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High phosphorus diet increases preproPTH mRNA independent of calcium and calcitriol in normal rats

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High phosphorus diet increases preproPTH mRNA independent of calcium and calcitriol in normal rats. Phosphorus retention favors secondary hyperparathyroidism by decreasing calcitriol synthesis and serum calcium levels. However, a direct effect of high extracellular phosphorus on parathyroid (PTH) function, gene expression, and cell proliferation is still controversial. Normal rats were fed standard (St; 0.6% calcium, 0.6% phosphorus) or high phosphate (HP) diet (0.6% calcium, 1.2% phosphorus) for 18 days. To rule out transient decreases in serum calcium or calcitriol levels, sets of animals were sacrificed at different time periods after the last feeding (2, 4, 8, 12 or 24 hr). The HP diet led to hyperphosphatemia and secondary hyperparathyroidism and maximum differences in PTH levels were observed eight hours after feeding (St 29.4 ± 15 vs. HP 87.9 ± 56 pg/ml, mean ± SD; $P = 0.01$). High PTH levels induced by the HP diet prevented both hypocalcemia and low calcitriol levels at each study point. The HP diet also promoted a significant increase of PTH mRNA levels that peaked about eight hours after feeding (100% increase). This was confirmed at the cellular level by *in situ* hybridization. Parathyroid glands from animals fed the HP diet showed a 25% increase in volume with respect to the St diet ($P = 0.01$), and a typical pattern of hyperplasia was found. Parathyroid vitamin D receptor (VDR) mRNA levels were not modified by the HP diet. In conclusion, parathyroid gene expression per cell and parathyroid cell hyperplasia are stimulated by high dietary phosphorus independently of calcium and calcitriol. This effect is not mediated by alterations in the gene expression of the parathyroid calcitriol receptor. Our findings emphasize the importance of the control of hyperphosphatemia in chronic renal failure patients.

Secondary hyperparathyroidism is a common finding in patients with renal failure [1]. Hypocalcemia, phosphorus retention, and a deficit of calcitriol are important factors involved in the genesis of renal hyperparathyroidism [2]. Hypocalcemia stimulates parathyroid hormone (PTH) secretion and it has been shown to increase rat PTH mRNA levels *in vivo* [3–5]. Calcitriol decreases PTH gene transcription both *in vivo* [6] and *in vitro* [7, 8]. Phosphorus retention favors secondary hyperparathyroidism by decreasing calcitriol synthesis [9] and serum calcium levels [10]. However, a direct effect of high extracellular phosphorus on parathyroid function is still controversial. Lopez-Hilker et al [11] have shown in dogs with experimental chronic renal failure that phosphorus restriction decreases PTH levels independently of changes in serum calcium and calcitriol levels. More recently Kilav, Silver and Naveh-Many [12] have reported a post-transcriptional decrease in PTH mRNA levels in rats fed a low phosphorus diet; however, in this study a separate effect of phosphorus restriction from increased serum calcium and calcitriol levels was only observed in second generation vitamin D-deficient rats one day after feeding a low calcium and phosphorus diet [12]. Moreover, the fact that phosphorus restriction decreases PTH gene expression does not necessarily imply that the opposite occurs with a phosphorus load. For example, hypocalcemia increases PTH mRNA levels [3–5], while hypercalcemia from periods of six hours to three weeks has no effect on PTH mRNA levels *in vivo* [3, 13].

Our previous studies in rats with normal renal function have shown that a high phosphorus diet induces secondary hyperparathyroidism without decreasing serum calcium or calcitriol levels [14, 15]. The goal of the present study was to evaluate the effect of a high phosphorus diet on PTH gene expression and on parathyroid gland size and cell number in normal rats. To rule out transient decreases in serum calcium or calcitriol levels and to determine the time of maximal PTH mRNA response to a high phosphorus diet, sets of animals were sacrificed at different time periods after feeding. Finally, the molecular mechanisms involved in the genesis of secondary hyperparathyroidism induced by an increased dietary phosphorus have not been established. We have previously reported that the parathyroid gland calcium receptor gene expression is not regulated by increased dietary phosphorus [16]. Thus, in the present study we also investigated whether vitamin D receptor (VDR) gene expression in the parathyroid gland is regulated by a high phosphorus diet.

