

Increased Parathyroid Hormone Gene Expression in Secondary Hyperparathyroidism of Experimental Uremia Is Reversed by Calcimimetics: Correlation with Posttranslational Modification of the *Trans* Acting Factor AUF1

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Most patients with chronic kidney disease develop secondary hyperparathyroidism with disabling systemic complications. Calcimimetic agents are effective tools in the management of secondary hyperparathyroidism, acting through allosteric modification of the calcium-sensing receptor (CaR) on the parathyroid gland (PT) to decrease parathyroid hormone (PTH) secretion and PT cell proliferation. This study showed that rats that were fed an adenine high-phosphorus diet had increased serum PTH and PTH mRNA levels at 7 and 21 d. For studying the effect of activation of the CaR by the calcimimetics R-568 on PTH gene expression, R-568 was given by gavage to uremic rats for the last 4 d of a 7-d adenine high-phosphorus diet. R-568 decreased both PTH mRNA and serum PTH levels. The effect of the calcimimetic on PTH gene expression was posttranscriptional and correlated with differences in protein-RNA binding and posttranslational modifications of the *trans* acting factor AUF1 in the PT. The AUF1 modifications as a result of uremia were reversed by treatment with R-568 to those of normal rats. Therefore, uremia and activation of the CaR mediated by calcimimetics modify AUF1 posttranslationally. These modifications in AUF1 correlate with changes in protein-PTH mRNA binding and PTH mRNA levels.

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The elevated parathyroid hormone (PTH) and disordered mineral metabolism associated with secondary hyperparathyroidism (HPT) complicate the clinical course of most patients with late-stage chronic kidney disease (CKD) and, when advanced, are associated with markedly increased morbidity and mortality (1). The hallmark of secondary HPT is the high levels of circulating PTH, which result from increased PTH secretion, increased PTH gene expression and synthesis, and increased parathyroid gland (PT) cell proliferation (2). The elucidation of PTH gene regulation in CKD is central to the understanding and control of the pathogenesis of secondary HPT (2–4). A limited number of preformed secretory granules contain mature PTH in the PT, and the increased PTH secretion demands the synthesis of new hormone (5–7). Accordingly, treatments that are designed to regulate PTH gene expression and translation may be of substantial clinical benefit.

Data from clinical trials have demonstrated that calcimimetic therapy can reduce PTH, serum calcium and phosphorus, and the calcium-phosphorus product ($\text{Ca} \times \text{P}$) (8,9) and lead to the achievement of Kidney Disease Outcomes Quality Initiative

target levels for PTH and $\text{Ca} \times \text{P}$ in many more patients (10). In addition, calcimimetics, which act through the allosteric modulation of the calcium-sensing receptor (CaR), have been shown effectively to decrease PT cell proliferation in a rat model of secondary HPT (11). Therefore, there is considerable interest in determining the mechanisms by which this novel therapeutic class regulates PTH. To date, there have been no reports on the effects of calcimimetics on PTH gene expression. In this study, we examined the effect of the calcimimetic R-568 on PTH mRNA levels, protein-RNA binding, and posttranslational modifications of the PTH mRNA binding protein AUF1.

Materials and Methods

Experimental Procedures

Adult male Sabra rats (weight 100 to 120 g) were fed a control diet or a uremia-inducing 0.75% adenine, high-phosphorus (1.5%) diet. A portion of the rats that received the adenine diet were treated with R-568 20 mg/kg by oral gavage twice daily as indicated in each figure legend. For the different experiments, four to five rats were used in each group. Each experiment was repeated at least three times. On days 3, 7, and 21 after initiation of the adenine diet, microdissected PT or thyroparathyroid tissue was removed under ketamine anesthesia, and blood samples were taken for serum creatinine, PTH, $1,25(\text{OH})_2$ vitamin D_3 , calcium, and phosphorus levels. Serum PTH was measured by a radiomimetic assay (Immutopics, San Clemente, CA), and $1,25(\text{OH})_2$ vitamin D_3 was measured by a radioreceptor assay (Incstar Corp., Stillwater, UK). All animal experiments were approved by the appropriate Institutional Animal Care and Use Committees.

