EMT regulators by applying proteomic or phosphoproteomic analysis. Pi-mediated EMT may result in differential effects, such as lethal, metastatic, or fibrogenic. *In vitro* assays (such as invasion and migration assays) and animal models (genetically or dietary-induced high Pi) are desired to demonstrate these Pi-related phenotypes of EMT. Finally, it will be critical to determine the factors involved in Pi-mediated EMT's differential effects. High Pi-induced changes in the microenvironment may be one of the key driving factors.

8.6 Concluding Remarks

EMT plays essential roles in biological (organogenesis and wound healing) and pathological (tumorigenesis and fibrogenesis) cellar and tissue events. It can be regulated by canonical TGF-β signaling and noncanonical pathways (such as RTK, PI3K/AKT, Wnt, Notch signaling). Recently, TGF-β induced EMT has shown to have a higher potential of cell death, indicating a novel function of EMT as a lethality mediator. Dietary phosphate overload directly causes severe cytotoxicity (cell stress and apoptosis), which is partly linked to elevated Pi-mediated EMT. Further insights into the molecular details of Pi-medicated lethal EMT will assist us to understand the pathology of Pi toxicity, and likely to offer novel therapeutic targets for the clinical management of phosphate toxicity-induced tissue and organ damages.

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9 Phosphate and Endothelial Function: How Sensing of Elevated Inorganic Phosphate Concentration Generates Signals in Endothelial Cells

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Abstract

Present in all cells, inorganic phosphate (Pi) is involved in regulating a wide range of fundamental cellular processes including energy homeostasis; nucleotide, nucleic acid and phospholipid metabolism; and signalling through protein phosphorylation events. However, at excess concentrations, Pi is known to exert adverse effects on cells, particularly on endothelial cells. This review gives a brief overview of the functional effects of elevated extracellular Pi concentration on mammalian cells and tissues *in vitro* and *in vivo*. We then address the cardiovascular effects of elevated extracellular Pi concentration *in vitro* and *in vivo*, emphasising that effects have been reported *in vivo* even within the top end of normal range for plasma [Pi]. Cardiovascular sites of action of Pi are then considered, with a focus on the role of soluble Pi in endothelial dysfunction. The regulation of intracellular Pi concentration by Pi transporter proteins in mammalian cells is described, followed by consideration in detail of how changes in Pi concentration are sensed in mammalian cells and how these trigger functional effects in endothelial cells.

Keywords

Phosphate · Microvesicles · Angiogenesis · Endothelial-mesenchymal transition · Signaling

9.1 Introduction

Comprising about 1% of an individual's body weight, phosphorus is one of the most abundant minerals in the human body. Predominantly found in conjunction with calcium (hydroxyapatite), or in cells as organic phosphates in carbohydrates, proteins, nucleic acids and lipids, only a very small part (~0.1%) of phosphorus present in extracellular fluids occurs in the form of inorganic phosphate (Pi). Plasma Pi (comprising dihydrogen orthophosphate $H_2PO_4^-$ and monohydrogen orthophosphate HPO_4^{2-} in equilibrium at a normal physiological pH) ranges

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between 0.8 and 1.5 mM, but this range can be exceeded transiently following ingestion of a phosphate enriched diet [1], or chronically under certain pathological conditions such as chronic kidney disease (CKD) [2]. It should also be noted that other pathophysiological disorders, such as hypoxia/ischemia [3] and chemotherapy in cancer patients resulting in tumor lysis syndrome [4, 5], may lead to a large-scale Pi generation from cytosolic organophosphorus metabolites, such as ATP and phosphocreatine, resulting in localised and systemic hyperphosphatemia. Even though Pi plays a pivotal role in all cells, in processes as disparate as energy homeostasis; nucleotide, nucleic acid and phospholipid biosynthesis and signalling through protein phosphorylation events; excess concentrations of extracellular Pi are known to exert pathological effects on cells including cells of the vasculature (both the endothelial cells [1, 6–8] and the underlying smooth muscle cells [9–11]) and can also disturb bone and mineral metabolism in humans [12].

Commonly in CKD patients, hyperphosphatemia drives soft-tissue calcification [9, 10, 13, 14], through insoluble calcium phosphate deposition, osteogenic transformation in vascular smooth muscle [9, 10, 13, 15] and increased vascular stiffness [16]. This chapter will focus principally on the endothelial effects of elevated *soluble* extracellular Pi in the concentration range ~1 mM to ~2.5 mM (i.e. from normal human plasma Pi concentration to the elevated concentrations that transiently follow ingestion of a dietary Pi load or that may occur chronically in disorders such as CKD). Interesting and important effects also occur outside this range, but will not be discussed here as they may be qualitatively different – for example because of ATP depletion as a consequence of severe Pi depletion (<<1 mM Pi) or because of the substantial complexing of ionised Ca^{2+} by Pi ions that occurs at very high Pi concentrations >>2.5 mM [17].

A further reason for focussing here on the extracellular Pi concentration range 1–2.5 mM is that, in recent years, serum or plasma Pi concentrations even within the top end of normal range have been shown to be associated with poor cardiovascular outcomes [18–23]. In a population-based study consisting of 8953 participants with normal kidney function, high serum Pi levels greater than 1.3 mM were shown to be associated with elevated low-grade albuminuria [19] which *per se* increases the risk of CV events and mortality [24]. In another population-based cohort study consisting of 13,340 subjects, higher serum Pi levels were shown to be associated with increased CV risk (i.e. carotid intima-media thickness (cIMT)) independent of participants' eGFR, hypercholesterolemia, diabetes, age, sex, and hypertension) [21]. Additionally, in a study on patients with coronary disease but no record of overt hyperphosphataemia, it has been shown that higher Pi levels well within the normal range are associated with greater all-cause mortality and adverse cardiovascular outcomes [22]. Foley, el al., 2009, demonstrated that there is a link between higher serum Pi levels and increased coronary atherosclerosis in a large population of young adults with no concomitant kidney disease and overt hyperphosphataemia [23]. Notably, there may be sex differences in the relationship between serum Pi and cardiovascular mortality with clear associations in men but less consistent associations in women [25, 26].

Taken together, these data imply that high Pi levels within the normal range (e.g. >1.3 < 1.5 mM) may nevertheless accelerate the onset and development of CV morbidity in the general population with or without kidney disease. Indeed in 2012, Ellam and colleagues reviewed Pi as a "new cholesterol" meaning that (by analogy with LDL cholesterol), intervention to manage Pi even at levels not regarded as particularly high, may benefit the general population and prevent development of atherosclerotic vascular mortalities [18]. It has therefore been suggested that modern diets containing large amounts of canned products and prepared food which are rich in Pi and/or preservatives containing Pi, may be a suitable target for intervention to manage serum Pi levels with possible vascular benefits even in the general population [18].

Harmful effects of Pi *in vivo* have been confirmed by specific manipulation of phosphate status in experimental animals. Hyperphosphatemic Klotho knockout mice demonstrated features of premature aging and a reduced life span. Klotho and NaPi-IIa double knockout mice (in which deletion of NaPi-IIa transporters of the SLC34 gene family favoured Pi excretion), reversed these pathological features. However, feeding these double-knockout animals with high dietary Pi supplements restored premature aging features, indicating that premature aging in these animals is predominantly a manifestation of Pi toxicity [27]. Furthermore, Yamada, et al. demonstrated that on feeding adenine-induced CKD rats with high (1.2%) dietary Pi for two months (and manifesting hyperphosphatemia; Table 2 in [14]), serum and tissue levels of TNF-α were significantly increased. In this study the apparent Pi toxicity involved premature aging phenotypes, vascular calcification, malnutrition, and mortality without any effect on kidney function [14]. All of the observed Pi-induced changes were blunted after feeding CKD rats on the 1.2% phosphorus diet with 6% lanthanum carbonate as a Pi binder [14].

These emerging effects on CV risks of higher serum Pi levels within the normal range might be attributable to indirect cardiovascular effects of phosphate-responsive hormones (i.e. phosphatonins) rather than to direct action by Pi on the cells of the cardiovascular system. Such indirect endocrine effects of Pi may include (but are not restricted to) inhibition of 1,25-dihydroxyvitamin D synthesis [28] and increased secretion of PTH [29] and FGF23 both of which are considered as predictors of cardiovascular mortality in populations with or without kidney disease [18]. Nevertheless, there is also mounting evidence of direct toxic effects of excess soluble Pi on mammalian cells. These might include impaired cell signalling, increased cell death, impaired fertility, renal fibrosis, osteoblastic transformation of smooth muscle cells, premature aging, angiogenesis, carcinogenesis, tumour progression, endothelial–mesenchymal transition, generation of procoagulant endothelial microvesicles (MVs) and enhanced systemic inflammation and malnutrition [6, 7, 10, 11, 16, 27, 30–35]. The implications of such effects for a direct role of Pi in endothelial dysfunction are therefore discussed in the next section. (The more specific question of how a relatively modest increase in extracellular Pi concentration could generate a biologically significant intracellular signal in endothelial cells is discussed later in Sect. 9.5).

9.2 Pi and Endothelial Function – What Pi Does to Endothelium *In Vitro* and *In Vivo*

9.2.1 Effects on Vasodilation

Important early evidence of direct endothelial effects of Pi came from work by Shuto et al. who showed that ingestion of a high phosphate diet impaired flow-mediated dilation (FMD) of the brachial artery at 2 h after a meal [1]. Nishi et al. also reported that excessive dietary phosphate intake can acutely (within 1–4 h) impair endothelial function in healthy people as determined by decreased FMD [36]. Levac et al. showed that an acute oral phosphate load resulted in impaired FMD even in the absence of measurable changes to plasma phosphate levels [37]. A possible mechanism for this effect was proposed by Di Marco et al. who demonstrated that in cultured human coronary artery endothelial cells, exposure to high phosphate media directly results in stiffened endothelial cells which could, in turn, reduce mechanosensing and nitric oxide generation [16]. Consistent with this, studies on cultured human umbilical vein endothelial cells (HUVECs) show inhibition of endothelial nitric oxide synthase (eNOS) expression/activity in response to Pi [8]. In addition to effects on endothelium-dependent vasorelaxation, Six et al. have also shown that a high phosphate diet

increases phenylephrine-induced vasoconstriction in mice [38]. Thus effects of Pi on vascular function likely involve both endothelial cells and vascular smooth muscle.

9.2.2 Oxidative Stress and Cell Survival

Oxidative stress is a condition whereby the production of reactive oxygen species (ROS) exceeds antioxidant defenses [39]. A further potentially important mechanism by which a Pi-load could induce dysfunction in endothelial cells is by triggering oxidative stress, resulting in apoptosis. It has been reported that an extracellular Pi-load induces apoptosis in HUVECs [8] and triggers mitochondrial oxidative stress and subsequently cellular apoptosis in the human endothelial cell line EA.hy926 *in vitro* [7]. However, work elsewhere using the same cell line failed to detect these effects [6]. It has also been reported that a high extracellular Pi concentration induces autophagy in endothelial cells via the inhibition of Akt/mTOR signalling, a process that the authors suggested may serve as a protective mechanism that shields endothelial cells from high Pi-induced apoptosis [40]. A further distinct response was reported by Olmos et al. who demonstrated that a high extracellular Pi concentration induces oxidative stress and cellular senescence in endothelial cells via upregulation of endothelin-1 (ET-1) [41]. Thus several Pi-mediated effects have been described in endothelial cells. The reason for discrepant, sometimes conflicting results from different laboratories is currently unknown. While some may arise from differences in the origin of the endothelial cells studied, this does not explain the difference reported between [7] and [6], possibly indicating technical differences. Technical variables that may merit future investigation include the precise timing of the addition of the Pi load to the culture medium, which could affect precipitation of biologically active factors such as calcium phosphate nanocrystals [42]; and the presence or absence of trace impurities in the added Pi load, for example arsenate and arsenite which are structurally and chemically similar to Pi and may promote oxidative stress in endothelial cells [43]. These suggest that there is a need in future studies to take such variables into consideration to avoid results variation between laboratories.

9.2.3 Microvesicles

Membrane-derived microvesicles (MVs) are sub-micron (<1 μm diameter) vesicles which are shed from plasma membrane in response to apoptosis or cellular activation (notably in platelets, endothelial cells, and leukocytes) [6, 7] (Reviewed in [44]). It has been demonstrated *in vitro* that an extracellular Pi-load consistently leads to liberation of endothelial MVs in human coronary artery endothelial cells [16], in human EA.hy926 cells [6] and in HUVECs (Supplemental data in [6]). This has also recently been demonstrated in response to hyperphosphatemia in the rat partial nephrectomy model of CKD *in vivo* [35]. It is worth noting that, once released from endothelial cells, MVs may exert effects elsewhere in the vasculature as they are potently procoagulant [6], and carry miRNA cargo [45], capable of delivering miRNAs to other cells, including feedback effects on the endothelium [46–48]. This Pi-induced release of MVs may partly explain the high concentration of pro-coagulant endothelial MVs reported in circulation in hyperphosphatemic CKD patients *in vivo* [49]. Similarly this has shown that MVs induce reactive oxygen species production and cell cycle arrest in cultured endothelial cells and this may contribute to Pi-induced effects on oxidative stress and senescence [50]. Shedding of MVs in response to Pi may also be sufficient to deplete the cell-surface expression of biologically important endothelial proteins. In this regard, Di Marco et al. showed in human coronary artery endothelial cells [16] that a higher Pi milieu induces a decrease in cellular Annexin II protein level which these authors attributed to shedding of

Annexin II in endothelial MVs rather than reduced synthesis or increased degradation of the Annexin II protein within the cells.

9.2.4 Angiogenesis

New blood vessel development (angiogenesis) is a complex and highly regulated process which involves the proliferation, migration, and re-modelling of endothelial cells from pre-existing blood vessels. Pro-angiogenic genes such as forkhead box protein C2 (FOXC2), osteopontin, and VEGF-α have been shown to be influenced by an elevated Pi concentration [9, 10, 13, 14]. However, the precise effect of Pi is dependent upon the origin of the endothelial cells that are being studied. Even though an increase in extracellular Pi concentration has been reported to *promote* the angiogenic potential of cancer cells through a mechanism requiring FOXC2 and osteopontin (OPN, a secreted cytokine like factor) [51], hyperphosphataemia has also been reported to *impair* endothelial cells' angiogenic competence by inducing alteration in the structure and functionality of endothelial cells and thus contributing to endothelial dysfunction [16]. It should be noted that the effect of higher extracellular Pi in promoting the angiogenic potential of cancer cells reported by Lin and colleagues [51] differs from the reported Pi-induced impairment of endothelial cells' angiogenic potency [16]. In [51] it was the conditioned medium from Pi-loaded cancer cells that promoted angiogenic markers in HUVECs: however in [16] the reported effect of Pi on impairment of angiogenetic potency (cell migration and tube formation) was the consequence of direct effect of Pi-loaded medium on human coronary artery endothelial cells (HCAECs) and EA.hy926 human endothelial cells. It has been suggested that the down-regulation of Annexin II that occurs under these conditions (as described in Sect. 9.2.3 above) is an important contributor to this angiogenic impairment in view of Annexin II's angiogenesis-promoting effects [16].

9.2.5 Endothelial-Mesenchymal Transition

Endothelial–mesenchymal transition (EndoMT) [52, 53] is a biological mechanism involving endothelial cell transformation into mesenchymal cells in which endothelial cells lose their specific morphology and markers and obtain myofibroblast-like features [52]. EndoMT is distinct from epithelial-mesenchymal transition which is a fundamental part of metazoan embryogenesis and characterises the structural development of organs [54]. EndoMT has been shown to be associated with the loss of common endothelial cell antigenic markers such as CD31, CD144, and von Willebrand factor (vWF) and resulting instead in acquisition of the expression of mesenchymal marker proteins for example smooth muscle actin (α-SMA), vimentin, fibronectin, calponin and fibroblast-specific protein-1 (FSP1) [52, 53]. A wide range of signalling pathways have been associated with the initiation and progression of EndoMT during both development and disease conditions. Transforming growth factor β (TGF-β) signalling is a potent inducer of EndoMT [55]. The activation of TGF-β signalling results in EndoMT by inducing accumulation of nuclear transcription factor complexes (SMADs) that induce the expression of the mesenchymal transcription factors (e.g. Snail, Twist, and Slug) which initiate EndoMT [55, 56]. Whether the TGF-β-induced EndoMT can be directly affected by intracellular rises in Pi in endothelial cells remains unclear. Such interaction between TGF-β and Pi merits further investigation however in view of the induction of EndoMT by Pi discussed below, and reports that TGF-β increases expression of SLC20A1 (GLVR1) Pi transporters in chondrogenic ATDC5 cells [57].

A chronic Pi load applied to coronary endothelial cells has been reported to induce EndoMT even in the absence of added TGF-β [53]. Tan and colleagues reported that applying an elevated extracellular Pi concentration results in an increased Pi influx which results in phosphorylation and activation of the DNA methyltransferase (DNMT1), which is recruited to the RAS protein

activator like 1 (RASAL1) promoter by histone deacetylase 2 (HDAC2) and hence induces RASAL1 promoter CpG island hypermethylation which has been implicated in pathological EndoMT in cardiac pathologies such as Calcific Aortic Valve Disease and cardiac fibrosis [53, 58]. However, such promotion of EndoMT cannot be assumed to occur in all endothelial cells. Wang, et al. demonstrated that in retinal microvascular endothelial cells autophagy can induce a reduction in Smad3 phosphorylation and prevent its association with Smad4 to be translocated to the nucleus and in this way inhibits the transcription of specific genes such as snail (the master regulator of EndoMT), slug and twist [59]. As discussed in Sect. 9.2.2 above, a higher extracellular Pi concentration can inhibit Akt/mTOR signalling in endothelial cells and subsequently induces protective autophagy in the cells [40]. It remains to be determined whether the direct effect of Pi on the induction of EndoMT or the indirect suppressive effect through the induction of autophagy dominates under these conditions.

9.3 How Pi Concentration Is Regulated in Mammalian Cells

Pi is a major intracellular anion in mammalian cells. The negative resting membrane potential inside the cells tends to repel this negatively charged anion from the cytosol into extracellular fluid. To overcome this tendency, and therefore maintain a cytosolic Pi concentration of the order of millimolar inside the cells, mammalian cells express active Pi transporters in their plasma membrane which accumulate Pi anions in the cytosol against the electrical gradient of the membrane potential [60]. In humans, transporter proteins (solute carriers, SLCs) are encoded by genes which are classified in 65 SLC gene families [61]. Of these, three gene families – SLC20, SLC34 (and possibly SLC17) encode active Pi transporters (i.e. "pumps") that are expressed in the plasma membrane and pump Pi into the cytosol. These Na^+-dependent transporters perform secondary active co-transport of Pi anions and Na^+ into the cell, thus using the electrochemical gradient of Na^+ that exists across the plasma membrane to drive Pi into the cell. Besides these plasma membrane Na^+-dependent Pi transporters, there are other (non – Na^+-dependent) Pi transporters that are expressed in intracellular compartments, for instance in mitochondria (where Pi transport is coupled to H^+ and dicarboxylate ions) and the endoplasmic reticulum (where transport is coupled to Ca^{2+}-ATPase) [60, 62, 63]. However, the role of these intracellular Pi transporters in the regulation of intracellular Pi concentration is less clear.

Even though the influx of Pi through active Na^+-linked Pi transporters into mammalian cells clearly plays a role in the regulation of the intracellular Pi concentration, it is not obvious at first sight how changes in the influx of Pi through these transporters could alter the intracellular Pi concentration (and hence exert functional effects on the cell) when the extracellular Pi concentration is varied in the range 1–2.5 mM as discussed in Sects. 9.1 and 9.2 above. An apparent problem is that these transporters, for example the ubiquitously expressed transporters of the SLC20 gene family (Pit1/SLC20A1 and Pit2/SLC20A2), have a transport Michaelis constant (K_M) for Pi which is << 1 mM i.e. the transporters are saturated with Pi at physiological extracellular Pi concentration, and are operating near their V_{MAX} in the Pi concentration range 1–2.5 mM. It might be expected therefore that the intracellular Pi concentration would be unaffected by raising the extracellular concentration from 1 mM to 2.5 mM. However, mathematical modelling of intracellular Pi concentration [63] suggests that, at steady-state, the response of the intracellular Pi concentration to the extracellular concentration could in principle depend upon a *combination* of influx of Pi through active Na^+-linked symporters balanced by efflux of Pi through a passive "back-leak" transporter. This "pump-leak" model is shown schematically in Fig. 9.1. According to this model, at concentrations of extracellular Pi > > the K_M of the influx transporter for Pi, the behaviour of the corresponding steady-state intracellular Pi concentration would depend on the relative magnitude of the K_M for Pi on the influx

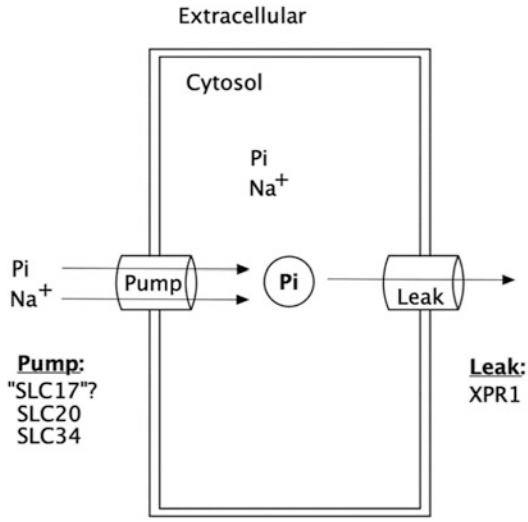

Fig. 9.1 Schematic representation of a "Pump-Leak" theoretical model for the regulation of intracellular Pi concentration in cells [63]**.** At steady state, active Na^+-linked influx of Pi into the cell through transporters (such as those of the SLC20 and SLC34 families) is balanced by passive efflux of Pi through a "leak" transporter such as XPR1

transporter and the K_M for Pi on the "back-leak" transporter. If the K_M of the influx transporters performing Na^+-dependent active pumping of Pi into the cell is significantly less than the K_M of the "leak" transporters, then the intracellular Pi concentration should show little change when extracellular Pi is increased. In contrast, if the K_M of the "pump" is similar to that of the "leak" transporters, an increase in extracellular Pi concentration over the range ~1–2.5 mM is predicted to lead to a commensurate increase in the intracellular concentration [63].

The relatively recent identification of a member of the SLC53 gene family (SLC-53A1 or XPR1), initially identified as the cell surface receptor for xenotropic and polytropic murine leukemia retrovirus (X/P-MLV) [64], as an efflux transporter [65] has now provided a plausible molecular basis for the "pump-leak" model (Fig. 9.1). Furthermore, the discovery of the responsiveness of the efflux transport activity of XPR1 to ambient Pi concentration sensed via InsP6 kinase/InsP8 signalling [66] (as described in Sect. 9.4 below) has revealed a further level of molecular control over the regulation of intracellular Pi concentration.

