Lanthanum carbonate decreases PTH gene expression with no hepatotoxicity in uraemic rats

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Abstract

Background. Lanthanum (La) carbonate is an effective phosphate-binder, used to reverse hyperphosphataemia due to chronic kidney disease. Some recent studies in rodents have cast safety uncertainties. The aims of this study were to examine the effects of La on parathyroid hormone (PTH) gene expression and hepatic toxicity.

Methods. Uraemia was induced in rats by a 0.3% adenine-containing diet for 2 weeks. Thereafter, rats were maintained for 4 weeks on an adenine diet with 1.5 or 3% La. Normal, uraemic, and uraemic rats fed a low-phosphorus (P) diet served as controls.

Results. Azotaemia developed in all adenine-fed rats. La of 3%, but not 1.5% La, decreased serum P to normal in uraemic rats. Both La 1.5 and 3% decreased urine P excretion. Plasma PTH was tripled in uraemic compared with normal rats. Both the 3.0% La and the low-P diet decreased PTH to normal. PTH mRNA content was increased 2-fold in uraemic compared with normal rats. The 3% La or the low-P diet decreased PTH mRNA levels to normal in uraemic rats. Liver enzymes were normal in all groups. Adenine-induced uraemia decreased liver weight with no additional effect of La. Liver weights corrected for body-weights were the same in all study groups, including the La group. Therefore, La had no effect upon absolute or corrected liver weight. Liver magnetic resonance imaging and microscopy did not reveal toxic changes due to La.

Conclusions. These findings confirm that in rats with adenine-induced uraemia, the P-binder La reverses the hyperphosphataemia and hyperparathyroidism. They are the first demonstration that La decreases PTH gene expression. Importantly, we found no evidence of drug-induced liver toxicity.

Keywords: chronic kidney disease; gene expression; hyperphosphataemia; lanthanum carbonate; phosphate binders; secondary hyperparathyroidism

Introduction

The elevated parathyroid hormone (PTH) and disordered mineral metabolism associated with secondary hyperparathyroidism complicate the clinical course of most patients with chronic kidney disease (CKD) and, when advanced, are associated with markedly increased morbidity and mortality [1]. The hallmark of secondary hyperparathyroidism is the high level of circulating PTH, which results from increased PTH secretion, increased PTH gene expression and synthesis, and increased parathyroid gland (PT) cell proliferation [2–4]. Phosphate (P) is a major regulator of serum PTH [5–7]. Hyperphosphataemia, a factor that elevates PTH, is associated with increased mortality in patients with CKD [1]. It is not practicable to reduce serum P by dietary restriction only, because such a diet would induce protein malnutrition [8]. Thus, P binders that bind dietary P and decrease its absorption are the mainstay in the management of CKD-associated hyperphosphataemia and secondary HPT.

Lanthanum (La) carbonate is a new non-calcium (Ca)-based P binder. It has been shown to safely decrease serum P and PTH in patients on dialysis [9–13]. However, recent studies, in which La was given to rodents, have stirred a safety debate concerning possible effects on the liver. Specifically, Lacour and colleagues [14], as well as Slatopolsky et al. [15], reported increased accumulation of La in livers of uraemic rats. One study also reported that livers of La-fed uraemic rats were smaller than uraemic -control livers [14]. Thus, our aim was to examine the effects of La on mineral metabolism in uraemic rats and to assess potential liver toxicity.
Materials and methods

Animal care and procedures

The experimental protocol was generally based on the adenine-arm of the study by Lacour and colleagues [14] (Figure 1). Male Sprague–Dawley rats (300–350 g) were fed a normal control diet containing 0.65% P (TD.05397, Teklad, Madison, WI) or a uraemia-inducing 0.3% adenine diet (TD.05393). After 2 weeks, some of the uraemic rats were switched to a 0.3% adenine diet that also included 1.5% La (TD.05395), 3.0% La (TD.05394) or low (0.03%) P content (TD.05400). All diets contained 1.0% Ca.