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Nuclear Run-on Transcription Assays

Nuclei were prepared from pooled thyroparathyroid tissue from five rats in each treatment group (adenine diet and adenine diet + R-568), and nuclear run-on transcription assays were performed as described previously (12). Elongated ³²P-labeled RNA was extracted by TRI reagent (Molecular Research Center, Cincinnati, OH) and resuspended in 300 μ l of hybridization buffer (7% SDS, 10% polyethylene glycol [8000], and 1.5% saline-sodium phosphate-EDTA). Equivalent amounts of labeled RNA from each condition (1 to 2 \times 10⁶ cpm) were hybridized to linearized cDNA (5 μ g) for PTH, vitamin D receptor, β actin, L32 ribosomal protein, and pBluescript II KS (Stratagene, La Jolla, CA), which were immobilized to Hybond filters using a slot blot apparatus. Hybridization was performed at 65°C for 72 h. The filters were washed and autoradiographed.

RNA Transcripts and Probes, Cytoplasmic Protein Purification, Ultraviolet Cross-Linking, and RNA Electrophoretic Mobility Shift Assays

Labeled and unlabeled RNA was transcribed from linearized plasmid that contained the PTH mRNA 3'-UTR in PCR II as before (13). The RNA was used for the ultraviolet (UV) cross-linking and RNA electrophoretic mobility shift assays (REMSA) that were performed with S100 PT cytosolic proteins using our published methods (13).

One- and Two-Dimension Gels

S-100 protein extracts from PT glands were run on one- (1-D) and two-dimension (2-D) SDS gels, and Western blots were performed with a mAb for AUF1 that was provided by G. Dreyfuss (University of Pennsylvania, Philadelphia) as described previously (14). The mAb for α -tubulin was purchased from Sigma (St. Louis, MO).

Statistical Analyses

Results were analyzed by one-way ANOVA with the *post hoc* Bonferroni multiple comparison test to determine the significance of differences between means. $P < 0.05$ was considered statistically significant. Results are expressed as mean \pm SD.

Table 1. Serum chemistry in control rats, uremic rats, and uremic rats that were treated with the calcimimetic R-568 for the indicated times^a

| | 3 Days | | N | 7 Days | | R-568 (4 d) | 21 Days | | |
|---------------------------------|-----------------|------------------------------|-----------------|------------------------------|-----------------------------|------------------|-------------------------------|-----------------------------|--------------|
| | Normal | Uremic | | Uremic | Uremic | | Normal | Uremic | R-568 (14 d) |
| Creatinine (mg/dl) | 0.25 \pm 0.04 | 0.62 \pm 0.12 ^b | 0.27 \pm 0.03 | 0.60 \pm 0.07 ^b | 0.66 \pm 0.13 | 0.25 \pm 0.02 | 1.31 \pm 0.2 ^b | 1.12 \pm 0.14 | |
| Calcium (mg/dl) | 10.2 \pm 0.32 | 9.4 \pm 0.14 ^b | 10.4 \pm 0.26 | 9.8 \pm 0.52 | 9.08 \pm 0.64 | 9.92 \pm 0.12 | 7.9 \pm 0.08 ^b | 7.0 \pm 0.48 ^c | |
| Phosphorus (mg/dl) | 9.01 \pm 0.34 | 9.1 \pm 1.3 | 9.8 \pm 0.51 | 10.75 \pm 1.95 | 12.96 \pm 2.22 | 10.44 \pm 1.62 | 15.53 \pm 2.61 ^b | 17.57 \pm 1.36 | |
| PTH (pg/ml) | 56 \pm 28 | 70 \pm 39 | 56 \pm 28 | 338 \pm 113 ^b | 29 \pm 12 ^c | 56 \pm 28 | 928 \pm 142 ^b | 657 \pm 170 ^c | |
| 1,25(OH) ₂ D (pg/ml) | ND | ND | 152 \pm 51 | 116 \pm 64 | 163 \pm 57 | 152 \pm 51 | 41.5 \pm 29.7 ^b | ND | |
| PTH mRNA | 1 \pm 0.16 | 1.3 \pm 0.14 | 1 \pm 0.17 | 1.9 \pm 0.05 ^b | 0.6 \pm 0.12 ^c | 1 \pm 0.24 | 2.8 \pm 0.29 ^b | 2.3 \pm 0.2 ^c | |
| n | 5 | 5 | 4 | 4 | 4 | 5 | 5 | 4 | |

^aPTH mRNA levels are corrected for 18S RNA and expressed as percentage of normal rats. PTH, parathyroid hormone; ND, not done.

