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Hipometilación del gen de la PTH por elevado fósforo de la dieta: un posible agravante epigenético de la severidad del hiperparatiroidismo secundario en la enfermedad renal crónica

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Hypomethylation of the PTH gene due to high dietary phosphorus: a possible aggravating of severe secondary hyperparathyroidism in chronic renal failure

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Summary

Introduction: Hyperphosphataemia aggravates both parathyroid hyperplasia and PTH secretion in patients with chronic kidney disease (CKD). Hyperplasia is associated with decreases in calcium receptor expression (CaSR), vitamin D (VDR) and α-Klotho, inducing resistance of the parathyroid gland to respond both to treatment and to increases in FGF23. This study examined the possible epigenetic contributions of raised phosphorus to aggravate secondary hyperparathyroidism (SHPT) in patients with (CRD).
Material and methods: The degree of methylation was compared by pyrosequencing of bisulfite in CpG-rich sequences of the promoters in the CaSR, VDR, PTH and α-Klotho genes in parathyroid gland DNA from uremic rats fed a normal and high phosphorus diet.
Results: The diet rich in phosphorus increased PTH expression and caused a marked reduction in the degree of methylation in the promoter of the PTH gene. In contrast, the promoter regions of the CaSR, VDR and α-Klotho genes did not show significant differences in the percentage of methylation between the two groups of rats. Thus, it was not the determining mechanism for the decrease of the expression of these genes observed in the SHPT.
Conclusions: The epigenetic alterations induced by the phosphorus rich diet in SHPT, particularly the PTH gene hypomethylation, could contribute to the increases that occur in the synthesis and secretion of this hormone. The identification of the mechanisms involved would allow better treatments for SHPT to be designed in the early stages of CKD.

Key words: *DNA methylation, PTH, chronic kidney disease, parathyroid glands, hiperphosphataemia.*

Introduction

Secondary hyperparathyroidism (SHPT) is a common complication of chronic renal disease (CRD) characterized by hyperplasia of the parathyroid glands and increases in the synthesis and secretion of parathyroid hormone (PTH). Raised serum levels PTH cause alterations in bone remodeling and phospho-calcium homeostasis that increase both fracture propensity and vascular calcification, a process that aggravates the morbidity and mortality of patients with renal problems[1].

In the course of CRD, the most important stimuli for the development of SHPT are decreases in circulating levels of calcium, nutritional vitamin D and its hormonal form, calcitriol, as well as raised serum phosphorus, when levels are below the upper limit of the normal range[2].

It is noteworthy that the degree of parathyroid hyperplasia in CRD is also associated with a proportional decrease in the parathyroid expression of calcium and vitamin D receptors (CaSR and VDR)[3]. These reductions diminish the gland's ability to suppress both cell proliferation and PTH secretion rates in response to changes in circulating levels of calcium and vitamin D induced by treatment to correct hypocalcemia or Vitamin D deficiency. An additional aggravating factor to parathyroid dysfunction of CRD is the early decrease of the anti-aging molecule, α-Klotho, in the membrane of parathyroid cells[4]. This reduction leads to an ineffective suppression of PTH synthesis and secretion by the phosphaturic FGF23 hormone, since α-Klotho acts as a co-receptor bound for cellular signals of the FGF23 complex with its specific receptor FGFR[5,6].

We now know that, in addition to the defects in the transcriptional control of the PTH, CaSR and α-Klotho gene due to calcitriol deficiency, or to decreased levels of its receptor, VDR, in the parathyroid gland hyperplastic,[3,4] epigenetic modifications such as hypermethylation of the CaSR, VDR or α-Klotho genes in their promoter regions may also contribute to renal parathyroid dysfunction. Interest in the epigenetics of SHPT in CRD arose from the evidence of the critical role of hypermethylation of tumor suppressor genes in processes of exacerbated cell proliferation[7,8], as occurs in nodular SHPT. This form of SHPT is similar in its development to a benign endocrine tumor, with a very serious adverse impact in the progression of SHPT, renal and vascular damage, as well as in the survival of the renal patient who develops resistance to treatment[9,10].