Methods

Animals and diets

Animals were housed and handled in compliance with standard animal welfare guidelines, under the supervision of a veterinarian. Ninety male Wistar rats weighing 200 to 300 g were studied. Half the rats received a standard diet (St, 0.6% calcium, 0.6% phosphorus) and the other half a high phosphorus diet (HP, 0.6% calcium, 1.2% phosphorus; ICN Biomedicals, Cleveland, OH, USA) for 18 days. Both diets had the same vitamin D content (100 IU/100 mg). Rats were pair-fed with 16 g of food daily, and received water *ad libitum*. Rats ingesting less than 10 g per day were removed from the study. At the end of the experiment and

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after the usual morning feeding on day 18, sets of 9 rats were sacrificed at 2, 4, 8, 12 or 24 hours later for each dietary group. At each time, blood was drawn by aortic puncture under pentobarbital anesthesia (Abbott Laboratories, Chicago, IL, USA). The parathyroid glands were excised with the aid of a dissecting microscope at 4, 8 and 12 hours after feeding, and pooled in sets of six glands (3 rats) for Northern blot analysis. A total of four additional Northern blots, for comparison of PTH mRNA levels between St and HP diet groups were performed eight hours after food intake (24 additional rats: 12 in each dietary group). Therefore, at this time point, PTH mRNA levels were measured in a total of seven experiments in each group.

Rat preproPTH cDNA probe

A rat preproPTH cDNA probe was obtained by RT-PCR. Briefly, cDNA synthesis reactions were carried out using about 5 μg total RNA from parathyroid glands, following standard methods [17]. A 284 cDNA fragment was obtained by PCR using the sense primer PTH1, 5′CTCTCTGAGAGTCATTGTATG3′ and the antisense primer PTH2, 5′CTCCTTCTTGGTGGGCCT-CTG3′ [18]. Amplification reactions were carried out in a final volume of 50 μl containing 10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM Mg Cl_2, 0.1% Triton X-100, 100 μM dNTPs, 1 μM each primer, 1 μl cDNA sample, and 1 U *Taq* DNA polymerase. The amplifications were performed on a DNA thermal cycler (Perkin-Elmer/Cetus Corp, USA) with the following temperature profile: 94°C (1 min), 60°C (1 min), 72°C (1 min), 30 cycles.

The PCR product was cloned in pCRII vector (In Vitrogen Corp., CA, USA), and the insert was confirmed by di-deoxinucleotide termination sequencing (Sequenase, Amersham, IL, USA). Using plasmid DNA as template, PCR reactions were carried out to amplify the specific insert that was used as probe. PCR products were purified with Sephadex G-50 (Pharmacia Biochemicals, WI, USA) [19]. About 25 ng of the amplified fragments were labeled by random priming using the Prime-a-Gene kit (Promega Corp, WI, USA) and ^{32}P-dCTP 3000 Ci/mmol (Amersham, Germany). Specific activity of probes were typically about 10^8 cpm/μg.

Northern analysis

Pools of 6 parathyroid glands (3 rats) were processed. Total RNA was isolated by extraction with acid-guanidine isothiocyanate-phenol-chloroform [20]. The yield of RNA was determined by spectrophotometry [19]. Typically one pool of 6 glands yielded 6 μg RNA.

Five micrograms of total RNA from parathyroids were denatured with formamide in the presence of ethidium bromide and loaded onto a submarine 1.2% agarose minigel with 1.1% formaldehyde. After electrophoresis, gels were photographed with 665 type Polaroid film. The resolved RNA was transferred to Hybond-N nylon membranes (Amersham, Germany) by capillary blotting, and cross linked to the membranes by UV. The uniformity of RNA transfer was checked by UV visualization of the filters. The membranes were prehybridized, hybridized and washed as described elsewhere [21]. The membranes were exposed to Cronex X-ray film (Dupont, DE, USA) during 16 hours. To quantitate the mRNA levels in each blot, X-ray films were subjected to densitometry using a GS-670 imaging densitometer (BioRad Labs, CA, USA). Since the total RNA was stained with ethidium bromide, the intensity of the rRNA bands under UV light was proportional to the amount of RNA loaded per lane. Therefore, the negative film from a Polaroid 665 picture was scanned with a densitometer and the density of the 28 s band was used to normalize the loaded mRNA.

