Regulation of PTH mRNA stability by the calcimimetic R568 and the phosphorus binder lanthanum carbonate in CKD

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Nechama M, Ben-Dov IZ, Silver J, Naveh-Many T. Regulation of PTH mRNA stability by the calcimimetic R568 and the phosphorus binder lanthanum carbonate in CKD. Am J Physiol Renal Physiol 296: F795–F800, 2009.—Secondary hyperparathyroidism is characterized by increased parathyroid hormone (PTH) mRNA stability that leads to increased PTH mRNA and serum PTH levels. PTH gene expression is reduced by the calcimimetic R568 and the oral phosphorus binder lanthanum carbonate (La). Changes in PTH mRNA stability are regulated by the binding of trans-acting stabilizing and destabilizing factors to a defined cis element in the PTH mRNA 3'- untranslated region (UTR). Adenosine-uridine (AU)-binding factor 1 (AUF1) is a PTH mRNA-stabilizing protein, and K-homology splicing regulatory protein (KSRP) is a destabilizing protein that targets mRNAs, including PTH mRNA, to degradation by the ribonuclease complex exosome. We now show that KSRP-PTH mRNA binding is decreased in parathyroids from rats with adenine-induced chronic kidney disease (CKD) where PTH mRNA is more stable. KSRP-PTH mRNA binding is increased by treatment with both R568 and La, correlating with decreased PTH gene expression. In vitro degradation assays using transcripts for PTH mRNA and rat parathyroid extracts reproduce the differences in mRNA stability in vivo. Accordingly, PTH mRNA is destabilized in vitro by parathyroid extracts from CKD rats treated with R568 or La compared with parathyroid extracts from untreated CKD rats. This destabilizing effect of R568 and La is dependent on KSRP and the PTH mRNA 3'-UTR. Therefore, the calcimimetic R568 and correction of serum phosphorus by La determine PTH mRNA stability through KSRP-mediated recruitment of a degradation complex to the PTH mRNA, thereby decreasing PTH expression.

K-homology splicing regulatory protein; protein-ribonucleic acid interactions; calcium receptor; secondary hyperparathyroidism; parathyroid hormone; chronic kidney disease

MANY PATIENTS WITH CHRONIC kidney disease (CKD) develop secondary hyperparathyroidism (2HPT) with disabling systemic complications (19). Oral phosphorus binders, such as lanthanum-carbonate (La), are used to decrease serum phosphate and subsequently serum PTH in patients with CKD (8, 13). Calcimimetics activate the parathyroid calcium receptor (CaR), leading to decreased serum PTH and hence control of the 2HPT (4, 21, 22).

In experimental models, serum calcium and phosphate, as well as CKD, regulate serum PTH levels and PTH gene expression. The increase in PTH gene expression in dietary-induced CKD and hypocalcemia is posttranscriptional (14, 20, 31), as is the decrease in PTH gene expression due to dietary-induced hypophosphatemia. The differences in PTH mRNA stability in these models are mediated by regulated protein-PTH mRNA interactions that determine the accessibility of PTH mRNA to the degradation machinery (18, 25). We have previously identified a conserved cis-acting adenosine-uridine (AU)-rich element (ARE) in the PTH mRNA 3'-untranslated region (UTR) and the trans-acting factors that bind to it. The PTH mRNA 3'-UTR cis-acting element is a 63 nucleotide sequence that is both necessary and sufficient to confer regulation of PTH mRNA stability by parathyroid extracts from hypocalcemic and hypophosphatemic rats (15, 20). AU-binding factor 1 (AUF1) and upstream of N-ras (Unr) are PTH mRNA-stabilizing proteins (10, 29) and K-homology splicing regulatory protein (KSRP) destabilizes PTH mRNA (25). AUF1 either stabilizes or destabilizes other mRNAs, depending on the cellular system (9, 16). KSRP recruits the ribonuclease complex, the exosome, to target mRNAs including PTH mRNA for degradation (12, 25). We (23, 25) have recently shown that in the parathyroid the balanced interaction of AUF1 and KSRP determines PTH mRNA stability and the response to hypocalcemia, hypophosphatemia, and CKD.