In contrast, evidence shows the significant impact of mild increases in α-Klotho gene methylation induced by aging in the brain and by uremic toxins in the kidney, both in α-Klotho expression in the cell membrane and in its anti-oxidant and anti-inflammatory functions which have not been studied in the parathyroid gland[11,12].

Another important epigenetic modification for controlling SHPT is the global hypomethylation of the PTH gene, demonstrated exclusively in the parathyroid tissue[13]. Although the degree of global hypomethylation of the PTH gene is similar in glands with normal function and hyperfunctioning glands[13] this finding suggests that a differential methylation process of this gene in its promoter regions may contribute to the severity of SHPT.

As phosphorus retention by the diseased kidney is the major risk factor for directly exacerbating the degree of parathyroid hyperplasia, stabilizing the messenger RNA of PTH, secretion of PTH into the circulation, and increasing FGF23 in the CRD, by non-transcriptional mechanisms, this study aimed to assess the possible contribution of epigenetic alterations induced by elevations in serum phosphorus to the severity of parathyroid dysfunction in a murine model of CRD. To do this, we compared the degree of methylation of the promoters of the CaSR, VDR, Klotho and PTH genes in uremic rats fed diets with normal or high phosphorus content and their association with the severity of SHPT.

Material and methods

Experimental Study

For the study, 4-month-old male Wistar rats from the University of Oviedo animal lab were subjected to a nephrectomy (NX) of 7/8 consisting of the elimination of three quarters of the left kidney and total resection of the right kidney[14].

Immediately after nephrectomy, a group of uremic animals continued with the maintenance diet for rodents with normal (N) content in phosphorus (P) (0.6%, NX-NP group), while the other group of nephrectomized animals received a diet with high (E) phosphorus content (0.9%; NX-EP group) for 20 weeks.

At the time of sacrifice, carried out under CO_2 anesthesia and by exsanguination, serum was collected to determine general markers of CRD grade and alterations in bone and mineral metabolism and also parathyroid glands in each experimental group (14 glands of 7 rats per group) stored at -80°C until use.

Analysis of methylation of the promoters of the genes under study by bisulfite pyrosequencing

To extract genomic material from the rat parathyroid glands, the phenol-chloroform method was used. The DNA extracted from the parathyroid glands was treated with sodium bisulfite following EZ DNA Methylation-Gold™ Kit D5005" instructions (Zymo Research, Orange, USA). A specific polymerase chain reaction (PCR) was then performed with biotinylated primers followed by the pyro-sequencing protocol (PyroMark QUIAGEN® Q24), which consists of denaturing the double strands of the PCR products to obtain single chains, one of them labeled with biotin. The biotinylated strand was used as a template to bind the sequencing primer. The methylation pattern of the promoter region of the PTH, VDR, CaSR and Klotho genes between the initiation of transcription to the 5' end was analyzed with Pyromark 2.0.6 software using the pairs of primers indicated in table 1.

Statistic analysis
For the analysis of the results the statistical program SPSS 17.0 was used. For the quantitative variables analyzed we used Student's t. Statistically significant differences were considered when values of $p<0.05$.

Results

Biochemical data
Biochemical data from both experimental groups are presented in table 2. As expected, animals fed the high phosphorus diet (NX-EP) presented a greater impairment of renal function measured as serum urea and creatinine, regarding the values of these parameters in the group of uremic rats fed with the diet with normal phosphorus content (NX-NP).

Although no significant differences were found in serum calcium levels between the two experimental groups, the combination of lower renal function and high dietary phosphorus led to marked increases in circulating levels of phosphorus and FGF23 on the order of 2 and 3 times higher than the values of these parameters in uremic animals fed with normal phosphorus. Consequently, the degree of SHPT was also higher in uremic animals with high phosphorus in the diet, showing 40-fold higher serum levels of PTH.