In situ hybridization

To obtain a probe for *in situ* hybridization, the 284 bp preproPTH cDNA fragment was subcloned into pGEM4 vector (Promega, WI, USA) and the orientation was confirmed by sequencing. The plasmid was linearized by using either *Bam*HI or *Bst*XI, and *in vitro* transcription was carried out according to previously published methods [22], using SP6 and T7 RNA polymerase to generate sense and antisense RNA preproPTH probes, respectively.

In situ hybridization was carried out on 16 rats (8 fed with St and 8 with HP) as previously described [23]. Animals were sacrificed eight hours after the last feeding. Briefly, thyroparathyroid tissue was dissected and fixed in 4% phosphate-buffered paraformaldehyde for two hours. It was then subjected to automated ethanol dehydration and paraffin-embedding. Five micrometer-thick sections were mounted on glass slides coated with 2% aminopropyltriethoxysilane (Sigma Chemicals, St. Louis, MO, USA). Sections were then dewaxed, hydrated and prehybridized with 50% formamide, 0.75 M NaCl, 25 mM piperazinediethanesulfonic acid, 25 mM EDTA, 1× Denhardt's [19], 100 mM dithiothreitol, 0.2% SDS, and 300 μg/ml salmon sperm DNA. Next, the hybridization mixture was applied, containing 3×10^4 cpm/μl of ^{35}S-labeled preproPTH antisense RNA. The sections were dehydrated in a graded series of ethanol and vacuum dried. The sections were dipped in 50% aqueous solution of photographic emulsion NTB-2 (Eastman Kodak, Rochester, NY, USA) at 45°C, exposed for seven days at 4°C, developed with D19 developer (Kodak) and fixed with Rapid Fix (Kodak). The sections were counterstained with hematoxylin, dehydrated and mounted. Sections were examined and photographed with a photomicroscope using bright and dark field condensers. Sense RNA PTH probe controls were also carried out. Sections from St and HP diet-fed rats were processed simultaneously to minimize experimental variations.

Parathyroid sizing and histology

Standard paraffin-embedded 4 to 5 μm thick tissue sections were studied. Assuming an elliptical shape, parathyroid gland volume was derived from the measurement of both the large (a) and small (b) radius at the maximal diameter section of the gland. The estimated gland volume was: $4/3\pi ab^2$. All measurements were performed with the aid of a computer-assisted image analysis system (Microsciences Corp, GA, USA). Twenty rats, 10 fed with St and 10 with HP diet as described above, were used for this analysis. Animals were sacrificed eight hours after the last food intake.

Ribonuclease protection assay (RPA)

To study the steady state level of the rat parathyroid VDR mRNA, a RT-PCR generated 220 bp VDR cDNA fragment was cloned in pSK Bluescript vector (Stratagene, La Jolla, CA, USA), and antisense riboprobe was obtained by *in vitro* transcription as described above for *in situ* hibridization. The probe was purified by gel electrophoresis. Ribonuclease protection assay was performed using the RPAII kit from Ambion (Austin, TX, USA).

Table 1. Biochemical data of normal rats fed with standard (St) or high phosphorus (HP) diet and sacrificed on day 18, 2, 4, 8, 12, or 24 hours after food intake. $N = 9$ at each time point. X ± SE