9.4 How Changes in Pi Concentration Are Sensed in Mammalian Cells

Even though mechanisms for the sensing of Pi by bacteria [67], yeast [68, 69] and plants [70] have been described in detail, corresponding explanations for how changes in Pi availability are sensed in mammalian cells have only recently emerged. Bon and colleagues have demonstrated a direct sensing mechanism for extracellular Pi concentration by SLC20 transporters through transport-independent signalling to ERK [71]. Binding of extracellular Pi to SLC20 transporters in the plasma membrane (i.e. a heterodimer of PiT-1/SLC20A1 and PiT-2/SLC20A2) in murine pre-osteoblastic MC3T3-E1 cells stimulated Pi-dependent ERK1/2 phosphorylation and subsequently resulted in up-regulation of gene expression for the mineralization inhibitors matrix Gla protein and OPN. It is worth noting that this effect of Pi occurred in the concentration range 1–10 mM even though (as pointed out in Sect. 9.3) the transport K_M of these transporters for Pi is <<1 mM. The transport-independence of this signalling effect through PiT1/PiT2 hetero-dimers apparently overcomes this K_M problem through binding of Pi to putative "Pi-sensing" sites in the heterodimer which are distinct from the amino acid residues responsible for Pi transport.

In a more recent study, Wilson, et al. reported intracellular sensing of ambient Pi concentration by inositol hexakisphosphate kinase (IP6K) signalling to XPR1 [66]. Using HCT116 cells (a human colon cancer cell line), which expresses PiT1/SLC20A1 but no other inwardly directed Pi-transporters, the authors demonstrated that deletion of IP6K1/2 (proposed to be involved in regulation of mammalian intracellular Pi concentration) results in an increase in free measurable intracellular Pi through a regulatory effect on the plasma membrane Pi exporter (XPR1). It is interesting to note that this novel and important role of

IP6K1/2 in Pi-sensing in HCT116 cells may be distinct from the biological role of IP6 kinases in other cell types. IP6K1 also regulates polyphosphate (PolyP) levels in eukaryotic cells [72], and it has recently been shown that high extracellular Pi concentration *in vitro* increases platelet PolyP content in a Pi-transport and IP6K dependent manner [73]. In contrast the HCT116 cells that show the IP6K1/2-dependent Pi-sensing and regulatory effect possesses no detectable PolyP [66].

While the two mechanisms above are clearly important in sensing Pi in mammalian cells, they have not yet been investigated in endothelial cells. A further Pi sensing mechanism has however been demonstrated in EA.hy926 human endothelial cells in which it was shown that raising extracellular Pi concentration can increase the intracellular Pi concentration by transport through active Na^+-linked PiT1/SLC20A1 transporters, thus allowing Pi to inhibit intracellular phosphoprotein phosphatases and subsequently induce a global increase in both protein Tyr and protein Ser/Thr phosphorylation [6, 74, 75].

9.5 Amplification of Pi Signals in Endothelial Cells

A number of phosphoprotein phosphatases expressed in mammalian cells are directly inhibited by physiological Pi concentrations [6, 74–78], potentially acting as sensors of intracellular Pi concentration which are directly capable of affecting intracellular signalling through protein phosphorylation. There have been no recent studies of the enzymology of this process. However, older literature (much of it pre-dating detailed molecular characterisation of the enzymes and the genes that encode them), contains reports of inhibition of protein tyrosine phosphatases and protein serine/threonine phosphatases by physiologically relevant Pi concentrations *in vitro* [6, 74, 75, 77]. By itself this is of limited value in explaining effects of Pi on endothelium, especially the observation (noted in Sect. 9.1) that even modest increases in plasma Pi concentration *in vivo* are associated with cardiovascular effects. This implies the existence not just of a Pi sensor but also some form of amplification. Such a mechanism has now been described in EA.hy926 human endothelial cells [74]. The phosphoprotein phosphatase PP2A, that accounts for the majority of protein Ser/Thr phosphatase activity in eukaryotic cells [79], is directly inhibited by Pi acting on the enzyme protein [74]. A substrate for PP2A in these cells is the phosphorylated (activated) form of the regulatory nonreceptor tyrosine kinase Src [80] (Fig. 9.2). Dephosphorylation of Src's carboxy-terminal Tyr-530 and autophosphorylation on Tyr-419 activates the Src kinase [81]. Consequently inhibition of PP2A by Pi increases phosphoactivation of Src. Src can then further inhibit PP2A by inhibitory phosphorylation of the PP2A-C catalytic sub-unit of the phosphatase [74]. Together the mutual effects of PP2A-C and Src on one another may allow the initial direct inhibitory effect of Pi ions on PP2A-C to be amplified (Fig. 9.2).

9.6 How Pi Signals Trigger Functional Effects in Endothelial Cells

In principle the Pi/PP2A/Src regulatory cycle described above has multiple targets in the control of cell signalling: PP2A is a ubiquitously expressed phosphatase which is involved in the regulation of a wide range of signals and responses, including neural development; Akt, NF-kB and MAPK signalling; apoptosis, and cell cycle progression [79], whereas Src plays a crucial role in cellular processes as diverse as cell proliferation, cell survival and drug resistance [80, 82, 83] and angiogenesis [84]. In EA.hy926 human endothelial cells a potentially important target of this Pi/PP2A/Src cycle is death-associated protein kinase 1 (DAPK-1) [74] (Fig. 9.2). Wang, el al. demonstrated that the activation of Src results in inhibitory phosphorylation of DAPK-1 [85]. DAPK-1 is a 160 kDa, serine/threonine protein kinase that is involved in a number of cellular processes including tumor suppressive function, apoptosis and autophagy

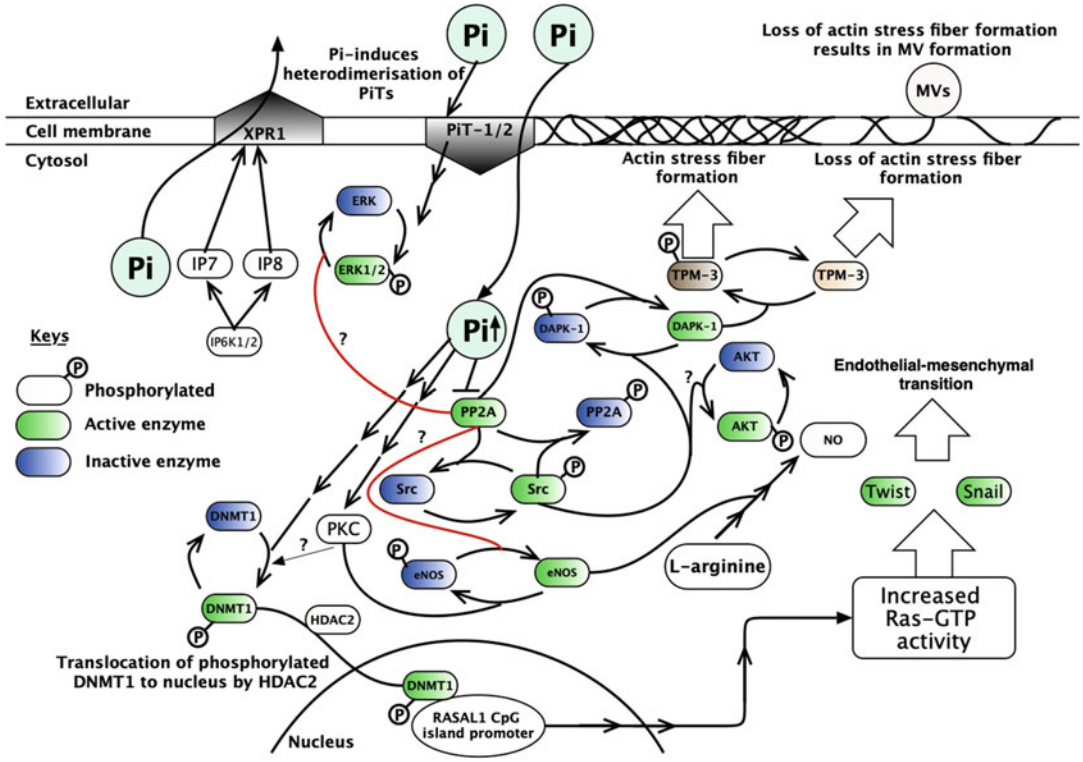

Fig. 9.2 Pi-sensing mechanisms and proposed interplay between different pathways which elicit biological responses to elevated extracellular Pi concentration in mammalian cells. The involvement of the pathways, molecules and effector enzymes shown here is based on data presented in [1, 53, 66, 71, 74]. Abbreviations: DNMT1, DNA methyltransferase; HDAC2, histone deacetylase 2; RASAL1, RAS protein activator like 1

[85, 86]. The kinase activity of this enzyme has been previously shown to be reduced by phosphorylation [85, 87], and subsequent proteasomal degradation of the phosphorylated DAPK-1 protein reduces the kinase activity of this enzyme even further. Houle, et al. reported that DAPK-1 phosphorylates cytoskeletal regulatory protein Tropomyosin [88], and this phosphorylation has been shown to protect the integrity of the cell membrane by inducing actin-stress fibre formation. Conversely, hypophosphorylation of -Tropomyosin-3 (TPM-3) (which has been shown to be triggered by Pi-induced PP2A inhibition [74]), results in the loss of actin stress fibre formation and an associated increase in membrane blebbing and MV generation [74] (Fig. 9.2). Thus in endothelial cells Pi-induced signalling through PP2A/Src and DAPK-1 culminates in cytoskeleton disruption and generation of pro-coagulant microvesicle (MVs) as outlined in Sect. 9.2.3 above.

9.7 Directions for Future Work

In addition to Pi signalling through the PP2A/Src/DAPK-1/TPM-3 pathway described above, it will be of interest in future work to investigate whether the Pi/PP2A/Src cycle plays any role in the other functional effects of Pi loading that were described above in Sect. 9.2, for example the Pi-induced inhibition of eNOS in Sect. 9.2.1. Previous work [1] demonstrated that this occurs as a result of inhibitory phosphorylation at Thr-497 of eNOS via protein kinase C (PKC) (Fig. 9.2). PP2A is a key determinant of eNOS

dephosphorylation and enzyme activity [89], suggesting that a possible contributor to inhibitory phosphorylation of eNOS induced by Pi may be the inhibition by Pi of PP2A. However, a possible countervailing effect may arise from the accompanying Src activation induced by Pi [74] because (at least in EA.hy926 endothelial cells and HUVECs [90]) Src activation results in eNOS activation via PI3K/Akt.

Further work is also needed to clarify whether Pi affects protein phosphorylation simply by inhibiting phosphoprotein phosphatases. Firstly, even for a given phosphatase, this may vary depending on the phosphoprotein substrate. Even though the activity of two phosphoprotein phosphatases (PPP-I and PPP-II, EC 3.1.3.16) from rabbit liver has been shown to be inhibited by Pi and by pyrophosphate (PPi) while using casein as a substrate, Pi was found to activate these enzymes when a histone was the substrate [91]. Furthermore, in addition to rapid direct inhibitory effects of Pi on phosphoprotein phosphatases, countervailing compensatory effects may also occur. In EA.hy926 cells prolonged Pi-loading led to compensatory upregulation of low molecular weight protein tyrosine phosphatase (LMW-PTP)) [6].

More generally, as at least three distinct Pi sensing mechanisms have now been described in different cell types (i.e. SLC20/PiT heterodimer signalling to ERK; IP6K signalling to XPR1; and PP2A/Src signalling (Fig. 9.2), an important priority for future work is to determine whether all 3 mechanisms are expressed together in endothelial cells and, if so, how these three pathways interact. For example, in addition to activating Akt as discussed above, in cerebral ischemia [92] Src also activates ERK, through decreasing PP2A activity, suggesting potential interaction between Pi signalling through the SLC20/PiT heterodimer and PP2A/Src pathways.

Finally, even though it has been shown that high serum Pi (or a Pi-dependent hormonal response derived from it) is sufficient to induce a marked increase in circulating pro-coagulant MVs *in vivo* [35] (indicative of endothelium dysfunction), it must be acknowledged that much of the knowledge related to Pi-mediated effects on the endothelium stems from *in vitro* monoculture systems. However, we know that monoculture does not reflect the *in vivo* condition. In particular, we know that adjacent vascular smooth muscle cells are also responsive to Pi and could indirectly influence endothelial cells through paracrine mechanisms (and vice-versa). *In vitro* co-culture systems could aid in better understanding the interplay between smooth muscle cells and the endothelium in response to Pi.

9.8 Conclusions

The potent biological effects described in this review underline both the importance of Pi in normal cell function and in the pathological responses of endothelial cells to hyperphosphatemia. In particular, because of the almost universal role of protein phosphorylation signals in regulating cell function, the recent evidence that elevation of the intracellular Pi concentration profoundly affects protein phosphorylation, at least partly through its direct action on phosphoprotein phosphatases, has far-reaching implications both in endothelial cells and more widely throughout human cell biology.

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Common Dietary Sources of Natural and Artificial Phosphate in Food

10

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Abstract

The Recommended Dietary Allowance (RDA) for phosphate in the U.S. is around 700 mg/day for adults. The majority of healthy adults consume almost double the amount of phosphate than the RDA. Lack of awareness, and easy access to phosphate-rich, inexpensive processed food may lead to dietary phosphate overload with adverse health effects, including cardiovascular diseases, kidney diseases and tumor formation. Nutritional education and better guidelines for reporting phosphate content on ingredient labels are necessary, so that consumers are able to make more informed choices about their diets and minimize phosphate consumption. Without regulatory measures, dietary phosphate toxicity is rapidly becoming a global health concern, and likely to put enormous physical and financial burden to the society.

Keywords

RDA · Adequate Intake · Processed food · CKD · Phosphate additives

10.1 Introduction

Phosphorus is a mineral found almost exclusively in the phosphorus-containing compound phosphate (PO_4). Throughout this chapter, the term phosphate will be used most typically to describe this element [9]. Chronic kidney disease (CKD) is increasingly recognized as a serious public health issue with the incidence rising worldwide [2, 3, 27]. CKD and declining renal function are associated with impaired phosphate and calcium homeostasis, hormonal imbalance and progressive pathology of the cardiovascular and skeletal systems [10]. Hyperphosphatemia is linked to vascular calcification in CKD and an independent risk factor for cardiovascular mortality in hemodialysis patients [18]. Furthermore, serum phosphate, even when within the normal range, has been associated with cardiovascular events and all-cause mortality, both in healthy subjects and in patients with CKD [8]. In these contexts, restriction of dietary phosphate is an important intervention in the prevention of CKD and cardiovascular diseases. However, estimation of the dietary intake of phosphate is complicated by inaccuracies in nutrient database due to varied use of phosphate-containing food additives

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[35]. In recent years, the amount of phosphate intake has increased worldwide, especially in countries with high consumption of processed food [9]. Unlike sodium and other food components, phosphate is usually not listed as an ingredient, making it difficult for patients to avoid phosphate-rich food and drinks [7]. This chapter will focus on common dietary sources of natural and artificial phosphate in food within the U.S. and Japan.

10.2 Recommended Dietary Allowance (RDA) in USA

In the United States, the average person consumes far more phosphate than the Recommended Dietary Allowance (RDA) (700 mg/d for adult), although very few individuals exceed the tolerable upper limit (4000 mg/d for adults <70 year old) [34]. Moore et al. evaluated the association of dietary phosphate intake and mortality by using a nationally representative sample of healthy participants in the NHANES III [20]. The estimates of usual phosphate intake are based on information from 24-h recall data, using a validated method, with second-day recalls from a subset of the surveyed population to assess intra-individual variation [20]. Twenty-four-hour recall data from the recently completed NHANES (2009–2010) were used to show the percentage of phosphate in various food categories. Milk and dairy had the highest percentages, followed by meat and poultry. The average phosphorus intake from both foods and supplements was 1,301 mg/24 hours for women and 1,744 mg/24 hours for men, as estimated by analyzing 2013–2014 NHANES data [20]. However, several lines of evidence suggest greater intakes of phosphate than those shown in the NHANES [12]. The evidence of underestimation came from comparisons of direct chemical analyses of foods with the available estimates from the nutrient database used in the NHANES [23].

10.3 Adequate Intake (AI) in Japan

In Japan, the Recommended Dietary Allowances (RDA) was first established in 1970, after which a revision was made every 5 years. The concept of Dietary Reference Intakes (DRIs) was introduced in the sixth revision of RDA (2000–2004). DRIs were established on a scientific basis, utilizing domestic and foreign research investigations and data that are available [32]. The Adequate Intake (AI) is a quantity that is sufficient to maintain a satisfactory nutritional status of a particular gender and age group. In general, the AI is determined based on epidemiological studies that estimate nutritional intake of healthy individuals. UL means tolerable upper intake level: AI of phosphate intake in adult male is 1000 mg/day, UL is 3000 mg/day. The AI values are higher than the RDA in the EU and US (~700 mg/day). These values are based on the report of the National Nutritional Survey in Japan (NNSJ), which is initiated to obtain factual information on the nutritional health, and actual food consumption and food requirements in Japan [39]. Food intake survey by weighed food records in three consecutive representative days were conducted by specially trained dietary interviewers. NNSJ showed that phosphate intake is 1000 ~1100mg /day in 2010 [21]. However, these intake values are not estimated dietary phosphate intake obtained from nutrient content database with direct chemical analyses.

10.4 Common Phosphate-Containing Food

The American and Japanese diet's phosphate content have been increasing due to the greater consumption of food processed with phosphate additives [4, 37, 38]. Two main sources of dietary phosphate are organic, which includes animal and vegetarian proteins, and inorganic, which are mainly food additives. In general, foods high in

protein like meats, milk, eggs, and cereals are naturally high in phosphate and have represented the main source of dietary phosphate. In natural foods, phosphate is present both as inorganic ions and as a constituent of phosphoproteins, and also as membrane phospholipids, adenosine triphosphate, adenosine diphosphate, DNA, and RNA [14]. Protein-rich foods, such as meat, contain natural phosphate compounds (nucleotides, phospholipids, etc), along with naturally occurring orthophosphate. Besides the natural dietary phosphate present in food, phosphate is contained in functional food additives. Phosphate-containing additives work by sequestration of meat ions and dissociation of the actomyosin complex, bringing about an increase in water-holding capacity [1]. The direct identification of added phosphate in meat products is difficult. It represents the so-called hidden phosphate.

In contrast, plant phosphate is included in beans, cereals and nuts in the form of phytic acid or phytate [19]. In humans, the bioavailability of phosphate derived from plant foods is relatively low (less than 50%) [5]. Therefore, if phosphate content is "apparently" high in plants, the amount of phosphate actually absorbed from the intestinal tract may be less in vegetable proteins compared to animal proteins [19]. In addition, processing food by cooking, soaking, or fermenting is known to hydrolyze phytic acid. Therefore, a large amount of phosphate is likely to be bioavailable from cooked or fermented full-grain or legume foods than from raw or unprocessed foods [5].

Meat and milk products are important sources of dietary phosphate and protein. The use of phosphate additives is common both in processed cheese and meat products. Recently, Karp et al. reported that measuring in vitro digestible phosphate content of foods may reflect the absorbability of phosphate [15, 16] Measurements of in vitro digestible phosphate content of foods support that there is better phosphate absorbability in foods of animal origin than of plant origin [15, 16]. When healthy people ingested the same amount of phosphate from plant foods or animal foods, urinary excretion of phosphate was higher in the case of ingestion of meat [19]. Moe et al. demonstrated that meals having equivalent amounts of phosphate but of different sources (meats compared with grains) resulted in serum phosphate differences [19]. These data suggest the importance of bioaccessible phosphate content, not just total phosphate content in foods.

10.5 Inorganic Phosphate Additives

Inorganic Phosphate is the main component of most additives and is usually in the form of phosphoric acid, phosphates, or polyphosphates in processed foods. The food industry uses them in food processing for a variety of reasons (i.e, to extend conservation, enhance color, improve flavor, and retain moisture). No limit is given in regards to the amount of phosphate-containing preservatives. Currently, only a technological limit of 5 g/kg of food exists [7]. In the United States, the dietary phosphate burden from phosphate-containing preservatives has increased dramatically from an average of 470 mg/d in the 1990s to more than 1000 mg/d for a typical American diet in recent years [12]. In general, foods high in protein like meats, milk, eggs, and cereals are naturally high in inorganic phosphate and traditionally have represented the main source of dietary inorganic phosphate. However, this is changing as inorganic phosphate is currently being added to a large and increasing number of processed foods [36]. Preservatives are largely used in meat products (eg, chicken nuggets and hotdogs), processed cheese spreads, pasta, cooked and frozen dishes, puddings, sauces, bakery products, partially cooked and frozen foods, and soft drinks. As a result, depending on the food choices, additives may increase the inorganic phosphate intake by as much as 1.0 g/day and up to 100% of inorganic phosphate in processed foods may be absorbed [5].

The absorbability of phosphate may differ substantially among different plant foods. Despite high total phosphate content, legumes and seeds may be relatively poor phosphate sources, which could be used in the diets of patients with CKD [5]. In addition, most beverages contain little to no protein. Hence, any phosphate content is

almost entirely from additives. As a consequence, patients who consumed beverages with a high phosphate content had serum phosphate levels that were quite high although their nutritional status may have been inferior [14]. In soft drinks and beer, all phosphate were digestible. Total phosphate content in soft drinks is from phosphoric acid, which is likely to degrade efficiently in the intestine. In beer, phosphate is from the grain used in preparing beer; in Finland, this is most often barley. On the basis of the results, it seems that during malting and other processing of grain when preparing beer, phosphate is efficiently released from the grain in a highly digestible form, resulting in even higher phosphate content than in soda beverages [16].

In Japan, although the risk of food additives attracted attention in the 1970s, there has been no study on phosphate additives [13]. However, there are many kinds of used food additives. Therefore, it is extremely difficult to show the content of food additives from the nation's foods precisely. In addition, Japanese eating habits vary, and even vary by generation. For example, elderly Japanese may enjoy traditional Japanese meals (fish and rice) whereas the younger generation likes Western dietary patterns of intake common to many Americans. Daily Intake of Food Additives in Japan was determined from 1976 to 2000 by the Market Basket Method [13]. For this method, researchers purchased about 250 foods reflecting the average eating habits in different parts of Japan and examined the quantity of additives included in the food. The study showed that the average phosphate additives, specifically polyphosphate and pyrophosphate, was 259 mg/day in 2013. However, a large-scale survey for phosphate additives in Japan has not been published.