^b $P < 0.05$, for the uremic rats *versus* the rats that were fed a control diet (normal).

^c $P < 0.05$, for the uremic rats that were given R-568 *versus* the uremic rats. The results are from a representative experiment using the number of rats as shown in this experiment.

Results

Biochemical Effects of the Adenine Diet and R-568

Uremia was induced by feeding rats an adenine, high-phosphorus diet, as demonstrated by a 2.5-fold increase in serum creatinine levels at days 3 and 7, with a five-fold increase at 3 wk (Table 1). Serum calcium was significantly decreased in uremic rats at days 3 and 21. Serum phosphorus was significantly increased from baseline at day 7. Serum 1,25(OH)₂ vitamin D₃ levels were significantly decreased in the uremic rats at 21 d but not at the earlier time points. Serum PTH was not significantly increased at day 3 but was increased from day 7 onward, reaching a 17-fold increase relative to control rats at day 21. PTH mRNA levels were increased also from day 7 with a two-fold increase, with only a moderate further increase at 21 d (2.8-fold increase) (Figure 1). The addition of R-568 for the last 4 d of the 7-d treatment regimen resulted in a decrease in serum PTH and PTH mRNA levels to normal levels (Table 1, Figure 2). At 21 d, the adenine-fed rats had a marked increase in serum PTH levels, and administration of R-568 for the last 14 d led to a moderate but significant decrease in serum PTH levels compared with uremic rats (Table 1). The 2.8-fold increase in PTH mRNA levels in the adenine-fed rats at 21 d also was only moderately decreased by R-568 (Table 1). These results show that the effects of uremia and the calcimimetic are due to a combination of differences in PTH mRNA levels and subsequent events such as translation, protein degradation, and finally secretion.

Nuclear Transcription Run-on Experiments

To determine the mechanism of the effect of R-568 on PTH gene expression in uremia, we performed nuclear transcript run-on experiments. Nuclear transcript run-ons showed that

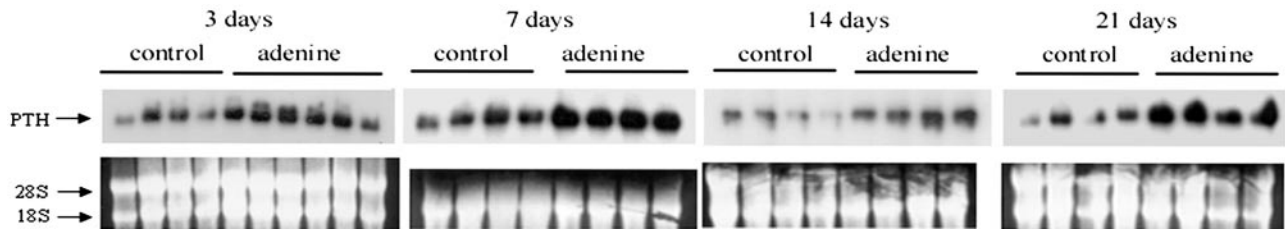


Figure 1. Rats with adenine-induced chronic renal failure have increased parathyroid hormone (PTH) mRNA levels. Rats (four in each group) were fed the diets for the indicated times when thyroparathyroid tissue was removed and RNA was extracted and analyzed for PTH mRNA by Northern blots. Each lane represents PTH mRNA from a single rat. The differences in PTH mRNA for control rats at the different time periods are a result of different exposure times of the autoradiograms. Bottom panels show ethidium bromide staining of the membrane demonstrating equal loading of the gels as visualized by the 28 and 18S ribosomal RNA. Quantification of the autoradiographs is shown in Table 1. Adenine high-phosphorus diet model of chronic renal failure led to an increase in PTH mRNA levels at 7, 14, and 21 d.