	2 hours	4 hours	8 hours	12 hours	24 hours
Creatinine *mg/dl*					
St	0.25 ± 0.02	0.22 ± 0.03	0.21 ± 0.01	0.21 ± 0.01	0.26 ± 0.03
HP	0.24 ± 0.02	0.25 ± 0.03	0.23 ± 0.02	0.22 ± 0.02	0.25 ± 0.02
Total calcium *mg/dl*					
St	9.93 ± 0.08	9.76 ± 0.26	9.96 ± 0.09	9.84 ± 0.16	9.50 ± 0.10
HP	9.80 ± 0.12	9.71 ± 0.18	9.78 ± 0.10	9.60 ± 0.16	9.84 ± 0.14
Ionized calcium *mg/dl*					
St	4.48 ± 0.11	4.40 ± 0.11	4.24 ± 0.11	4.48 ± 0.23	4.84 ± 0.15
HP	4.36 ± 0.11	4.36 ± 0.08	4.20 ± 0.19	4.44 ± 0.15	4.92 ± 0.15
Phosphorus *mg/dl*					
St	7.39 ± 0.30	7.84 ± 0.47	7.83 ± 0.28	7.19 ± 0.39	7.24 ± 0.52
HP	8.36 ± 0.29[b]	8.56 ± 0.33	8.90 ± 0.32[b]	6.9 ± 0.25	6.30 ± 0.32
PTH *pg/ml*					
St	28.50 ± 10.20	24.05 ± 3.60	29.43 ± 4.90	21.10 ± 4.90	28.42 ± 12.10
HP	73.40 ± 20.60	51.80 ± 11.33[b]	87.90 ± 18.57[a]	60.40 ± 16.13[b]	67.84 ± 12.60[b]
Calcitriol *pg/ml*					
St	38.60 ± 13.78	37.40 ± 3.27	38.20 ± 3.27	28.90 ± 2.51	32.30 ± 2.20
HP	42.40 ± 9.50	39.50 ± 8.53	58 ± 9.53	40.97 ± 9.39	34.60 ± 5.30

[a] $P = 0.01$, [b] $P < 0.05$ versus St

Samples with about 10 μg parathyroid RNA were incubated with the ^{32}P-UTP-labeled VDR riboprobe (100,000 cpm per sample), in 20 μl of hybridization buffer and processed following the manufacturer's protocol. The gels were dried and expossed at −80°C to Cronex X-ray film for at least 24 hours. Densitometric analysis was carried out as described above for Northern blot. In each case, 1 μg RNA was simultaneously processed using a 297 bp rat β-actin riboprobe to normalize VDR measurements. Positive and negative (RNase-minus) controls were also performed. Pools of four glands (2 rats) were processed, and a total of two repeated experiments were performed in each dietary group.

Biochemical determinations

Serum calcium, phosphorus and creatinine were measured by standard autoanalyzer procedures. Ionized calcium was measured by means of a selective electrode (ABS, Copenhagen, Denmark). Rat intact PTH was determined by an immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA, USA), and serum levels of calcitriol with a radioreceptor assay (Nichols Institute).

Statistical analysis

The results are presented as the mean ± SE. Student's unpaired two-tailed *t*-test was used to compare two group means. A P value < 0.05 was considered significant. All statistical analyses were performed with SPSS 6.1.2 for Windows (Chicago, IL, USA) [24].

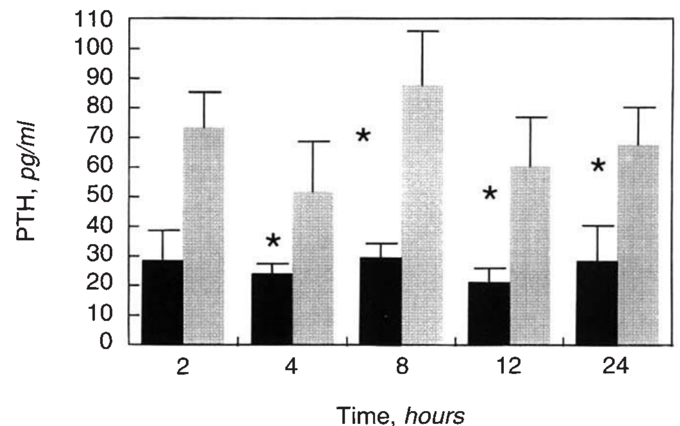

Fig. 1. *Time course of PTH levels in rats fed with a standard (■) or high phosphorus (▨) diet.* Levels were obtained 2, 4, 8, 12, or 24 hours after food intake on day 18 (X ± SE, $^*P < 0.05$).