10.6 Phosphate Intake and Bioaccessibility

As mentioned above, dietary phosphate assessment is complicated by inaccuracies in nutrient database due to the widespread and varied use of phosphate-containing food additives, as well as the regional and seasonal variation in naturally occurring phosphate (organic phosphate) in various crops [12]. Oenning et al. showed a significant 20–25% underestimation of phosphate content [23]. In addition, direct chemical analyses showed that a majority of chicken products (fresh frozen chicken products in Midwestern grocery stores) contained one or more phosphate-containing ingredients, contributing a mean of 84 mg phosphate/100 g serving (range = 12–165-mg/100 g serving). Sherman et al. reported similar observations in the content of phosphate in meat, poultry, and fish products [33]. The cumulative impact of added phosphate in many food categories can significantly raise actual phosphate intake well beyond food intake estimates with existing database information [5].

A major barrier to studying the relation between dietary phosphate and various health outcomes is the current inaccuracy in estimating dietary phosphate intake, especially in terms of bio-accessibility [12]. For the nutrient database, it is necessary for the values to account for the bioaccessibility of phosphate, not just total phosphate. Of relevance, phosphate is primarily absorbed from the intestinal tract where about 70% of the intake is believed to be used. The absorption mechanism of phosphate is in the intestinal tract, but the mechanism is complicated by factors that affect absorption, such as vitamin D [17]. As described above, phosphate forms (organic and inorganic phosphate) are also important in understanding the absorption of phosphate.

10.7 Food Additives and Health

Recent studies have revealed that high phosphate intake is associated with increased mortality in a healthy US population [6]. Increased dietary phosphate intake may be detrimental even in the absence of high serum phosphate concentrations [26]. Serum phosphate concentrations are effectively regulated by fibroblast growth factor 23 (FGF23), a hormone that increases urinary phosphate excretion [28, 29]. Although individuals with normal kidney function are able to regulate serum phosphate levels in a physiologic range by increasing FGF23 concentrations,

long term effects may lead to left ventricular hypertrophy, heart failure, and mortality. More recently, studies showed that consumption of a diet rich in phosphate based food additives while stable for calcium for 1 week increased circulating FGF23, osteopontin and osteocalcin concentration relative to baseline values, and decreased mean sclerostin concentrations in healthy individuals [11]. Similar results were observed in animals fed diets with increased phosphate content; findings showed substantial decreases in bone mineral density (BMD) and structural indices. FGF23, osteopontin, and sclerostin are critically involved in regulation of bone and mineral metabolism and are associated with bone and cardiovascular disease [11]. These results indicate that high phosphate additives intake may have adverse effects in individuals with normal kidney function. In addition, Moore et al. showed that dietary intake from foods that are sources of inorganic phosphate have a greater impact on serum phosphate than do foods that are sources of mainly organic phosphate, which supports the importance of accounting for phosphate bioaccessibility in studies on dietary phosphate [20].

10.8 Conclusion and Perspective

Phosphate intake is an important issue of phosphate balance in CKD patients [22, 30, 31]. Phosphate toxicity can cause a wide range of organ damage, including renal injury, cardiovascular damage and accelerate aging processes (Fig. 10.1) [24, 25]. The information of phosphate content and phosphate type (organic and inorganic) in foods is not exactly observable. Phosphate in food additives is almost completely reabsorbed in the intestine. It is important to consider that inorganic phosphate is not only contained in natural food, but also inorganic phosphate added in processing food. In addition, since phosphate does not have mandatory labeling, accurate grasp of the phosphate content, which is present in many processed foods, is difficult to obtain. Even if the food is listed as having been prepared with phosphate salts by the United States Food and Drug Administration (FDA), the manufacturer is not obliged to display the phosphate content in food. To prevent excess phosphate intake above nutrient needs of healthy adults, the labeling of phosphate content and a database with accurate phosphate availability are required [9].

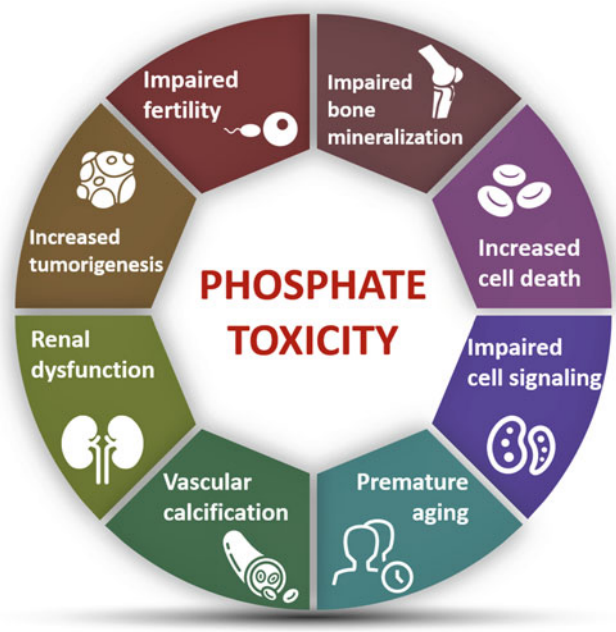

Fig. 10.1 Partial list of various pathologies induced by phosphate toxicity

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Phosphate Is a Cardiovascular Toxin

11

Maren Leifheit-Nestler, Isabel Vogt, Dieter Haffner, and Beatrice Richter

Abstract

Phosphate is essential for proper cell function by providing the fundamentals for DNA, cellular structure, signaling and energy production. The homeostasis of phosphate is regulated by the phosphaturic hormones fibroblast growth factor (FGF) 23 and parathyroid hormone (PTH). Recent studies indicate that phosphate induces phosphate sensing mechanisms via binding to surface receptors and phosphate cotransporters leading to feedback loops for additional regulation of serum phosphate concentrations as well as by phosphate itself. An imbalance to either side, enhances or reduces serum phosphate levels, respectively. The latter is associated with increased risk for cardiovascular diseases and mortality. Hyperphosphatemia is often due to impaired kidney function and linked to vascular disease, hypertension and left ventricular hypertrophy. In contrast, hypophosphatemia either due to reduced dietary intake or intestinal absorption of phosphate or hereditary or acquired renal phosphate wasting, may result in impaired energy metabolism and cardiac arrhythmias. Here, we review the effects and its underlying mechanisms of deregulated serum phosphate concentrations on the cardiovascular system. Finally, we summarize the current therapeutic approaches for both lowering serum phosphate levels and improvement of cardiovascular disease.

Keywords

Phosphate · Fibroblast growth factor 23 · Parathyroid hormone · Vascular disease · Left ventricular hypertrophy · Intervention

11.1 Physiology of Phosphate Homeostasis

The element phosphorus is essential for normal cell function and occurs in combination with oxygen by generating phosphate. In the body, phosphate is found as structural element in teeth and bone in the form of hydroxyapatite and as phospholipids in cell membranes. Furthermore, DNA is a chain of PO_4^{3-}-containing molecules. Phosphate is involved in energy production as energy-carrying molecule adenosine triphosphate (ATP) and in the activation of enzymes, hormones and signaling cascades. In red blood cells, the salt 2,3-diphosphoglycerate controls the oxygen release to tissue by hemoglobin.

Serum phosphate levels are subject to tightly regulated homeostasis determined by dietary

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intake, intestinal absorption and renal glomerular filtration and tubular reabsorption. Active transport of phosphate is facilitated via type II sodium-dependent phosphate co-transporters NaPi-2b in the brush border membrane (BBM) of enterocytes and NaPi-2a and NaPi-2c in the BBM of renal proximal tubule cells. Type III sodium-coupled phosphate transporters, PiT1 and PiT2, initially discovered as retroviral receptors, are ubiquitously expressed in mammalian tissue [1, 2]. Low dietary phosphate upregulates the expression of PiT1 in the renal proximal tubule cell [3]. Both phosphaturic hormones parathyroid hormone (PTH) and fibroblast growth factor (FGF) 23 downregulate PiT2 in proximal tubules of the kidney [4, 5]. For now, the contribution of PiTs for renal and intestinal phosphate transport is not fully understood. The tubular reabsorption of phosphate is regulated by FGF23 and PTH, which regulate the renal production and secretion of active vitamin D ($1,25(OH)_2D_3$). The latter in turn enhances intestinal absorption of dietary phosphate in the jejunum and ileum by stimulating NaPi-2b-dependent phosphate transport. In the kidney, PTH decreases the abundance of NaPi-2a and NaPi-2c in the proximal tubule BBM leading to reduced tubular reabsorption of phosphate.

Normal serum phosphate levels in healthy adults are 2.5–4.5 mg/dL [6]. Hyperphosphatemia is defined as serum phosphate levels more than 4.5 mg/dL. For hypophosphatemia, it is distinguished between mild (2–2.5 mg/dL), moderate (1–2 mg/dL) and severe (<1 mg/dL).

11.1.1 FGF23

The main source of FGF23 is the bone where it is synthesized by osteocytes and osteoblasts due to different stimuli, among phosphate is one of them. However, the mechanisms how exactly phosphate affects FGF23 expression in bone are still unknown. FGF23`s main target organ are the kidneys where it binds to a complex of FGF receptor 1 (FGFR1) and its specific co-receptor klotho in renal proximal tubule cells [7]. The FGF23/FGFR1/klotho complex activates an intracellular cascade involving signal transduction via fibroblast growth factor receptor substrate (FRS) 2a and extracellular signal-regulated kinases (ERK) 1/2 affecting serum phosphate levels by two mechanisms. First, FGF23-mediated ERK1/2 activation inhibits both the expression of NaPi-2a and NaPi-2c and their abundance in the renal BBM. The decreased bioavailability of the transporters reduces renal phosphate reabsorption in the proximal tubules and thereby increases phosphate excretion in urine. Second, through the activation of the same pathway, FGF23 downregulates the expression of CYP27B1, encoding for 1α-hydroxylase, which converts prohormone 25-hydroxyvitamin D3 into the active form $1,25(OH)_2D_3$ [8], and upregulates the expression of CYP24A1, which encodes for the catabolic enzyme 24-hydroxylase. Reduced renal synthesis of active vitamin D leads to decreased serum $1,25(OH)_2D_3$ levels and thus, a low abundance of NaPi-2b in the gastrointestinal tract limiting phosphate uptake [9]. Both the suppression of renal phosphate reabsorption and reduction of intestinal phosphate absorption mediated by FGF23 lower the phosphate level in the serum.

11.1.2 PTH

PTH is the second phosphaturic hormone regulating phosphate homeostasis. High serum phosphate concentrations stimulate the secretion of PTH in the parathyroid glands that increases renal phosphate excretion. Thereby, PTH binds to the G-protein coupled PTH receptor 1 (PTHR1) that increases intracellular cyclic adenosine monophosphate (cAMP) levels and subsequently activates protein kinase A (PKA), protein kinase C (PKC) and ERK1/2 [10–12]. Activation of these signaling cascades leads to the internalization of NaPi-2a and NaPi-2c co-transporters from the BBM, resulting in renal phosphate wasting [13, 14]. To note, PTHR1 expression is found at both the apical and the basolateral membranes of renal proximal tubular cells [15]. In addition to maintaining phosphate homeostasis, PTH further interacts with FGF23 in the parathyroid gland.

Similarly to the kidney, FGF23 also binds to and activates the FGFR1/klotho complex in the parathyroid. The subsequently induced ERK1/2 signaling promotes the expression of the transcription factor early growth response 1 (Egr-1), which in turn decreases the transcription and secretion of PTH. *In vitro* and *in vivo* studies have shown that PTH induces the FGF23 expression in bone that initiates a negative feedback loop [16]. Thereby, FGF23 mRNA expression is increased via activation of intracellular PKA and WNT pathways by PTHR1. Furthermore, PTH affects bone mass by stimulating both bone resorption and bone formation. During chronic hyperparathyroidism, the catabolic actions of PTH reduce the bone mineral density causing an increased risk for fractures because of a net loss of bone mass [17]. During increased bone turn over, the liberation of calcium-phosphate product leads to an increase of serum phosphate levels and, over time, possibly to soft tissue calcifications. Interestingly, intermittent, low doses of PTH improve bone formation via stimulation of osteoanabolic WNT signaling [18, 19]. Due to the missing phosphaturic effect of PTH during hypoparathyroidism, patients display hyperphosphatemia [20]. Thus, hypoparathyroidism causes a reduction in bone remodeling with abnormalities in bone strength and microarchitecture as well as increased bone density [21].

11.1.3 Phosphate

Phosphate sensing has been extensively studied in bacteria and yeast (excellently reviewed in [22]). Since these unicellular organisms use types of phosphate transporters for phosphate sensing, it might be considered to hypothesize similar mechanisms for mammalian cells. *In vitro* data by Bon et al. support this idea. The authors show that by deleting PiT1 or PiT2, the phosphate-induced ERK1/2 activation as well as increase in gene expression of matrix Gla protein (MGP) and osteopontin were blunted [23]. Furthermore, overexpressing phosphate transport-deficient PiT mutants rescued the activation of ERK1/2. Even in the absence of phosphate transport activity, the heterodimerization of PiT1 and PiT2 was regulated by extracellular phosphate. These data suggest that the phosphate sensing function of PiT co-transporters does not depend on cellular phosphate uptake, but on phosphate binding. In a follow-up study, the authors addressed the question if PiT1 and PiT2 contribute to the regulation of FGF23 secretion from bone. *Ex vivo* results suggest that PiT1 deletion in long bones does not contribute to the phosphate-mediated FGF23 secretion [24]. In contrast, by using *ex vivo* organ culture of long bones from PiT2 knockout animals, the authors showed that PiT2 is involved in the phosphate-dependent secretion of FGF23. They conclude that at least in hyperphosphatemic situations PiT2 plays a role for phosphate-mediated sensing to induce osseous FGF23.

Recently, it was shown that unliganded FGFR1 serves as phosphate-sensing receptor regulating the production of FGF23 in bone [25]. *In vivo* and *in vitro* data show that phosphate does not directly increase the expression of FGF23, but the expression of the enzyme encoded by the N-acetylgalactosaminyltransferase (GALNT) 3 gene via induction of the ERK1/2 signaling pathway. FGF23 is posttranslationally modified via several steps. GALNT3 initiates the O-glycosylation of FGF23, which prevents its cleavage and increases the bioavailability of active FGF23 in the serum [26, 27]. The authors show that inhibition and deletion of FGFR1 in osteoblasts/osteocytes prevented the dietary phosphate-induced GALNT3 expression in bone and subsequent elevation of serum FGF23 levels [28]. Thus, high phosphate load activates ligand-independent FGFR1-mediated intracellular signaling events in bone cells resulting in increased serum FGF23 levels to regulate serum phosphate levels.

The calcium-sensing receptor (CaSR) is the main controller of PTH secretion [29]. Centeno et al. identified the CaSR in the parathyroid as phosphate sensor [30]. Here, the authors show that extracellular phosphate is a noncompetitive antagonist of CaSR resulting in phosphate-stimulated PTH secretion. Phosphate binds to phosphate-binding sites in the CaSR causing a

conformational change of the receptor [30]. Phosphate-induced CaSR inhibition leads to its open, inactive form that permits increased PTH secretion. In turn, PTH stimulates renal phosphate excretion to normalize serum phosphate levels.

Taken together, most likely several molecules function as phosphate sensing receptors and interfere in the feedback of phosphate regulation. More studies are needed to clarify the involved players and signaling events.

11.2 Phosphate-Related Cardiovascular Disease

Impaired phosphate homeostasis can result in hyper- or hypophosphatemia causing multiple phosphate-related disorders, such as cardiovascular diseases (CVD). Hyperphosphatemia can develop as a result of increased phosphate intake, defects in bone mineralization, genetic disorders in genes of phosphaturic hormones or an impaired renal phosphate excretion [31]. Most studies, analyzing the cardiovascular (CV) risk of phosphate were obtained from chronic kidney disease (CKD) patients, because the progressive loss of kidney function leads to the development of hyperphosphatemia that is associated with vascular calcification, left ventricular hypertrophy (LVH) and increased CV mortality in CKD patients [32–36]. In the general population, adverse effects of high phosphate levels gained attention as the average dietary phosphate intake of 1200 mg greatly exceeds the recommended dietary allowance (RDA) of 700 mg in the Western population [37]. Epidemiologic studies show an association of high serum phosphate levels with an increased all-cause and CV mortality in the general population [37–39]. In subjects with preserved renal function, increased serum phosphate levels associate with an increased risk for vascular calcification even within normal phosphate range [40–43]. The higher risk of coronary artery calcification (CAC) was observed in young white and African-American adults (mean age: 25.2 years) [40] as well as in Korean participants with mean age of 40.8 [42] or 53.5 [43] years, respectively, suggesting an association of high phosphate with vascular calcification irrespective of age and ethnic origin. High phosphate levels enhance the ankle brachial index indicating that phosphate contributes to peripheral arterial stiffness [41]. In participants without pre-existing CVD or CKD, high serum phosphate and dietary phosphate intake correlate with an increased left ventricular (LV) mass and prevalence of LVH [44–46]. In a small cohort of 20 healthy young adults, controlled high dietary phosphate intake over 11 weeks increased systolic and diastolic blood pressure (BP) [47]. The underlying mechanisms of multiple hyperphosphatemia-related CV events and increased mortality are not completely understood and therefore focus of research.

11.2.1 Mechanisms of Phosphate-Induced Vascular Calcification

Phosphate-induced vascular calcification occurs predominantly in form of pathological calcium phosphate depositions in the medial layer of the arteries. Medial vascular calcification is a common health risk in CKD, diabetes and aging but also high serum phosphate levels in the general population are associated with CAC. Vascular smooth muscle cells (VSMCs) play a key role in the onset and progression of phosphate-induced vascular calcification. In high phosphate conditions, VSMCs undergo osteochondrogenic transdifferentiation promoting calcification by reducing calcification inhibitors, stimulating extracellular matrix (ECM) remodeling, apoptosis, senescence, and pro-inflammatory responses [48, 49].

VSMCs incorporate phosphate via PiT1 and PiT2 [50]. PiT1 also acts as phosphate sensor and mediates vascular calcification via activation of ERK1/2 signaling independent of phosphate uptake. In transport-deficient PiT1 mutants that cannot incorporate phosphate and wild type VSMCs, high phosphate levels promote ERK1/2 signaling and osteochondrogenic differentiation, which is attenuated by knockdown of PiT1 [51, 52]. In contrast, knockdown of PiT2

increased phosphate-induced calcification by lowering osteoprotegerin (OPG) in VSMCs suggesting a protective function of PiT2 in phosphate-mediated calcification [53] (Fig. 11.1). It is discussed that Toll-like receptor 4 (TLR4) might be involved in extracellular phosphate-sensing as it is upregulated under high phosphate conditions *in vitro* in VSCMs. Likewise, knockdown of TLR4 by siRNA reduces calcification and expression of inflammatory cytokines in VSMC [54].

11.2.1.1 Phosphate-Dependent Osteochondrogenic Transdifferentiation of VSMCs

High extracellular phosphate levels activate several intracellular pathways stimulating the transdifferentiation from contractile into osteochondrogenic VSMCs cells. The nuclear factor "'kappa-light-chain-enhancer" of activated B cells (NF-κB) is a key regulator of phosphate-induced osteochondrogenic transdifferentiation and subsequent vascular calcification (Fig. 11.1). Phosphate activates NF-κB signaling in VSMCs via serum- and glucocorticoid-inducible kinase (SGK1) [55], oxidative stress [56] or inflammatory pathways [57]. Activation of NF-κB promotes the expression of osteochondrogenic transcription factors, such as msh homeobox 2 (MSX2) and core-binding factor subunit α1 (CBFA1) and concurrently decreases the expression of the VSMC-specific marker SM22α [54, 55, 57]. The osteochondrogenic transcription factors further increase the expression of alkaline phosphatase (ALP) that promotes mineral deposition in VSMCs [57, 58]. Inhibition of NF-κB is sufficient to reduce calcification during high phosphate conditions in VSMCs *in vitro* and in a CKD mouse model *in vivo* [54, 59].

Furthermore, activation of WNT/β-Catenin signaling by phosphate contributes to osteochondrogenic transdifferentiation and vascular calcification (Fig. 11.1). In VSMCs, high phosphate induces β-Catenin activity and downstream MSX2, CBFA1 and PiT1 expression [60, 61]. Besides the induction of transdifferentiation, WNT signaling promotes calcification by stimulating the expression of the matrix metalloproteinases MMP2 and MMP9 in VSMCs [62]. Rodent CKD models fed with a high phosphate diet show an association of WNT/β-Catenin activation and aortic calcification [61, 63]. The lentiviral knockdown with short hair pin RNA of β-Catenin attenuates vascular calcification *in vivo* [61]. Likewise, inhibitors of WNT/β-Catenin signaling such as secreted frizzled-related proteins [64], Dickkopf-related protein 1 [65] or the knockdown of WNT8b [66] reduces phosphate-induced calcification in VSMCs.

Experimental studies suggest that interaction of WNT/β-Catenin and peroxisome proliferator-activated receptor gamma (PPARγ) signaling contribute to calcification during hyperphosphatemia [67]. In the differentiation of mesenchymal stem cells PPARγ and WNT act antagonistically. PPARγ activates adipocyte differentiation and suppresses osteoblast differentiation, whereas WNT/β-Catenin signaling promotes osteogenesis and inhibits PPARγ expression [68]. Hyperphosphatemia induces calcification by downregulating the expression of PPARγ and its downstream target klotho in VSMCs and rodent CKD. The PPARγ agonist Rosiglitazone stimulates the expression of klotho and reduces vascular calcification *in vitro* and *in vivo* [69, 70]. Other PPARγ agonists, Pioglitazone and Ginsenoside Rb 1, exert anti-calcific effects by inhibiting WNT/β-Catenin signaling [67, 71].