Rats with adenine high phosphorus diet-induced CKD have 2HPT with increased PTH mRNA and serum PTH levels (18, 32). The 2HPT can be corrected by the calcimimetic R568 or correction of the serum phosphate by La (2, 18, 27). The regulation of PTH gene expression by R568 is posttranscriptional. This regulation correlates with differences in protein-RNA binding and posttranslational modifications of the trans-acting factor AUF1 in the parathyroid glands. AUF1 modifications due to CKD are reversed by R568 to those of rats fed a control diet (18). In addition to AUF1, the binding of the mRNA decay-promoting protein KSRP is also regulated in 2HPT (25). KSRP-PTH mRNA interaction is decreased in glands from calcium-depleted or CKD rats, where PTH mRNA is more stable, and increased in parathyroid glands from phosphorus depleted rats in which PTH mRNA is unstable compared with controls. In contrast, AUF1-PTH mRNA interactions in these parathyroid extracts are increased in calcium-depleted or CKD rats and decreased in phosphorus-depleted rats. In transfected cells, KSRP overexpression and knockdown experiments show that KSRP decreases PTH mRNA stability and steady-state levels through the PTH mRNA 3'-UTR ARE (25). Therefore, hypocalcemia, hypophosphatemia, and CKD regulate PTH mRNA half-life and the interactions of AUF1 and KSRP with the PTH ARE and thus PTH gene expression.

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We have now studied the mechanism of the effect of the calcimimetic R568 and correction of the serum phosphate by La to decrease PTH gene expression in CKD. We show that parathyroid extracts from CKD rats given R568 and La decrease PTH mRNA stability in vitro compared with extracts from untreated CKD rats, correlating with PTH mRNA levels in vivo. In addition, KSRP-PTH mRNA interactions are increased in the parathyroids of R568- or La-treated rats compared with untreated CKD rats. Finally, the differences in in vitro degradation of PTH mRNA by parathyroid extracts is dependent on KSRP and the PTH mRNA 3′-UTR, which contains the KSRP-binding sequence.

**MATERIALS AND METHODS**

**Animals.** Male Sabra rats were fed either a control or adenine-rich (0.75%) diet with high phosphorus content (1.5%; Harlan Teklad, Indianapolis, IN) for 14 days (18). The CKD-induced 2HPT was prevented by daily intraperitoneal R568 (5 mg·kg<sup>−1</sup>·day<sup>−1</sup>). Other rats received the adenine high phosphorus diet supplemented with La (3%; Ref. 2). At 2 wk, serum urea, creatinine, phosphate, calcium, and PTH levels were measured (2).

**RNA immunoprecipitation.** RNA immunoprecipitation (RIP) was performed on cross-linked thyroparathyroid glands (a pool of 6) as previously described (25). Equal amounts of whole cell extracts were immunoprecipitated with protein A-agarose-bound anti-KSRP antibodies (Bethyl, Montgomery, TX) or IgG as a negative control. RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH), reverse transcribed with random hexamer polymerase (25).

**Protein extractions.** Postmitochondrial extracts were prepared from parathyroid glands (pools of 6) and incubated on ice for 10 min in an extraction buffer containing 0.25 M sucrose, 30 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and a protease inhibitor mix. Tissue samples were homogenized, and the supernatant was cleared by centrifugation at 15,000 g for 15 min (4°C).

**In vitro degradation assays.** Radiolabeled transcripts (200,000 counts/min) were incubated with 40 μg protein extract from parathyroid glands in a volume of 50 μl as described previously (25). At timed intervals, samples were removed, RNA extract, separated on agarose gel, and analyzed by autoradiography.

**Plasmids.** The pBluescript II KS plasmid containing the full-length rat PTH cDNA including a stretch of ~150 dT nucleotides that by [α-<sup>32</sup>P]UTP and T3 RNA polymerase (25).

**Immunobots.** Proteins were analyzed by SDS-PAGE immunobots, as described elsewhere (29).

**Antibodies.** Anti-KSRP was a kind gift from R. Gherzi. The anti α-tubulin was from Sigma (St. Louis, MO).

**Table 1.** Plasma biochemistry at 2 wk

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine, μmol/l</th>
<th>Calcium, mmol/l</th>
<th>Phosphorus, mmol/l</th>
<th>PTH, pg/ml</th>
<th>Urea, mmol/l</th>
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<tr>
<td>Normal</td>
<td>0.20±0.08*</td>
<td>2.69±0.09</td>
<td>2.26±0.18*</td>
<td>153±68</td>
<td>ND</td>
</tr>
<tr>
<td>CKD</td>
<td>0.91±0.27</td>
<td>2.66±0.23</td>
<td>3.85±0.59</td>
<td>1,975±157</td>
<td>28.0±1.4</td>
</tr>
<tr>
<td>CKD + La</td>
<td>0.61±0.09</td>
<td>2.38±0.13</td>
<td>1.90±0.25*</td>
<td>1,460±121</td>
<td>29.7±1.4</td>
</tr>
<tr>
<td>CKD + R568</td>
<td>0.66±0.14</td>
<td>1.74±0.06*</td>
<td>2.51±0.13*</td>
<td>203±96</td>
<td>34.4±1.8*</td>
</tr>
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Data are means ± SE. ND, not determined; PTH, parathyroid hormone; La, lanthanum carbonate. *P < 0.05, compared with the chronic kidney disease (CKD) group.