Methylation of the promoter regions of the genes under study
Figure 1 shows the percentages of methylation of the CpG sites in the parathyroid DNA included in the promoter regions of the CaSR, VDR and Klotho genes. The low percentage of methylation, less than 5% in both experimental groups, prevents any comparison of possible differential epigenetic alterations attributable to the high phosphorus in the diet compared to a normal phosphorus intake.

In contrast to these genes that decrease with SHPT progression, Figure 2 shows that in the two CpGs of the study area of the PTH gene promoter, in the parathyroid glands of normal-phosphorus-fed uremic rats there was a percentage of methylated parathyroid DNA greater than 40%. More importantly, for the same baseline grade of renal damage and duration of uremia (20 weeks), elevated phosphorus in the diet was associated with a significant 80% decrease in methylation of that region of the PTH promoter. This decrease is in line with increases in PTH levels in the serum of these animals 40 times higher than serum PTH in animals with the same basal grade of uremia fed a diet with normal phosphorus.

Discussion

This study is the first to show a possible epigenetic association between CRD hyperphosphatemia and SHPT severity: hypomethylation of the PTH gene. In addition, our results corroborate the findings of other researchers that decreases in parathyroid content of CaSR and VDR cannot be attributed to an epigenetic process of silencing by hypermethylation of these genes, both critical for effective treatment of SHPT. Our findings also question the contribution of α-Klotho promoter hypermethylation in membrane α-Klotho declines that occur with SHPT progression.

In general, CpG island hypermethylation in promoter regions results in silencing the transcription of genes. Our results indicate a degree of methylation of less than 5% in CaSR and VDR, both of which regulate normal parathyroid function and the development of resistance to treatment. In addition, high dietary phosphorus, which led to significant increases in both the degree of renal damage and the SHPT of these nephrectomized animals, did not induce significant changes in the

Table 1. Pair of primers used in mutilation studies

Primer	Sequence	MR_f	Size	CpG
Klotho F1	TGGAAAGTTTAGAATGGGAGAAAG			
Klotho R1	CCCTTTACCTTCCAAAAACTAAT	51.3	121 pb	5
Klotho SQ	GGGAAAGTAGGTGTTTTATT			
CaR FW1	AGTTTGGGAATGGTTATAGTT			
CaR RV1	CTCCCTAAATCTCTCAAATCAACCTTTA	52.7	169 pb	8
CaR SQ1	TAGGTGGTTTGGGGG			
PTH RW1	GGATTTTGAGTTTTGGGTTAGTTTGAT			
PTH RV1	ACCTAAATTTCATATACAAACCTTTTACT	52.9	360 pb	2
PTH SQ1	ATTTGAAATTTTAGAGGAGTG			
VDR FW1	AGGAATGTTAGGTAGGAGAGA			
VDR RV1	CCTTAAAAACCCTACCTTATAAAAAACTCT	52.6	344 pb	6
VDR SQ1	GATATTATTAAAGATTGT			

MT_f: PCR annealing temperature; F1: direct primer; R1: reverse primer; SQ: sequencing primer.

degree of methylation of CaSR or VDR, as also demonstrated by other investigators in murine models[15]. These did not measure the degree of global methylation of these two genes in human parathyroid glands from normal subjects or with variable grade of SHPT and primary, in which the contribution of hyperphosphatemia was not the main objective of the epigenetic analysis[16,17].

Regarding the degree of methylation of the anti-aging gene α-Klotho, its methylation degree was also less than 10%, and no significant differences were observed in the methylation percentage of the CpGs induced by high phosphorus in the diet, at least in the area of the promoter studied in the parathyroid glands from both experimental groups of uremic rats. These findings are not surprising, since in cells of the distal renal tubule, which is where the Klotho gene is expressed predominantly in a normal kidney, there seems to be a mechanism that actively protects the α-Klotho promoter from the methylation of CpGs sequences[18]. In other tissues, with low expression of α-Klotho, as in brain, breast, stomach, colon, skeletal muscle or skin, there also appears to be a similar mechanism of protection of α-Klotho levels preventing their methylation. It is also important to note that several authors have observed that a low degree of methylation appears to be sufficient to cause significant differences in the degree of gene expression. In fact, King et al. have shown in the brain of aged monkeys that a small 0.4% increase in CpG island methylation led to 20% declines in gene expression, corroborating that the degree of methylation of CpGs sequences may be involved in downward regulation of the Klotho gene associated with aging[13].