Phosphate-mediated downregulation of AKT signaling might as well contribute to vascular calcification in VSMCs [72, 73]. Upregulation of AKT signaling by the farnesyl transferase inhibitor 277 prevents osteochondrogenic differentiation and mineral deposition in VSMCs [73]. Extracellular acid loading stimulates AKT phosphorylation and could reduce phosphate-mediated apoptosis and calcification [72]

11.2.1.2 ECM Remodeling During Hyperphosphatemia

The ECM is a highly structured network composed of fibrous proteins and proteoglycans [74]. Remodeling of the ECM by phosphate creates an environment that fosters the development of vascular calcification in which elastin

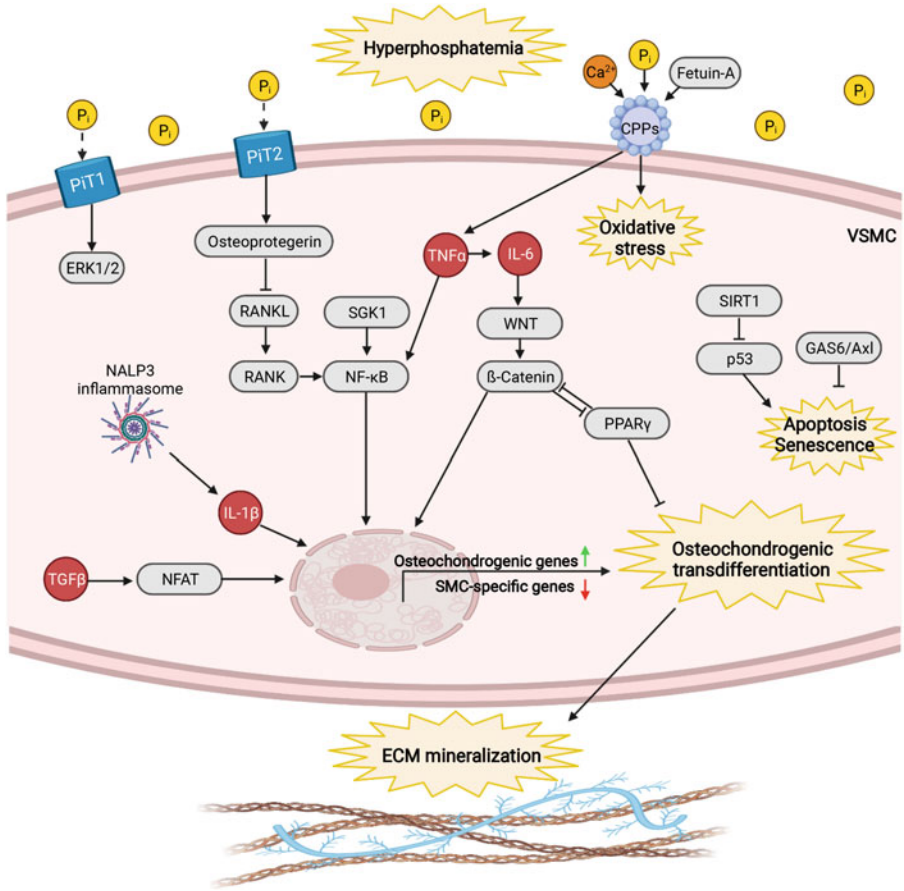

Fig. 11.1 Hyperphosphatemia-induced mechanisms in vascular smooth muscle cells contributing to vascular calcification. High extracellular phosphate (P_i) stimulates the production of inflammatory cytokines, oxidative stress, apoptosis, senescence and osteochondrogenic transdifferentiation in VSMCs, which together contribute to increased ECM mineralization. The simplified schematic illustration shows the main pathways involved in P_i-mediated VSMC dysfunction. P_i sensing and uptake via PiT1 promotes osteochondrogenic transdifferentiation, whereas PiT2-mediated increase in osteoprotegerin protects from calcification. P_i, TNFα and IL-6 promote osteochondrogenic transdifferentiation by upregulating the expression of osteochondrogenic genes and downregulating SMC-specific genes via NF-κB and WNT/β-Catenin signaling. Osteochondrogenic transdifferentiation is also stimulated by P_i-induced TGFβ/NFAT and NALP3/IL-1β activation. P_i stimulates apoptosis by downregulating GAS6/Axl and senescence by downregulating SIRT1 expression. Incorporated in CPPs, P_i induces oxidative stress and inflammation in VSMCs. (This figure was created with BioRender.com)

degradation via MMPs plays a crucial role. MMP2 and MMP9-knockout mice are rescued from calcium chloride induced aortic injury and neither develop elastin degradation nor calcification [75, 76]. Hyperphosphatemia predominantly triggers ECM remodeling and calcification in the medial layer of vasculature [77–79]. Phosphate-induced expression of MMP2, MMP9 and the cysteine protease cathepsin S promote degradation of extracellular matrix proteins and thereby calcification *in vitro* and *in vivo* [80–82]. Elastin-derived peptides only induce calcification under high phosphate conditions in VSMCs and in uremic mice suggesting that elastin-derived peptides are necessary but not sufficient to initiate calcification [76, 83]. In CKD patients on dialysis,

increased MMP2 expression correlates with elastic fiber disorganization and calcification [84].

High phosphate levels are further associated with an increased collagen I (Col I) expression in clinical and experimental studies [85–88]. Col I promotes matrix vesicle-mediated mineralization and accelerates vascular calcification *in vitro* [88, 89]. Additionally, phosphate mediates collagen cross-linking by upregulating the enzymes lysyl hydroxylase 1 (PLOD1) and lysyl oxidase (LOX). Inhibition of collagen synthesis or cross-linking reduce phosphate-induced calcification in VSMCs [86, 90].

11.2.1.3 Phosphate-Induced Apoptosis, Senescence and Autophagy

Another mechanism how phosphate promotes calcification is apoptosis. In arteries of children with CKD, dialysis induces VSMCs apoptosis, followed by osteochondrogenic differentiation and subsequent severe calcification [91]. Further studies give evidence that VSMCs apoptosis occurs before the onset of calcification and inhibition of apoptosis with a caspase inhibitor can reduce calcification [92, 93]. In VSMCs, elevated phosphate levels induce apoptosis by downregulating the survival pathway mediated by growth arrest-specific gene 6 (Gas6) and its receptor Axl (Fig. 11.1). Statins could stabilize Gas6 mRNA and thereby have anti-apoptotic and anti-calcific effects in phosphate-induced calcification [94].

Senescence describes a permanent cell arrest that occurs in age or due to stress. In VSMCs and CKD rats, hyperphosphatemia increases the senescence-associated β-galactosidase activity that is associated with calcification. Phosphate mediates senescence via downregulation of Sirtuin 1 expression and subsequent activation of p53 and p21 [95–97] (Fig. 11.1).

Autophagy counteracts phosphate-induced vascular calcification by inhibiting osteochondrogenic transdifferentiation and matrix vesicle release [98–100]. In high phosphate diet fed mice and VSMCs under high phosphate culture conditions, autophagy is increased indicating an endogenous mechanism to protect from calcification. However, a recent study suggests that high phosphate inhibits VSMCs autophagy via upregulation of OGlcNAc transferase and subsequently the Hippo-YAP pathway promoting phosphate-induced calcification [101]. Although, the direct effect of phosphate on autophagy needs further investigation, induction of autophagy might be an interesting therapeutic strategy to reduce phosphate-induced calcification.

11.2.1.4 Regulation of Endogenous Calcification Inhibitors by Phosphate

Extracellular fluids are supersaturated with calcium and phosphate and therefore circulating calcification inhibitors are necessary to prevent spontaneous ectopic precipitation. Pyrophosphate, fetuin-A, vitamin K dependent MGP, osteopontin (OPN) and osteoprotegerin are endogenous calcification inhibitors [102, 103].

In end-stage kidney disease (ESKD), i.e., in patients on dialysis, reduced plasma pyrophosphate levels are associated with arterial calcification [104–106]. Experimental studies show that high dietary phosphate intake reinforces aortic calcification in mice lacking ectonucleotide pyrophosphatase phosphodiesterase, the enzyme that synthesizes extracellular pyrophosphate [107]. *In vitro*, physiological levels of pyrophosphate are sufficient to prevent calcium phosphate deposition in VSMCs under normal phosphate concentrations but not in hyperphosphatemia [108]. *In vivo*, daily injections of pyrophosphate could prevent aortic calcification in uremic rodent models [109, 110].

Fetuin-A is a liver-derived circulating glycoprotein that builds complexes with calcium and phosphate to prevent ectopic precipitation. Recently, it was shown that fetuin-A levels decrease with the progression of CKD [111]. Experimental studies in uremic rodents show that low fetuin-A levels promote the precipitation of calcium phosphate and high levels protect from calcification [112, 113]. In fetuin-A deficient CKD mice, additional challenge with a high phosphate diet induces more profound calcification than in wild type CKD mice

[114]. However, several clinical studies confirm a correlation of low circulating fetuin-A levels with increased calcification in dialysis patients [111, 115–117], whereas others did not find a correlation of fetuin-A or OPN with calcification [118].

MGP is another endogenous calcification inhibitor synthesized by VSMCs and chondrocytes [119]. High extracellular phosphate stimulates the expression of MGP by activating the ERK1/2-Fra-1 pathway *in vitro* [120–122] that might counteract phosphate-induced calcification. However, the activity of MGP is vitamin K dependent. It was shown in CKD and ESKD patients that dephosphorylated uncarboxylated MGP (dp-ucMGP), the inactive form of MGP, is associated with vitamin K deficiency and vascular calcification [123–125], indicating that MGP cannot exert its anti-calcific function in these patients due to the lack of vitamin K.

OPN is an inhibitor of hydroxyapatite crystal formation *in vivo* and *in vitro* that is induced by hyperphosphatemia [126–128]. Physiologically, OPN is not found in arteries but under pathological conditions, such as ESKD, it localizes to areas of calcification [129]. Several studies demonstrate that high levels of OPN protect from calcification, whereas OPN deficiency promotes calcification [130–132].

OPG, a soluble receptor of the TNF-receptor family, acts anti-calcific by inhibiting osteoclastogenesis. OPG binds to the Receptor Activator of NF-κB Ligand (RANKL) and thereby prevents its interaction with the RANK receptor. Inhibition of the RANK-RANKL interaction downregulates NF-κB signaling as key regulator of phosphate-induced osteochondrogenic differentiation and vascular calcification [133, 134]. Consequently, knockdown of OPG leads to medial calcification that is further enhanced by high dietary phosphate intake in mice [135]. In contrast, clinical studies show that elevated OPG levels are associated with increased vascular calcification and mortality in CKD patients [136–139]. However, increasing OPG levels might rather be a compensatory mechanism to inhibit progression of calcification instead of being the underlying cause for it.

11.2.1.5 Hyperphosphatemia-Associated Inflammatory Pathways

Pro-inflammatory proteins and cytokines are associated with vascular calcification in CKD patients [140–142] and promote vascular calcification in VSMCs *in vitro* [143–145]. In patients on chronic hemodialysis, increased C-reactive protein (CRP) levels associated with more severe vascular calcification [141]. Already in early CKD stages, medial calcification of the aorta is accompanied by vascular inflammation and osteochondrogenic differentiation of VSMCs [142]. Experimental studies reveal that high phosphate levels directly stimulate the expression of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), IL-1β, IL-18 and transforming growth factor β (TGFβ) [146–151]. High cytokine levels activate several intracellular pathways that contribute to osteochondrogenic transdifferentiation and the development of vascular calcification (Fig. 11.1).

TNFα activates NF-κB signaling in VSMCs that promotes the osteochondrogenic transdifferentiation by upregulating the osteogenic transcription factor MSX2 and ALP activity [57]. In a diabetic mouse model, increased TNFα levels induce vascular calcification via bone morphogenetic protein 2 (BMP2), MSX2 and WNT signaling [152, 153]. Besides activating pro-calcific intracellular pathways, TNFα stimulates the expression of the pro-inflammatory cytokine IL-6 [154]. It is under debate whether IL-6 alone is sufficient to induce vascular calcification or just in an inflammatory environment. Sun et al. found that IL-6 induces calcification via BMP2 in VSMCs under otherwise normal non-calcific culture conditions [145]. In contrast, in the study of Deuell et al. TNFα, but not IL-6 alone, was sufficient to induce mineralization of VSMCs in high phosphate but not control media. However, co-incubation of TNFα-treated VSMCs with IL-6 further increased calcification suggesting a synergistic effect. TNFα and IL-6 also interfere with RANKL-NF-κB signaling. RANKL stimulates the expression of TNFα and IL-6 in macrophages and *vice versa*, neutralization of TNFα, IL-6 or both cytokines reduce RANKL-

mediated calcification in co-cultures of macrophages and VSMCs [155]. Another study shows that neutralization of IL-6 further prevents RANKL-mediated expression of the osteogenic markers CBFA1 and BMP2 and the calcification inhibitor OPN [156]. In summary, both, TNFα and IL-6, interfere with NF-κB and WNT signaling to induce vascular calcification.

Furthermore, high phosphate activates the NALP3 inflammasome resulting in higher expression of IL-1β and calcification in VSMCs. Inhibition of the NALP3 inflammasome mitigates IL-1β secretion and calcification [144]. Phosphate also stimulates TGFβ expression in VSMCs [150, 157] and TGFβ promotes osteochondrogenic gene expression via upregulating the transcription factor NFAT5 and its downstream target SOX9 [157, 158]. Moreover, TGFβ contributes to remodeling of the ECM by induction of Col I, fibronectin, osteocalcin and OPN [150, 159].

11.2.1.6 The Role of Calciprotein Particles in Vascular Calcification

In calcium and phosphate supersaturated fluids, circulating calcification inhibitors are necessary to prevent spontaneous ectopic precipitation. Fetuin-A, MGP and GRP form primary calciprotein particles (CPP) with calcium and phosphate to enable efficient clearance of amorphous calcium phosphate clusters. The primary CPPs can undergo ripening into a highly structured more stable crystalline form called secondary CPPs [160, 161]. High dietary phosphate intake increases serum CPP levels in experimental as well as clinical studies [162, 163]. In serum of dialysis patients, predominantly primary CPPs occur, most likely, because the ripening process is time-consuming and primary CPPs are rapidly cleared in the liver by sinusoidal endothelial cells. Secondary CPPs are cleared by liver-resident Kupffer cells via class A scavenger receptor-mediated endocytosis [160, 164, 165]. Clinical studies give evidence that increased CPP levels are associated with aortic stiffness in pre-dialysis CKD patients and coronary atherosclerosis [166, 167].

The predisposition of serum to form crystals can be assessed by the T_{50} test *in vitro*. In this test, serum is supersaturated with calcium and phosphate to determine the necessary time to convert 50% of primary into secondary CPPs. Shorter T_{50} times represent a higher propensity of the serum to form crystals. In CKD patients, lower T_{50} times are associated with progression of vascular calcification, increased atherosclerotic CV disease events and CV and all-cause mortality [166, 168–171].

In vitro studies show that secondary but not primary CPPs cause calcification of VSMCs by activating inflammatory pathways and inducing oxidative stress (Fig. 11.1). Treatment of VSMCs with secondary CPPs stimulates TNFα and activates NF-κB signaling promoting osteochondrogenic transdifferentiation [148]. In macrophages, secondary CPPs also stimulate pro-inflammatory TNFα and IL-1β [172]. In CKD patients, there is evidence that CPPs directly induce pro-calcific pathways but at least the T_{50} test serves as a marker for the progression and appearance of CV complications.

11.2.2 Hyperphosphatemia-Associated Left Ventricular Hypertrophy

Elevated serum phosphate levels are associated with an increased LV mass and incidence of LVH in the general population [44–46]. In a large prospective study with 4005 healthy young adults, 4.5% develop LVH during a 5-year follow-up. The development of LVH correlates with increased baseline phosphate levels assessed at the beginning of the study [46]. Additionally, Dhingra et al. found a higher prevalence of incident heart failure and increased LV mass in subjects with high serum phosphate levels in a prospective study with 3300 participants [44]. Besides elevated serum phosphate levels, high dietary phosphate intake is associated with an increasing LV mass in a multi-ethnic cohort of 4494 healthy participants [45]. Hyperphosphatemia is also a common CV risk factor in CKD patients because of the insufficient renal

phosphate clearance, and correlates with an increased LV mass [173, 174]. Nevertheless, these studies have limitations i.e., the serum FGF23 levels were not measured. FGF23 is a well-known risk factor and inducer for LVH [173–178]. Thus, it is not possible to discriminate whether cardiac hypertrophy is directly mediated by phosphate or phosphate-induced FGF23 elevation. Furthermore, vascular calcification, especially medial calcification, not only leads to vascular stiffness, but also to LVH and systolic hypertension [179]. The structural changes by calcium-phosphate deposition in the vasculature leading to CAC can be diagnosed and quantified by electron beam computer tomography. Functional changes are measured by pulse wave velocity indicating vascular stiffness (Fig. 11.2d–e).

So far, experimental studies could also not clarify whether phosphate directly causes LVH. In some studies, feeding of a 2% high phosphate diet to wild type mice for 12 weeks induce LVH and cardiac fibrosis [180–182], whereas in others the cardiac function is not altered [183, 184]. Grabner et al. suggest that a high

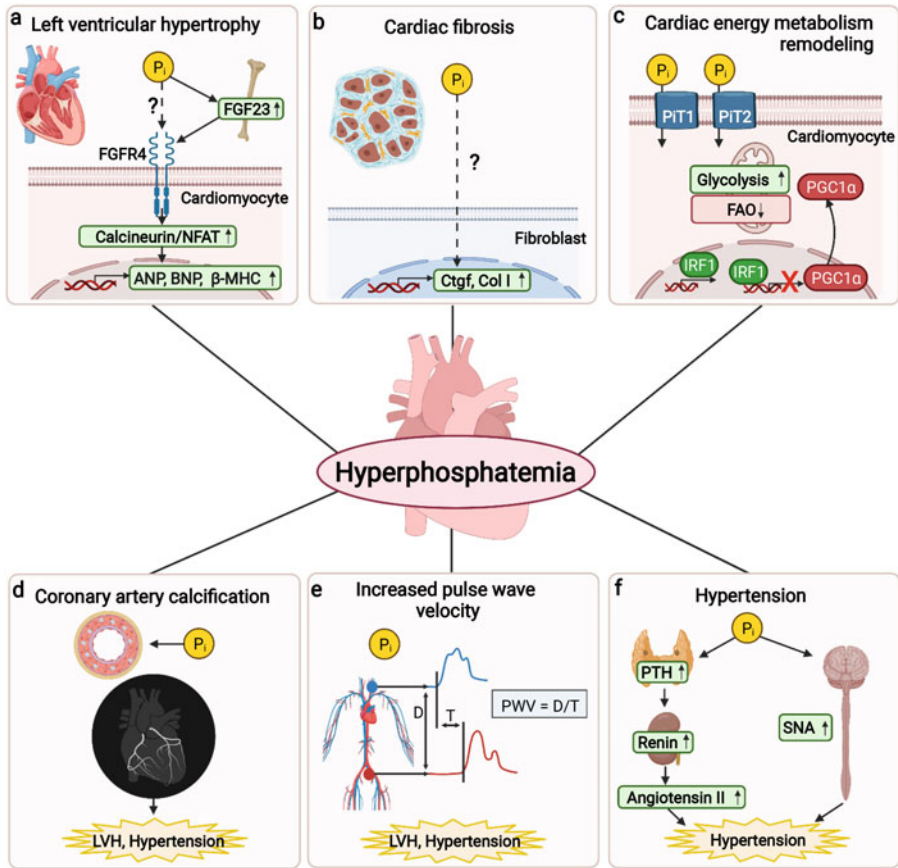

Fig. 11.2 Hyperphosphatemia-associated cardiac diseases. (**a**) P_i induces LVH via FGF23/FGFR4/calcineurin/NFAT signaling in cardiomyocytes or an unknown direct mechanism. (**b**) High P_i stimulates the expression of fibrotic genes Ctgf and Col I in cardiac fibroblasts via unknown mechanisms, (**c**) P_i promotes cardiac energy metabolism remodeling by entering cardiomyocytes via PiT1 and PiT2 to activate IRF1 expression that inhibits PGC1α. Downregulation of PGC1α reduces FAO and increases glycolysis. (**d**) Medial calcification, diagnosed by electron beam computer tomography and (**e**) increased pulse wave velocity, leads to LVH. (**f**) P_i triggers hypertension via PTH-mediated activation of the RAAS system and increased sympathetic nerve system (SNA) activity. (This figure was created with BioRender.com)

phosphate diet increases FGF23 and promotes LVH via FGFR4 [180] (Fig. 11.2a). This supports the hypothesis that phosphate mediates LVH only indirectly via FGF23. FGF23 promotes LVH by activating the PLCγ/calcineurin/NFAT pathway via FGFR4 [175]. *In vitro* studies in cardiac fibroblasts show pro-fibrotic effects of high phosphate that stimulates the expression of connective tissue growth factor and Col I but not Smad2/3 [181]. Thus, the contribution of phosphate for the development of cardiac fibrosis remains unsolved (Fig. 11.2b).

In the studies of Amann et al. and Neves et al., a 1.2% high phosphate diet for 8 weeks only promotes the development of LVH and cardiac fibrosis in uremic but not wild type rats [185, 186]. In CKD mice, feeding of a 2% high phosphate diet for 12 weeks further enhance LVH compared to a normal phosphate diet [187]. Huang et al. show that phosphate alone induces the hypertrophic markers ANP, BNP and β-MHC in the embryonic rat cardiomyocyte H9c2 cell line and in primary neonatal rat ventricular myocytes *in vitro*. Additionally, high phosphate disturbs the myocardial energy metabolism in H9c2 cells *in vitro* and in CKD mice *in vivo*. Phosphate enters cardiomyocytes via PiT1 and PiT2 and increases the expression of interferon regulatory factor 1 (IRF1) (Fig. 11.2c). IRF1 inhibits the expression of peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α) and thereby, induces the switch from fatty acid oxidation to glucose dependent metabolism [187]. Together, these studies indicate that phosphate might only induce LVH under certain metabolic conditions. The source of phosphate and further composition of the diet, such as calcium or vitamin D content, could influence phosphate absorption and related effects. Inorganic phosphate is absorbed by over 90% in the intestine, organic phosphates only by 40–60% [188]. Therefore, phosphate composition of the diet plays a crucial role in the actual phosphate absorption and could influence phosphate-mediated effects. Furthermore, experimental studies give evidence that low vitamin D concentrations reduce high phosphate diet-induced LVH, but augment fibrosis. In contrast, high vitamin D concentrations promote high phosphate diet-induced cardiac hypertrophy and attenuate fibrosis [182]. Future studies need to investigate whether phosphate could cause LVH directly or indirectly and trigger cardiac remodeling only under certain metabolic conditions.

11.2.3 The Role of Hyperphosphatemia in Hypertension

Hyperphosphatemia is a potential risk factor for the development of hypertension [189]. In a large cohort of more than 9000 hypertensive participants, higher baseline serum phosphate levels are associated with increased systolic BP after 5-year follow-up. However, the poor BP control in patients with elevated baseline serum phosphate level does not correlate with the increased mortality in these patients [190]. A study in CKD patients reveals that serum phosphate levels only associate with higher systolic and diastolic BP in diabetic but not in non-diabetic CKD patients [191]. Also in the general population, high dietary phosphate intake for 6 weeks increases systolic and diastolic BP in young healthy adults that is not reversed by a concomitant vitamin D treatment for another 5 weeks. Reducing the high dietary phosphate load to a normal diet reverse the phosphate-induced elevation in BP after 2 months. Mohammad et al. suggest activation of the sympathetic nerve system as the underling mechanism for phosphate-induced hypertension and pulse rate elevation [47]. This study is limited by its small cohort size of 20 subjects, so the results have to be validated in larger cohorts.