**RESULTS**

La and R568 decrease PTH mRNA half-life by parathyroid extracts. CKD and 2HPT were induced by an adenine-rich high phosphorus diet (18). At 2 wk, there was an increase in serum creatinine, phosphate, and PTH levels. Treatment with La and even more so by R568 (Table 1) significantly decreased serum PTH levels in the CKD rats (Table 1). La decreases serum phosphate and thereby leads to a decrease in serum PTH (2). The decrease in serum PTH by R568 occurred even in the presence of hypocalcemia, which is an active stimulus for PTH secretion. La and R568 decrease both serum PTH and PTH mRNA levels, and the decrease in the PTH mRNA level by R568 was shown to be posttranscriptional (2, 18). We measured PTH mRNA stability using an in vitro degradation assay (IVDA) with parathyroid extracts from CKD rats with and without R568 or La treatment. Polyadenylated transcripts for

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*Fig. 1. Lanthanum carbonate (La) and R568 decrease parathyroid hormone (PTH) mRNA half-life by parathyroid extracts with no effect on the levels of the trans-acting proteins K-homology splicing regulatory protein (KSRP) and adenosine-uridine (AU)-binding factor 1 (AUF1). A: representative in vitro degradation assays (IVDAs) using parathyroid extracts from CKD rats with or without treatment with La or R568 and full-length polyadenylated radiolabeled transcript for PTH mRNA. RNA was fractionated by gel electrophoresis and autoradiographed. Amount of intact PTH RNA remaining with time is shown. Half-life (t<sub>1/2</sub>) calculated after quantification is shown bellow the gel. Similar results were obtained in 3 repeat experiments. B: Western blot analysis of parathyroid extracts as in A for KSRP, AUF1, and α-tubulin.*

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*Statistical analyses. Values are reported as means ± SE. One-way ANOVA with Dunnett post hoc analysis was used to assess differences from the uricemic control group (SPSS 13.0; SPSS Chicago, IL). A two-sided P value was considered significant when <0.05.*

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full-length PTH mRNA were incubated with parathyroid extracts to measure the amount of intact transcript remaining with time. The IVDA has been shown to reproduce differences in mRNA stability that occur in vivo, and specifically, the differences in PTH mRNA stability induced by calcium and phosphate depletion (11, 20). Parathyroid extracts from both La- and R568-treated rats led to a more rapid PTH mRNA decay compared with untreated CKD rats (Fig. 1A). This decrease in mRNA stability correlated with the decrease in PTH mRNA levels after La and R568 (2, 18; and Fig. 2A). There was no difference in protein levels of the trans-acting factors KSRP and AUF1 in these parathyroid extracts (Fig. 1B). These results

