Adequate phosphate binding with lanthanum carbonate attenuates arterial calcification in chronic renal failure rats

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Abstract

Background. Hyperphosphataemia is a risk factor for arterial calcification contributing to the high cardiovascular mortality in patients with chronic kidney disease. Calcium-based phosphate binders can induce hypercalcemia and are associated with progression of vascular calcification. Therefore, the effect of lanthanum carbonate, a non-calcium phosphate binder, on the development of vascular calcification was investigated in uraemic rats.

Methods. Chronic renal failure (CRF) was induced by feeding rats an adenine-enriched diet for 4 weeks. After 2 weeks, 1% or 2% lanthanum carbonate was added to the diet for 6 weeks. Calcification in the aorta, carotid and femoral arteries was evaluated histomorphometrically, biochemically


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and by ex vivo micro-CT. Chondro-/osteogenic conversion of vascular smooth muscle cells was also analysed in the rat aorta.

Results. Treatment with 1% lanthanum carbonate (1% La) did not reduce vascular calcification, but in the 2% lanthanum carbonate (2% La) group vascular calcium content and area% Von Kossa positivity were decreased compared with control CRF rats. The aortic calcified volume measured with ex vivo micro-CT was significantly reduced in rats treated with 2% La. Although calcification was inhibited by treatment with 2% La, the chondrocyte transcription factor sox-9 was abundantly expressed in the aorta.

Conclusion. Treatment of CRF rats with 2% La reduces the development of vascular calcification by adequate phosphate binding resulting in a decreased supply of phosphate as a substrate for vascular calcification.

Keywords: cardiovascular disease; hyperphosphataemia; media calcifications; phosphate binders; uraemia

Introduction

Impaired phosphate excretion due to renal failure results in hyperphosphataemia, leading to secondary hyperparathyroidism and the development of renal osteodystrophy. In addition, clinical studies revealed that excessive serum phosphate concentrations and an increased calcium × phosphorus product are associated with the development of vascular calcifications [1,2] and increased cardiovascular mortality [3,4] in patients with renal failure. The role of phosphate in the calcification process is further supported by in vitro experiments. Incubation of aortic smooth muscle cells with high phosphate levels, transported into the cell via the sodium-dependent phosphate co-transporter Pit-1, results in osteochondrogenic transdifferentiation and the formation of hydroxyapatite crystals [5].

Dietary phosphate restriction and phosphate clearance during dialysis in patients with end-stage renal disease are insufficient to achieve serum phosphate concentrations within the normal range. In order to adequately lower serum phosphate levels and prevent the development of secondary hyperparathyroidism, these patients are often treated with calcium-containing phosphate binders, frequently combined with 1,25-(OH)2 vitamin D3. Despite the efficient phosphate-binding capacity of these phosphate binders, this treatment strategy may induce hypercalcaemic episodes, which are associated with an increased prevalence and progression of vascular calcification [1,6,7]. Indeed, cell and organ culture experiments showed that calcium is another important inducer of arterial smooth muscle cell calcification [8,9].

Sevelamer hydrochloride, a non-calcium phosphate binder, has been shown to attenuate the progression of vascular calcification in pre-dialysis patients [10], as well as in prevalent [11] and incident [12] haemodialysis patients. However, from clinical studies it is unclear whether this is due to phosphate binding without increasing the calcium load or to its lipid-lowering effects.

Lanthanum carbonate has proven to be an efficacious and well-tolerated non-calcium phosphate binder. Since lanthanum carbonate does not induce hypercalcaemic episodes, nor exerts any effect on lipid metabolism, this compound allows for the investigation of the isolated effect of phosphate binding on the development of arterial calcification.