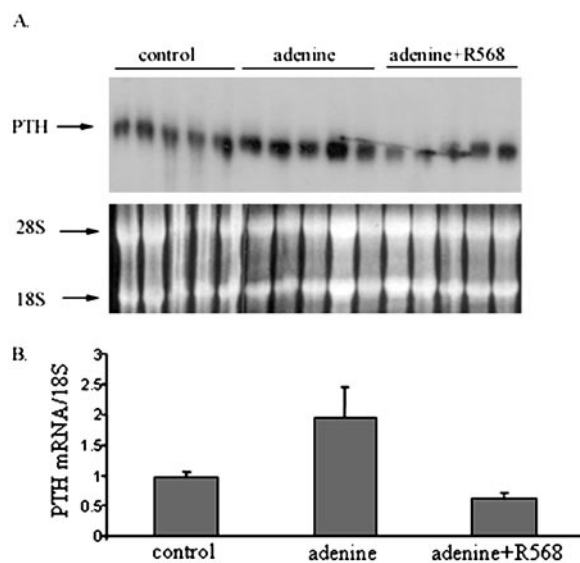


Figure 2. The calcimimetic R-568 decreased PTH mRNA levels in rats with adenine-induced chronic renal failure. Rats (five in each group) were fed control diets or adenine high-phosphorus diets for 7 d, and R-568 was given for the last 4 d. (A) Northern blot for PTH mRNA levels. Each lane represents mRNA from a single rat. Bottom panel shows ethidium bromide staining of the membrane demonstrating equal loading of the gels as visualized by the 28 and 18S ribosomal RNA. (B) Quantification of the autoradiogram shown in A of PTH mRNA levels normalized to 18S RNA. The PTH mRNA/18S of control rats is represented as 1.0. Rats with adenine-induced chronic renal failure have increased PTH mRNA levels that are decreased after treatment with R-568.

the PTH gene transcription rate was the same using PT nuclei from uremic rats at day 7 and uremic rats that were fed the calcimimetic R-568 for the last 4 d of the 7-d adenine diet (Figure 3), despite the decrease in PTH mRNA levels (Figure 2). There was also no change in transcription rates for control genes (vitamin D receptor, β -actin, and L32). These results indicate that the decrease in PTH gene expression as a result of R-568 in this model of adenine-induced experimental uremia is posttranscriptional.

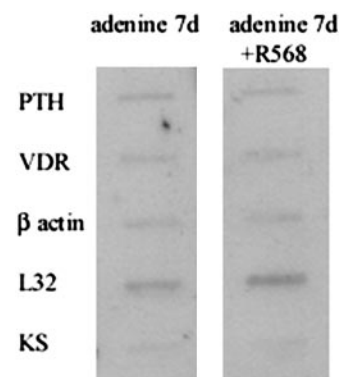


Figure 3. The effect of R-568 on PTH gene expression is posttranscriptional. Nuclei from pooled thyroparathyroid tissue (five rats in each group) of rats that were fed adenine high-phosphorus diets for 7 d without or with the calcimimetic R-568 were prepared, and nuclear run-on transcription assays were performed. The transcription rates for PTH and the control genes, vitamin D receptor (VDR), β -actin, and L32 ribosomal protein were the same in rats with adenine-induced chronic renal failure and in rats that had chronic renal failure and were given R-568. The blue script KS plasmid (KS) was used for nonspecific hybridization. These experiments are representative of three repeat experiments with different parathyroid gland (PT) nuclear preparations.