Fig. 3. Decrease in PTH mRNA half-life by parathyroid extracts from CKD rats treated with La or R568 is dependent on KSRP and the PTH mRNA 3′-untranslated region (UTR) in IVDAs. A: IVDA with mock or KSRP-depleted parathyroid extracts from CKD rats and CKD rats after La or R568. Extracts were incubated with a full-length PTH mRNA transcript (top) or a truncated polyadenylated transcript lacking the 3′-UTR (bottom). B and C: quantification of the results in A, presented as % intact transcript remaining with time, of the full-length PTH mRNA transcript (B) and the transcript without the 3′-UTR (C) incubated with the different parathyroid extracts described in A. Open symbols represent mock depleted parathyroid extracts, and closed symbols extracts after KSRP depletion [KSRP(−)].
are in agreement with our previous publications (29) where we have shown differences in binding of AUF to PTH mRNA but not in protein levels. KSRP-PTH mRNA interaction is decreased by CKD and increased after La and R568. CKD leads to increased binding of AUF1 and decreased binding of KSRP to the PTH mRNA 3'-UTR compared with control rats (25). R568 decreases AUF1-PTH mRNA binding (18). To study the role of KSRP in the decreased PTH mRNA levels observed with R568 or La treatment, we performed RIP assays (Fig. 2). Parathyroid glands were cross-linked and then input and KSRP-bound RNA was analyzed by quantitative RT-PCR. PTH mRNA was increased by CKD and decreased by La and R568 treatment in the input (Fig. 2A) as before (2, 18). In contrast, the amount of KSRP-bound PTH mRNA was decreased by CKD and increased by La and R568 (Fig. 2B). The differences in KSRP-PTH mRNA interactions are consistent with the destabilizing role of KSRP (12). It is noteworthy that the R568-induced KSRP-PTH mRNA interaction was greater than that after correction of the serum phosphate by La (Fig. 2B), although they led to a similar decrease in PTH mRNA (Fig. 2A). This disparity underlines the complexity of protein-RNA interactions determining PTH mRNA decay. Together, these results demonstrate the contributions of KSRP-PTH mRNA binding to PTH mRNA levels and serum PTH in 2HPT and its correction by La and R568. Decrease in PTH mRNA half-life by parathyroid extracts from CKD rats treated with La or R568 is dependent on KSRP and the PTH mRNA 3'-UTR. To further study the function of KSRP in these models, we immunodepleted KSRP from parathyroid extracts and determined the effect of KSRP depletion on PTH mRNA decay by IVDA. PTH mRNA was not stable in the IVDA with mock-depleted parathyroid extracts from both La- and R568-treated CKD rats compared with parathyroid extracts from untreated CKD rats (Fig. 3A, top, and B, open symbols). R568 was more potent than La in decreasing PTH mRNA stability (Fig. 3B). The amount of intact PTH mRNA at 90 min in parathyroid extracts from untreated CKD rats was 80%, after La 65%, and after R568 45% (Fig. 3B). These findings are in agreement with the observation that R568 is more potent in decreasing serum PTH levels (Table 1). Furthermore, R568 led to a greater increase in binding of KSRP to PTH mRNA than after correction of the serum phosphate by La (Fig. 2B). Immuno-depletion of KSRP had no effect on PTH mRNA decay in CKD parathyroid extracts (Fig. 3, A and B). This result is consistent with the finding that in CKD KSRP-PTH mRNA interaction is minimal (Fig. 2B). In contrast, KSRP depletion prevented PTH mRNA decay by parathyroid extracts from both La- and R568-treated CKD rats (Fig. 3A, top, and B, filled symbols). This effect of KSRP depletion is consistent with the increased KSRP-PTH mRNA interaction observed by RIP after La and R568 (Fig. 2B). It suggests that KSRP is necessary for PTH mRNA decay by parathyroid extracts from R568- and La-treated rats. Depletion of KSRP would allow less KSRP-PTH mRNA binding, and this may block the recruitment of the degradation machinery by KSRP to PTH mRNA. KSRP binds PTH mRNA through the PTH mRNA 3'-UTR that also mediates the decrease in PTH mRNA levels after KSRP overexpression (25). We therefore performed the IVDAs with a truncated polyadenylated PTH mRNA transcript that lacks the 3'-UTR. The stability of the truncated PTH mRNA transcript was the same using mock-depleted parathyroid extracts from CKD rats with or without La and R568 treatment (Fig. 3A, bottom, and C, open symbols). Thus the regulation of PTH mRNA stability by La and R568 in these extracts is dependent on the 3'-UTR. In addition, KSRP depletion had no effect on the decay of the truncated PTH mRNA transcripts compared with mock-depleted extracts (Fig. 3A, bottom, and C, filled symbols). Therefore, the decreased PTH mRNA stability by parathyroid extracts of the La- or R568-treated CKD rats is dependent on both the PTH mRNA 3'-UTR and KSRP.