However, experimental animal studies support the idea that phosphate stimulates the sympathetic nerve system (Fig. 11.2f). Likewise to the clinical study of Mohammad et al., feeding of a high phosphate diet activates the sympathetic nerve system and increases BP in healthy rats [192]. Independent of kidney function, a high phosphate diet also augments phenylephrine-induced vasoconstriction in aortic rings from

mice *ex vivo* [193]. Another mechanism of phosphate-induced hypertension might be the activation of the renin angiotensin aldosterone system. High dietary phosphate intake increases renin expression via PTH and subsequently angiotensin II to induce hypertension in healthy rats [194]. The effect of hyperphosphatemia on hypertension and its underlying mechanisms has to be confirmed in larger clinical trials to develop suitable therapeutic strategies.

11.3 Hypophosphatemia and CVD Risk

The mechanisms causing the development of hypophosphatemia are decreased intestinal absorption, shift of extracellular phosphate into cells and urinary phosphate loss. A reduced phosphate intake can be caused by severe malnutrition and in patients with symptoms that affect their food intake, including vitamin D deficiency and alcohol [6, 195]. Thereby, vitamin D deficiency is associated with the development of CVD risk factors, e.g., hypertension [196]. This is supported by the results found in vitamin D receptor null mice developing high BP, cardiac hypertrophy and fibrosis [197, 198]. Furthermore, it is known for a long time that chronic heavy drinking is a cause for hypertension, arrhythmias and heart failure, thus, increases the risk for death and CVD [199]. Hypophosphatemia caused by redistribution of extracellular phosphate into cells does not mean a depletion of total body phosphate. The mechanisms causing internal redistribution can be respiratory alkalosis, catecholamines, administration of hormones and other drugs (e.g., insulin), alcohol and rapid cell proliferation.

Hereditary causes of hypophosphatemia are X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR) and autosomal recessive hypophosphatemic rickets (ARDR) [200]. Furthermore, abundant expression of FGF23 in certain tumors can cause renal phosphate-wasting, so called tumor-induced osteomalacia (TIO).

Heart failure patients have an incidence of about 13% for hypophosphatemia [201]. Data investigating the association of hypophosphatemia with cardiomyopathy in regards for mortality are rare. Rozentryt et al. report that among their cohort with 722 heart failure patients and a 2-year follow-up, even the adjusted analysis for mortality was similar for patients with hypophosphatemia compared with patients having normal values of serum phosphate [202]. Hypophosphatemia is associated with an impairment of energy metabolism and the induction of arrhythmias [203]. Several studies discuss cardiac arrest as severe symptom of hypophosphatemia with the possibility leading to death [204–206]. Interestingly, several studies indicate a reversibility of cardiac impairments due to phosphate repletion [207, 208]. O'Connor et al. were the first showing that repletion of serum phosphate levels by potassium phosphate solution in seven patients with severe hypophosphatemia improved myocardial stroke work [207]. Returning serum phosphate levels to normal significantly increased the mean LV stroke work as determined by thermodilution technique. Zazzo et al. reported improved cardiac performance after normalizing serum phosphate concentrations in patients with moderate or severe hypophosphatemia by giving intravenous glucose phosphate during a short time [208]. By measuring cardiac output via thermodilution they demonstrate increased cardiac index, systolic index and LV stroke volume index after phosphate load. In contrast, several other cases show no evidence for an association between phosphate depletion and cardiac dysfunction at all [209, 210]. Thereby, Rasmussen et al. investigated the effect of moderate phosphate depletion (minimum serum phosphate levels of 1.46 mg/dL) induced by glucose infusion after surgery for myocardial performance [209]. The authors found no effect on stroke volume and cardiac output as well as unaltered mean arterial pressure concluding glucose-induced hypophosphatemia lacks affecting cardiac performance. Interestingly, Davis et al. show that only in cases of severe hypophosphatemia (0.9 ± 0.15 mg/dL), but not in patients with moderate hypophosphatemia (1.4 ± 0.11 mg/dL), the LV performance improved by corrections of serum phosphate levels

[210]. Thus, they hypothesized that the conflicting results are caused by the differences in serum phosphate levels among the different studies. To note, none of the hypophosphatemic patients in this cohort presented with clinical signs of congestive heart failure.

XLH is caused by inactivating mutations in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) causing high serum FGF23 concentrations [211, 212]. For patients with XLH, the presence of CV complications is still under debate and varies in its characteristics. Alon et al. describe the presence of hypertension with the need for treatment in 8 out of 41 pediatric XLH patients [213]. Echocardiography revealed that two out of the eight hypertensive patients had LVH. Interestingly, all eight children with hypertension had hyperparathyroidism. Nehgme et al. investigated 13 patients with XLH, and despite no CV symptoms and normal heart structure as well as LV function, determined by cardiac ultrasonography, seven children presented LVH [214]. This was independent of the parathyroid status. During a maximal exercise stress test, all XLH patients with LVH had an abnormal diastolic BP response, but there was no significant correlation between these two parameters. Furthermore, cardiac calcification was not observed in these patients. More recent studies by Nakamura et al. (22 adult XLH patients) and Hernández-Frías et al. (24 pediatric patients) support the findings that characteristic symptoms of XLH patients can be the development of both hypertension and LVH [215, 216]. Only Hernández-Frías et al. reported the serum FGF23 concentrations in their XLH patient cohort (278.18 ± 294.45 pg/mL) and found no correlation with the observed echocardiographic parameters in the XLH patient cohort. Other studies give contradictory results in regards of CVD development in XLH patients [25, 217, 218]. Takashi et al. found no presence of LVH and FGF23 correlation with LVH-related parameters determined by echocardiography and electrocardiography in adult patients with FGF23-related hypophosphatemic rickets/osteomalacia [25]. In a cohort of pediatric patient with X-linked hypophosphatemic rickets, no evidence for LV dysfunction could be revealed by Doppler echocardiography [218]. Likewise, a study in adult patients with clinically significant hypophosphatemia, Vered Z et al. did observe normal LV function demonstrated using M-mode echocardiography and radionuclide ventriculography [217]. Interestingly, three patients displayed right ventricular dysfunction despite normal BP. Again, discrepancies might be explained by the initial serum phosphate concentration in the respective cohorts (Vered I et al.: lowest was 1.5 mg/dL; Vered Z et al. range between 1 and 2 mg/dL).

In line with clinical observations, investigations in the Hyp mouse, the animal model of XLH, show conflicting results too. In several studies, no signs of pathological remodeling were observed determined by histological analyses, gene expression analyses and echocardiography [219, 220]. Additionally, Liu et al. reported that tail cuff measurements did not reveal changes in systolic BP in male Hyp mice compared to wild type littermates [220]. In contrast, others showed that Hyp mice present LVH and hypertension [221, 222]. In another mouse model of XLH, the $Phex^{C733RMhda}$, bearing an amino acid substitution that causes mutations in the coding region for the large extracellular catalytic domain of the PHEX protein, despite high serum FGF23 levels in 27 weeks old mice, no LVH or altered cardiac function was present [223]. Importantly, the BP was not changed between the genotypes. Hypophosphatemic rickets in ARHR are caused by inactivating mutations in the gene dentin matrix protein 1 (DMP1) that encodes for a non-collagenous bone matrix protein in osteoblasts and osteocytes. This mutation leads to elevated serum FGF23 levels in patients and in animal models with DMP1 mutation, which causes the observed hypophosphatemia [224–226]. Wacker et al. showed that Dmp1 null mouse did not present an indication of cardiac hypertrophy, despite the increase of the heart weight to body weight ratio [200]. The authors did not observe the induction of classic pro-hypertrophic genes in cardiac tissue and they found no difference between wild type and Dmp1 null mice in *ex vivo* whole heart

contractility test. The conflicting results of the different publications urge for the need of more studies to have a better understanding for the relationship between hypophosphatemia and cardiac complications.

11.4 Therapeutics Interventions to Prevent Phosphate-Dependent CVDs

Both, low and high phosphate levels may have severe effects on cardiac function. Thus, the detection of phosphate imbalance and its treatment is of high importance. Understanding the mechanisms of phosphate homeostasis shows that serum phosphate balance depends on an interplay between bone, kidneys and intestinal tract regulated by a tight endocrine system. Since phosphate, FGF23, PTH, $1,25(OH)_2D_3$ and calcium have a tight relation/interconnection to each other, changing one parameter influences all, making a therapeutic intervention rather complicated.

11.4.1 Treatment of Hypophosphatemia

Severe symptoms of phosphate depletion, such as cardiac arrhythmia and muscle weakness, were seen when serum phosphate levels are below 1 mg/dL [210]. However, most patients with hypophosphatemia are asymptomatic, but require the correction of the underlying cause, such as magnesium deficiency [195], which mostly normalizes serum phosphate levels. Oral phosphate supplementation is favorized for mild and moderate hypophosphatemia. Hereby, sodium phosphate tablets, potassium phosphate tablets or skim/cow milk are available phosphate sources. Corrections of the phosphate imbalance led to a reversibility of the myocardial dysfunction as shown in feeding experiments with dogs and rats [227, 228]. Phosphate depletion in dogs caused a decline in stroke volume and peak blood flow velocity as well as a decrease in maximum LV time rate change of pressure [227]. The values returned to control values after dietary phosphate repletion improving cardiac performance.

Intravenous supplementation of phosphate is used for patients with severe hypophosphatemia and in cases when oral supplementation is not possible. After a successful increase of serum phosphate concentration to >1 mg/dL, the intravenous administration can be switched to oral supplementation. Repletion of serum phosphate levels by intravenous administration of potassium phosphate solution or glucose phosphate in patients with moderate or severe hypophosphatemia improves cardiac performance [207, 208]. Thereby, normalizing serum phosphate concentrations increases mean LV stroke work, cardiac index, systolic index and LV stroke volume index after phosphate load. Furthermore, in early stages of sepsis low serum phosphate concentrations are associated with a high incidence for cardiac arrhythmias in patients without previous cardiac pathological conditions [229]. It has been shown that intravenous phosphate replacement in adult septic patients with hypophosphatemia, but without pre-existing cardiac condition, is associated with a significant reduction of incidence of arrhythmia (38% vs. 64% in non-treated group, $p = 0.04$) [203]. Hypophosphatemia is also a common problem after cardiac surgery [230]. Treatment of hypophosphatemia of a patient, who developed cardiac failure after cardiac surgery, by giving intravenous phosphate resulted in an improved cardiac index, indicating the consideration of preoperative serum phosphate measurements and postoperative management of hypophosphatemia [231]. In the case report of Frustaci and colleagues, the patient's characteristics were severe hypophosphatemia and impaired LV function measured by echocardiography [232]. Correction of serum phosphate levels by administration of potassium phosphate improved the myocardial contractility (ejection fraction increased from 40% to 72%).

All in all, ATP is essential for cardiac function, phosphate depletion will decrease cardiomyocyte function leading to cardiac failure, thus, management of hypophosphatemia is crucial for

improving patient health. Hence, phosphate supplementation is not predictable, serum phosphate levels should be tightly controlled to avoid hyperphosphatemia or other electrolyte imbalances.

11.4.2 Treatment of Hyperphosphatemia

Increased serum phosphate levels are associated with a higher risk for CVD in the general population [233, 234]. Even more serious is hyperphosphatemia for patients with impaired kidney function. Thereby, the prevention and intervention of hyperphosphatemia is essential.

11.4.2.1 Phosphate Restricted Diet

The KDIGO (Kidney disease Improving Global Outcomes) guidelines suggest restricted dietary phosphate intake to attenuate hyperphosphatemia combined with other treatments for CKD patients [235]. In uremic rats, it has been shown that low dietary phosphate restriction reduces the perivascular fibrosis and cardiomyocyte size compared to rats on high phosphate diet [236]. Additionally, the mortality was reduced in rats under phosphate restriction. However, despite the reduction of serum FGF23 and phosphate levels in non-dialysis patients on a very low phosphate diet [237], more studies are needed to investigate the association between dietary phosphate uptake and improvement for general and CV outcome in the CKD cohort as well the general population.

11.4.2.2 Phosphate Binders Targeting Intestinal Phosphate Absorption

Phosphate binders prevent the intestinal phosphate absorption by binding phosphate, forming a nonabsorbable complex that is excreted in the feces. Different types of phosphate binders are approved to treat hyperphosphatemia in advanced stages of CKD, including calcium-containing phosphate binders (calcium acetate, calcium carbonate) and non-calcium-containing binders (sevelamer carbonate, lanthanum carbonate, ferric citrate). KDIGO guidelines suggest oral phosphate binder administration for patients with CKD stages 2–4 when serum phosphate levels are >4.6 mg/dL [238]. Calcium-containing phosphate binders appear to have pronounced adverse effects on vascular calcification and can cause hypercalcemia in patients requiring dialysis. The study of Block et al. compared lanthanum carbonate, sevelamer carbonate and calcium acetate in a randomized, double blind, placebo-controlled trial within a cohort of moderate to advanced CKD patients [239]. The authors show treatment with phosphate binders resulted in the progression of CAC and abdominal aortic calcification. Interestingly, Russo et al. studied the combined effect of low phosphate diet (<800 mg/d) with calcium carbonate or sevelamer in 90 pre-dialysis CKD patients with stable serum phosphate concentration [240]. This treatment combination reduced the progression of CAC, even in the calcium carbonate group, suggesting a relevance for avoiding CAC in patients not requiring dialysis. Despite beneficial effects of the phosphate binders sevelamer and ferric citrate on serum FGF23 levels [238, 241, 242] observations on cardiac function and performance were not objective of these studies. In a single-blinded, placebo-controlled, 3-month-study, Isakova et al. studied the effect of phosphate restricted diet and lanthanum in respect to serum FGF23 and cardiac function [243]. The study included 39 patients with CKD stages 3 or 4 with normal serum phosphate levels. The authors report that the combination of 900 mg phosphate-restricted diet plus lanthanum decreased serum FGF23 levels. However, echocardiography revealed no changes in ejection fraction or LV wall thickness between groups.

Ex vivo studies on aortic rings show that sevelamer treatment improved the CKD-induced endothelial dysfunction, aortic systolic expansion rate and pulse wave velocity [193]. In a murine model of chronic renal failure (CRF), Maizel et al. investigated the effects of sevelamer on CVD [244]. This model is characterized by the absence of hypertension and aortic calcification and by 6 weeks after surgery, CV abnormalities, such as LVH, diastolic dysfunction and aortic stiffness, have been developed in these mice. Six weeks

after initiation of renal failure, CRF and sham-operated mice received regular chow or supplemented with 3% sevelamer for 14 weeks. Sevelamer reduced serum phosphate levels and improved aortic systolic expansion rate, pulse wave velocity and diastolic function, while LVH was not altered in CRF mice after 8 weeks of treatment. However, after 14 weeks of treatment, sevelamer prevented the progression of LVH in CRF mice. Furthermore, the multiple regression analysis revealed that serum phosphate concentrations, but not serum FGF23 levels, are associated with the LV diastolic function and mass in this mouse model. These results indicate that the phosphate binder sevelamer has beneficial effects on CV complications associated with renal impairment by lowering serum phosphate levels.

The iron-based intestinal phosphate binder ferric citrate hydrate further has positive effects on iron-related parameters, which in turn helps to normalized serum FGF23 levels [245]. Since iron deficiency stimulates FGF23 transcription and occurs frequently in CKD patients, correction of CKD-related anemia via increased hemoglobin, ferritin and transferrin saturation causes reduced serum FGF23 levels [246, 247]. Interestingly, an experimental study in Col4a3 knockout mice, a model for progressive CKD, showed that early initiation of ferric citrate slowed CKD progression, improved cardiac function and overall prolonged the survival of these mice [248]. Col4a3 knockout mice develop CKD-related anemia, have elevated serum phosphate concentrations and serum FGF23 levels rise by 6 weeks of age. In this study by Francis and colleagues, administration of ferric citrate rescued the iron deficiency and anemia regardless of the CKD stage. When starting the treatment in early CKD, but not in late CKD, both serum phosphate and FGF23 levels could be reduced. Furthermore, knockout mice on control chow developed hypertension and had significant lower ejection fraction, stroke volume and cardiac output compared to wild type mice on control diet. Feeding of ferric citrate mitigated the observed systolic dysfunction and lowered the BP in Col4a3 knockout mice. Summarizing that the combination of corrected serum phosphate levels, serum FGF23 levels and parameters of the iron metabolism might be beneficial for heart and kidney function.

Taken together, phosphate binders are well tolerated, efficacious, without safety concerns and commonly used to treat hyperphosphatemia among renal failure patients requiring dialysis. However, experimental and clinical studies about the impact of phosphate binders for CVD outcome in the CKD and in the general population are missing.

11.4.2.3 Drugs Targeting the Phosphate Transporters in the Intestine

Active intestinal phosphate absorption is mediated by the transporter sodium/hydrogen ion-exchange isoform 3 (NHE3) and novel minimally systemic NHE3 compounds have been developed. Tenapanor (previously referred to as RDX5791 or AZD1722) acts locally in the gut, inhibits NHE3 and therefore intestinal sodium and phosphate absorption. Spencer et al. showed that inhibition of intestinal sodium absorption by tenapanor has protective effects on sodium-driven cardiac and renal damage in rodents with CKD [249]. In a follow-up study, the authors showed that the inhibited sodium absorption in the intestine is accompanied by an inhibition of dietary phosphate uptake [250]. They show that administration of tenapanor increased fecal phosphate excretion and reduced urinary phosphate excretion accompanied by reduced ectopic calcification in CKD rats. In addition to the reduced hyperphosphatemia, CKD rats treated with tenapanor displayed reduced serum FGF23 levels and reduced cardiac hypertrophy. However, clinical trials investigating the benefits of tenapanor on cardiac function for patients displaying hyperphosphatemia are not available.

11.4.2.4 Drugs Targeting the Renal Phosphate Transporters

Studies in NaPi-2a knockout mice demonstrated that this cotransporter implements for roughly 70% of the renal phosphate reabsorption via the BBM of the proximal tubule [251]. The development of specific inhibitors against NaPi-2a might

be useful to manage normal phosphate balance. A compound inhibiting NaPi-2a, named BAY767, showed in preclinical testing induction of phosphaturia and reduction of vascular calcification [252]. Of course, the value of this promising approach to treat hyperphosphatemia and associated CV dysfunctions must be tested in humans. Here, it should be considered that others hypothesized that NaPi-2c, not NaPi-2a, has a greater role for renal phosphate reabsorption in man [253]. As a second scenario, it is also possible that NaPi-2c expression increases for compensating the loss of NaPi-2a [254]. Maybe a combined approach of inhibiting both intestinal absorption and renal reabsorption could be beneficial to treat hyperphosphatemia and consequently reduce the risk for CV events and mortality.

11.4.2.5 Magnesium

A promising approach to prevent vascular calcification seems to be the administration of magnesium supplements, which are either inorganic (like magnesium chloride, magnesium oxide) or organic (magnesium citrate or magnesium glutamate) salts. *In vitro* studies showed that phosphate-induced calcification in VSMCs was reduced by treatment with magnesium [122, 255]. In bovine VSMCs, magnesium inhibited the beta-glycerophosphate-induced cell calcification, whereby, magnesium prevented the formation of secondary CCPs, which is a driving factor for vascular calcification [256]. In a register-based cohort with Japanese hemodialysis patients, increasing serum magnesium levels attenuate the risk for CV mortality associated with serum phosphate concentrations [257]. In rats with adenine diet-induced kidney disease, low-dose (375 mg/kg) and high-dose (750 mg/kg) of magnesium citrate significantly decreased serum phosphate levels compared to the untreated adenine diet alone [258]. The adenine diet-induced extensive vascular calcification was inhibited by magnesium citrate in a dose-dependent manner suggesting a protective role of magnesium citrate in calcification. The authors demonstrate that the underlying protective mechanism of magnesium is an increase of alpha smooth muscle actin and a decrease of pro-chondrogenic runt-related transcription factor-2 expression in aortas. This indicates magnesium inhibits the cellular transformation of VSMCs into osteoblast-like cells. Interestingly, Diaz-Tocados et al. reported that increasing dietary magnesium supplementation from 0.1% to 0.6% reversed established vascular calcification in 5/6 nephrectomized rats [259]. The serum phosphate levels in uremic rats were reduced by dietary magnesium, but the levels were still significantly higher compared to sham-operated animals. In addition, increased dietary magnesium reduced the BP in nephrectomized rats compared to controls. Furthermore, the beneficial action of magnesium was not restricted to oral application only, because intraperitoneally administered magnesium decreased vascular calcification too. Kaesler and colleagues reported reduced vascular calcification in heart aorta and kidney after treating 5/6 nephrectomized mice with either magnesium carbonate alone or the combination of magnesium carbonate plus nicotinamide [260]. The treatment significantly lowered serum FGF23 levels, but had only a slight effect on serum phosphate concentrations. Despite these promising results for attenuating calcification, single treatment of either magnesium carbonate or nicotinamide alone induced renal NaPi-2b expression and increased intestinal NaPi-2b expression, which in turn might enhance phosphate absorption and reabsorption. In summary, a combined strategy of magnesium carbonate and nicotinamide or another therapy reducing serum phosphate levels might be suitable to treat vascular calcification and control phosphate levels.

In an open-label, randomized, controlled trial in patients with CKD stages 3–4 presenting risk factors for calcification, orally administered magnesium oxide was effective to slow the progression of CAC [261]. Over the 2-year trial, the serum phosphate levels were unchanged by magnesium administration (3.5 mg/dL at baseline and 2 years later). Contrary to the results found for CAC, progression of thoracic aorta calcification was not suppressed by magnesium oxide. The authors speculate that thoracic aorta is resistant

to uremic-induced calcification, because of the cellular origin of the VSMCs resulting in a less likelihood to undergo osteoblastic transdifferentiation. Furthermore, is has been reported that higher dialysate magnesium prolongs the conversation of primary CCP into secondary CPP in ESKD patients [262]. The conversation time, measured by T_{50} test, indicates the risk for vascular calcification, whereby a longer time means a lower risk. Additionally, serum phosphate levels were reduced by higher dialysate magnesium.

Despite the promising results from experimental and human studies, more investigations are need to clarify the role of magnesium for reducing the risk of CVD development and/ or progression.