Protein–PTH mRNA Interactions

Posttranscriptional regulation of PTH gene expression correlates with changes in protein–RNA binding that determine PTH mRNA stability (15); therefore, we performed REMSA and UV cross-linking binding studies. We used an *in vitro*-synthesized, labeled transcript for the PTH mRNA 3'-UTR and PT cytosolic extracts from rats that were fed the control diet; adenine, high-phosphorus diet for 7 d; and adenine, high-phosphorus diet with R-568 given for the last 4 d. There was increased protein binding by both REMSA and UV cross-linking of PT extracts from uremic rats compared with control-fed rats (Figure 4). R-568 led to a decreased PT extract–PTH RNA binding (Figure 4), correlating with PTH mRNA levels *in vivo*. The representative REMSA in Figure 4A showed that under conditions in which the control-fed rat PT extracts showed

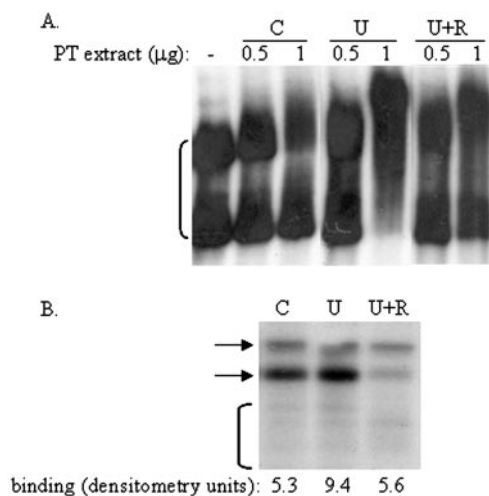


Figure 4. Adenine-induced uremic rat parathyroid cytosolic extracts show increased binding to the PTH mRNA 3'-UTR transcript that is decreased by R-568. (A) RNA electrophoretic mobility assay (REMSA) using a PTH mRNA 3'-UTR transcript without (–) and with 0.5 and 1.0 µg of PT cytosolic extract from control (C) rats that were fed an adenine high-phosphorus diet for 7 d without (U) or with R-568 for the last 4 d (U+R). The bracket shows the free probe that runs as two bands as a result of two conformations of the transcript. Note the shifted complex at 1 µg of uremic PT extract that is corrected by R-568. (B) Ultraviolet cross-linking of 10 µg of the same proteins as in A. The two arrows and the bracket denote the protein–RNA bands. The intensity of the bands was quantified by densitometry and expressed as arbitrary units below the gel. The gels are representative of three repeat gels with different PT extracts.

minimal binding (0.5 and 1.0 µg of protein), there was increased protein–PTH mRNA 3'-UTR complex formation with extracts from PT from uremic rats. Specifically, there was no free probe when 1.0 µg of PT extract from uremic rats was examined, indicating that all of the probe was bound (Figure 4A). In the PT extracts from control rats at 1.0 µg, there was still a significant amount of free probe. The free probe ran as two bands that are a result of different foldings of the RNA. R-568 led to a decrease in protein binding to a level that was almost identical to that of control animals (Figure 4A). In the UV cross-linking analysis, protein extracts are incubated with the RNA probe and UV cross-linked, and then the free probe is digested by RNase and the complexes are analyzed on a denaturing gel. UV cross-linking of PT protein extracts and the PTH mRNA 3'-UTR transcript results in three protein–RNA bands (15). We have identified by affinity chromatography and mass spectrometry that the smaller band on UV cross-linking is AUF1 (13). In some UV cross-linking gels, AUF1 runs as several bands that represent its four isoforms (Figure 4B) (13). UV cross-linking showed an approximately two-fold increase in binding with PT extracts from uremic rats compared with PT extracts from control rats. The binding with PT proteins from rats that were given R-568 decreased to levels of PT extracts from control rats (Figure 4B). The gel shown in Figure 4B is representative of three repeat experiments, and the differences

in binding indicated in the figure below the gel were obtained by densitometry of all of the protein–RNA bands combined. The differences in binding by UV cross-linking analysis were complementary to the results with the REMSA that is performed under nondenaturing conditions.