The present study investigates whether lanthanum carbonate treatment can reduce the development of vessel calcifications in rats subjected to adenine-induced chronic renal failure (CRF), a model that typically develops media calcifications. Transdifferentiation of arterial smooth muscle cells towards a chondro-/osteogenic phenotype is a well-known mechanism underlying the process of arterial calcification [13,14] driven by phosphate. Therefore, the effect of phosphate binding on this conversion process was investigated by analysis of the expression of the osteoblast transcription factor core binding factor alpha1 (cbfa-1) and the chondrocyte-specific markers sox-9 and collagen II. Finally, recent reports highlighting the interaction between vessel calcification and bone turnover [15–17] prompted analysis of bone status in CRF rats treated with lanthanum carbonate.

Methods

Experimental design

Experimental procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals 85-23 (1996) and approved by the University of Antwerp Ethics Committee.

Before the start of the study, 10-week-old male Wistar rats (Iffa Credo, Brussels, Belgium) were conditioned on a high-phosphorus diet [1.03% phosphorus, 1.06% calcium and 19% protein (protein source: soya)] (SS-NIFF Spezialdiäten, Soest, Germany) for 2 weeks. To induce CRF, rats were fed a diet containing 0.75% adenine (0.92% phosphorus, 1.0% calcium and 19% protein) for 4 weeks [18,19]. Two weeks after starting adenine feeding, 2% cellulose (CRF control group), 1% lanthanum carbonate (1% La) or 2% lanthanum carbonate (2% La) was added to the diet. After adenine withdrawal, these agents were further administered in combination with a high-phosphorus diet (1.03% phosphorus, 1.06% calcium and 19% protein) until sacrifice after 8 weeks of CRF. In order to accurately measure lanthanum concentrations in various organ samples, the treatment diet was replaced by the lanthanum-free high-phosphorus diet 24 h before sacrifice, i.e. before the last blood sampling at Week 8, to avoid contamination of tissue samples with exogenous lanthanum from the powdered diet. Twenty animals were included in each study group. Blood sampling via the tail vein and 24-h urine collections were performed before the equilibration period, before the start of adenine feeding (pre-CRF) and before sacrifice. Additional 3-h urine collections and blood sampling were performed before the start of treatment at Week 2 and during treatment at Week 5.

At sacrifice, animals were exsanguinated through the retro-orbital plexus after anaesthesia with sodium pentobarbital (Nembutal, Ceva Santé Animale, France) 60 mg/kg via intraperitoneal injection.

Serum and urine biochemistry

Serum creatinine, calcium and phosphate concentrations were measured on a Vitros 750 XRC (Ortho Clinical Diagnostics, Rochester, NY) autoanalyser system. Urinary calcium was analysed with flame atomic absorption spectrometry (Perkin-Elmer, Wellesley, MA, USA), and urinary phosphate was measured with the Ecoline⃝ Phosphate kit (DiaSys, Holzheim, Germany). Serum PTH was measured with the rat PTH–IRMA kit (Immunotech Inc, San Clemente, CA, USA). A radioimmunoassay kit (Biosource, Nivelles, Belgium) was used for the measurement of serum 1,25-(OH)2 vitamin D.

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Serum/tissue lanthanum measurement

Serum samples were initially freeze dried at −48 °C for 72 h. After adding 300 µl of high purity HNO₃ and 50 µl of H₂O₂, the mixtures were further digested by heating in a microwave in three successive steps at 80, 160 and 240 Watt. The digests were then diluted to the initial volumes giving final acid concentrations of 1–2%. Tissue samples were wet-weighed and digested in 65% nitric acid at 60 °C overnight. The lanthanum concentration in the digests was measured using ICP-MS (Model 810, Varian Inc, Australia). The serum and tissue lanthanum concentrations are expressed as microgram/l and microgram/g wet tissue respectively.

Evaluation of vascular calcification

Calcium content. The left carotid and femoral arteries and the proximal abdominal aorta were weighed and subsequently digested in 65% nitric acid. The total calcium content was measured with flame atomic absorption spectrometry. Results were expressed as milligram calcium per gram wet tissue.