Other authors have also reported small differences (from 1 to 4.5%) in the degree of renal methylation in nephrectomized mice[13], similar to the parathyroid methylation values obtained in this study (2-5%). However, high dietary phosphorus did not lead to significant increases in the degree of methylation of this gene in parathyroid tissue.

Undoubtedly, the most important finding of this study has been the identification, for the first time, of an association between hyperphosphatemia and a decrease in methylation of the PTH promoter in the 350 nucleotide sequence preceding the start of transcription. Although studies of almost two decades ago, using techniques that preceded the development of pyrosequencing, demonstrated a global hypomethylation of the PTH gene exclusive of parathyroid tissue, but without significant differences between glands with normal or hyperfunctioning glands, the result of this study adds a possible epigenetic modification to the known post-transcriptional mechanisms induced by high phosphorus to markedly increase the synthesis and secretion of PTH, such as the stabilization of messenger RNA of PTH or the induction of secretory pathways[19-21]. It is important to note that the significant hypomethylation of PTH gene induced by high phosphorus in the diet could contribute in part to the marked elevations in serum PTH levels in this murine model of advanced experimental renal disease. However, we can not rule out with these results that, in fact, the normal or low phosphorus of the diet is the cause of the greater methylation of the PTH gene during the 20 weeks of uremia studied in this paper. In fact, phosphorus restriction in the diet does not affect the intraglandular content of PTH, but the capacity of the parathyroid cell for secretion into the circulation[22].

An important limitation of this study is that the impact of the methylation of these two CpGs on the transcription of the PTH gene has not been considered. Therefore, we can only postulate a potential mechanism to be analyzed in greater depth in the future to identify a cause-effect relationship between hypomethylation development of the PTH gene and its molecular control mechanisms, which would allow the incorporation of new therapeutic strategies for SHPT control in CRD.

In conclusion, these findings suggest that the development of epigenetic alterations such as the significant hypomethylation of the PTH gene, in advanced stages of parathyroid gland dysfunction in experimental renal disease, could contribute to the increase of both synthesis and secretion of PTH. The design of studies to obtain conclusive evidence of the impact that hypomethylation has on the synthesis of PTH and that lead to the identification of the molecular mechanisms responsible for this epigenetic modification induced by the high phosphorus in the diet is the first step for designing innovative therapeutic strategies for the effective treatment of SHPT from early stages of CRD.

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Conflict of interest: The authors affirm that they have no conflict of interests in this paper.

The handling of experimental animals has been carried out in accordance with the provisions of current legislation (European Union Directive 2010/63/EU and Royal Decree 53/2013 of 1 February).

Table 2. General biochemical markers and mineral metabolism

	Urea (mg/dL)	Creatinine (mg/dL)	Ca (mg/dL)	P (mg/dL)	PTH (pg/mL)	FGF23 (pg/mL)
NX-NP	104 ± 32	1.0 ± 0.3	11.7 ± 1.1	5.8 ± 1.2	44 ± 23	378 ± 103
NX-EP	201 ± 51	2.1 ± 0.4	10.5 ± 1.1	12.8 ± 1.9	1,762 ± 493	1,029 ± 101
P value	0.001	0.001	0.066	0.001	0.001	0.001

Figure 1. Degree of methylation of the CpGs sites preceding the initiation of promoter transcription of the A) CaSR (-172 to -3) genes; B) VDR (-533 to -189) and C) α-Klotho (-217 to -96) in parathyroid glands from nephrectomized (NX) rats fed a diet with normal phosphorus content PN (NX-PN) or high (NX-PE) for 20 weeks. At the bottom of each gene, the analyzed area of the promoter and the number of CpG sites present in the fragment are plotted