Posttranslational Modifications of AUF1

To study the expression of these PTH RNA binding proteins, we performed 1-D and 2-D Western blots for AUF1 of PT proteins from control and uremic rats and from uremic rats that were treated with R-568 using the 7-d model. The 1-D gel showed the four isoforms of AUF1 with no apparent differences among the PT extracts of the control rats, uremic rats, and uremic rats that were given R-568 (Figure 5A). In contrast, the 2-D pattern for AUF1 was very different between PT extracts from control and uremic rats (Figure 5B). In the PT extracts from uremic rats, there are additional spots that were absent in the normal PT extracts. Intriguingly, treatment with R-568 reversed the effect of uremia on the AUF1 protein (Figure 5B). The differences in the 2-D mobilities represent differences in posttranslational modifications of AUF1. In the second dimension, a sample of the PT extracts from uremic rats that were treated with R-568 was run alongside and separated only by its molecular weight to identify the four AUF1 isoforms (Figure 5B, right). The comparison between the 1-D and 2-D gels suggests that isoforms p37, 40, and 42 but not 45 were targets for posttranslational modification in uremia. The nature of these posttranslational modifications remains to be determined.

Discussion

Elevation in serum PTH is a well-characterized complication of uremia. In this study, we explored the temporal relationship between uremia and secondary HPT, the mechanisms underlying these increases, and the effect of calcimimetic treatment on these processes. At 7 and 21 d, there was a progressive increase in serum PTH that was greater than the increase in PTH mRNA levels at these time points. These results suggest that in experimental uremia, serum PTH is regulated at a number of levels. These include PTH mRNA levels together with an even greater effect at later stages, such as PTH translation, degradation, secretion, and parathyroid cell proliferation. The quantitative contribution of each stage remains to be determined. In this study, we examined the contributions of protein–PTH mRNA binding and posttranslational regulation of the PTH mRNA binding protein AUF1 in adenine-induced renal failure and its correction by the calcimimetic R-568.

We showed previously in the remnant kidney model of experimental uremia that PTH mRNA levels were increased *via* a posttranscriptional mechanism but without a difference in serum calcium and phosphate at 3 wk (16). It is interesting that in that model, protein–PTH RNA binding studies showed no differences as a result of uremia, but the PTH transcript was more stable in an *in vitro* degradation assay. In the model used in our study, uremia that was induced by an adenine, high-phosphorus diet resulted in an increased creatinine already at 3 d. In this model, the increased PTH mRNA levels correlated with changes in PT protein–PTH RNA binding and modifica-

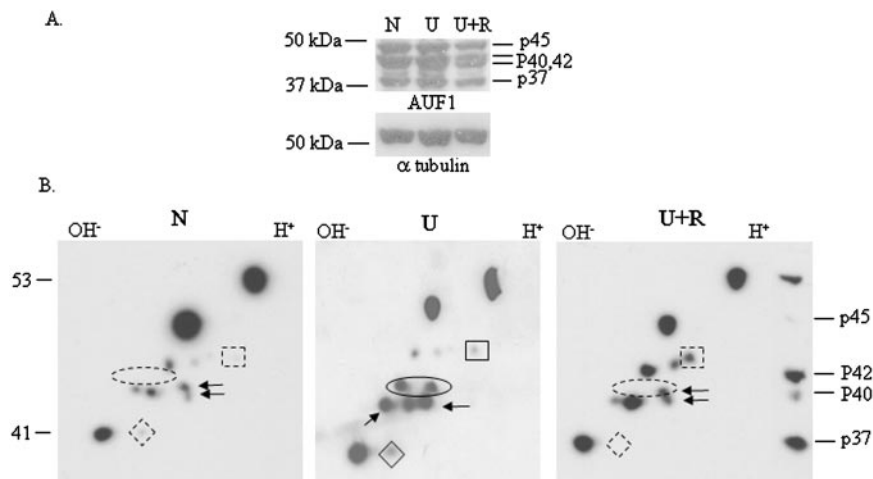


Figure 5. Adenine-induced uremia and the calcimimetic R-568 have no effect on AUF1 protein levels but modify AUF1 posttranslationally in the parathyroid. One-dimensional (A) and two-dimensional (B) Western blots of 50 or 200 μ g, respectively, of parathyroid extracts from rats that were fed a normal (N) or an adenine high-phosphorus diet for 7 d without (U) or with calcimimetic R-568 (U+R) for the last 4 d. The membranes were immunoblotted for AUF1 (A and B) or tubulin (A, bottom). There was no difference in AUF1 protein levels in the one-dimensional gel (A). Two-dimensional gels (B) showed differences in AUF1 forms. Spots that differ between the blots are indicated by a square, an ellipse, and a diamond, with the broken lines indicating spots that are either absent or markedly reduced. The acid-base direction is indicated above the gels. In gel B, the panel of U+R also shows a sample of the extract that was separated only by molecular weight to identify the four isoforms of AUF1 (shown on the right). Molecular weight markers are shown on the left. Similar findings were found in a repeat experiment.

