Persistent Downregulation of Calcium-Sensing Receptor mRNA in Rat Parathyroids when Severe Secondary Hyperparathyroidism Is Reversed by an Isogenic Kidney Transplantation

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Abstract. Experimental severe secondary hyperparathyroidism (HPT) is reversed within 1 wk after reversal of uremia by an isogenic kidney transplantation (KT) in the uremic rats. Abnormal parathyroid hormone (PTH) secretion in uremia is related to downregulation of CaR and vitamin D receptor (VDR) in the parathyroid glands (PG). The aim of this investigation was to examine the expression of CaR and VDR genes after reversal of uremia and HPT in KT rats. 5/6 nephrectomized rats were kept on a normal or high-phosphorus (hP) diet for 8 wk to induce severe HPT (n = 8 in each group). In another group of seven uremic hP rats, uremia was reversed by an isogenic KT and PG were harvested within 1 wk posttransplant. Plasma urea, creatinine, total calcium, phosphorus, and PTH levels were measured. Parathyroid CaR and VDR mRNA were measured by quantitative PCR. Uremic hP rats had significantly elevated levels of creatinine, urea, and phosphorus (P < 0.001) and developed significant hypocalcemia (plasma calcium 1.83 ± 0.2 mmol/L; P < 0.001) compared with normal control rats. After KT, the levels were normalized from day 3 to 7: creatinine from 0.117 ± 0.016 to 0.050 ± 0.002 mmol/L; urea from 23 ± 4 to 7 ± 0.3 mmol/L; phosphorus from 3.9 ± 0.6 to 1.5 ± 0.06 mmol/L; calcium from 1.8 ± 0.2 to 2.5 ± 0.02 mmol/L. Plasma PTH levels fell from 849 ± 224 to a normal level of 38 ± 9 pg/ml (P < 0.01). In uremic rats on a standard diet, CaR mRNA was similar to that of normal control rats, whereas VDR mRNA was significantly decreased. In uremic rats kept on hP diet, CaR mRNA was significantly decreased to 26 ± 7% of control rats (P = 0.01) and VDR mRNA reduced to 36 ± 11% (P < 0.01). In KT, previously hP uremic rats, both CaR mRNA and VDR mRNA remained severely reduced (CaR, 39 ± 7%; VDR, 9 ± 3%; P < 0.01) compared with normal rats. In conclusion, circulating plasma PTH levels normalized rapidly after KT, despite persisting downregulation of CaR and VDR gene expression. This indicates that upregulation of CaR mRNA and VDR mRNA is not necessary to induce the rapid normalization of PTH secretion from hyperplastic parathyroid glands.

In chronic uremia, the parathyroid gland (PG) function is abnormal, resulting in an increase in parathyroid hormone (PTH) biosynthesis and secretion and parathyroid cell hyperplasia (1,2,3). The molecular basis for the abnormal PTH secretion still remains partly obscure. It presumably involves dysfunction of the mechanism by which the parathyroid cells sense changes in plasma calcium (4). PG from patients with severe secondary or tertiary hyperparathyroidism (HPT) have an elevated set-point for Ca\(^{2+}\) in vitro (5). A substantial reduction in the expression of the Ca\(^{2+}\)-sensing receptor (CaR) protein and mRNA has been demonstrated in hyperplastic parathyroid glands from uremic patients (6,7). 1,25(OH)\(_2\)D\(_3\) dramatically decreases PTH gene transcription (8), and it has been proposed that this sterol also influences the sensitivity of the parathyroid cells to Ca\(^{2+}\) (9). Changes in the vitamin D receptor (VDR) concentration of the parathyroids would allow for a modulation of the effect of 1,25(OH)\(_2\)D\(_3\). A reduction of VDR protein and mRNA has been found in glands from uremic patients with severe secondary HPT (10). In experimental uremia in 5/6 nephrectomized rats, a significant HPT is induced, which becomes far more severe when the uremic rats are kept on a high-phosphorus (hP) diet (11,12). In such uremic HPT rat models, a reduced VDR mRNA has been demonstrated in the parathyroids by some investigators (13). A decrease of the CaR protein and mRNA expression has further been observed in the parathyroid glands of hyperphosphatemic uremic rats but not in uremic rats kept on standard diet (14).

