

The different effects of high phosphate levels in chronic kidney disease

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INTRODUCTION

As renal function declines, the accumulation of phosphate adversely affects mineral homeostasis and bone turnover. The consequences of this imbalance include secondary hyperparathyroidism (2°HPT), osteodystrophy, and vascular calcification. High phosphate levels are a key factor in the pathogenesis of 2°HPT. Hyperphosphatemia is common in uraemic patients due to a reduced excretion of phosphate and because routine dialysis cannot remove the absorbed phosphate. Studies in uraemic rats have shown that phosphate loading accelerates development of 2°HPT, whereas dietary phosphate restriction can prevent and even arrest 2°HPT in animal models of renal

failure. In addition, hyperphosphatemia has been shown to be a significant predictor of mortality in dialysis patients. Therefore the reduction of phosphate levels is an important therapeutic target in the management of uraemic patients.

PHOSPHATE AND PARATHYROID FUNCTION

Patients with renal failure develop 2°HPT. A decrease in the serum concentration of calcium and vitamin D and a rise in serum phosphate levels are the key factors in the pathogenesis of the 2°HPT. In addition, calcium and vitamin D receptors (CaR, VDR) have been reported to be decreased in parathyroid glands from urae-

mic patients and animals with experimental uraemia. This may contribute to the progression of 2° HPT^{1,2}.

Studies in patients and animals have shown that secondary hyperparathyroidism in chronic renal failure is stimulated by dietary phosphate loading and ameliorated by dietary phosphate restriction³⁻⁶. The effect of phosphate on parathyroid function is indirect and direct: indirect, through the inhibition of vitamin D synthesis and the induction of hypocalcemia due to a decreased calcaemic response to PTH and the precipitation of calcium; a direct effect of phosphate on parathyroid function has been shown more recently in vivo and in vitro studies. These studies have demonstrated a direct effect on PTH synthesis and secretion and on parathyroid cell proliferation⁷⁻¹⁰.

Phosphate and PTH secretion and synthesis

In vivo and *in vitro* studies show that high phosphate directly stimulates parathyroid hormone (PTH) secretion. Almadén *et al.*⁷ demonstrated that intact rat parathyroid glands incubated in a high phosphate medium increased PTH secretion, despite no change in the ionized calcium concentration in the medium. Further demonstration of a direct effect of phosphate on PTH secretion was obtained in vitro in hyperplastic parathyroid tissue from hemodialysis patients with severe hyperparathyroidism⁸ and in in vivo studies in hemodialysis patients¹¹ and in dogs¹² respectively.

Different groups have also shown a direct effect of phosphate on PTH secretion, and that it is observed only in tissue preparations rather than in dispersed parathyroid cells in culture. Nielsen *et al.*¹³ showed that the stimulation of PTH secretion by phosphate was observed only

in parathyroid tissue with intact architecture; however, both dispersed cells and tissue preparations responded to changes in the calcium concentration. In a report by Roussanne *et al.*¹⁴, the observation of an effect of phosphate on PTH secretion was suggested to be possible as a result of the presence of cell clusters with close cell-to-cell interaction. However, there is not a clear explanation of why cell-to-cell interaction is important to observe an effect of phosphate on PTH secretion. Sun *et al.*¹⁵ demonstrated that parathyroid cells in close proximity are stimulated to secrete more PTH and suggest the presence of a paracrine interaction among parathyroid cells. Intercellular communication may be also required to observe an effect of phosphate on PTH secretion. In a recent study, a pseudogland model of parathyroid tissue grown in collagen illustrates the importance of the 3-D tissue architecture in parathyroid gland function¹⁶.

In vivo studies have demonstrated that the effect of phosphate on PTH secretion is dose-dependent, even though the degree of PTH response to phosphate is much lower than to calcium^{11,12}.