tions of the *trans* acting factor AUF1. In both models, there was a posttranscriptional increase in PTH gene expression. The differences between the two models may be that in the remnant kidney model used, there were more moderate increases in serum creatinine and PTH levels. In the adenine high-phosphate model, the increased creatinine levels already at 3 days may have contributed to the differences in protein–RNA interactions and AUF1 modifications shown in our study. Similar changes in protein–RNA binding and posttranslational modifications of AUF1 were shown by dietary-induced hypocalcemia with attendant hyperphosphatemia in rats with normal kidney function (14). Moreover, recombinant AUF1 stabilized a PTH transcript in an *in vitro* degradation assay with parathyroid cytosolic proteins (13). Together, these models underline the importance of serum calcium and phosphorus to the pathogenesis of secondary HPT of CKD and the role of protein–RNA binding and mRNA levels. The causal relationship among RNA binding, AUF1 modifications, and PTH mRNA stability in uremia needs further study.

The management of calcium and phosphorus metabolism and secondary HPT in patients with CKD is a significant clinical problem and is associated with an alarming increase in the risk for morbidity and mortality (1). Calcimimetics have been shown to decrease significantly serum PTH levels and, presumably through this mechanism, simultaneously reduce serum calcium, phosphorus, and $\text{Ca} \times \text{P}$ in patients who are on dialysis (1,17). Initially, calcimimetics were thought to mediate their effects solely through a decrease in PTH secretion (18) (an immediate effect) and PT cell proliferation (11,19). The evidence presented in this report demonstrates that calcimimetics also mediate reductions in serum PTH levels by decreasing PTH

gene expression. The signal transduction pathway whereby the CaR regulates PTH secretion is not totally defined and even less so for PTH mRNA stability and PT cell proliferation. The CaR regulation of PTH secretion correlates with its activation of signal transduction pathways that result in increased inositol triphosphate production, the release of Ca^{2+} from intracellular stores, and the activation of the mitogen-activated protein kinase pathway (20). We showed previously that activation of the CaR regulates PTH gene expression by a mechanism involving activation of the protein phosphatase 2B calcineurin that correlates with posttranslational modifications in AUF1 (14). Moreover, calcineurin β knockout mice had higher basal levels of PTH mRNA, suggesting that calcineurin β is essential to PTH gene expression (14). It is interesting that calcineurin β also determined the response of the renal Na/Pi type II co-transporter to changes in serum phosphate (21), suggesting an important role for Ca^{2+} calmodulin activation of calcineurin in the signal transduction after changes in serum Ca^{2+} and phosphate.

The calcimimetic-mediated decrease in PTH mRNA levels may be of particular clinical relevance because the PT possesses only a limited amount of preformed PTH secretory granules (22). Studies in the bovine PT have demonstrated that the gland contains enough preformed PTH to maintain secretion for only 90 min after the initiation of hypocalcemia (7). Accordingly, more prolonged secretion, as would be observed in patients with secondary HPT, is dependent on increased PTH synthesis and decreased PTH breakdown in the PT. The hypocalcemia-induced increase in PTH gene expression by stabilization of the PTH transcript may have an important role in this effect (2). Calcimimetics decrease PTH gene expression posttranscription-

ally. 1,25(OH)₂ vitamin D₃ potentially decreases PTH gene transcription (23). Our studies suggest that the combined use of 1,25(OH)₂ vitamin D₃ and a calcimimetic may be an optimal choice to decrease PTH gene expression and PTH secretion.

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