**DISCUSSION**

mRNA decay is a major mechanism controlling gene expression. mRNA-binding proteins associate with mRNAs to coordinately regulate their localization, translation, and/or degradation. The binding of stabilizing and decay-promoting proteins to target mRNAs determines the accessibility of these mRNAs to the degradation machinery and hence mRNA half-life and steady-state levels. The regulation of PTH gene expression by calcium, phosphorous, and CKD is mediated by differences in PTH mRNA stability that are dependent on the regulated interactions of cis elements in the PTH mRNA 3'-UTR and trans-acting factors that bind to it (15, 23). In the present study, we show that PTH mRNA decay in vitro is increased by parathyroid extracts from CKD rats given R568 and La, compared with extracts from untreated CKD rats, correlating with the decreased steady-state PTH mRNA and serum PTH levels in vivo. This rapid decay of PTH mRNA by parathyroid extracts from R568- and La-treated rats is dependent on KSRP and the PTH mRNA 3'-UTR. Calcimimetics and oral phosphorus binders are potent regulators of 2HPT in CKD patients and are an essential part of
the therapeutic strategies for these patients. In experimental models, both calcimimetics and phosphorus binders are effective in decreasing serum PTH, PTH gene expression, and parathyroid cell proliferation (3, 7, 18). R568 acts directly to activate the parathyroid calcium receptor, even in the presence of hypocalcemia, overriding the relaxation of the CaR by the low serum calcium. The decrease in serum calcium due to R568 may represent the action of the calcimimetic on the calcium receptor in the renal tubules (26) or an effect on bone due to decreased serum PTH resulting in a “hungry bones phenomenon” as in dialysis patients given calcimimetics (17). Both R568 and La decreased serum phosphate (Table 1). The effect of R568 on serum phosphate reflects decreased bone resorption secondary to the reduction in serum PTH. La is a phosphorus binder and hence decreases serum phosphate levels leading to a decrease in serum PTH. The oral La is unlikely to have a direct effect on the parathyroid because the gastrointestinal absorption of La is very low and almost all circulating La is protein bound (1, 3). It is not possible to exclude the possibility that the reduction in serum phosphate contributes to the effects of both R568 and La on the parathyroid. Rodriguez et al. (28) showed that treatment with R568 increased vitamin D receptor mRNA and protein levels in normal rat parathyroid glands in vivo and in human parathyroid glands with diffuse but not nodular hyperplasia, suggesting that the calcimimetic R568 exerts a stimulatory effect on vitamin D receptor expression in the parathyroid glands. The effect of vitamin D on PTH gene expression is transcriptional (30) and would not be reflected in the posttranscriptional studies performed here. A role for FGF23 in these studies remains to be determined.

We (18) have previously shown that adenine-induced CKD leads to alterations in protein-PTH mRNA binding and post-translational changes in the PTH-mRNA-binding protein AUF1. The AUF1 modifications as a result of CKD were reversed to those of normal rats by treatment with R568. These modifications in AUF1 correlate with changes in protein-PTH mRNA binding and PTH mRNA levels. Therefore, uremia and activation of the CaR-mediated by calcimimetics modify AUF1 posttranslationally. AUF1 is a stabilizing protein for PTH mRNA (29). In contrast, KSRP is a PTH-mRNA-binding and -destabilizing protein (25). KSRP binds to ARE-containing mRNAs and recruits the ribonuclease complex, the exosome, to degrade these mRNAs (5, 6, 12).

We have now studied the molecular mechanism of how R568 and La decrease PTH mRNA levels and stability and, as a result, serum PTH. We show in adenine-induced CKD that the calcimimetic R568 and correction of the serum phosphate by La regulate serum PTH and PTH mRNA levels. R568 led to a greater decrease in serum PTH than La, although the decrease in PTH mRNA was similar in both groups. This may be because the comparison between serum PTH and PTH mRNA levels is qualitative rather than quantitative. The differences may reflect in part the differences in the sensitivities between the assays. One assay is an immunoassay and the other is quantitative RT PCR.

We have identified KSRP as a protein that responds to CKD and R568 or La treatment in the parathyroid by altered interaction of this protein with PTH mRNA. We show that R568 and La regulate PTH mRNA stability by parathyroid extracts in vitro by IVDA and PTH mRNA-KSRP interaction in the parathyroid in vivo. We demonstrate by IVDA that the accelerated decay of PTH mRNA by parathyroid extracts of R568- and La-treated rats is dependent on KSRP and requires an intact PTH mRNA 3′-UTR. In CKD, there is increased binding of AUFI and decreased binding of KSRP to PTH mRNA resulting in increased PTH mRNA and serum PTH levels (25, 29). We show that in both R568- and La-treated CKD rats, KSRP-PTH mRNA interactions are increased compared with untreated CKD rats. This would lead to more rapid decay of the PTH mRNA (Fig. 4). The changes in binding of the trans-acting factors to the PTH mRNA 3′-UTR, therefore, determine PTH gene expression in CKD and after management of the 2HPT by both calcimimetics or phosphorus binders.

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GRANTS

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REFERENCES

13. Joy MS, Finn WF. Randomized, double-blind, placebo-controlled, dose titration, phase III study assessing the efficacy and tolerability of lantha-