The direct stimulatory effect of phosphate on PTH secretion is relatively rapid. It was observed after 2 hours in the *in vitro* setting (personal observation). This issue has been recently addressed by the Slatopolsky's group¹⁷. These authors showed that oral phosphate increased PTH release more rapidly than previously reported in parathyroid culture models. In uraemic rats adapted to a high phosphate diet (HPD) and trained to ingest food during a 2-hour feeding period each morning, a switch to a meal of low phosphate diet (LPD), caused a decrease in 80% of serum PTH within the 2-hour feeding period with no change in plasma calcium but a 1 mg/dl fall in plasma phosphate. In contrast, HPD gavage increased PTH by 80% within 15

minutes with no change in plasma phosphate or calcium. Furthermore, duodenal and intravenous infusion of sodium phosphate increased PTH within 10 minutes, whereas infusion of sodium chloride had no effect.

Phosphate does not only stimulate PTH secretion but also its synthesis. *In vivo* studies show that in rats, high dietary phosphate produced an elevation in serum PTH and an increase in PTHmRNA¹⁸⁻²⁰. Almaden *et al*,⁸ were able to show stimulation of PTH secretion and PTHmRNA by high phosphate concentration in vitro in hyperplastic parathyroid tissue from hemodialysis patients with severe hyperparathyroidism. Silver and collaborators have characterized the mechanisms of PTH mRNA regulation by phosphate and calcium. Phosphate regulates the PTH gene post-transcriptionally by modulation of the binding of parathyroid cytosolic proteins, trans factors, to a defined cis sequence in the PTH mRNA 3'-untranslated region (UTR), thereby determining the stability of the PTHmRNA²¹.

Phosphate and parathyroid hyperplasia

Hyperplasia of the parathyroid gland develops in azotemic patients as chronic renal failure evolves. The knowledge of the cellular and molecular mechanisms underlying the regulation of parathyroid cell proliferation is poorly understood. However, it is well accepted the progression of parathyroid hyperplasia as a consequence of the same factors enhancing the PTH secretion, a decrease in the serum concentration of calcium and vitamin D and a rise in serum phosphate levels, which together or separately may stimulate parathyroid cell proliferation.

The effect of phosphate on parathyroid cell proliferation has been broadly addressed as

manoeuvres with the dietary phosphate in normal and uraemic rats have become a suitable model of experimental hyperparathyroidism with clear evidence of parathyroid hyperplasia.

In uraemic rats, a low phosphate diet prevented the parathyroid proliferation^{18, 24}. Conversely, dietary phosphate loading and/or hyperphosphatemia, even in the absence of changes in serum vitamin D values, increase parathyroid cell proliferation and stimulate the development of parathyroid gland hyperplasia^{9,22-24}. A study from our laboratory²³ showed that in normal rats a high phosphate diet stimulated the parathyroid cell proliferation as early as 24 hours after initiation of the high phosphate diet.

Diminished CaR in 2° HPT has been associated to increased parathyroid cell proliferation. Slatopolsky's group have studied the effect of phosphate on the parathyroid CaR expression²⁴ and found that high dietary phosphate stimulates both parathyroid cell proliferation and a fall in CaR. Parathyroid proliferation precedes down-regulation of the CaR in uraemic rats fed a high phosphate diet. The down-regulation of the CaR occurred in chronic renal failure only when the parathyroid glands were hyperplastic, suggesting that the CaR reduction is a consequence rather than the cause of parathyroid cell proliferation²⁵. Restriction of dietary phosphate, which inhibits parathyroid cell hyperplasia, prevented down-regulation of the CaR²⁶. In addition, after hyperplasia is developed in uraemic rats, down-regulation of the parathyroid CaR can be reversed by phosphate restriction. Nonetheless, as the restoration of the CaR is relatively slow and does not appear to play a role in the more rapid normalization of PTH and arrest of parathyroid hyperplasia, the authors conclude that the improvement of the parathyroid function with dietary phosphate restriction in renal failure may be due, only in part, to increased CaR expression.

The specific mechanism(s) by which a high phosphate diet increases parathyroid cell proliferation still remain unclear. In a study in azotemic rats, Dusso *et al.*²⁷ showed that a high phosphate diet increases parathyroid cell proliferation by increasing TGF- α whereas a low phosphate diet decreased parathyroid cell proliferation by stimulating the expression of the cyclin-dependent kinase inhibitor p21/WAF; however they could not establish a relationship between the high phosphate and the expression of p21.