Results

After 18 days on a St or HP diet, groups of nine animals were sacrificed at 2, 4, 8, 12 or 24 hours after the last feeding. As shown in Table 1, total and ionized serum calcium, as well as calcitriol levels, were not different between the two groups at each study point. However, serum phosphorus was greater at 2, 4, and 8 hours in the HP group, although significant differences were only observed at 2 and 8 hours after feeding. At 12 and 24 hours after feeding serum phosphorus levels were slightly lower in the HP group compared to St, perhaps as a result of the phosphaturic effect of the peak in the PTH synthesis observed in this group eight hours after feeding. PTH levels were significantly greater in the HP group at each study point except at two hours, reaching a maximum difference eight hours after last food intake (Table 1 and Fig. 1). Northern blot analysis showed that PTH mRNA levels were greater at each study point in the HP as compared to the St group (Fig. 2), and that the maximum difference in the PTH mRNA/28S RNA ratio was observed eight hours after feeding (Fig. 3). The mean PTH mRNA/28S RNA ratio of the HP group was twice that of the St group for seven repeated experiments in which animals were sacrificed eight hours after feeding (Fig. 4). To confirm this PTH mRNA increase at the cellular level, paraffin-embedded thyroparathyroid sections were analyzed by *in situ* hybridization. The preproPTH mRNA signal was uniformly distributed in all the parathyroid cells and low background was measured in the surrounding thyroid tissue. The preproPTH mRNA signal was stronger in parathyroid cells from rats fed with the HP as compared to St diet (Fig. 5).

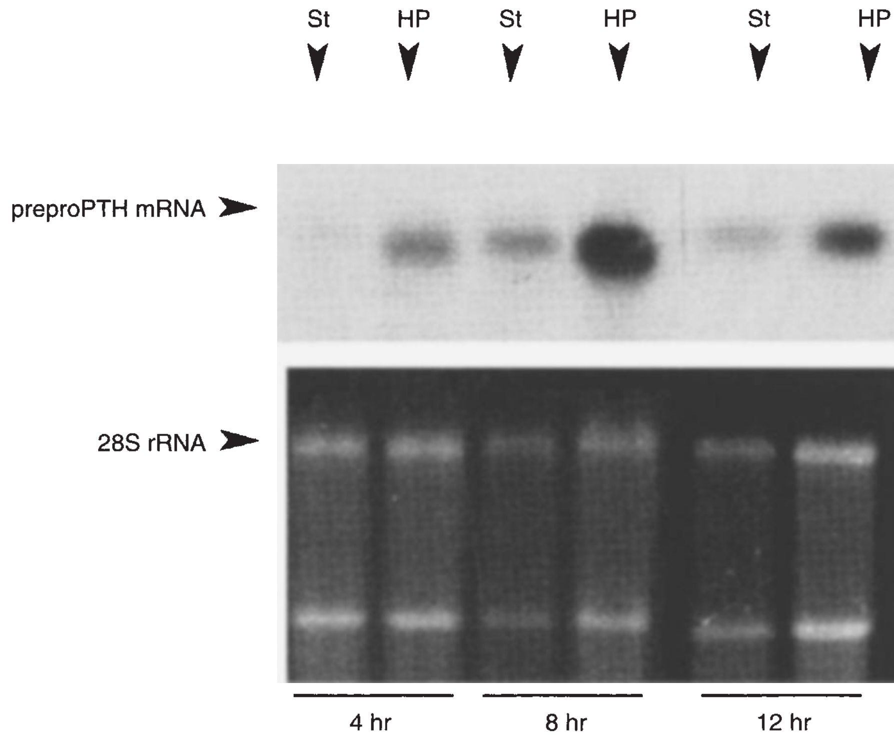

Fig. 2. *Effect of dietary phosphorus on rat preproPTH mRNA levels measured by Northern blot analysis.* Rats had been fed a standard (St) or high phosphate (HP) diet for 18 days and sacrificed 4, 8, or 12 hours after last feeding. For each time point the first lane is St and the second HP. Total RNA from parathyroid tissue was hybridized with a rat cDNA probe. 28S rRNA (stained with ethidium bromide) was used to normalize mRNA levels. Each lane represents total RNA from six parathyroid glands (3 rats).