We have previously shown that experimental, secondary HPT due to long-term (20-wk) uremia was reversed very rapidly after reversal of uremia by an isogenic kidney transplantation (KT). Even severe secondary HPT due to long-term
experimental uremia and hyperphosphatemia was very rapidly reversible. In both conditions, the circulating levels of PTH became normal within 1 wk after normalization of kidney function by the isogenic KT (11). The mechanism behind this rapid reversal of secondary HPT has, however, not been clarified.

The aim of the present investigation, therefore, was to study the time course of this normalization of the circulating levels of PTH, which takes place very rapidly after normalization of the GFR in previously uremic rats, and to examine whether such a normalization of PTH levels was associated with an upregulation of CaR mRNA and VDR mRNA levels in the PG of these previously uremic rats.

Materials and Methods

Animals

Inbred, male DA rats weighing 250 g at the start of the study were used. The experimental studies on the rats were performed in accordance with the NIH Guidelines for Care and Use of Laboratory Animals and were approved by our institution.

Uremia

Chronic renal failure (CRF) was induced by a one-step 5/6 nephrectomy procedure. A group of CRF rats (n = 8) was kept on a standard diet containing 0.9% calcium, 0.7% phosphorus, and 1000 IU vitamin D/kg. Another group of CRF rats (n = 15) was kept on hP diet containing 0.9% calcium, 1.5% phosphorus, and 1000 IU vitamin D/kg to induce severe HPT. Eight rats of the hP group were sacrificed, at which time PG were removed. The other seven rats were used for KT; after reversal of uremia, the rats were sacrificed and the PG were removed. The duration of uremia was 8 wk. A group of normal control rats (n = 8) was kept on standard diet. The rats were allowed free access to food and water.

Reversal of Uremia and Secondary HPT by an Isogenic KT

In a group of seven CRF rats kept on hP diet, uremia was reversed by an isogenic KT. The kidney was transplanted into the left orthopic side with end-to-end anastomoses of the blood vessels and of the ureter. We have previously described this model in detail (15). The advantage of using an isogenic KT for reversal of uremia is that there are no rejection episodes of the transplanted kidney and no need to use immunosuppressive treatment. After KT, the rats were on a standard diet. Blood (400 μl) was drawn daily from the tail of the transplanted rats, and plasma levels of total calcium, phosphorus, urea, and creatinine were monitored to determine when a normal kidney function was reestablished. Plasma PTH levels were also measured daily. As soon as plasma urea was normalized, the PG were removed by microdissection, frozen, and stored in liquid nitrogen until analysis.

Determination of CaR mRNA and VDR mRNA in the PG: RNA Isolation and RT-PCR

One milliliter of phenol-guanidine isothiocyanate solution (TRI-Reagent, Sigma, St Louis MO) was added to the PG. The glands were ultrasonicated for 5 min at 4°C to allow for complete cell rupture. Total RNA was then extracted according to a modified Chomczynski and Sacchi’s protocol (16). Extracted total RNA was dissolved in nuclease free water (Promega, Madison WI) and heated for 10 min at 60°C. Total RNA was quantified by spectrophotometry (17). VDR versus actin and CaR versus actin were amplified separately with the Access-PCR System (Promega) (18) using specific primers, as listed in Table 1 and 100 ng of total RNA per sample.

Each sense primer was marked with 6FAM fluorochrome. The PCR were performed when the reactions were in the linear range after 25 cycles, DNA amplifications were processed by a Gene Scan Analyzer, ABI Prism 310 (Perkin Elmer, Foster City, CA). The pair bases standard (size standard) was marked with a different type of fluorochrome and run in parallel. The graphic representation of the relative expression of the genes is an electropherogram. Data were analyzed using Gene Scan version 3.1/1998 (Perkin Elmer). The amount of VDR/actin mRNA and CaR/actin mRNA were expressed as percent of the control group (normal rats).