11.5 Conclusions

Serum phosphate levels are regulated by FGF23, PTH and phosphate itself and are linked to the development of CVD. Hyperphosphatemia is a risk factor for the initiation of vascular calcification, LVH, fibrosis and hypertension. The main process of phosphate-induced vascular calcification is the osteochondrogenic transdifferentiation of VSMCs resulting in ECM remodeling, apoptosis, senescence and pro-inflammatory responses. In contrast, the mechanisms underlying phosphate-induced LVH, fibrosis and hypertension are poorly understood. Hypophosphatemic patients have a higher risk to develop cardiac arrhythmias. Keeping serum phosphate levels within the normal range is crucial for the CV outcome of patients especially suffering from renal failure. Despite several therapeutic approaches to normalize serum phosphate concentrations, only few studies present data on the impact of the treatment for associated CVD. Thereby, calcium free phosphate binders, such as sevelamer, combined with a second approach to lower serum levels of phosphate show promising results. Furthermore, ferric citrate potentially acts on two routes to prevent CVD. First, it functions as a phosphate binder reducing serum phosphate, and second, it helps to overcome iron deficiency and subsequently reducing serum FGF23 levels, a risk factor for LVH. Strategies to lower the intestinal absorption or renal reabsorption of phosphate via blocking the specific phosphate cotransporters are another important intervention. However, it must be demonstrated that this approach results in prevention or improvement of CVD. The most promising intervention for hyperphosphatemia-related CVD for now seems to be normalizing magnesium concentrations in the serum. Experimental studies indicate that dietary magnesium supplementation is beneficial for CV calcification. Recent clinical studies do also point in this direction. However, for all the discussed interventions to lower serum phosphate levels, the value for improvements of CVD, such as reducing the mortality risk, slow down the progression, prevent disease induction, reverse the disease outcome, are still not completely known. Summarizing the current knowledge of phosphate-induced CVD and therapeutic options reveals the urgent need for more experimental and clinical investigations addressing underlying mechanisms and especially CV outcomes of therapeutic strategies in patients suffering from out-off range serum phosphate levels.

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Coordination of Phosphate and Magnesium Metabolism in Bacteria

12

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Abstract

The majority of cellular phosphate (PO_4^{-3}; Pi) exists as nucleoside triphosphates, mainly adenosine triphosphate (ATP), and ribosomal RNA (rRNA). ATP and rRNA are also the largest cytoplasmic reservoirs of magnesium (Mg^{2+}), the most abundant divalent cation in living cells. The co-occurrence of these ionic species in the cytoplasm is not coincidental. Decades of work in the Pi and Mg^{2+} starvation responses of two model enteric bacteria, *Escherichia coli* and *Salmonella enterica*, have led to the realization that the metabolisms of Pi and Mg^{2+} are interconnected. Bacteria must acquire these nutrients in a coordinated manner to achieve balanced growth and avoid loss of viability. In this chapter, we will review how bacteria sense and respond to fluctuations in environmental and intracellular Pi and Mg^{2+} levels. We will also discuss how these two compounds are functionally linked, and how cells elicit physiological responses to maintain their homeostasis.

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Keywords

Phosphorus · Magnesium · Bacteria · Cytotoxicity · Transport · *Salmonella*

12.1 Phosphorus Acquisition in Bacteria

Phosphorus (P) is an intrinsic component of all living cells. The importance of this chemical element is illustrated by its various structural and biochemical functions. P is a structural constituent of lipids and complex carbohydrates, including glycolipids, lipopolysaccharides (LPS) and (lipo)teichoic acids. These molecules are major structural components of biological membranes and cell walls, and are required for the formation of cellular boundaries and membrane-bound organelles. P in phosphoryl groups enables the implementation of phosphorelays and the establishment of signal transduction cascades [41]. P is also a component of nucleotides. In addition to their role as biosynthetic precursors for polymers, nucleotides can act as second messengers, promoting the transfer of information in signaling networks [14, 46, 53, 136], and store and release chemical energy to be used in energy-dependent processes. Finally, the structural role of P as a component of nucleic acids enables the storage, expression and transmission of genetic information. How do bacterial cells acquire P from their environment?

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Although bacteria are able to acquire and metabolize P from organic molecules such as organophosphates (phosphate esters, C-O-P bonds) or phosphonates (C-P bonds) [75, 115, 119, 120, 130], these organisms typically prefer to utilize inorganic orthophosphate (PO_4^{3-}, Pi, phosphate) as their P source (Fig. 12.1). In this context, the acquisition of P relies primarily on inorganic Pi transporters present at the cytoplasmic membrane. Three biochemically distinct classes of Pi transporters have been identified in bacteria: NptA, Pit, and Pst (Fig. 12.2a). NptA (Na-dependent phosphate transport) belongs to the type II sodium/phosphate (Na/Pi) cotransporters family. Homologs of this transport family are common in eukaryotic organisms, where they have been extensively studied [7, 47, 125]. Studies of bacterial NptA homologs are scarce [57, 61, 137]. Nonetheless, the biochemical characterization of the NptA homolog from *Vibrio cholerae* indicates that like its eukaryotic counterparts, this transporter has a low affinity for Pi (Km of ~300 μM) and uses a sodium (Na^+) gradient to facilitate the movement of Pi into the cell at a stoichiometry of 3:1 (Na^+:Pi), and a rate of ~9 pmol Pi/min/mg of protein [61].

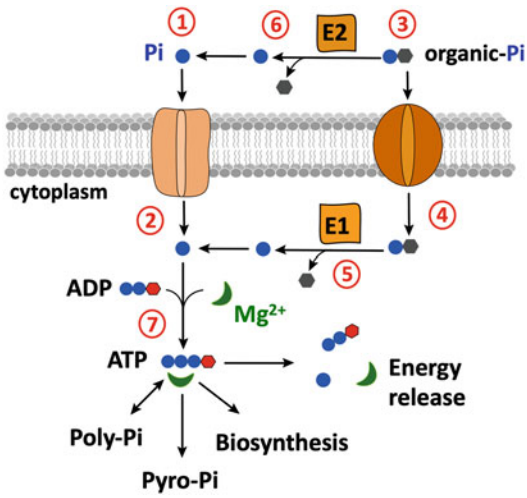

Fig. 12.1 Schematic representation of bacterial Pi metabolism. Inorganic Pi is the preferred P-source **(1)**. Pi is translocated into the cytoplasm by specialized membrane-bound transporters **(2)**. Alternatively, bacteria can use organic-P sources, such as organophosphate and phosphonates **(3)**, which are transported into the cytoplasm by a variety of dedicated membrane transporters **(4)**. Enzymatic Pi extraction from organic molecules can occur either in the cytoplasm **(5)** by intracellular enzymes **(E1)**, or extracytoplasmically **(6)** by specialized enzymes **(E2)**. Pi is assimilated by the cell during the synthesis of ATP **(7)**. Intracellular ATP functions as the P-donor molecule, serving as the source of Pi for the biosynthesis of inorganic (e.g., pyro-Pi and poly-Pi) and organic (e.g., DNA and lipids) molecules, and powering energy dependent processes **(7)**

The Pit (phosphate inorganic transport) family is widespread among all domains of life [76, 85, 113]. Pit systems use the proton motive force to transport soluble, neutral divalent metal-phosphate complexes (Me:Pi) across biological membranes. Bacterial Pit transporters have low affinity for Pi (Km ≈ 1.9–25 μM) and can transport Pi at a rate of 19–58 nmol Pi /min/mg of dry cell [45, 77, 114, 127]. This system can mediate both the efflux and homologous exchange of Me:Pi complexes [114], and displays some promiscuity towards non-Pi oxyanions such as arsenate [128] (Fig. 12.2a). In *Escherichia coli*, the Pit transporter PitA is often thought to function as a constitutive housekeeping Pi transporter. PitA expression is, nonetheless, regulated: *pitA* transcription is stimulated by exposure of cells to zinc-phosphate ($Zn_3(PO_4)_2$) salt [54]. Furthermore, PitA expression is post-transcriptionally repressed during Mg^{2+} starvation [134].

In addition to NptA and Pit, bacteria often encode inducible, ATP-dependent Pi transporters. The Pst system is a complex of four proteins (PstSCAB) that can transport Pi with high affinity (Km 0.18–0.40 μM Pi) and at a rate of ~16 nmol Pi/min/mg of dry cell [127] (Fig. 12.2a). PstS is an extracytoplasmic substrate-binding protein. PstC and PstA are transmembrane components that comprise the Pi channel, and PstB harbors the ATP-binding domain needed for coupling energy hydrolysis with the release of Pi into the cytoplasm [38, 92]. The Pst system is highly specific for Pi, efficiently discriminating it from other inorganic oxyanions such as arsenate and

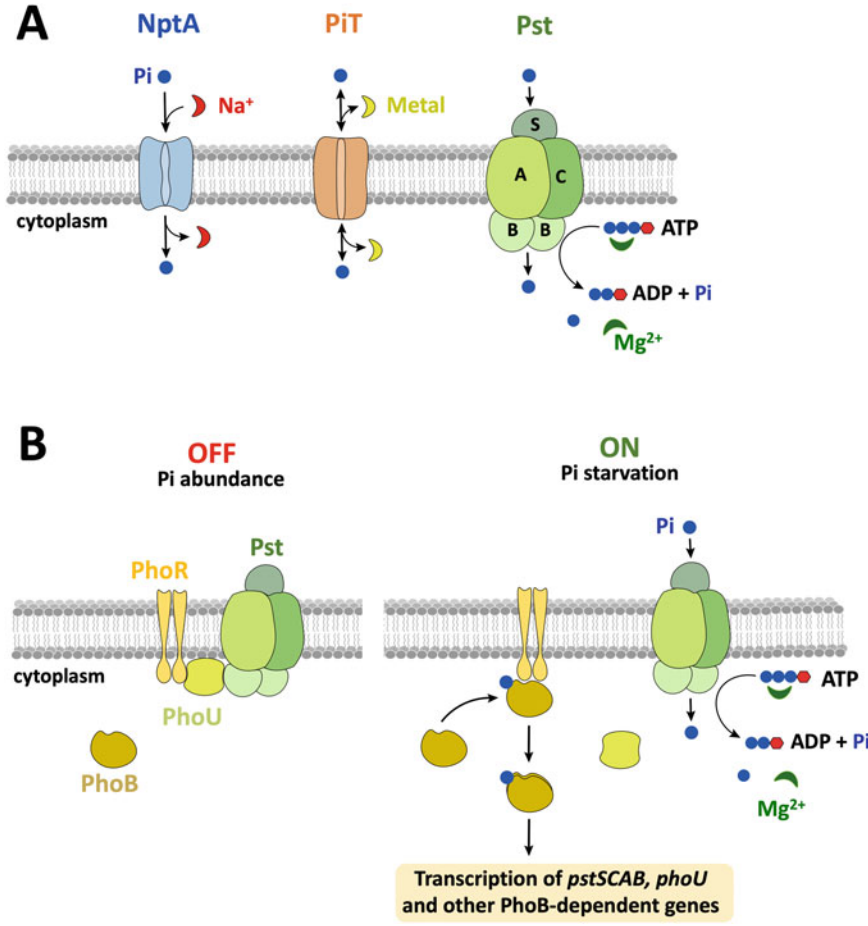

Fig. 12.2 (a) Cartoon depicting the known classes of Pi transport systems found in bacteria: NptA, Pit and Pst. NptA is a sodium/phosphate symporter. Pit functions as a metal:phosphate (M:Pi) proton symporter (proton not shown for simplicity). Pst is a multi-component, high affinity, ATP-dependent Pi transporter. PstS is the extracytoplasmic Pi-binding protein, which binds and delivers Pi to the transmembrane channel comprised of the PstC and PstA components. PstB is responsible for the ATP binding, hydrolysis, and release of ADP that powers Pi transport. **(b) Schematics depicting the multicomponent signal complex controlling Pi-responsive PhoB/PhoR two-component system activity.** (Left-hand side panel) During growth in Pi abundant conditions, the PhoU regulatory protein interacts with and represses the activity of the Pst transport system and PhoR kinase activity. (Right-hand side panel) A decrease in cytoplasmic Pi levels alters or disrupts the PhoU interaction with Pst and PhoR. Pst begins to import extracellular Pi while PhoR phosphorylates its cognate response regulator, PhoB. PhoB-P activates the transcription of its target genes, including the *pstSCAB-phoU* operon, *phoBR*, and other genes required for Pi acquisition

sulfate [6, 77, 94, 127]. Noteworthy, Pst-coding genes are usually arranged in an operon (*pstSCAB-phoU*) along with a regulatory accessory gene *phoU*, which codes for a peripheral membrane protein [110]. The *pstSCAB-phoU* operon is transcriptionally activated by the PhoB/PhoR two-component system (see below).

12.2 Sensing and Responding to Pi Starvation

When Pi is plentiful, bacteria can maintain adequate supplies of cytoplasmic Pi using their housekeeping, low affinity Pi transporter systems. However, when Pi is limited and these

transporters can no longer keep up with cellular demand, bacteria activate a Pi starvation response. In *E. coli* and most bacteria, this response is governed by the PhoB/PhoR two-component signal transduction system [27, 33, 38, 40, 52, 80, 119–121, 129].

PhoR is a membrane-bound homodimeric, bifunctional histidine kinase/phosphatase. PhoB is its cognate transcriptional response regulator, comprised of an N-terminal receiver domain and a C-terminal DNA-binding domain. Scarce environmental Pi promotes the kinase state of PhoR. PhoR autophosphorylates at a conserved histidine residue and subsequently transfers this phosphoryl group to the receiver domain of PhoB [30, 73, 121]. Phosphorylated PhoB (PhoB-P) binds to DNA sequences known as Pho boxes [3, 35, 74]. Binding of PhoB-P to Pho boxes located at the promoter regions of target genes results in either recruitment of RNA polymerase and transcriptional activation of downstream gene(s), or interference with RNA polymerase and downregulation of gene expression [9, 13, 29, 56, 72, 79, 132, 135]. Abundant environmental Pi promotes the phosphatase state of PhoR, leading to the dephosphorylation of PhoB-P and inactivation of the Pi starvation response [16, 38, 100, 119–121].

While the phosphorylation events controlling the activation state of PhoB/PhoR are well understood, how this two-component system senses environmental Pi is still unclear. Unlike other prototypical bifunctional histidine kinases/phosphatases, PhoR does not contain a sizable extracytoplasmic domain, which is typically involved in the recognition of extracellular ligands [97]. Hence, PhoR is unlikely to directly detect Pi via extracytoplasmic sensory domain(s). Rather, the ability of PhoB/PhoR to respond to Pi requires the Pst transport system and the PhoU protein. PhoU mediates a physical interaction between PhoR and the PstB component of the Pst system, creating a multicomponent signaling complex capable of modulating PhoB/PhoR activity in response to environmental Pi [37, 38, 107, 110, 120, 121] (Fig. 12.2b). Pi limitation is presumed to disrupt (or alter) this signaling complex, promoting the kinase activity of PhoR, phosphorylation of PhoB and activation of Pi starvation response (Fig. 12.2b).

It is also unclear whether Pi starvation is sensed by the extracytoplasmic or cytoplasmic portions of this multicomponent signaling complex. The extracytoplasmic sensing model relies on two lines of evidence. First, intracellular Pi levels have been measured by ^{31}P NMR and shown only a modest decrease under conditions in which PhoB is activated [91]. However, given the lack of positive and negative controls in this study, it is difficult to determine whether the observed differences are biologically significant or if larger discrepancies in intracellular Pi concentrations would be revealed by the experimental set up. Second, certain mutations in the PstC and PstA components of the Pst system have been shown to abolish Pi transport activity while retaining Pi sensing capacity [24, 25]. This argument makes the assumption that an inactive Pst system would lead to a decrease in intracellular Pi levels—that is, it does not consider that cells can compensate for a decrease in intracellular Pi, resulting from a defective Pst system, by upregulating other transporters such as PitA [15, 49].

The cytoplasmic sensing model has been evoked to account for several experimental results that can only be explained if Pi starvation is being sensed by the cytoplasmic portion of the PhoR-PhoU-Pst signaling complex. For example, when metabolic mutants of *E. coli* are grown in medium containing high Pi, PhoB/PhoR is activated under conditions that promote an expansion of cytoplasmic pools of ATP [126]. Similarly, a decrease in cytoplasmic concentrations of Mg^{2+} activates PhoB/PhoR in both *E. coli* and *Salmonella enterica* (*Salmonella*) grown in high Pi media [15, 87, 90]. Notably, cytoplasmic Mg^{2+} starvation destabilizes the bacterial ribosomal subunits [90]. Decreased ATP consumption by functionally compromised ribosomes causes a rise in cytoplasmic ATP concentrations and, presumably, a concomitant decrease in levels of free Pi that is proposed to activate the PhoB/PhoR two-component system. This model is independently supported by the demonstration that antibiotics that inhibit translation cause a rise in

intracellular ATP levels, resulting in PhoB/PhoR activation in both *E. coli* and *Salmonella* grown in high Pi medium. Importantly, activation of PhoB/PhoR by translation inhibitors is not observed if the rise in ATP levels is prevented by inducing the expression of an ATPase prior to antibiotic treatment [87]. Finally, in *Salmonella*, inactivation of the *pitA* transporter causes an increase in the basal level of *pstSCAB* transcription, presumably because this mutation lowers the intracellular concentration of Pi [15].

Which genes are expressed in response to Pi starvation? In *E. coli*, the PhoB regulon contains at least 38 genes including: *phoBR*, for the positive autoregulation of the system; *phoA*, an extracytoplasmic alkaline phosphatase; *phoE*, an outer membrane porin channel for anions (including Pi); *pstSCAB-phoU*, coding the high affinity Pst system and the chaperon like inhibitory protein PhoU (see above); *phnCDEFGHIJKLMNOP*, phosphonate uptake and breakdown genes; *ugpBAECQ*, genes for glyceraldehyde-3-phosphate uptake plus a phosphodiesterase that hydrolyzes glycerophosphoryl diesters (deacylated phospholipids) (For reviews see [38, 92, 119, 120]). Notably, this response goes beyond the mere acquisition of environmental Pi and alternative P sources. Some PhoB-activated genes allow *E. coli* to scavenge Pi from its own cellular components. For example, the PhoB activated *waaH* gene encodes a glucuronic acid transferase. WaaH modifies the core region of the *E. coli* LPS, replacing a Pi residue, which can be re-purposed by the cell, with a glucuronic acid [4, 60]. Pi-starvation driven changes in cell composition have also been documented in the distantly related species *Bacillus subtilis*, and may be a general strategy adopted by bacteria to reallocate assimilated Pi [10, 11, 68, 96].

Importantly, the genes within the PhoB regulon are neither expressed at the same time, nor at the same levels. An elegant analysis of the PhoB-dependent temporal patterns of gene expression revealed the existence of *early* genes, encoding proteins required for PhoBR autofeedback regulation and Pi transport (i.e. *phoBR* and *pstSCAB-phoU*); and *late* genes, encoding proteins required for the acquisition and utilization of alternative, typically organic phosphorus sources (i.e. *phoA*, and *ugp* and *phn* operons) [35]. This temporal expression pattern can be explained, in large part, by variations in the DNA sequence of Pho boxes, which produce different binding affinities to PhoB-P. While the promoter regions of genes needed at the initial stages of starvation usually have high affinity Pho Boxes, requiring lower cytoplasmic concentrations of PhoB-P for transcriptional activation, the promoter regions of genes needed at the later stages of the response have low affinity Pho Boxes, requiring higher concentrations of PhoB-P [35].

12.3 Phosphate Cytotoxicity via Pst

While P is essential, excessive transport of Pi into the cytoplasm is toxic. In some cases, toxicity is mediated by the co-transported counterions, rather than Pi itself. For instance, some Pi transporters import Pi:Me salts (Fig. 12.2a). Excessive transport of Pi:Me ($Pi:Co^{2+}$; $Pi:Cu^{2+}$; $Pi:Mn^{2+}$; $Pi:Zn^{2+}$) salts by the Pho84 transporter of *Saccharomyces cerevisiae* results in metal toxicity [55, 81, 95]. An analogous phenomenon occurs in the PitA transporter of *E. coli*, which can render cells liable to Zn^{2+} poisoning [5]. Toxicity mediated by these transport systems likely reflect the fact that relatively small disturbances in the intracellular concentrations of these metals are sufficient to promote unproductive mismetallation events that hinder enzymatic reactions [34].

In bacteria, Pi toxicity *per se* has been observed in cells experiencing uncontrolled Pst-mediated Pi uptake resulting from either (1) missense mutations in Pst components that increase Pi influx [122], (2) null mutations in *phoU* that derepresses the PhoB/PhoR regulon [93, 107], or (3) over-expression of the Pst system [15]. The PstA and PstC proteins form the Pst Pi channel that spans through the cytoplasmic membrane [92, 122] (Fig. 12.2a). In *E. coli*, certain amino acid substitutions within PstC transmembrane helices cause a severe growth defect when the Pi concentration in the medium is raised to

levels approaching that of the cytoplasm. These substitutions are thought to lock the transport systems into an "open" conformation that allows unrestricted Pi uptake. In agreement with this notion, the Pi sensitivity of cells harboring these PstC variants is suppressed by additional amino acid substitutions in PstC that abolish Pi transport, presumably by locking the channel into a "closed" conformation [122].

The involvement of Pst in potentiating Pi toxicity is also inferred from genetic studies of the *phoU* regulatory gene (Fig. 12.2b). Pioneer work in *E. coli* established that inactivation of *phoU* results in a severe growth defect due to increased Pst expression and transport activity. Specifically, the growth impairment of *phoU* mutants is suppressed by secondary mutations that inactivate components of the Pst transporter (*pstB* or *pstSCAB*), or prevent its expression (*phoB* or *phoR*) [107], and it is improved by reducing the concentration of Pi in the medium [93]. Importantly, this relationship is not restricted to *E. coli*: *phoU* mutations also lead to growth impairment in species where PhoU participates in the repression of PhoB/PhoR and Pst transport activity. These include *Pseudomonas aeruginosa* [2], *Synechocystis* sp. [78], *Streptococcus pneumoniae* [137], *Staphylococcus epidermidis* [118], *Mycobacterium marinum* [117], *Sinorhizobium meliloti* [28] and *Caulobacter crescentus* [69]. In *C. crescentus*, *phoU* inactivating mutations are conditionally lethal. These genetic lesions can only be introduced in strains already containing mutations in *phoBR* or *pstSCAB* [69]. Similarly, in *Sinorhizobium meliloti*, *phoU* can only be inactivated if cells are grown in medium containing low concentrations of Pi, or if the Pst transporter is also inactivated [28].

The dysregulation of Pst-mediated Pi uptake observed in *phoU* mutants has pleiotropic effects on cellular physiology, reflecting the pervasive role of Pi in the cell. In addition to hindering growth, mutations in *phoU* are frequently linked to increased sensitivity to antibiotics and other environmental stresses [2, 117, 137]. Furthermore, these mutations lead to elevated intracellular ATP [117, 118] and over accumulation of polyphosphate (poly-Pi) [2, 28, 48, 69, 78, 117]. Notably, secondary mutations that hinder poly-Pi synthesis do not suppress the lethality of *phoU* mutations in *C. crescentus* [69], indicating that toxicity does not originate from poly-Pi accumulation.