The effect of phosphate on cell proliferation has been also evidenced in *in vitro* studies in which parathyroid cells were cultured from parathyroid glands of patients with secondary hyperparathyroidism²⁸. In that study, a high phosphate concentration in the medium increased parathyroid cell proliferation after a long period of incubation.

Phosphate and resistance to vitamin D

Hyperphosphatemia is associated to parathyroid resistance to treatment with vitamin D. Thus, control of serum phosphate is required to achieve a proper response of the PTH secretion to vitamin D in uraemic patients²⁹.

In a recent study³⁰, we analyzed the rate of parathyroid cell proliferation and the response to vitamin D (the inhibition of cell proliferation by vitamin D) of hyperplastic parathyroid tissue from parathyroidectomized patients with 2°HPT *in vitro*. The results showed that both parathyroid cell proliferation and impaired inhibition of vitamin D were inversely correlated not only with the female gender, but also with the level of the pre-parathyroidectomy serum phosphate (figure 1). It is possible that a high serum phosphate concentration imprints the parathyroid cell with signals that counteracts the suppressive effect

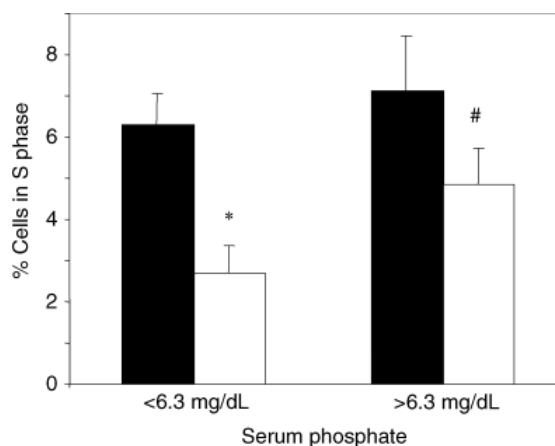


Figure 1. The effect of pre-PTX serum phosphate values on human parathyroid cell proliferation *in vitro*. Cell proliferation (% cells in S phase) was determined in parathyroid tissue from 47 glands (from 19 patients) incubated for 24 hours in medium without (dark bars) or with (white bars) 10^{-7} mol/L calcitriol. Groups are divided by the mean pre-PTX serum phosphate value (6.3 mg/dL). Values for percent cells in S phase are given as the mean \pm SE. *P < 0.01 vs. (-calcitriol); #P < 0.05 vs. (+calcitriol) of serum phosphate <6.3 mg/dL. From Almadén *et al.* *Kidney International* 64 (6), 2311-2317, 2003.

of calcitriol *in vitro*. Moreover, in the same study, vitamin D treatment did not significantly reduce PTH values and parathyroid cell proliferation in normal rats on a high phosphate diet. These results are in agreement with clinical studies showing that vitamin D fails to control secondary hyperparathyroidism in patients with high serum phosphate levels³¹⁻³³.

The mechanism involved in the phosphate-induced resistance to vitamin D is unclear. Vitamin D is known to decrease cell proliferation by inhibiting the oncogene c-myc³⁴, which then results in the stimulation of p21/WAF³⁵. Thus, as deduced from the study by Dusso *et al.*²⁷, it is possible that dietary phosphate loading could overcome the stimulatory effect of on p21/WAF to stimulate parathyroid cell proliferation.

Phosphate and cell signaling

The effects of phosphate on parathyroid function may be mediated through a specific molecule capable of sensing variations in phosphate levels. One candidate molecule would be a Na⁺-Pi cotransporter, termed rat PiT-1, that has been isolated from rat parathyroids³⁶. The amount of PiT-1 mRNA in the parathyroids was much greater in rats fed a low-phosphate diet than in those fed a high-phosphate diet, indicating that PiT-1 may contribute to the effects of phosphate on parathyroid function. Thus, it has been proposed that this transporter may function as a putative "phosphate sensor" for the parathyroid cell³⁷.