The maintenance period lasted four additional weeks, during which rats were regularly weighed and clinically examined. During the final week, several rats underwent liver-oriented magnetic resonance imaging (MRI) (GE Signa Milwaukee, WI) under brief intraperitoneal ketamine-xylazine anaesthesia. Calculation of the liver fat was quantified on T1-weighted gradient-echo MR images as the percentage of relative signal intensity loss of the liver on out of phase images, with the following formula: 100 x (SI_{in} - SI_{out})/SI_{in}, where SI is the signal intensity of the liver, SI_{in} the in-phase signal intensity, and SI_{out} the out-phase signal intensity. The scan parameters were gradient-echo T1 imaging with repetition time (TR) 125 ms, time of echo (TE) 3.9 ms for in-phase and 6.6 ms for out-phase. Field of view (FOV) was 14 x 10 cm, slice thickness 3 mm with no intersection gap and matrix 256 x 160. Some rats were placed in metabolic cages for 6–7 days for acclimatization before metabolic studies. Urine was collected during the last 24 h.

At the completion of the study period the rats were weighed, and anaesthetized by intraperitoneal ketamine injection. All further procedures were performed to ensure that there was no cross-contamination of La from organ to organ and from the surroundings, especially external contamination from the rats’ fur, operating equipment and gastrointestinal tract. The abdominal cavity was opened, and blood was collected by exsanguination of the abdominal aorta. Plasma was separated, and placed at 4°C until La content determination.

The Institutional Animal Care and Use Committee approved all animal experiments.

Biochemical analyses

Ca, P and creatinine (Cr) were measured in urine and plasma samples, while plasma was also assayed for activity of liver enzymes, including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALKP) and gamma-glutamyl transpeptidase (γ-GTP). Measurements were conducted using an automated chemistry analyser (Kryptor, Vitros 950, Rochester, NY). Plasma PTH was measured by a Rat PTH Immunoradiometric Assay (Cat.# 50-2000, Immutopics Inc., San Clemente, CA). Creatinine clearance (CrCl) was defined as urine-volume x [urine Cr] x [plasma Cr]^{-1}/24 h. Tubular reabsorption of phosphate (TrP) was calculated as: 100 x (1 – Cp/CCr), where Cp is P clearance, defined in the same way as CrCl.

Lanthanum content determination

Frozen liver and plasma samples were mailed on dry ice for La content determination at the Centre for Analytical Sciences, University of Sheffield, UK. Results are displayed as microgram per kilogram wet-liver-weight and microgram per litre plasma, respectively.

PTH gene expression studies

Total RNA was extracted from thyroparathyroid complexes by TRI-Reagent (Molecular Research Center, Cincinnati, OH), and resuspended in water. Thyroparathyroid RNA of 2 μg from each rat (quantified by Nanodrop ND-1000, Prolab Biotechnologie, Erlangen, Germany) underwent reverse transcription with oligo-dT and random hexamer primers (M-MLV RT, Promega Corporation, Madison, WI), while the remaining RNA was blotted on Hybond-N membranes (Amersham Biosciences, Buckinghamshire, UK), and hybridized to rat PTH and the control gene ubiquitin C (Ubc), which we found to be similarly expressed across the study groups, spanned large intronic areas to prevent amplification of genomic DNA. Amplicon specificity was ascertained by melting curve analysis as well as length evaluation by ethidium bromide staining of agarose gels. The sequences for rat PTH sense and antisense primers [16] were TTGTCTCTTACCAGGCGAGAT and TTTGCCAGGTTTGTCATATA, respectively. The primers for rat Ubc sense and antisense were designed using Primer Express.
software v2.0 (Applied Biosystems, Foster City, CA), and were TAAGACCATCACCTGGAGGTC and GGATGCCTCCTTGTCCT, respectively. Quantification was done by the standard-curve method.

Statistical analyses

Values are reported as mean ± SEM, unless stated otherwise. One-way ANOVA with Dunnett post hoc analysis was used to assess differences from the uraemic control group (SPSS 13.0, SPSS Inc., Chicago, IL). A two-sided P-value was considered significant when <0.05.