In *Salmonella*, overexpression of the Pst transporter from an inducible promoter is sufficient to hinder growth and induce a rise in cytoplasmic ATP levels [15]. These phenotypes are also accompanied by a global decrease in translation and the transcription of the *mgtCBRU* operon, encoding genes that respond to cytoplasmic Mg^{2+} starvation (see below). Surprisingly, the effect of *pstSCAB* overexpression on growth can be drastically improved by enzymatically decreasing intracellular ATP concentration through the controlled expression of soluble components of the F1Fo ATPase [15]. Excessive Pi, therefore, becomes toxic following its incorporation into ATP, and this toxicity can be remediated by providing cells with Mg^{2+} [15].

12.4 The Role of Mg^{2+} on the Utilization of Assimilated Pi

Pi imported into the cytoplasm is incorporated into biomass primarily through the synthesis of ATP. ATP functions as the main P-carrier molecule in the cell, mediating the transfer of Pi in many biosynthetic reactions, and serving as the main source of phosphoanhydride bonds used to power energy-dependent processes (Fig. 12.1) [70, 120]. In bacteria and other living organisms, most of the assimilated Pi is found in ATP, and ribosomal ribonucleic acids (rRNA), the most abundant RNA species in cells [12, 31, 39, 89].

Following assimilation, the negative charges from Pi residues in biomolecules often undergo charge neutralization by cytoplasmic cationic species. Mg^{2+} plays a pivotal role in this process due to its high charge density (the highest among all biologically relevant cations), and its presence as the most abundant divalent cation in cells [116] (Fig. 12.1). The Mg^{2+} concentrations in bacteria are estimated to be around 75–100 mM, of which

two-thirds (50–65 mM) are intracellular and one-third (25–35 mM) are bound to cell envelop components such as the LPS of Gram-negative species [89]. Half of the intracellular Mg^{2+} is complexed to nucleoside-triphosphates (NTPs), mainly ATP [89]. Importantly, it is this ATP:Mg^{2+} salt, rather than the ATP anion, which serve as the substrate for most ATP-dependent enzymatic reactions [71, 108].

The assembly of rRNAs into functional ribosomes also requires the association of Mg^{2+} ions to reduce the electrostatic repulsion among Pi residues present in the sugar-phosphate backbone, enabling the compaction of the rRNA into stable structures and the association of ribosomal proteins [59]. A prototypical bacterial ribosome binds approximately 170 Mg^{2+} ions [98]. During rapid exponential growth, ribosomes pools are at their highest and are estimated to chelate the equivalent of 25% of the total intracellular Mg^{2+} [89]. Importantly, Mg^{2+} associates with ribosomes in a specific manner [58, 123, 124]. Compared to other common biological cations such as Na^+, K^+, Ca^{2+}, or polyamines [32], Mg^{2+} has a greater affinity for oxygen atoms in phosphodiester bonds. Mg^{2+} binds oxygen atoms with a well-defined geometry, bringing them into its first hydration shell. Consequently, rRNA:Mg^{2+} complexes are also stabilized by non-electrostatic components of the binding such as polarization, charge transfer and exchange interactions [86].

Given the central role of Mg^{2+} on the function of assimilated Pi, it is not fortuitous that ATP and rRNA, the largest cytoplasmic reservoirs of Pi, are also the largest cytoplasmic repositories of Mg^{2+} [89]. How do bacteria maintain adequate concentrations of Mg^{2+} in their cytoplasm? Do they alter their Pi metabolism in response to insufficient Mg^{2+}?

12.5 Sensing and Responding to Mg^{2+} Starvation

Bacterial cells have also evolved means to sense and respond to decreases in the concentration of extra- and intracellular Mg^{2+}. In many bacterial species, the Mg^{2+} starvation response is controlled by the PhoP/PhoQ two-component signal transduction system. This regulatory system has been extensively studied in *Salmonella*, from which most of our knowledge is derived (for extensive reviews see [42, 43]. PhoQ is a membrane-bound, homodimeric, bifunctional histidine kinase/phosphatase. PhoQ senses extracytoplasmic Mg^{2+} through a patch of acidic amino acid residues located at its extracytoplasmic domain [18, 20, 36]. Binding of Mg^{2+} to this acidic patch forms salt bridges that reduce electrostatic repulsion against the negatively charged cytoplasmic membrane, stabilizing PhoQ in a rigid conformation that favors its phosphatase activity. In this conformation, PhoQ maintains its cognate DNA-binding transcriptional regulator PhoP in an inactive, unphosphorylated state [17, 20, 36, 66, 102] (Fig. 12.3).

As the concentration of extracellular Mg^{2+} decreases, the Mg^{2+} bridges between the acidic amino acid patch of PhoQ and the outer leaflet of cytoplasmic membrane are destabilized, promoting conformational changes favoring a kinase state [20, 36]. PhoQ undergoes autophosphorylation and, subsequently, transfers its phosphoryl residue to PhoP. Phosphorylated PhoP (PhoP-P) binds with high-affinity to target DNA sites, named PhoP boxes [65, 66, 102, 131]. The majority of PhoP boxes are located at promoters, where binding of PhoP-P stimulates the transcription of downstream genes through physical interactions with RNA polymerase [22, 84, 102, 138, 139] (Fig. 12.3).

The response to Mg^{2+} starvation orchestrated by PhoP/PhoQ occurs in an orderly manner, with transcription of individual genes being induced at specific stages of the stress, in a fashion resembling the PhoB/PhoR response to Pi starvation (see above). However, unlike PhoB [35], the time and order of transcriptional activation of genes within the regulon is not dictated solely by the affinity of PhoP-P to PhoP boxes. In *Salmonella*, the expression order of genes is influenced by additional regulatory mechanisms, including the existence of Mg^{2+} sensing RNAs and other regulatory elements at the 5′-end of transcripts [26, 50, 62, 63, 82, 99, 106], transcriptional silencing by nucleoid associated proteins

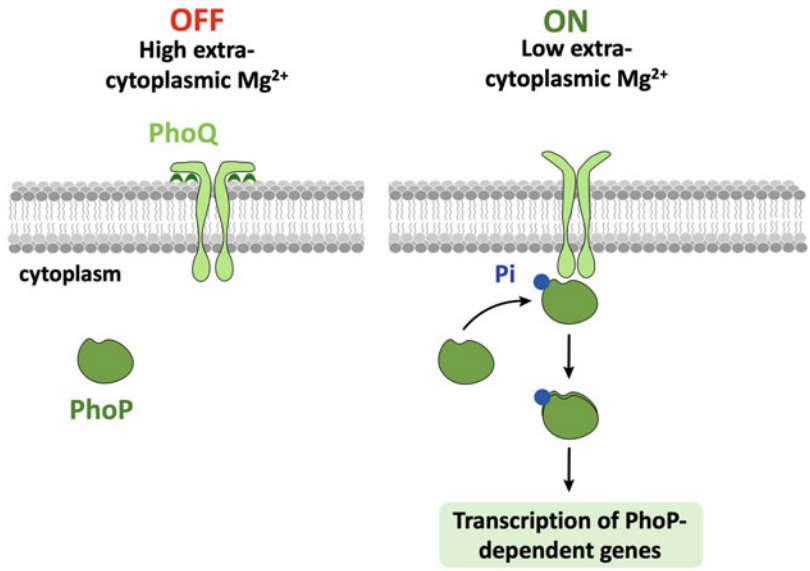

Fig. 12.3 Representation of the Mg^{2+}-sensing PhoP/PhoQ two-component system. (Left-hand side panel) High extracellular Mg^{2+} levels stabilize the sensor protein PhoQ in a rigid conformation. This state promotes PhoQ phosphatase activity, and maintains its cognate response regulator, PhoP, in an inactive, unphosphorylated state. (Right-hand side panel) Low extracytoplasmic Mg^{2+} activates PhoQ kinase activity, increasing the intracellular concentration of PhoP-P, which, in turn, promotes transcription initiation at PhoP-dependent promoters, such as those involved in adaptation to low Mg^{2+} environments (e.g., cell envelope remodeling, Mg^{2+} scavenging, etc.)

[23, 140], and modulation of PhoQ activity by members of the regulon [67, 83]. Nonetheless, the timely expression of genes in the regulon is linked to their physiological functions. For instance, during the early stages of Mg^{2+} starvation, PhoP activates the expression of several enzymes involved in the remodeling of the cell envelope. These proteins carry out covalent modifications that replace negatively charged Pi-containing residues in the LPS. The result is a reduction in the net negative charge of the outer membrane, which facilitates charge neutralization when Mg^{2+} is limited, and frees Mg^{2+} ions bound to the LPS to be imported into the cytoplasm [8, 19, 44, 51, 101, 105, 112].

The transcription of genes responding to physiological disturbances caused by prolonged Mg^{2+} starvation is often conditioned by additional events taking place in the cytoplasm. For instance, while phosphorylation of PhoP resulting from low extracytoplasmic Mg^{2+} sensing by PhoQ causes transcription to initiate at the promoters of the *mgtA* gene and the *mgtCBRU* operon, transcription elongation into their corresponding coding regions takes place only when cells also experience a decrease in free cytoplasmic Mg^{2+} [26, 106]. Therefore, MgtA and the proteins within the *mgtCBRU* operon are expressed following a substantial period of starvation, when the housekeeping transporter, CorA, is no longer able to maintain an adequate supply of Mg^{2+} to the cytoplasm. Whereas *mgtA* and *mgtB* encode high affinity, ATP-dependent Mg^{2+} transporters [103, 104, 111], *mgtC* encodes a protein that reduces ATP levels by inhibiting Pi uptake via an unidentified Pi transporter [15, 63, 64, 88]. (MgtR and MgtU encode regulatory proteins that control the degradation of MgtA, MgtB, and MgtC [1, 21, 133]). Hence, MgtA and MgtB import extracellular Mg^{2+} into the cytoplasm and MgtC prevents the sequestration of this scarce ion by ATP molecules

[63, 64]. Notably, the transcription of a subset of PhoP-activated genes requires the activity of the MgtA transporter. The importation of extracellular Mg^{2+} by this transporter increases the levels of PhoP-P, presumably by removing inhibitory Mg^{2+} from the vicinity of PhoQ [83].

12.6 Coordination of Pi and Mg^{2+} Homeostasis in Bacteria

Pi toxicity resulting from increased Pst transporter activity explains why this transporter is not constitutive, being only expressed in low Pi environments. However, this does not reveal a biologically relevant context where Pi toxicity would occur. Do natural populations of bacteria experience Pi toxicity? If so, under what circumstances? In *Salmonella*, prolonged Mg^{2+} starvation causes a decrease in cytoplasmic Mg^{2+}. At the initial stages of cytoplasmic Mg^{2+} starvation, the assembly of ribosomal subunits is compromised while Mg^{2+}-dependent enzymatic reactions remain unaffected (Fig. 12.4a). This phenomenon likely reflects differences between the Mg^{2+} requirements of the ribosome and enzymes. Whereas the ribosome has a high, continuous requirement for Mg^{2+} (>170 ions for their structural stabilization), enzymes have a low and transient need for this cation (one or a few ions during each catalytic cycle). Consequently, cells experience a decrease in translation efficiency, but can still carry out other biosynthetic reactions such as ATP synthesis and transcription [90]. Because translation is the most expensive anabolic activity in the cell, consuming over 70% of the ATP that is utilized in biosynthetic processes [89, 109], this ribosome assembly defect leads to a reduction in ATP consumption and a concomitant rise in cytoplasmic ATP concentration [15, 87, 90] (Fig. 12.4a). This sets in motion two chains of events.

First, as a substantial portion of intracellular Pi comprise phosphoryl residues in ATP, a decrease in ATP hydrolysis from translation reactions reduces the intracellular recycling of Pi. This presumably decreases cytoplasmic Pi concentrations, which triggers PhoB/PhoR activation [87]. PhoB promotes Pst expression, causing an increase in Pi uptake, and ATP synthesis [15]. Increased ATP concentrations stimulate rRNA synthesis, the rate-limiting step in ribosome biosynthesis. However, newly synthesized rRNA cannot assemble into functional ribosomes due to insufficient Mg^{2+} [90] (Fig. 12.4a). Consequently, cells are subjected to a vicious cycle, whereby the transport of Pi by Pst increases cytoplasmic ATP levels and rRNA synthesis, promoting additional chelation of Mg^{2+} that further destabilizes the ribosomes and stimulates Pst expression [15, 90].

Second, this reduction in translation efficiency also triggers the expression of genes responding to cytoplasmic Mg^{2+} starvation. Inefficient translation promotes transcription elongation into the coding regions of *mgtA* and *mgtCBRU* through a number of regulatory mechanisms acting at their 5′-mRNA leaders [62, 63, 82, 99, 106]. MgtA and MgtB import Mg^{2+}, promoting the stabilization of ribosomes [90] (Fig. 12.4b). MgtC reduces ATP levels [63] by hindering the activity of a yet unidentified Pi transport system [15] (Fig. 12.4b). By inhibiting Pi uptake, MgtC hampers all ATP generating reactions in the cell. On the one hand, a reduction in ATP quenches rRNA synthesis, preventing further entrapment of Mg^{2+} by nascent rRNA (Fig. 12.4b). On the other hand, as new steady-state levels of ribosomes are reached, the degradation of excess rRNA and ribosomes liberates additional Mg^{2+} ions into the cytoplasm. Overall, the concerted activities of MgtA, MgtB and MgtC restore ribosomal function, effectively silencing PhoB/PhoR and inhibiting Pst expression [15, 87, 90].

12.7 Concluding Remarks

The utilization of assimilated Pi into ATP and ribosomes requires Mg^{2+}. Because cytoplasmic Pi is rapidly assimilated into ATP [15], cells coordinate Pi uptake with Mg^{2+} availability. In *Salmonella*, the response to insufficient cytoplasmic Mg^{2+} entails the inhibition of Pi uptake as a general strategy to prevent sequestration of

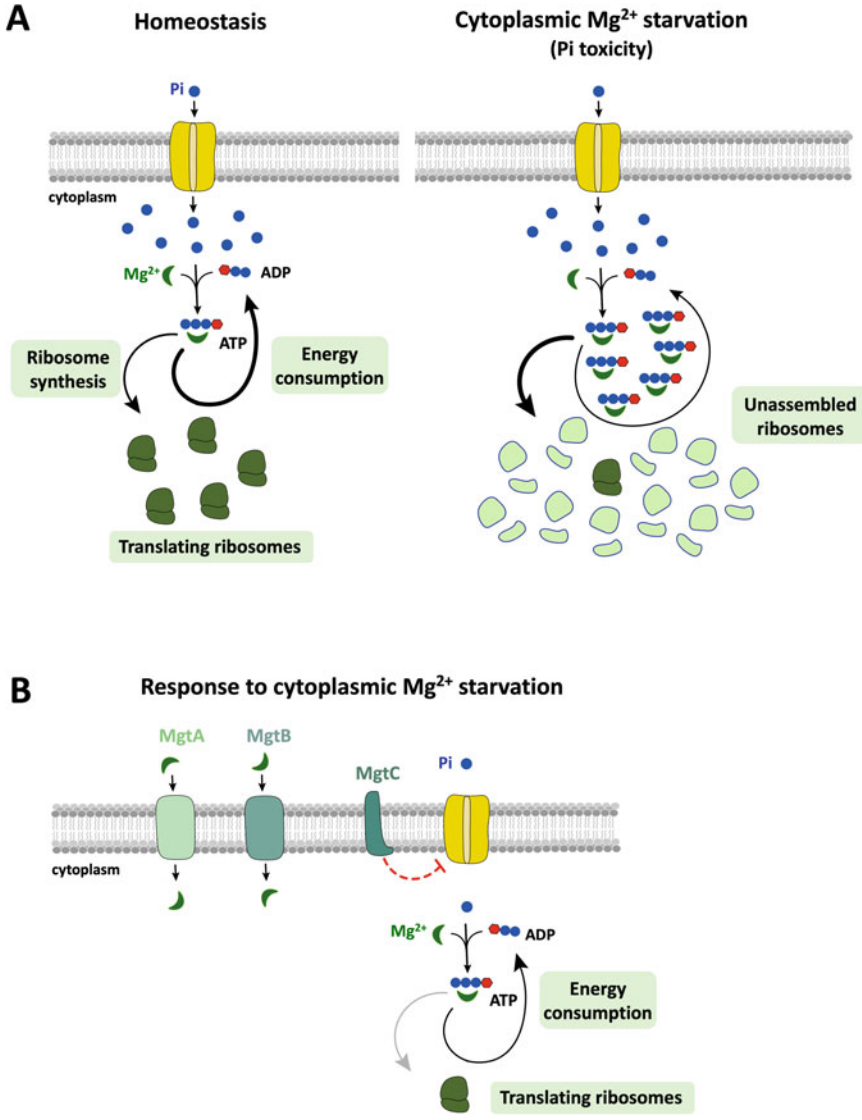

Fig. 12.4 (a) Model illustrating the interplay between Pi and Mg^{2+} metabolisms in bacteria. (Left-hand side panel) During homeostasis, Pi is translocated into the cytoplasm by dedicated membrane-bound transporters. Cells assimilate imported Pi through the synthesis of ATP, which is primarily neutralized by Mg^{2+} cations, yielding ATP:Mg^{2+}, the main physiological form of ATP. In enteric bacteria, ATP:Mg^{2+} stimulates rRNA synthesis, thus promoting ribosome biogenesis. Large amounts of Mg^{2+} ions are required to support assembled, translating ribosomes which consume the majority of intracellular ATP pools, thereby recycling Pi and Mg^{2+}. (Right-hand side panel) After exhausting the environmental Mg^{2+}, cells eventually experience a shortage in cytoplasmic Mg^{2+} levels. Insufficient cytoplasmic Mg^{2+} impairs ribosomal subunit assembly, lowering translation efficiency and ATP consumption. Yet, the combined activity of all Pi-assimilating reactions continues to promote ATP synthesis, which furthers rRNA transcription. This results in the futile accumulation of unassembled ribosomal subunits, which are now unable to hydrolyze ATP: Mg^{2+} to recycle Pi and Mg^{2+}. **(b) Cellular response to cytoplasmic Mg^{2+} starvation in *Salmonella*.** The decrease in cytoplasmic Mg^{2+} levels promotes transcription elongation into the coding regions of the PhoP-dependent *mgtA*, *mgtB*, and *mgtC* genes. MgtA and MgtB proteins import Mg^{2+} into the cytoplasm, and the MgtC protein reduces the uptake of Pi, thus, preventing its assimilation into Mg^{2+}-chelating ATP. The increase in free cytoplasmic Mg^{2+} in combination with a diminished pool of ribosomes restores translation, normalizing ATP hydrolysis and recycling of intracellular Pi and Mg^{2+}

already scarce Mg^{2+} by assimilated Pi. Interestingly, artificially increasing Pi intake through Pst expression induces the transcription of genes that respond to cytoplasmic Mg^{2+} starvation (see above). This occurs even when cells are grown in conditions where Mg^{2+} is plentiful [15]. Therefore, excessive cytoplasmic Pi mimics conditions resulting from insufficient cytoplasmic Mg^{2+}, and vice-versa. Given that Mg^{2+}, ATP and rRNA are indispensable constituents of every living cell, we anticipate that the inherent interplay among them hereby described is widespread in nature.

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Phosphate Dysregulation and Neurocognitive Sequelae

13

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Abstract

The endocrine regulator proteins, fibroblast growth factor 23 (FGF23) and Klotho have been well studied as mediators of phosphate metabolism. FGF23 has been implicated in the renal excretion of phosphate by limiting the docking of sodium-dependent phosphate transporters, Npt2a and Npt2c, into the luminal side of renal proximal tubular epithelial cells. By limiting Npt2a/c activity in the renal tubular epithelial cells, phosphate is reabsorbed at lower rates and is excreted at higher rates. The action of Klotho is relatively less understood but has been implicated as an FGF23 cofactor in receptor binding. Klotho is mostly synthesized in the distal tubules of the nephron relative to FGF23's activity in proximal renal tubules. The neurological sequelae due to alterations in the FGF23-Klotho axis may be explained by the direct effects of these phosphate-regulating proteins on neuronal tissues or by the roles of these proteins in phosphate metabolism. Hyperphosphatemia has been associated with vascular wall stiffness that may alter blood flow and weakenvessels in the brain. In contrast, hypophosphatemia may alter ATP usage and metabolism in the central nervous system (CNS), leading to neurological compromise. Altered levels of FGF23 and Klotho have both been associated with neurocognitive decline, clinical dementia, memory loss, and poor executive function in humans. Furthermore, FGF23 and Klotho dysregulation has been linked to structural and functional changes of the cardiovascular system with an increased risk of stroke. Subsequent research should focus on characterizing the neuropathology associated with alterations in the FGF23-Klotho system and dysregulated phosphate metabolism.

Keywords

Phosphate · Brain · Cognitive function · FGF23 · Klotho

13.1 Introduction

The metabolic regulation of phosphate relies on an interplay between hydroxypatite deposition and breakdown, intestinal absorption, and renal excretion. Within the intestines, phosphate is reabsorbed by a paracellular route through tight junctions and an active transport route through

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sodium-dependent phosphate transporters (Npt2b) [13, 17, 39]. Calcitriol and a low phosphate diet induce the expression of Npt2b on the apical membranes of intestine mucosal cells and therefore control active transport of phosphate [13, 40]. How the human body senses extracellular phosphate status is not completely elucidated, as a phosphate sensor is not yet identified but a likely mechanism could be mediated by parathyroid hormone (PTH) and its effects on bone and renal tubules. For example, high extracellular phosphate levels have been associated with elevated PTH, and in response, PTH suppresses Npt2a and Npt2c activities in proximal renal tubules [44]. Decreased Npt2a/c activity in the proximal renal tubules causes increased urinary excretion of phosphate. Similarly, the bone-derived fibroblast growth factor 23 (FGF23) and the membrane-bound protein, Klotho have augmentative actions with PTH on phosphate excretion within the renal tubules. FGF23 causes the suppression of proximal renal tubular Npt2a/c expression through direct inhibition or modulation of the parathyroid axis. This suppression causes decreased cotransport activity of Npt2a/c, which leads to decreased reabsorption of phosphate from the renal tubules. Murine and subsequent human genetic studies have linked hypophosphatemia with overexpression of FGF23, further characterizing the role of FGF23 in phosphate excretion [3, 7]. The actions of Klotho are relatively less understood, however, Klotho has been implicated as an FGF23 obligatory cofactor [47]. Conversely, Klotho is mostly expressed in the distal tubules while FGF23 is functional in the proximal tubules [16]. Klotho expression in the distal tubules may implicate this protein in an isolated action on phosphate metabolism that has yet to be fully explained. Additionally, Klotho knockout mice exhibited similar features as FGF23 knockout mice, both causing hyperphosphatemia and manifestations such as short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema [21, 32–34, 38]. The similar manifestations of FGF23 knockout and Klotho knockout imply a connection between the two molecules that control phosphate metabolism [28]. This article is shedding the light on the effects of hyperphosphatemia versus hypophosphatemia on the neuronal dysfunction and neurovascular complications, and discuss the effect of FGF23-Klotho axis on the neuronal functions.