Plasma Measurements

Plasma creatinine, urea, phosphorus, and total calcium were measured by an ETACHEM 250 Analyzer (Eastman Kodak, Rochester, NY), using a volume of 40 μl. Plasma PTH was measured in a volume of 100 μl by a rat PTH (IRMA) assay from Immunotopics (San Clemente, CA). The intra-assay coefficient of variance in our laboratory was 4%, and inter-assay coefficient of variance was 5%.

Statistical Analyses

The results are expressed as mean ± SEM. A one-way ANOVA and a post-hoc (Neuman-Keuls) test were used for the comparison of groups. P < 0.05 was considered significant.

Results

The plasma parameters of normal rats, CRF rats kept on standard diet, CRF rats kept on hP diet, and KT rats are shown in Table 2. In uremic rats kept on a hP diet, plasma creatinine and urea levels were significantly increased (P < 0.01 and < 0.001). The rats had significant hypocalcemia (P < 0.001), hyperphosphatemia (P < 0.001), and severe secondary HPT (P < 0.001) compared with normal rats. In a subgroup of seven CRF rats kept on hP diet and used for KT, the plasma parameters were: creatinine, 117 ± 16 μmol/L; urea, 23 ± 4 mmol/L; calcium, 1.83 ± 0.2 mmol/L; phosphorus, 3.9 ± 0.63 mmol/L; PTH, 849 ± 224 pg/ml. After a successful isogenic KT, plasma creatinine and urea levels became normal from the

<table>
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<tr>
<th>Table 1. Primer sequences</th>
<th>Sense</th>
<th>Anti-Sense</th>
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<tr>
<td>VDR</td>
<td>(6FAM)5’-TGAAGGCTGCAAAGGGTTTCTTACCGG-3’</td>
<td>5’-GGAGTGACTCTCTTATCATGCGGATG-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>(6FAM)5’-CGTCACCAACTGGAGCACAGCTGAGG-3’</td>
<td>5’-GGCGTACAGTACGACGCTTCAGG-3’</td>
</tr>
<tr>
<td>CaR</td>
<td>(6FAM)5’-ATTGAGGGGAGCCACCTGCTGCT-3’</td>
<td>5’-AAAGAGGGTGAGTGCGATGCCAAAGG-3’</td>
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posttransplant third to seventh day. Plasma PTH levels decreased gradually and became normal parallel to the normalization of the kidney function (Figure 1). Plasma calcium and phosphorus became normal as well (Table 2). A strong correlation was seen between the decline of plasma PTH levels and plasma urea levels, as monitored daily after KT ($r = 0.892$, $P < 0.0001$). Similar correlations were found between the decline of PTH and increase of calcium or fall of phosphorus levels ($r = 0.830$, $P < 0.0001$ and $r = 0.844$, $P < 0.0001$, respectively).

Figure 2 shows the results of CaR mRNA in the PG of normal rats, CRF rats kept on a standard diet, CRF high-phosphorous diet, and kidney transplanted previously uremic and hyperphosphatemic rats. The CaR mRNA levels of the uremic rats kept on a standard diet were similar to those of normal control rats, whereas uremic rats kept on hP diet had a significantly decreased CaR mRNA levels ($P < 0.001$). After reversal of uremia and of the secondary HPT by the isogenic KT, the CaR mRNA levels were still significantly decreased ($P < 0.001$) compared with normal rats and at the same low level of the uremic hyperphosphatemic rats, despite the normalization of circulating plasma PTH levels. Figure 3 shows a representative electropherogram of the CaR and actin in the different experimental groups.

Figure 4 shows the VDR mRNA levels of the PG from normal rats, uremic rats kept on a standard diet, uremic rats kept on hP diet, and kidney transplanted previously uremic and hyperphosphatemic rats. The parathyroid VDR mRNA levels were significantly decreased ($P < 0.01$) in both models of uremia and remained low after reversal of uremia by the isogenic KT. Figure 5 shows a representative electropherogram of the VDR and actin in the different experimental groups.