Ex vivo micro-CT. Prior to paraffin embedding, isolated proximal aortas, comprising the aortic arch and the thoracic aorta, were scanned for 50 min using a desktop micro-CT scanner (Skyscan 1076, Kontich, Belgium) with a 10-mega-pixel CCD camera and a micro-focal x-ray source with 5 µm spot size. A pixel size of 35 µm was selected, and a 1 mm aluminium filter was used. Virtual cross sections were reconstructed with Feldkamp’s cone beam algorithm. Vascular calcification was calculated as the number of voxels above a density threshold of 65 Hounsfield Units and expressed as calcified volume (mm³).

Histomorphometry. The right carotid and femoral arteries and the proximal aorta were fixed in neutral buffered formalin and cut into 2–3 mm thick rings that were embedded upright in the same paraffin block, so that every section comprised on average 16 cross sections for the aorta and 5 cross sections for the carotid and femoral arteries at different sites along the vessel. Four µm sections were stained with Von Kossa’s method. Vascular calcifications were then evaluated histomorphometrically with Kontron 400 2.0 image analysis software (Kontron, Nashville, TN, USA) at magnification 100 ×. The absolute areas of tissue and calcified tissue were summed for each animal, and the ratio was expressed as area% aortic calcification.

Immunohistochemistry

Immunohistochemical stainings for osteogenic transcription factor cbfa-1 (sc-8566) and the chondrocyte markers sox-9 (sc-17340) and collagen II (sc-7764) (Santa Cruz Biotechnology, CA, USA) were performed on aortic sections. For all immunohistochemical stainings, a biotinylated horse anti-goat IgG (Vector Laboratories, Burlington, CA, USA) was used as secondary antibody. Avidin and biotinylated horseradish peroxidase (VECTASTAIN® ABC KIT, Vector Laboratories) were added as signal amplifiers. 3-Amino-9-ethylcarbazole (AEC, Sigma-Aldrich, Bornem, Belgium) was used as chromogen. Sections in which the primary antibody was omitted served as negative controls. Per animal 12–26 aortic cross sections were scored semi-quantitatively for the expression of the respective proteins using a score system ranging from 0 to 3. Score 0 represents no expression, score 1 focal expression, score 2 partial expression taking up 20–80% of vessel circumference and score 3 circumferential expression (>80%). Within each group the percentage of cross sections per score was calculated.

Bone histomorphometry

The left tibia was fixed in Burkhardt’s solution, dehydrated and embedded in 100% methylmetacrylate (Merck, Hohenbrunn, Germany). Five micrometre sections were Goldner-stained for histomorphometric analysis of the proximal metaphysis with AxiosVision Release 4.5 software (Carl Zeiss, Germany). Bone parameters were calculated out of the primary measurements according to the American Society of Bone and Mineral Research standards [20]. Bone histomorphometric data of rats with normal renal function from previous studies served as reference values.

Statistical analysis

Data were expressed as mean ± standard deviation unless stated otherwise. Statistics were performed with SPSS 14.0. Differences between multiple time points for each study group were determined by the Friedman test, followed by a Wilcoxon signed-rank test with Bonferroni correction. Comparisons between the study groups for each time point were assessed using a Kruskal–Wallis test, followed by a Mann–Whitney U-test in combination with Bonferroni correction. Chi-square analysis was performed to compare the % calcified animals and the % cross sections with high (score 2 or 3) versus no or focal (score 0 or 1) protein expression. Spearman correlations were made between the serum phosphate levels and the calcification score and between the protein expression and the degree of calcification. A value of $P < 0.05$ was considered significant.

Results

The mortality in this study was minimal. One animal of the control group and one animal of the group treated with 2% La died 5 weeks after CRF induction.

Biochemical analyses

Serum and urine biochemistry is summarized in Table 1.