13.2 Hyperphosphatemia and Neuronal Dysfunction

The effects of hyperphosphatemia on the central nervous system (CNS) have been documented in humans, particularly in individuals with chronic kidney disease (CKD) and/or those on hemodialysis but have also been independently linked to acute/subacute neurological dysfunction and chronic cognitive decline. Other common causes of hyperphosphatemia are tumor lysis syndrome, increased exogenous phosphate ingestion (laxatives), vitamin D intoxication, pseudohypoparathyroidism, hypoparathyroidism, and genetic disorders such as Albright hereditary osteodystrophy [11]. The acute/subacute neurological symptoms of hyperphosphatemia are varied but can include paresthesia, seizures, reflex hyperexcitability, tetany, headaches, dizziness, delirium, and coma [11]. Elevated levels of phosphate in individuals with CKD have also been associated with cognitive impairment and poor performance on the Mini-Mental Status Examination (MMSE) [27]. Furthermore, higher phosphate levels have been associated with specific cognitive dysfunction, such as poor executive function, in older men when assessed with the Modified Mini-Mental Status Examination (3MS) and Trailmaking Test B (Trails B) cognitive function measures [45]. Higher serum phosphate levels have also been documented as a risk factor for incident dementia in those under 60 years old [24]. While cognitive decline is a frequently documented complication of elevated phosphate levels, vascular changes that cause neurological emergencies have also been documented in individuals with higher serum phosphate levels. In a study that measured the phosphate levels of 3437 individuals on hemodialysis, it was the individuals with the highest phosphate levels who were documented as

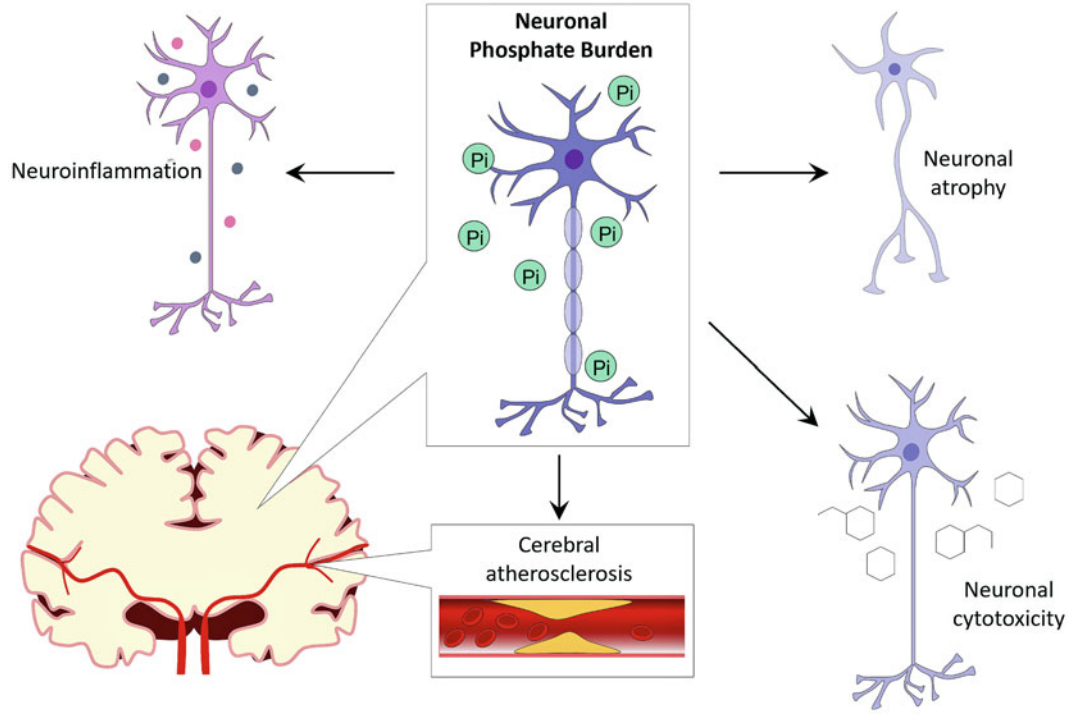

Fig. 13.1 Possible neuropathological events, initiated by neuronal phosphate burden, which can eventually contribute to overall declining brain function and cognitive impairment

experiencing future hemorrhagic brain infarctions [52]. Some of the possible effects of neuronal phosphate burden are outlined in Fig. 13.1.

13.3 Hyperphosphatemia-Induced Neurovascular Complications

It has been well-documented that chronically elevated levels of phosphate have a deleterious effect on brain tissue, but possible mechanisms of this damage have not been fully elucidated. The majority of the documented cases of hyperphosphatemia-induced vascular changes have been described in individuals with CKD as they are at the highest risk of retaining substantial levels of phosphate [5, 6, 35]. Systemically, hyperphosphatemia seems to have a calcific response in peripheral vasculature that may extend to the neurovasculature or act as a risk factor for cerebrovascular damage. Hyperphosphatemia causes vascular calcification by inducing vessel wall inflammation and converting vascular smooth muscle cells (VSMCs) into osteoblast-like cells [15, 48, 51]. The conversion of VSMCs to osteoblast-like cells is mediated by the effect of hyperphosphatemia on the sodium-dependent channels, PiT-1 and PiT-2 [6]. Hyperphosphatemia and hypercalcemia directly stimulate PiT-1 and PiT-2, which upregulates gene transformations that are associated with matrix deposition [6]. Once the VSMCs develop osteoblast-like properties, they deposit hydroxyapatite crystals into the vessel walls [15]. The calcific changes established by the phenotype conversion of VSMCs are identical to atherosclerosis and compromise the tunica intima and the tunica media [48]. Furthermore, entry of phosphate through PiT-1 and PiT-2 into the VSMCs can cause damage to these cells, eventual apoptosis, and compromise of vessel functions [51]. The high

phosphate microenvironment can also induce endothelial cell damage that further exacerbates vascular dysfunction.

Vessel wall inflammation may also have a prominent role in vascular changes associated with hyperphosphatemia. When rat models with induced CKD were fed with high-phosphate-diets, elevated levels of TNF-alpha were detected in serum and tissue, representing increased inflammatory responses in these rats relative to the control rats [51]. Furthermore, rats exposed to a high phosphate diet also had increased markers of general stress responses such as decreased body weight and lower serum albumin levels [51]. Hyperphosphatemic inflammation within vasculature may also be mediated by oxidant formation by mitochondria in VSMCs. When murine aortas were exposed to even low levels of phosphate, levels of the NADPH oxidase, Nox4 were elevated and subsequently stimulated oxidant formation, inflammation, and fibrosis in vitro [2]. The increases in inflammatory markers, oxidants, and stress responses may augment the atherosclerotic response associated with hyperphosphatemia. The increased inflammation associated with hyperphosphatemia may also directly affect nervous tissue and have a role in CNS impairment due to neuroinflammatory responses [26]. The neuroinflammatory responses associated with phosphate burden may be mediated by the production of oxidants, such as superoxide, by the mitochondria in neurons; as discussed above in the context of vasculature inflammation [2, 26].

While vessel wall compromise has been described as the leading cause of vascular damage due to hyperphosphatemia, endothelial dysfunction has also been associated with hyperphosphatemia in vitro. When bovine aortic endothelial cells were exposed to high levels of extracellular phosphate, there was an induction of reactive oxygen species (ROS) measured by tetrazolium blue assay and decreased production of nitric oxide by these cells [2, 43]. Due to the reduced levels of nitric oxide by the endothelial cells, there was also a decreased capacity of endothelial-dependent aortic rings to vasodilate [43].

Conclusively, vascular compromise is a well-documented complication of hyperphosphatemia and may play a significant role in central nervous system damage directly and indirectly. When the hyperphosphatemic-induced atherosclerosis of vasculature extends to the CNS, it may lead to vessel stenosis and chronically low oxygenation, vessel wall weakness, and hemorrhage, or acute downstream embolic episodes associated with plaque rupture. It has been established that individuals with peripheral artery disease and large-vessel disease have a higher risk of stroke, and this may also partly apply to artery disease induced by hyperphosphatemia [18, 49]. Furthermore, hypertension induced by hyperphosphatemic-induced atherosclerosis may increase the risk of lacunar infarcts and intracerebral hemorrhage. The possible mechanisms of CNS damage, due to phosphate burden, causing cognitive dysfunction are outlined in Fig. 13.2.

13.4 Hypophosphatemia and Neuronal Dysfunction

Hypophosphatemia has also been linked to changes in the CNS that have acute and chronic effects on neurological functions. Hypophosphatemia can occur due to poor dietary sources of phosphate, malabsorption associated with diarrhea and medication binding, kidney damage, primary or secondary hyperparathyroidism, and can occur in settings that cause intracellular shifting of phosphate, such as refeeding syndrome [10, 42]. While mild-moderate hypophosphatemia is often asymptomatic, the acute/subacute neurological symptoms of severe hypophosphatemia are altered mental status, numbness, and weak reflexes [42]. In a study enrolling patients with mild cognitive impairment and autosomal dominant dementia, the patients who were identified as having Aβ protein on PET scan had lower levels of serum phosphate relative to patients who did not express Aβ protein [30]. This finding may link low phosphate levels with the onset of more severe forms of dementia and cognitive impairment. Furthermore, low levels of phosphate have been linked with

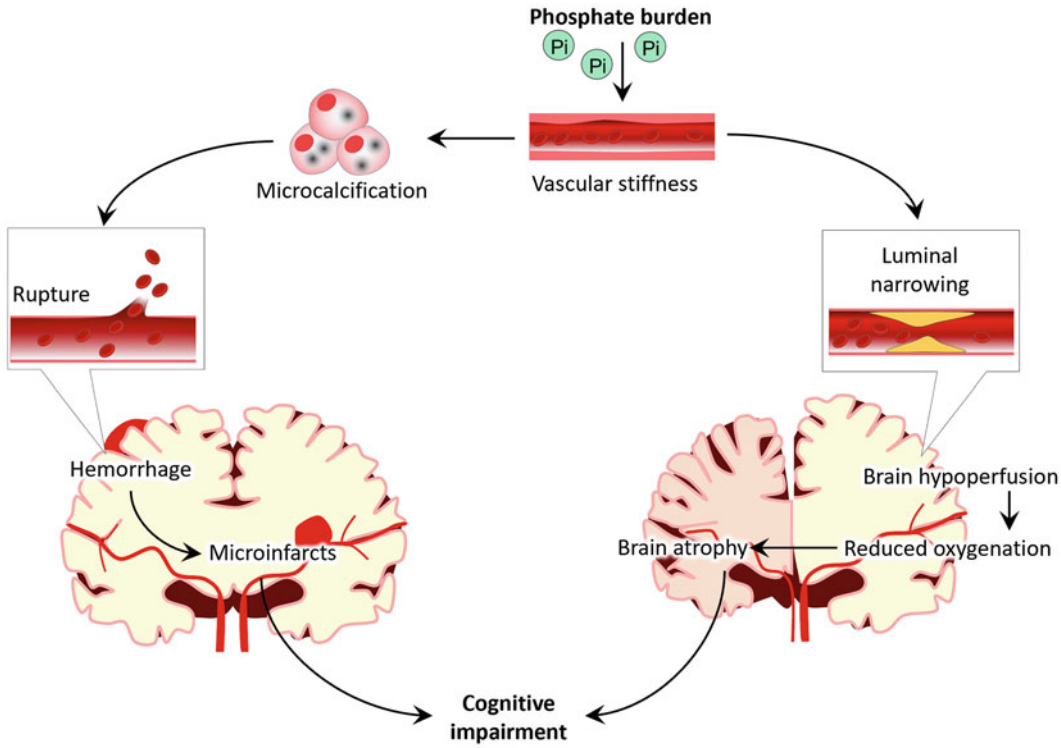

Fig. 13.2 Possible events in the brain, induced by phosphate burden, that could contribute to cognitive impairment

increased mortality in patients who have experienced an acute ischemic stroke and therefore may have a small role in neuronal energy use or vascular oxygen delivery [46].

13.5 Hypophosphatemia-Induced Neurovascular Complications

The mechanism by which hypophosphatemia causes neurological damage has not explicitly been elucidated yet, but it may be linked to decreased ability of nervous tissue to produce and use ATP, and thus nutrition of nervous tissue could be affected. In a study using induced hypophosphatemic mice, when insulin-stimulated rates of muscle ATP were measured between hypophosphatemic mice and controls, the hypophosphatemic mice had a 50% decrease in ATP synthesis [31]. Interestingly, resistant lactic acidosis is documented in multiple human case studies as a complication of hypophosphatemia which could represent altered energy metabolism as well [9]. Furthermore, lack of high-energy phosphate can decrease erythrocyte 2,3-DPG [41]. Reduced levels of 2,3-DPG cause a leftward shift of the hemoglobin dissociation curve that may lead to chronic decreases in oxygen delivery to nervous tissue and eventual neurological compromise [41]. It is plausible that the neurological manifestations associated with hypophosphatemia are the cumulative effects of chronic tissue deoxygenation and altered ATP production, although further research on this topic is necessary to understand the underlying pathology. The possible mechanisms of hypophosphatemia-induced cognitive impairment are outlined in Fig. 13.3.

13.6 FGF23-Klotho Axis and Neuronal Functions

While the FGF23-Klotho axis has been implicated in systemic phosphate metabolism,

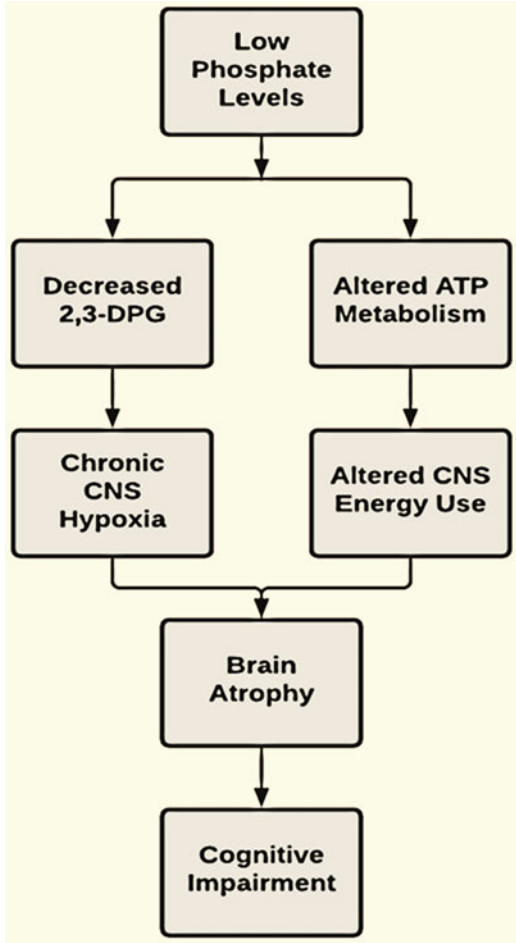

Fig. 13.3 Possible mechanisms of hypophosphatemic cognitive impairment

there has not been an explicit function of this axis uncovered in the CNS of humans. FGF23 was first found in the murine thalamic nuclei, which led to further studies addressing other locations within the CNS in which FGF23 can be found [53]. More recently, the presence of Klotho and FGF23 has been detected in the cerebrospinal fluid (CSF) of human children implicating the FGF23-Klotho axis in neurological activity outside of murine models [19]. Not only were FGF23 and Klotho identified in the CSF of human ventricles and sinuses, but a production locus for these proteins was found to be in the CNS as well, specifically from ependymal cells in the lateral ventricles [19]. Furthermore, Klotho and FGF23 have been uncovered in synapses of mice and within the hippocampus, implicating these proteins in possible neurotransmission and memory function [22, 23]. The finding of FGF23 and Klotho in neurotransmission and memory centers of the brain in murine models provides reason to study the possibility of the FGF23 and Klotho interaction further in the human nervous system.

13.7 FGF23 and Neuronal Functions

The possible functions of FGF23 in the CNS can be implied based on manifestations of the protein expression or suppression in murine models and human observations. When FGF23 deficiency was induced in murine models, mice showed decreased success in open-field velocity trials, and decreases in location memory tests after 5 weeks [22]. Furthermore, brain tissue analysis of the FGF23 deficient mice showed that hippocampal differentiation was impaired based on decreases in progenitor cell density in the dentate nucleus and subgranular zone [22, 29]. While FGF23 deficiency causes reduced hippocampal differentiation in murine models, FGF23 may also preserve hippocampal neuronal morphology and synaptic density [12]. When cultures of murine hippocampi are exposed to FGF23, synaptic density is increased, and enhancement and lengthening of dendrites occur [12]. The cognitive and neuro-morphological changes associated with deficiencies in FGF23 may be superimposed with hyperphosphatemia, as hyperphosphatemia has been correlated with multiple neurocognitive changes as mentioned above [24, 29, 45, 52]. However, FGF23 overexpression has been linked to CNS abnormalities and cognitive deficits in murine models, possibly due to effects of hypophosphatemia stimulated by the excretory actions of FGF23 [25]. Unlike the direct and beneficial effect of FGF23 on the murine hippocampus, FGF23 found in human plasma at elevated levels has been associated with poorer neurological outcomes. On MRI neuroimaging, 1170 individuals who were less than 40 years old and who had never been diagnosed with a stroke were more likely to have white matter

hyperintensity if they had elevated levels of FGF23 in the plasma [50]. Furthermore, an increased level of FGF23 in human plasma has been associated with poorer performance on several individual tests primarily focused on short delay and delayed recall memory in a population of 263 human participants who were pre-tested for eventual dialysis [8]. Regarding cerebrovascular emergencies in humans, elevated levels of FGF23 have been correlated with increased risk of stroke and intracranial hemorrhage, however, the population analyzed in this association also had reduced glomerular filtration rate (GFR), which may be a moderator or confounder of this finding [8]. It can be hypothesized that an elevated level of FGF23 is also associated with kidney disease, and the neurological effects of elevated plasma FGF23 cannot be separated from the neurological deficits associated with CKD and related uremia. Phosphate is often elevated in CKD, and since FGF23 is associated with phosphate excretion, its elevation may be compensatory. The CNS abnormalities associated with elevated FGF23 may also be explained by hypophosphatemia as rescue phosphate ingestion in mice with elevated FGF23 ameliorates the cognitive deficits that were previously expressed [25].

13.8 Klotho and Neuronal Functions

The coreceptor Klotho has been associated with changes in neuronal architecture and cognitive function in human and mouse models. Comparatively to FGF23 overexpression and the resultant white matter hyperintensities, deficiency in the Klotho protein has shown to be a significant risk factor for diffuse white matter lesions [20, 50]. Furthermore, elevated levels of Klotho have been associated with higher MMSE scores, possibly due to induction of phosphate clearance [20]. Low levels of Klotho also have a significant association with vascular dementia onset and progression of dementia to more severe forms in humans [4]. The association of low Klotho and dementia may also be explained by the effects of Klotho on the hippocampus in murine models. In Klotho knockout mice, synapses in the hippocampus and synaptophysin levels that represent vesicular transport are both reduced [23]. Furthermore, Klotho deficient mice showed reduced nerve terminals within the stratum lucidum in the hippocampus [23]. While Klotho and FGF23 have been associated with hippocampal structural and functional changes, it is important to note that Klotho has been associated with changes in hippocampal proliferation and differentiation, while FGF23 has only been associated with changes in proliferation, implying a possible augmentative effect on the hippocampus between the two proteins in murine models [22]. Moreover, using Xenopus oocyte models, injection of Klotho alone showed no increase in glutamate current but adding Klotho with EAAT3/4 amino acid transporters caused a significant increase in glutaminergic current across neuronal membranes and the blood-brain-barrier [1]. Therefore, the protective activity of Klotho may be associated with excitatory glutamate action only if the necessary proteins are available for the transport of amino acids. Klotho has also been described as an "immunological gatekeeper" at the interface between the immune system and the choroid plexus in mice [54]. This finding implies that Klotho depletion with age or genetic Klotho deficiencies in mice may be caused by immune-mediated neuropathogenesis beginning in the choroid plexus. Klotho-deficient mice also showed increased activation of microglia, further identifying immune mechanisms of hippocampal degeneration due to Klotho deficiency [54].

13.9 Summary

While the mechanisms by which FGF23 and Klotho cause neurological sequelae and morphological changes are not completely understood, it appears likely that part of these changes is superimposed with dysregulation of phosphate homeostasis. FGF23 has a direct and/or an indirect role on Npt2a/c expression in the kidney tubules and may work with PTH to cause phosphate excretion. Klotho acts as a cofactor for FGF23 in the proximal tubules and is produced

in the distal tubules, where it may have an isolated function in phosphate metabolism. FGF23 and Klotho have been isolated in brain tissue within the hippocampus, CSF, ventricles, and the cerebral neuron synapses of murine models. The locations of FGF23 and Klotho in the CNS may implicate these proteins in memory function, synaptic transmission, and immunoregulatory actions. Predictably, the cognitive decline and cerebrovascular changes associated with hyperphosphatemia and hypophosphatemia generally match the sequelae related to alterations in levels of FGF23 and Klotho. Hyperphosphatemia is associated with an increased risk of dementia, hemorrhagic stroke, isolated memory loss, and advancement of dementia [24, 29, 45, 52]. Hyperphosphatemia may also be associated with decreased brain growth, as observed in young mouse models [14]. Hypophosphatemia is associated with specific Aβ plaque deposition rates seen in individuals with Alzheimer disease, along with general dementia and ischemic stroke [30, 46, 52].

Future research should focus on quantifying and characterizing the relationships between FGF23, Klotho, and phosphate to uncover how altered phosphate concentrations affect neuronal functions. It is also important to further document the manifestations of Klotho and FGF23 deficiencies or excesses on the CNS, mainly in relation to phosphate levels. Finally, determining phosphate-independent effects of FGF23 and Klotho on neuronal growth, development, and maintenance will enhance our understanding of the pathophysiology of cognitive functions in general and beyond [36, 37].

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Index

M. S. Razzaque (ed.), *Phosphate Metabolism*, Advances in Experimental Medicine and Biology 1362,
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