Results

The adenine-induced uraemia model is associated with growth arrest

Following the 2-week induction period (Figure 1), rats consumed either a normal control (NC) diet, a uraemic control (UC) diet supplemented with 0.3% adenine, a uraemic diet with 1.5% (ULa1.5) or 3.0% (ULa3.0) La, or a uraemic diet with low P content (ULP). While NC rats gained weight throughout the study period (Figure 2), UC, ULa1.5 and ULa3.0 rats maintained a relatively steady weight, with minor differences amongst these subgroups. ULP rats lost ~20% of initial body weight. Variations in food consumption amongst the groups (data not shown) may partly explain inter-group weight differences. There was no mortality.

Kidney function is severely reduced in adenine-fed rats

As expected, plasma Cr was elevated in adenine-fed rats, without differences among the subgroups (Table 1). This finding was verified by measurement of CCr, conducted in metabolic cages during the last 24h, following a 5–6-day adaptation period. CCr (ml/min) was 1.52 ± 0.20 in NC rats, compared with 0.19 ± 0.03 in UC rats, 0.13 ± 0.04 in ULa1.5 rats and 0.18 ± 0.04 in ULa3.0 rats (n = 3 in each subgroup, P < 0.01 for the comparison between NC and other subgroups).

Plasma Ca and P levels in rats fed adenine diet with and without La

The adenine (UC) diet was associated with elevated plasma P levels (Table 1), which were normalized by ULa3.0 diet and markedly reduced by ULP diet. Plasma Ca was unchanged in UC and ULa1.5 rats, but was increased in the ULa3.0 and ULP subgroups [studies in metabolic cages implied that La treatment induced a dose-dependent increase in urinary Ca excretion (data not shown)]. Despite the increased serum Ca, the Ca–P product was significantly lower in the ULa3.0 and ULP rats compared with uraemic controls (Table 1). Studies in metabolic cages further elucidated the changes in P handling induced by the different diets (Figure 3). The UC diet was associated with unchanged 24-h urine P excretion and decreased TRP reflecting the increased serum P and Cr levels. In contrast, La-supplemented adenine diets caused dose-dependent reductions in urine P, associated with normalization of TRP.

Increased PTH levels in uraemic rats are decreased by La

Plasma PTH levels in normal (NC) rats were 51 ± 8 pg/ml (Table 1). Uraemia, induced by the adenine diet (UC), nearly tripled this level. ULa1.5 non-significantly decreased plasma PTH compared with the UC diet, whereas PTH levels were reduced in

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Cr (mmol/l)</th>
<th>Ca (mmol/l)</th>
<th>P (mmol/l)</th>
<th>Ca × P product (mmol²/l²)</th>
<th>PTH (pg/ml)</th>
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<tbody>
<tr>
<td>NC</td>
<td>20</td>
<td>52 ± 2*</td>
<td>2.66 ± 0.02</td>
<td>2.26 ± 0.04*</td>
<td>6.00 ± 0.12*</td>
<td>51 ± 8*</td>
</tr>
<tr>
<td>UC</td>
<td>20</td>
<td>301 ± 23</td>
<td>2.69 ± 0.03</td>
<td>3.20 ± 0.20</td>
<td>8.65 ± 0.58</td>
<td>139 ± 26</td>
</tr>
<tr>
<td>ULa1.5</td>
<td>10</td>
<td>377 ± 60</td>
<td>2.78 ± 0.06</td>
<td>3.27 ± 0.33</td>
<td>9.18 ± 0.99</td>
<td>99 ± 40</td>
</tr>
<tr>
<td>ULa3.0</td>
<td>15</td>
<td>365 ± 23</td>
<td>2.95 ± 0.05*</td>
<td>2.20 ± 0.25*</td>
<td>6.32 ± 0.64*</td>
<td>11 ± 3*</td>
</tr>
<tr>
<td>ULP</td>
<td>5</td>
<td>234 ± 26</td>
<td>3.16 ± 0.04*</td>
<td>0.97 ± 0.05*</td>
<td>3.08 ± 0.15*</td>
<td>6 ± 2*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.
NC, normal controls; UC, uraemic controls; ULa1.5, 1.5%-La treated uraemic rats; ULa3.0, 3.0%-La treated uraemic rats; ULP, low-P fed uraemic rats.

*P < 0.01 for the comparison with the uraemic (UC) group.
the ULa3.0 and ULP subgroups. PTH levels plotted against serum P showed an exponential relationship. This relationship between PTH and plasma P was shifted to the right by 3% La (Figure 4). This may reflect the correction of serum Ca and P in the 3% La group.