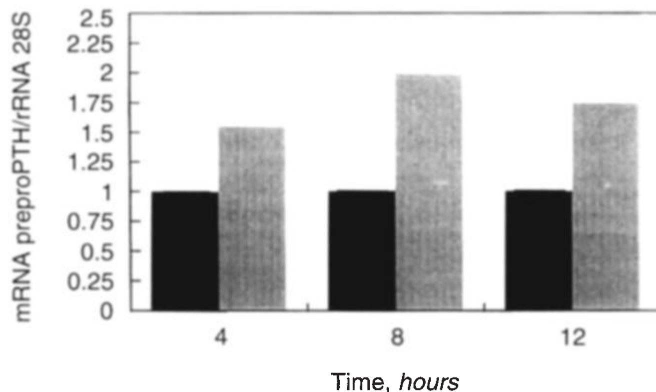

Fig. 3. *Densitometric mRNA prepro PTH/rRNA 28S ratio on standard (■) or high phosphate (▩) diet.* Rats were sacrificed 4, 8 or 12 hours after food intake on day 18. Data are the means of three experiments. High phosphate values were normalized to standard values.

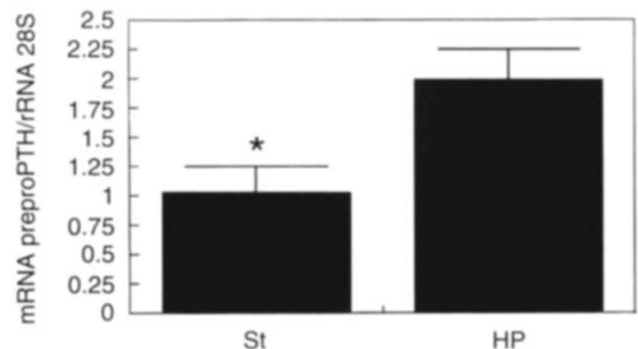

Fig. 4. *mRNA preprorRNA 28S ratio in rats under standard (St) or high phosphorus (HP) diet that were sacrificed 8 hours after feeding.* Data are the means ± SE of 7 repeated experiments (normalized to the mean value of St). $^*P < 0.05$.

Parathyroid glands from both groups showed uniform distribution of well differentiated cells with no morphological signs of nodular growth or clonal proliferation. However, rats fed with the HP diet showed a typical pattern of hyperplasia and were significantly larger than those from St rats. The mean derived gland volume was 25% greater in the HP group ($3.85 \pm 1.03 \times 10^8$ vs. $4.80 \pm 1.06 \times 10^8$ μm^3, respectively; $P = 0.01$). In addition, the number of cell nuclei per area unit (microscopic field of 3660 μm^2) was ascertained in both groups. Both St and HD groups showed similar values (65.63 ± 18.96 vs. 59.69 ± 11.79; $P = 0.22$).

VDR mRNA levels, as assessed by the RNAase protection assay, were not decreased in the HP as compared to the St group (Fig. 6). In fact, densitometric studies showed that the VDR mRNA/β-actin ratio was 1.2 times greater in the HP group.

Discussion

The present study demonstrates that a high phosphorus (HP) diet increases PTH gene expression per cell and induces parathyroid gland hyperplasia *in vivo* in the rat independently of changes in serum calcium or calcitriol levels. The effect of a high dietary