Discussion

In this study, we have found that the marked decrease in PTH secretion, which takes place after reversal of experimental uremia by an isogenic KT, occurs despite no changes in the expression of the CaR or VDR genes in the PG.

In uremic rats kept on hP diet, the circulating PTH levels were increased 16 times and the CaR mRNA levels decreased by approximately 60%. Three to seven days after KT, the PTH levels became normal, whereas CaR mRNA remained low, at the level of the hyperphosphatemic, uremic rats. Calcium is the major regulator of PG function, and this ion is the physiologic ligand for the CaR. Several lines of evidence support the role of the CaR as the key mediator of calcium-regulated PTH.

Table 2. Plasma parameters of normal rats, CRF rats kept on standard diet, high-phosphorous diet, and kidney transplanted rats

<table>
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<tr>
<th></th>
<th>Creatinine (μmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Calcium (mmol/L)</th>
<th>Phosphorus (mmol/L)</th>
<th>PTH (pg/ml)</th>
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<tr>
<td>Normal $n = 8$</td>
<td>71 ± 5</td>
<td>6 ± 0.2</td>
<td>2.56 ± 0.01</td>
<td>1.28 ± 0.07</td>
<td>70 ± 15</td>
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<tr>
<td>CRF standard diet, $n = 8$</td>
<td>96 ± 20</td>
<td>14 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.35 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.84 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>369 ± 58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRF high-phosphorous diet, $n = 15$</td>
<td>126 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.72 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1108 ± 205&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney transplanted $n = 7$</td>
<td>50 ± 2</td>
<td>7 ± 0.3</td>
<td>2.47 ± 0.03</td>
<td>1.55 ± 0.06</td>
<td>38 ± 9</td>
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<sup>a</sup> $P < 0.01$ versus normal and kidney transplanted rats.

Figure 1. Normalization of kidney function and circulating parathyroid hormone (PTH) levels after reversal of uremia by an isogenic kidney transplantation (KT). The line depicts plasma urea, and the bars depict plasma PTH levels. Uremic rats were kept on a high phosphorus (hP) diet to induce severe secondary hyperparathyroidism (HPT). The uremia and the secondary HPT were reversed 3 to 7 d after KT. Mean ± SEM; $n = 7$; <sup>*</sup>$P < 0.01$ versus levels of normal and KT rats.

Figure 2. CaR gene expression in the parathyroid glands (PG) of normal control rats, chronic uremic rats kept on a standard diet (CRF), chronic uremic rats kept on hP diet (CRFhighP), and KT previously uremic rats kept on a hP diet in which uremia was reversed by an isogenic KT (Transpl). Mean ± SEM; $n = 8–7$; <sup>*</sup>$P < 0.001$ versus control and CRF rats.

Figure 3. Representative electropherogram of the CaR and actin in the different experimental groups.

Figure 4. VDR mRNA levels of the PG from normal rats, uremic rats kept on a standard diet, uremic rats kept on hP diet, and kidney transplanted previously uremic and hyperphosphatemic rats. The parathyroid VDR mRNA levels were significantly decreased ($P < 0.01$) in both models of uremia and remained low after reversal of uremia by the isogenic KT. Figure 5 shows a representative electropherogram of the VDR and actin in the different experimental groups.
secretion (4). Two studies have demonstrated that expression of CaR is downregulated at both protein and mRNA levels in the hyperplastic PG of patients with chronic uremia (6,7). Similarly, a downregulation of the expression of CaR has been found in adenomas in PG of patients with primary HPT (7,19). In experimental uremia, a decrease of CaR mRNA was found in the parathyroids of rats kept on hP diet but not in rats kept on a standard diet (14). This observation is confirmed by the present results.