Renal function. After 2 weeks of adenine feeding, serum creatinine concentrations were four times higher compared to baseline values and remained significantly elevated in all groups until sacrifice, indicating that a stable, moderate-to-severe CRF was induced. Lanthanum carbonate treatment had no effect on renal function.

Mineral balance. CRF induction resulted in elevated serum phosphate concentrations, which were significantly increased at Week 5 with values up to 12.07 ± 3.44 mg/dl, and significantly lower serum calcium concentrations at Weeks 2, 5 and 8. Treatment with lanthanum carbonate prevented the induction of hyperphosphataemia with both 1% and 2% doses, which was accompanied by a partial restoration of the serum calcium levels at Week 5. However, at Week 8, serum phosphate was not different between the three study groups due to lanthanum carbonate withdrawal 24 h before blood sampling to avoid tissue lanthanum contamination from the powdered diet during sacrifice.

Administration of a high-phosphorus diet for 2 weeks before the start of CRF induction resulted in raised urinary phosphate concentrations (pre-CRF). After 8 weeks of CRF, urinary phosphate excretion was significantly lower in all groups compared to pre-CRF. Treatment with 2% La further decreased the already reduced phosphate excretion at Week 8. The urinary calcium concentration was significantly lower after 2 weeks on a high-phosphorus diet, just before the start of CRF induction (pre-CRF versus pre-equilibration values). Urinary calcium excretion was significantly increased under lanthanum carbonate treatment compared to pre-CRF baseline values, but no difference was noted between the three groups at Week 8. Renal insufficiency led to a striking reduction of $1,25-(OH)_2$ vitamin D at the study end, but no significant difference was observed between the treatment groups. Renal impairment caused severe hyperparathyroidism, as indicated by a dramatic increase in serum PTH levels from 88.5 ± 35.1 pg/ml at pre-CRF to 926 ± 516 pg/ml at the end of the experiment. Administration of either 1% La or 2% La had no lowering
Table 1. Serum and urine biochemistry. Immediately after sampling at week 2, lanthanum treatment was started and continued until 24 h before sacrifice.