The signal transduction mechanisms involved in the stimulation of PTH release by low extracellular calcium are increasingly understood; however, little is known about the intracellular signaling system involved in the regulation of PTH secretion by extracellular phosphate. Extracellular calcium concentration modulates PTH secretion via a G-protein-coupled calcium-sensing receptor³⁸. This effector system includes the hydrolysis of membrane phospholipids by phospholipase C (PLC), phospholipase D (PLD), and phospholipase A₂ (PLA₂) to generate the appropriate intracellular signals³⁹. The activation of PLA₂ leads to the formation of arachidonic acid (AA), a potent inhibitor of PTH release, which acts via the 12- and 15-lipoxygenase pathway^{40,41}. A study by Kifor *et al.*⁴² suggested that, in bovine parathyroid cells, the activation of CaR mediated the activation of PLA₂ through the MAP kinase cascade. Recently, we have also observed the involvement of the ERK1/2-MAPK in the regulation by calcium of both the PTH secretion and VDR gene expression via PLA₂-AA; the production of AA leads to ERK1/2-MAPK activation (unpublished data).

The results of our group showed that a high

phosphate concentration regulates PTH secretion through the PLA₂-AA signaling system. The results of an *in vitro* study in parathyroid tissue⁷ showed that despite the presence of high phosphate concentration in the medium, the addition of exogenous AA to the medium restored the capacity of a high calcium concentration to inhibit PTH secretion. Therefore, the addition of AA reversed the stimulatory effect of phosphate on PTH secretion. In a different study, Almaden *et al.*⁴³ demonstrated that the increase in PTH secretion induced by high extracellular phosphate was associated to a decrease in AA production by parathyroid cell. While in the presence of a normal phosphate concentration exogenous PLA₂ decreased PTH secretion, a high phosphate concentration prevented the inhibition of PTH secretion by exogenous PLA₂. The inhibitory effect of phosphate on AA production was not observed in other AA-producing tissue such as rat adrenal glomerulosa tissue.

Finally, in another work⁴⁴ we showed that in rat parathyroid tissue an increase in intracellular calcium activates PLA₂, resulting in increased AA production. Then, we evaluated the effect of an elevation of the intracellular calcium concentration on AA production in the presence of high extracellular phosphate levels. The results demonstrated that both the ionophore and thapsigargin were capable of inducing a marked increase in AA production, which was associated with a decrease in PTH secretion (figure 2). These results support the hypothesis that the reduction in AA production induced by high extracellular phosphate is due to an inadequate increase in cytosolic calcium in response to stimulation of CaR by calcium.

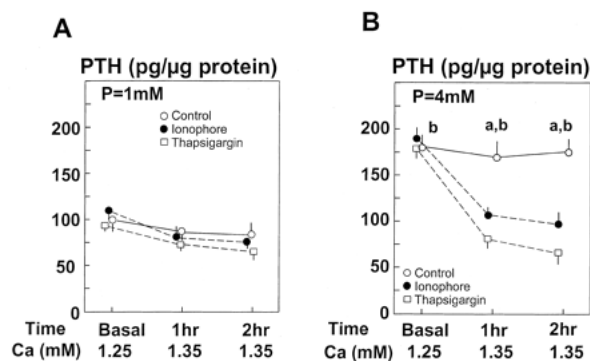


Figure 2. Effects of ionophore (A23187) (10 μ M) or thapsigargin (1 μ M), compared with control conditions, on PTH secretion by rat parathyroid glands incubated with a high calcium (Ca) concentration (1.35 mM), with normal (1 mM) (A) or high (4 mM) (B) phosphate (P) concentrations. Intact rat parathyroid glands were incubated for 1 h with 1.25 mM calcium (basal), and the same glands were then incubated for an additional 2 h with 1.35 mM calcium. Values are means \pm SEM ($n = 6$ for the two thapsigargin groups, $n = 11$ for all other groups). ^a $P < 0.05$ versus the same calcium concentration without ionophore or thapsigargin added. ^b $P < 0.05$ versus a phosphate concentration of 1 mM (shown in A). From Almadén et al. *J Am Soc Nephrol.* 13(3):693-8, 2001.