A linkage between the CaR and parathyroid cell proliferation in uremia has been suggested. This is based on the finding that the calcimimetic compound, NPS R-568, by acting directly on the CaR inhibited parathyroid cell proliferation in rats with renal insufficiency (21). It remains unclear, however, which are the molecular mechanisms that are involved in the CaR-regulated parathyroid cell proliferation in uremia. As such, it is not clear whether the proliferation of the parathyroid cells is the consequence of a downregulated expression of the CaR or whether there are decreased levels of the CaR because the parathyroid cells are proliferating rapidly and proliferating cells lack a well-formed CaR. Recent reports indicate that the proliferation of the PG may precede the downregulation of the CaR (22,23). In this study, we did not examine whether the parathyroid cell proliferation was inhibited after reversal of uremia.

The central observation of this study is that the CaR mRNA of the PG is severely decreased despite the fact that a dramatic decrease in the PTH levels occurs. This indicates that a reduced expression of the CaR gene does not impede the regulation of PTH secretion by extracellular calcium and might suggest the existence of a secretory mechanism in the parathyroid cell, which is not coupled to CaR and which responds to the reversal of uremia or to the simultaneous normalization of the plasma calcium and phosphorus levels. In the clinical situation, a decline of circulating PTH after KT might be partially due to an increased clearance of C-terminal PTH after restoration of normal kidney function. This could partly be explained by the co-measuring of some C-terminal PTH fragments besides the intact PTH molecule by the human PTH assays, which were used until recently (24). In the present rat model a decrease of

Figure 3. A representative electropherogram of the CaR mRNA and actin mRNA in the different experimental groups from Figure 2.

Figure 4. VDR gene expression in the PG of normal control rats, chronic uremic rats kept on a standard diet (CRF), chronic uremic rats kept on hP diet (CRFhighP), and KT, previously uremic rats kept on a high phosphorus diet in which uremia was reversed by an isogenic KT (Transpl). Mean ± SEM; n = 8–7; * P < 0.001 versus control rats.

Figure 5. A representative electropherogram of the VDR mRNA and actin mRNA in the different experimental groups from Figure 4.
PTH levels is caused by decreased secretion of the intact/N-terminal PTH. In the rat PTH assay used, the PTH molecule is captured by two antibodies against epitopes within the N-terminal part of the molecule and not crossreacting with C-terminal PTH, including the long 7 to 84 PTH fragment (25), known to be accumulated in uremia. On the other hand, secretion of non N-terminal PTH fragments will not be detected by this assay. It is a theoretical possibility that the normalization of the circulating PTH levels after experimental KT might be partially due to an increase of the intraglandular degradation of PTH 1 to 84 and to secretion of smaller PTH fragments.

Phosphorus controls PTH secretion by a posttranscriptional mechanism. Three in vitro studies have provided evidence for a direct stimulatory effect of phosphorus on the PG (26–28). Therefore, a reduction of plasma phosphorus after the experimental kidney transplantation might have been a factor contributing to the decrease in PTH secretion. This could be a direct action on the PG and/or mediated via an indirect effect on the increase in calcium. An effect of normalization of phosphorus on the amelioration of the skeletal resistance to PTH after KT is another important consideration in understanding the rapid decline in PTH levels post transplant.

In uremic rats on hP diet, Slatopolsky et al. (29) have previously shown that the PTH levels decreased when the rats were switched from a high- to a low-phosphorus diet, whereas the PTH content in the secretory granules of the parathyroid cells remained high. More recently, the same group (30) has shown in the same model of uremic rats that the CaR expression of the parathyroids was low and remained unchanged immediately after the decrease of the phosphorus content in the diet. The CaR expression did, however, become normal after the rats had been on a low-phosphorus diet for several weeks. The same might be the case in the present model of KT rats. The time course of the eventual upregulation of the CaR gene expression was, however, not the purpose of the present investigation. The CaR gene in the PG might only be slowly regulated. The CaR is probably an important part of the “calciosstat” of the organism, which determines the plasma Ca^{2+} level, which is a complex calcium homeostasis strives to maintain. As previously shown, activating and inactivating mutations of the CaR gene are associated with conditions of hypocalcemia and hypercalcemia, respectively (20). One does not expect dramatic changes to occur in the “calciosstat” on a minute-to-minute or day-to-day basis. Thus the regulation of the CaR gene is as yet only sparsely understood.