<table>
<thead>
<tr>
<th></th>
<th>Pre-equilibration</th>
<th>Pre-CRF</th>
<th>2-Week CRF</th>
<th>5-Week CRF</th>
<th>8-Week CRF ▲</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>CRF</td>
<td>0.38 ± 0.11a</td>
<td>0.42 ± 0.04</td>
<td>1.64 ± 0.32a</td>
<td>2.03 ± 0.42a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>0.38 ± 0.09</td>
<td>0.44 ± 0.07</td>
<td>1.78 ± 0.47a</td>
<td>2.22 ± 0.67a</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>0.34 ± 0.05a</td>
<td>0.45 ± 0.07</td>
<td>1.62 ± 0.40a</td>
<td>2.15 ± 0.39a</td>
</tr>
<tr>
<td>Phosphate (mg/dl)</td>
<td>CRF</td>
<td>7.65 ± 1.00</td>
<td>7.72 ± 0.76</td>
<td>8.25 ± 1.94</td>
<td>12.07 ± 3.44a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>7.58 ± 1.52</td>
<td>8.04 ± 0.79</td>
<td>9.36 ± 2.51</td>
<td>9.47 ± 2.24a</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>7.68 ± 1.02</td>
<td>8.17 ± 0.72</td>
<td>8.69 ± 2.07</td>
<td>8.32 ± 1.92a</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>CRF</td>
<td>9.91 ± 0.32</td>
<td>9.69 ± 0.24</td>
<td>8.06 ± 0.61a</td>
<td>5.29 ± 1.29a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>9.80 ± 0.19</td>
<td>9.68 ± 0.60</td>
<td>8.02 ± 0.57a</td>
<td>6.81 ± 1.00a</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>9.73 ± 0.35</td>
<td>9.76 ± 0.26</td>
<td>7.87 ± 0.56a</td>
<td>7.06 ± 1.83a</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>CRF</td>
<td>88.5 ± 35.1</td>
<td>87.9 ± 45.6</td>
<td>1137 ± 519a</td>
<td>1436 ± 736a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>152.9 ± 76.9</td>
<td>1371 ± 674a</td>
<td>1633 ± 995a</td>
<td>1633 ± 995a</td>
</tr>
<tr>
<td>1,25-(OH)2 vitamin D (pg/ml)</td>
<td>CRF</td>
<td>71.7 ± 2.9</td>
<td>73.7 ± 5.0</td>
<td>46.1 ± 20.1a</td>
<td>31.9 ± 20.2a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
<td>82.0 ± 11.3</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td>Phosphate (mg/24 h)</td>
<td>CRF</td>
<td>11.2 ± 6.0a</td>
<td>185.8 ± 28.4</td>
<td>63.1 ± 29.4a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>12.4 ± 4.3a</td>
<td>164.2 ± 32.4</td>
<td>63.9 ± 28.4a</td>
<td>39.3 ± 27.2a,b</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>15.0 ± 7.7a</td>
<td>190.7 ± 42.5</td>
<td>63.9 ± 28.4a</td>
<td>39.3 ± 27.2a,b</td>
</tr>
<tr>
<td>Calcium (mg/24 h)</td>
<td>CRF</td>
<td>2.99 ± 1.66a</td>
<td>1.09 ± 0.42</td>
<td>1.75 ± 1.05</td>
<td>2.40 ± 1.25a</td>
</tr>
<tr>
<td></td>
<td>1% La</td>
<td>2.16 ± 0.80a</td>
<td>1.12 ± 0.35</td>
<td>2.40 ± 1.25a</td>
<td>2.89 ± 1.98a</td>
</tr>
<tr>
<td></td>
<td>2% La</td>
<td>2.59 ± 1.13a</td>
<td>1.35 ± 0.62</td>
<td>2.40 ± 1.25a</td>
<td>2.89 ± 1.98a</td>
</tr>
</tbody>
</table>

La = lanthanum, CRF = chronic renal failure.

Table 2. Serum (µg/l) and tissue (µg/g wet weight) lanthanum concentration of untreated and lanthanum-treated CRF rats

<table>
<thead>
<tr>
<th></th>
<th>CRF</th>
<th>1% La</th>
<th>2% La</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.39 ± 0.40</td>
<td>0.34 ± 0.40</td>
<td>1.07 ± 0.92a</td>
</tr>
<tr>
<td>Aorta</td>
<td>0.61 ± 0.72</td>
<td>1.35 ± 2.35</td>
<td>0.90 ± 0.57a</td>
</tr>
<tr>
<td>Heart</td>
<td>0.14 ± 0.18</td>
<td>0.26 ± 0.33</td>
<td>0.17 ± 0.21</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.12 ± 0.16</td>
<td>0.44 ± 0.58a</td>
<td>0.51 ± 0.27a</td>
</tr>
<tr>
<td>Liver</td>
<td>0.09 ± 0.11</td>
<td>0.78 ± 0.26a</td>
<td>1.13 ± 0.41a</td>
</tr>
<tr>
<td>Femur</td>
<td>0.07 ± 0.07</td>
<td>0.42 ± 0.13a</td>
<td>0.86 ± 0.25a</td>
</tr>
</tbody>
</table>

CRF = chronic renal failure, La = lanthanum. 
P < 0.05 versus CRF group.

Vascular calcification

To evaluate the effect of lanthanum carbonate treatment on the development of ureaemia-related vascular calcification, both the area% Von Kossa positivity (Figure 1, upper graphs) and the calcium content (Figure 1, middle graphs) were measured in the aorta and the carotid and femoral arteries. Calcification in the proximal aorta was also measured by ex vivo micro-CT scanning (Figure 1, lower graph).