La decreases PTH gene expression

Thyroparathyroid PTH mRNA levels were increased in uraemia as measured by northern blot (Figure 5A), and real time PCR results (Figure 5B). Both display a 2-fold elevation in PTH mRNA levels due to uraemia. ULa3.0 and ULP diets decreased these elevated levels. Thus, the changes noted in plasma PTH were associated with alterations in PTH gene expression.

Lanthanum concentrations in livers of La-fed rats

Liver La content in uraemic rats that did not receive La was at detection limit. Liver La content in La treated uraemic rats is depicted in Table 2. La was also measured in plasma (Table 2).

La did not cause liver toxicity as shown by plasma biochemistry, magnetic resonance scans, gross morphology and light microscopy

In order to find whether increased La content is toxic to the liver, we initially measured plasma liver enzymes (Table 2). No abnormal values were found in association with either adenine or La supplementation. In addition, 3–5 rats of each group underwent liver-oriented magnetic resonance scans during the last week of the study period. There were no focal or generalized abnormalities in any study group (Figure 6A). Calculation of the signal intensity index ruled out infiltrative liver changes in adenine or La-treated rats; NC 15±1%, UC 17±2%, ULa3.0 14±3% and ULP 18±1%.

At necropsy, livers appeared grossly normal. The livers of uraemic rats were found to weigh less than the livers of normal rats (Figure 7). However, La supplementation did not further influence liver weight. Moreover, when corrected for body weight, all livers were alike, averaging 3% of total weight. Finally, although accentuation of the sinusoidal structure was noted in all uraemic rats, microscopic examination of haematoxylin–eosin stained liver sections revealed no evidence of drug-induced liver toxicity associated with either adenine or La treatment (Figure 6B).
Mild Kupffer cell hypertrophy and hyperplasia were observed, most notably in the ULP group.

**Discussion**

This study confirms the efficacy and safety of La carbonate treatment in hyperparathyroid uraemic rats. Adenine supplementation resulted in severe azotaemia, which was similar across study groups, regardless of other dietary manipulations (namely, P restriction or addition of La). Despite their similar degree of renal failure, 3% La corrected plasma P levels and the Ca x P product. A similar reduction in plasma P by 3% La was reported in 5/6 nephrectomized uraemic rats at 45 days by Slatopolsky et al. [15]. Lacour et al. [14]

**Table 2. Lanthanum contents and plasma liver enzymes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lanthanum content</th>
<th>Plasma, (µg/l)</th>
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<tbody>
<tr>
<td></td>
<td>Liver, (µg/kg wet)</td>
<td>Plasma, (µg/l)</td>
</tr>
<tr>
<td>NC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UC</td>
<td>0.08±0.08</td>
<td>0.05±0.03</td>
</tr>
<tr>
<td>ULa1.5</td>
<td>636±77*</td>
<td>0.87±0.19*</td>
</tr>
<tr>
<td>ULa3.0</td>
<td>662±62*</td>
<td>0.94±0.26*</td>
</tr>
<tr>
<td>ULP</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.
NC, normal controls; UC, uraemic controls; ULa1.5, 1.5%-La-treated uraemic rats; ULa3.0, 3.0%-La treated uraemic rats; ULP, low-P fed uraemic rats; AST, aspartate transaminase; ALT, alanine transaminase; ALKP, alkaline phosphatase; γGTP, gammaglutaryl transpeptidase; ND, not done.
*P < 0.01 for the comparison with the uraemic (UC) group.

![Fig. 6. Lanthanum was not hepatotoxic as shown by MRI and light microscopy. During the last week of the study, rats were anaesthetized and underwent longitudinal (A, top panel) and axial (A, bottom panel) magnetic resonance imaging. There were no focal, general or infiltrative changes (n=3 for each group, except n=5 in the 3% La group). Light microscopy (B) showed no signs of hepatotoxicity, as exemplified by representative portal triad (B, top panel) and central vein (B, bottom panel) structures (H&E staining, magnified 400×; n=10 for each group, except n=5 in the low P group).](image-url)
studied rats with experimental uraemia due to adenine or 5/6 nephrectomy, and 3% La led to a decrease in plasma P. Our results show that 1.5% La, unlike 3% La, decreased urine P with no change in plasma P. A sub-optimal La/P ratio in the 1.5% La group may explain the unchanged plasma P values compared with uraemic control rats.