One might speculate whether a persistent downregulation of the parathyroid VDR mRNA might have an influence on the persistent downregulation of the CaR mRNA, as shown in the present investigation. Regulation of the CaR gene by vitamin D has been suggested by Brown et al. (31), who found that the expression of the CaR gene was affected by the vitamin D nutritional status and was as such decreased in rats kept on a vitamin D–deficient diet. In the same study, it was shown that parathyroid CaR mRNA was upregulated by pharmacologic doses of 1,25(OH)_{2}D_{3}, given as a bolus. In contrast, another study by Rogers et al. (32) found no effect of pharmacologic doses of 1,25(OH)_{2}D_{3} on the expression of CaR mRNA levels, although 1,25(OH)_{2}D_{3} was administrated as a continuous infusion. In the present study in uremic rats kept on standard diet, VDR gene expression was decreased, whereas the CaR gene expression was normal. This result is in agreement with that of Rogers et al. (32), showing no effect of vitamin D on CaR gene regulation.

It is well documented that 1,25(OH)_{2}D_{3}, in pharmacologic doses is an important regulator of PTH gene (33). Whether, this is an expression of a physiologic role of 1,25(OH)_{2}D_{3} in the regulation of parathyroid function is uncertain, as it is still a question whether the physiologic effect of 1,25(OH)_{2}D_{3} is directly on the gene or mainly mediated via its calcemic effect (34). In the uremic rat models of the present study with high PTH levels stimulating the renal 1-α hydroxylase activity, it has previously been shown by our group (11) and by others (1), that circulating 1,25(OH)_{2}D_{3} levels are within normal range compared with normal rats. This level is, however, relatively low considering the extreme high PTH levels. In a setting of increased PTH secretion, a normal serum calcitriol value might be neither effective nor appropriate for an inhibitory effect on the PG. Furthermore, the activity of the sterol might be decreased due to decreased VDR expression in the PG. The reduced parathyroid VDR mRNA in uremic rats is confirmed by the present results. It is surprising that the circulating levels of PTH became normal despite low and unchanged expression of VDR mRNA after the experimental KT. It has previously been shown by Brown et al. (35) that calcium regulates the expression of VDR mRNA in rat parathyroids. They showed, that vitamin D–depleted rats, which received a high-calcium diet, had VDR mRNA levels similar to those of rats maintained on a normal vitamin D diet. Furthermore, the authors suggested that the upregulation of the PG’s VDR mRNA by pharmacologic doses of 1,25(OH)_{2}D_{3} might mainly be due to increased plasma calcium levels. In the present study, after KT, plasma Ca^{2+} levels are normalized and plasma 1,25(OH)_{2}D_{3} levels are normal, whereas the VDR mRNA levels in the parathyroids are persistently decreased. This difference could be due to the fact that the normalization of plasma Ca^{2+} after experimental KT in the present study was very fast in contrast to the response of chronic dietary treatment or to the pharmacologic effect of 1,25(OH)_{2}D_{3}. It is also possible that, despite persistently low VDR expression, the activity of 1,25(OH)_{2}D_{3} is improved in the PG of KT rats due to cessation of hypocalcemia. As such, it has been reported, that nuclear calreticulin in the parathyroids during hypocalcemia prevents the binding of the VDR-Retinoid X-receptor to the PTH gene, thereby inhibiting the downregulatory function of the sterol (36). The present results therefore underline the complexity of the coordinated regulation of the PG function by calcium and 1,25(OH)_{2}D_{3}. Finally, the CaR and VDR gene expressions are measured in the present investigation, whereas protein levels in the parathyroid cells or CaR protein density on the parathyroid cell membrane might have had a different expression from that of the mRNA levels.

In conclusion, after reversal of uremia by an experimental KT, even severe secondary HPT is reversed very quickly, within 1 wk, resulting in normal circulating levels of PTH,
Ca²⁺, and phosphorus, despite persistently decreased levels of CaR mRNA and VDR mRNA in the PG.

Acknowledgments

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References