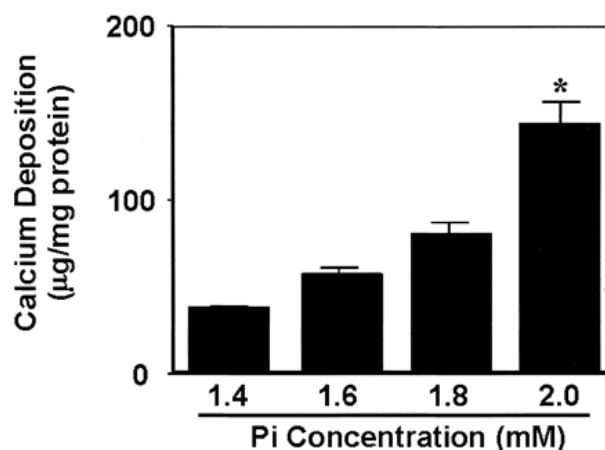
PHOSPHATE AND VASCULAR CALCIFICATION

Cardiovascular disease is the most frequent cause of mortality in patients with renal failure, showing a 30 fold increase in the mortality risk as compared to matched non uraemic individuals^{45,46}. Recent studies have shown that hyperphosphatemia is associated with soft-tissue and vascular calcification. Vascular calcification appears associated with cardiovascular disease which leads to increased mortality and morbidity in patients with renal failure⁴⁶⁻⁴⁸. Vascular calcification of the media affects 50% of patients in dialysis.⁴⁸

The uraemic state is associated with numerous metabolic abnormalities and disturbances of calcium and phosphate metabolism which contribute to the early development and progression of vascular calcification and atherosclero-

sis⁴⁹. Vascular calcification is associated with the alteration of mineral metabolism commonly found in dialysis patients such as hyperparathyroidism (HPTH), vitamin D therapy, high calcium-phosphate product, adynamic bone disease, duration of dialysis treatment, diabetes, increased phosphate concentration, and the dose of Ca-containing phosphate binders⁵⁰. Furthermore, other risk factors for atherosclerosis in uraemia such as abnormal lipid metabolism, the state of chronic inflammation and other "uraemia-related" factors may play a role in the formation of vascular calcification⁵¹.

It was previously thought that the precipitation of calcium phosphate was exclusively a passive process; however, it is now well established that vascular smooth muscle cells actively take up phosphate to form bioapatite⁵². Recent experimental data clearly define a role for phosphate in the mineralization of smooth



* $P < 0.01$ vs 1.4mM.

Figure 3. Effect of increasing extracellular (medium) inorganic phosphate concentrations on calcium deposition in human aortic smooth muscle cells in culture. Reprinted with permission from Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM: Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 87: E10-E17, 2000.

muscle cell (SMC), which undergo phenotypic conversion to osteogenic cell type in the presence of hyperphosphatemia; creating a predisposition for calcification⁵². Cells that are grown in the presence of higher Pi concentrations (up to 2 mmol/L) (figure 3), similar to those seen in individuals with hyperphosphatemia, show an increased deposit of calcium into vascular cells⁵³. In vitro data provide evidence that the phenotypic transformation of HSMC in response to hyperphosphatemia is mediated by the sodium-dependent phosphate co-transporter Pit-1, which moves extracellular phosphate into intracellular compartments. The increased intracellular phosphate serves as a signal for osteogenic gene expression (core-binding factor-1 genes, and downstream targets osteopontin and osteocalcin) and as a suppressor of SMC-specific gene expression, resulting in increased secretion of mineral-nucleating molecules (matrix vesicles, calcium-binding proteins, alkaline phosphatase, and collagen-rich extracellular matrix)⁵².

PHOSPHATE AND MORTALITY RISK

Recent studies have suggested that consequences of hyperphosphatemia reach beyond those of mineral metabolism and hyperparathyroidism. A high serum phosphate level has been recognized as a predictor of mortality in HD patients independent of PTH levels⁵⁴. A direct effect of hyperphosphatemia in the generation of vascular calcification may be a mechanism by which increased cardiovascular morbidity and mortality in dialysis patients are linked to hyperphosphatemia.^{53,55} Other mechanisms cannot be excluded.