PTH levels of uraemic rats were significantly lowered by 3.0% La, similar to PTH levels due to P restriction. In fact, these levels were not statistically different from the values in NC. However, the relatively high variability of the plasma PTH assay in its lower range does not allow us to determine whether La decreases PTH to levels lower than normal. PT PTH mRNA content was increased by uraemia and reduced by 3% La correlating with the changes in plasma PTH levels. The decreased PTH mRNA levels due to La were less than the effect on serum PTH levels. This may be a result of additional effects upon PTH translation and secretion. Further research is needed to clarify the mechanism of plasma PTH reduction and PTH mRNA down-regulation by La. Correlations of plasma PTH with P were shifted to the right in the 3% La group (Figure 4). This may reflect an effect of La upon serum Ca and P to decrease PTH levels.

It is clear that some percentage of La is absorbed from the diet. Indeed, in La-fed uraemic rats, La was measurable in plasma (although, in contrast to Lacour’s study [14], virtually no La was detected in La-naïve rats). Additionally, La deposited in livers of uraemic rats as expected (Zhang et al., 2006; J Microscopy, in press). The potential of La to adversely affect the liver was thoroughly investigated in our study.

First, plasma transaminase and cholestatic enzyme activities were assayed. These measurements disclosed no abnormal values. Then, we evaluated liver-oriented magnetic resonance images of La-treated and control rats. These disclosed no local or focal abnormalities. Neither was there evidence of metal or fatty liver infiltration. Next, livers were weighed at necropsy. Liver weight was similar in all uraemic rats, regardless of La supplementation. Furthermore, correction for body weight resulted in homogeneous results across all study groups, including normal rats (~3% of total body weight).

This contrasts with findings by Lacour et al. [14], which were further discussed by correspondence [17]. Correction for femur length instead of body weight may partly explain the diverse findings. In Lacour’s study [14], rats that had not been given La in their diet also had significant levels of La in their blood. This suggests that there was cross-contamination of food or feces from the La group to the control group, which can be a vexing problem in La studies where only a minute amount is absorbed.

Last, but not least, livers were carefully evaluated by light microscopy. While accentuation of the sinusoidal structure was noticed in all uraemic rats, and mild Kupffer cell hyperplasia and hypertrophy was noted especially in the P-restricted group, there were no signs of drug-induced liver parenchymal or bile duct toxicity. Similar findings were previously reported by Slatopolsky and coworkers [15], which underline the lack of hepatotoxicity of La in these uraemic models. Damment et al. [18] also found no evidence of adverse effects on liver after repeated intravenous administration of high doses to rats; in particular, there was no increase in unscheduled DNA synthesis in hepatocytes.

Study limitations include the absence of a high-P diet uraemic control group (due to the similarity with Lacour et al.’s [14] protocol). We are currently conducting mechanistic studies that integrate this control. Additionally, we did not examine the source for the mild elevation in serum Ca noted in the 3% La-fed uraemic rats. However, a similar finding was noted in other animal studies of non-Ca-containing P binders. Our urine metabolic studies imply a La-dose-dependent increase in urinary Ca excretion (data not shown). Thus, hypocalcaemia does not explain the mild hypercalcaemia. Mineral (re)absorption from bone and GI should probably be examined.

In summary, we found La, at a dose of 3%, to be an effective P binder in terms of plasma P, Ca × P product and PTH levels reduction. Plasma PTH decrements were linked to PTH mRNA down-regulation in the PTs. Efficacy was accompanied by increased liver La content. However, an extensive search for liver toxicity revealed no such signs, at least in this intermediate-term study.

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Conflict of interest statement. This work was supported in part by a grant from Shire Pharmaceuticals.

(See related article by Cozzolino and Brancaccio. Lanthanum carbonate—new data on parathyroid hormone control without liver damage. Nephrol Dial Transplant 2007; 22: 316–318.)
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