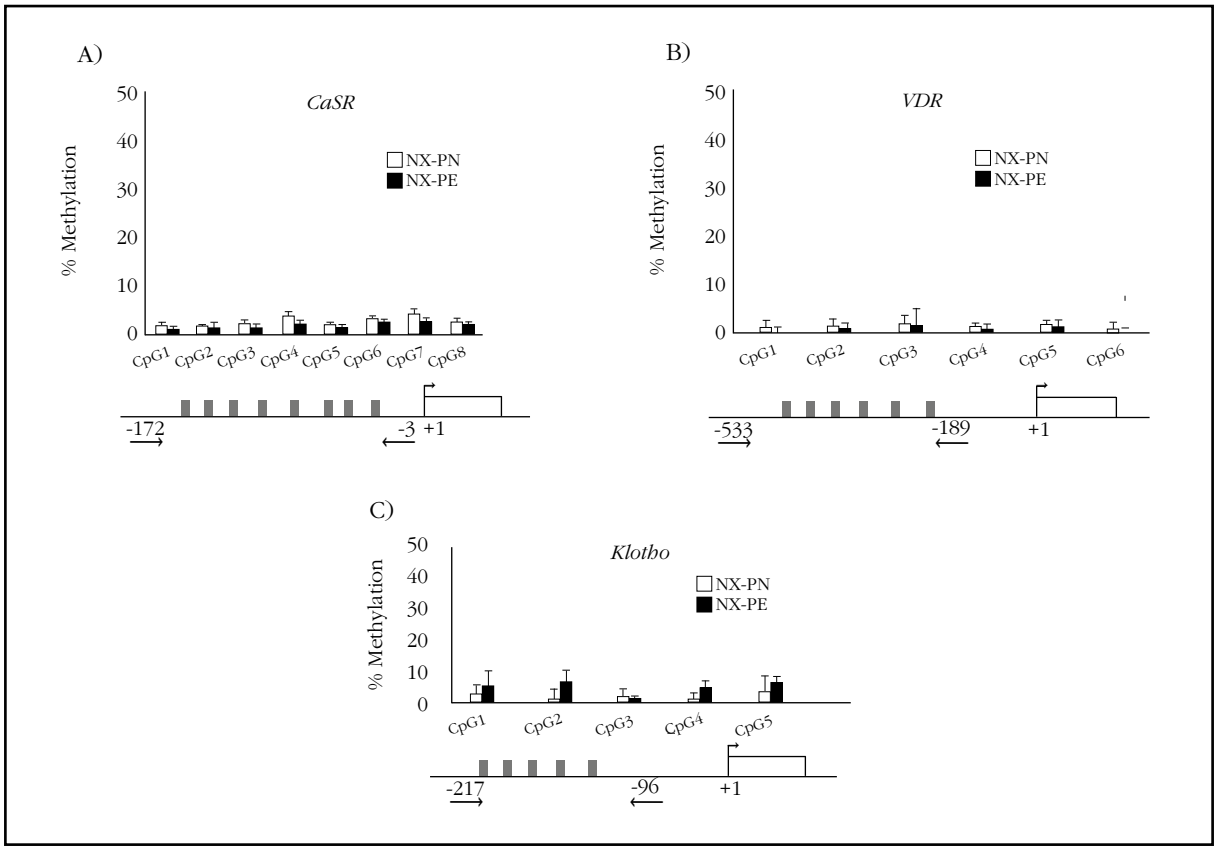

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Figure 2. Degree of methylation at the CpGs sites of the -342 to +18 region of the PTH promoter preceding the initiation of transcription in parathyroid glands from nephrectomized (NX) rats fed a diet with normal phosphorus content (NX PN) or high (NX-PE) for 20 weeks. (*P<0.05 with respect to NX-PN). At the bottom of the gene, the analyzed area of the promoter and the number of CpG sites present in this fragment