The cutoff value for serum phosphate concentration that predicted mortality was established at 6.5 mg/dL⁵⁴⁻⁵⁶ (figure 4). Nevertheless,

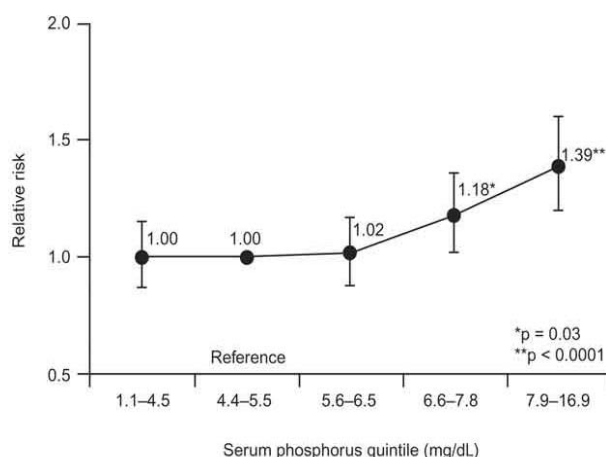


Figure 4. High serum phosphorus increases mortality risk. (Reproduced with permission from Block (20), © 1998 National Kidney Foundation).

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recent reports have progressively decreased this value. In a study by Rodriguez-Benot *et al.*⁵⁷ mild hyperphosphatemia came to be a mortality risk factor. The results obtained show that the cutoff value for serum phosphate that predicts a 2-fold increase in mortality is 5.0 mg/dL, the upper level of the established normal range (3.0 to 5.0 mg/dL) of serum phosphate concentrations (figure 5). These results are in agreement with those of a recent publication by Block *et al.*⁵⁸, where a serum phosphate level greater than 5.0 mg/dL was also associated with a significant increase in mortality. Finally, Kestenbaum *et al.*⁵⁹ have shown that even serum phosphate levels >3.5 mg/dl were associated with a significantly increased risk for death among a population of patients with chronic kidney disease.

Although mechanisms are not clearly established, these results support the need for a tight control of hyperphosphatemia to improve patient survival.

Survival functions stratified for serum phosphate level greater or less than 5.0 mg/dL (1.61 mmol/L) and derived from a Cox regression model after adjusting for age, sex, presence of diabetes, hemoglobin level, albumin level, nPCR, Kt/V, serum calcium level, and PTH level. For calculation of plots, means of covariates were entered as coefficients in model functions. Both phosphate as the mean of the last 6 months before the end of study or death [P (6m)] and phosphate as the mean of all determinations across time [P (av)] are plotted simultaneously to emphasize the similarities in the analysis between the 2 types of estimation of phosphate values. To convert phosphate in mg/dL to mmol/L, multiply by 0.3229

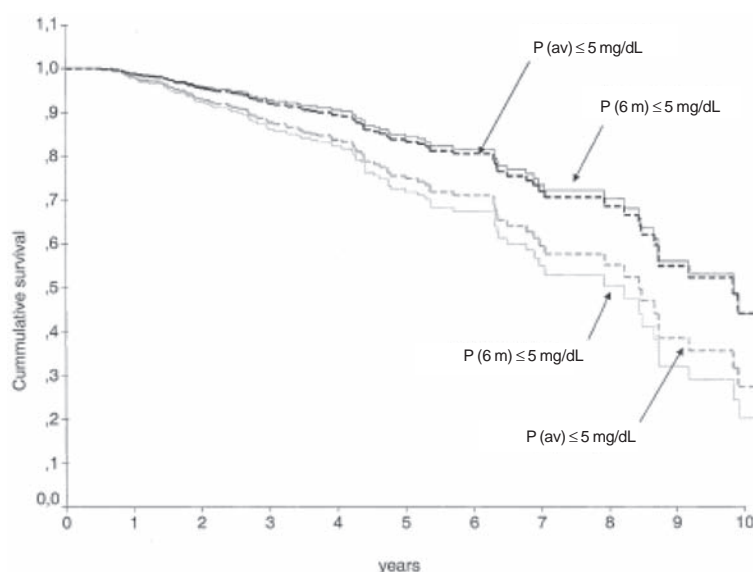


Figure 5.

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