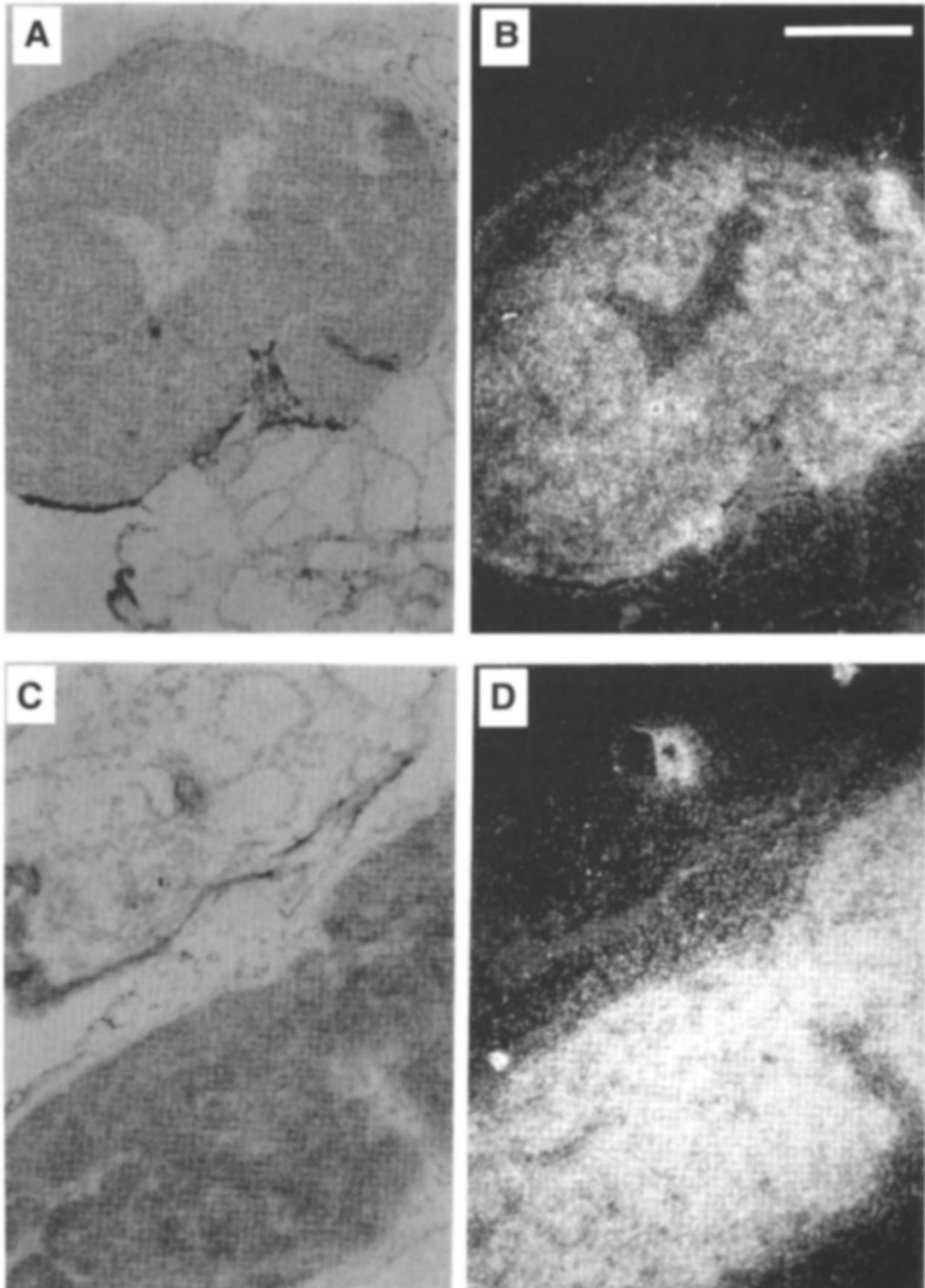

Fig. 5. *In situ hybridization of parathyroid-thyroid sections using a PTH riboprobe.* **A** and **B**. Parathyroid-thyroid tissue from a rat that had been fed a standard diet (bright and dark field condensers, respectively). **C** and **D**. Tissue from a rat that had been fed a high phosphate diet, showing the higher hybridization for PTH mRNA in the parathyroid and none present in the thyroid (magnification 138×; scale bar 200 μm).

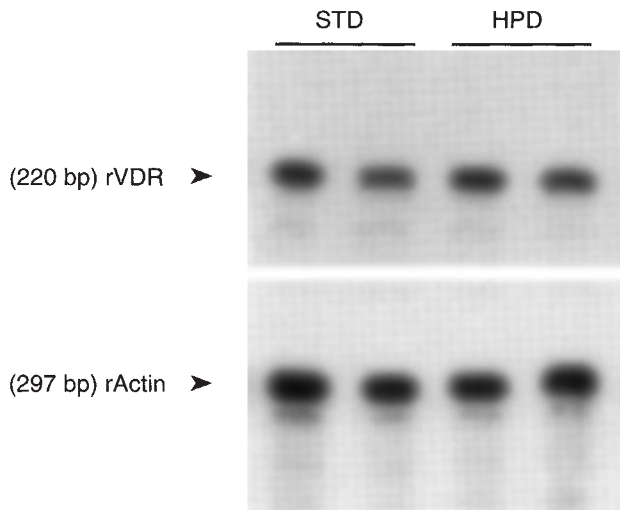

Fig. 6. *Effect of dietary phosphorus on VDR mRNA levels measured by RNAase protection assay.* Parathyroid RNA was hybridized with a rat VDR riboprobe and also with a β-actin riboprobe to normalize VDR measurements.

phosphorus is not mediated by changes in the gene expression of either the parathyroid vitamin D receptor (VDR), as shown in this study, or the parathyroid calcium receptor, as demonstrated in our previous study [16].

A high phosphorus diet has been used as the traditional method for the experimental induction of secondary hyperparathyroidism in renal failure [14, 25]. Phosphorus loading favors the development of secondary hyperparathyroidism by decreasing calcitriol synthesis [9] and serum calcium levels [10]. Consequently, it has been difficult to demonstrate an independent effect *in vivo* of phosphorus on parathyroid function in renal failure. In a recent study we evaluated the relative effects of PTH and dietary phosphorus loading on calcitriol levels in the rat [15]. In animals with normal renal function fed a high phosphorus diet (HP), the inhibitory effect of phosphorus loading on calcitriol production was counterbalanced by the concurrent increase in PTH levels that maintained normal calcitriol levels [15, 16]. In addition, hypocalcemia was not observed in this situation [15, 16]. However, transient decreases in calcium or calcitriol levels after feeding were not ruled out in these studies. The present study demonstrates that in a steady state, serum calcium and calcitriol levels are maintained within the normal range in rats with normal renal function (Table 1). The high PTH levels induced by chronic phosphorus loading prevented both hypocalcemia, presumably by increasing calcium mobilization from bone and decreasing urinary calcium excretion [14], and low calcitriol levels as a result of a normal renal reserve. Therefore, in our model an effect of chronic phosphorus loading on PTH secretion independent of hypocalcemia or low calcitriol levels could be demonstrated.

The HP diet also promoted an increase of PTH mRNA steady state levels (Fig. 4), which was confirmed at the cellular level by *in situ* hybridization (Fig. 5). Our time course study showed that hyperphosphatemia, circulating PTH levels and PTH gene expression peaked in parallel about eight hours after food intake (Table 1, Fig. 3), which is also consistent with a direct effect of phosphorus on PTH synthesis and secretion. Although phosphorus restriction decreases PTH mRNA levels by a post-transcriptional effect [12], a transcriptional effect of phosphorus loading cannot be completely ruled out, and future studies on recently transcribed mRNA levels are needed.

It is possible that phosphorus modulates parathyroid function by indirect mechanisms involving modifications of the gene expression of important receptors such as the calcium receptor (PCaR) [26] or the vitamin D receptor (VDR) [27]. We have shown that neither PCaR [16] nor VDR (Fig. 7) gene expression are regulated by phosphorus loading. However, a decrease or alteration at the protein level cannot be excluded. In fact, a right shift of the PTH calcium curve, making the parathyroid cell less sensitive to inhibition by calcium, has been reported *in vitro* in the presence of a constant calcium and increasing phosphorus concentrations in the incubation media [28]. This suggests that phosphorus may interfere with the parathyroid intracellular signaling pathway [28], including protein kinase-C stimulation [29]. However, the physiology of the phosphorus effect on the parathyroid cell remains to be elucidated.

In secondary hyperparathyroidism there is an increase in parathyroid cell number [30] and recently reported studies have contributed to our understanding of the mechanisms involved in the development of parathyroid cell hyperplasia secondary to chronic renal failure [31, 32]. Phosphorus restriction prevents and phosphorus loading stimulates parathyroid cell hyperplasia in normal [32] and azotemic rats [32, 33]. In one preliminary study the effect of a high dietary phosphorus on parathyroid cell proliferation was independent of calcium and calcitriol levels [33], as has been confirmed in the present study. This finding has important clinical implications since hyperphosphatemia correlates with a poor response to calcitriol therapy [34–37]. Whether high phosphorus levels play a role in the activation of immediate early genes [31] and in the recently demonstrated monoclonality of parathyroid cell growth in hemodialysis patients with refractory hyperparathyroidism [38] remains to be elucidated.

In conclusion, parathyroid gene expression per cell and parathyroid cell hyperplasia are stimulated by high dietary phosphorus independently of calcium and calcitriol. This effect is not mediated by alterations in the gene expression of the parathyroid calcium or calcitriol receptors. Our findings emphasize the importance of the control of hyperphosphatemia in chronic renal failure patients.

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