Treatment with 1% La did not reduce vascular calcification. In contrast, the calcified volume measured with ex vivo micro-CT was significantly reduced in the 2% La group in comparison with untreated CRF rats, which was further evidenced by the consistent decreasing trend in both the area% media calcification (Von Kossa) and the calcium concentration in the vessels. Considering a composite cut-off value of 0.5 area% Von Kossa positivity and 0.5 mg Ca/g wet tissue, 7/19 untreated CRF rats and 12/20 1% La-treated CRF rats developed vascular calcification, while only 1/19 of the animals treated with 2% La showed media calcification in the aorta (P < 0.05). The animal treated with 2% La that died before the end of the study did not show aortic calcification (0.23 mg Ca/g wet tissue and 0.07 area% media calcification), whereas the untreated rat that died 5 weeks after CRF induction had already developed severe media calcification (6.14 mg Ca/g wet tissue and 18.5 area% media calcification).

To investigate the role of phosphate in the development of vascular calcification, serum phosphate levels between calcified animals and non-calcified animals at Week 5 were compared in the groups treated with lanthanum carbonate. Serum phosphate concentrations were significantly higher in the calcified animals receiving lanthanum carbonate compared to non-calcified rats receiving the phosphate binder (P = 0.02) and correlated with the area% Von Kossa.
Fig. 1. Effect of lanthanum carbonate on artery calcification. Evaluation of vascular calcification in untreated (white diamonds) and 1% (grey diamonds)- or 2% lanthanum carbonate (black diamonds)-treated CRF rats. Individual area% Von Kossa positivity (upper row) and total calcium concentration (middle row) in the aorta and the carotid and femoral artery are presented. In the aorta, the calcified volume measured by ex vivo micro-CT is also plotted (lower row). *P < 0.05 versus CRF #P < 0.05 versus 1% La.

positivity ($r = 0.40, P = 0.01$), the calcium content ($r = 0.44, P = 0.01$) and the calcified volume ($r = 0.37, P = 0.02$) in the aorta.

Osteochondrogenic conversion markers in the aorta

Figure 2 (upper graphs) shows the extent of osteoblast and chondrocyte markers, as determined semi-quantitatively, in the aortic wall within each group. The chondrogenic transcription factor sox-9 was highly expressed in the aortic tunica media of the untreated CRF rats. The chondrocyte-specific matrix protein collagen II was also abundantly expressed, while the expression of the osteoblast transcription factor cbfa-1 was less pronounced. The expression of cbfa-1 and collagen II was significantly less pronounced in the aorta of rats treated with 2% La compared to the
Fig. 2. Expression of osteo-/chondrogenic conversion markers in the aorta. The extent of osteoblast and chondrocyte markers was determined semi-quantitatively with scores ranging from 0 (no expression) to 3 (circumferential expression). The upper graphs present the % cross sections with a respective score for each marker within each treatment group. The lower graphs show the degree of protein expression for calcified versus non-calcified animals. ∗P < 0.01: % cross sections with high expression (score 2 and 3) versus no or focal expression (score 0 and 1) between the groups.

untreated CRF group. However, sox-9 was expressed in the 2% La group to the same extent as in the untreated CRF rats, indicating that expression of a specific chondrocyte marker was also induced in rats where the development of media calcification was prevented by treatment with 2% La. The expression of all markers was significantly higher in the aorta of calcified versus non-calcified animals (Figure 2, lower graphs). However, the expression of cbfa-1 (r = 0.76, P < 0.01) and collagen II (r = 0.57, P < 0.01) were highly correlated with the presence of calcified areas, whereas sox-9 (r = 0.16, P < 0.01) was highly correlated to a much lower extent with vessel calcification and was also highly expressed in non-calcified animals as presented in Figure 2. Figure 3 shows the expression of the osteo-/chondrogenic markers (3F–H) in the aortic media of a calcified untreated CRF rat (3E). Significant expression of sox-9 (3C) is also present in the aortic media of an uncalcified animal treated with 2% La (3A), whereas collagen II (3D) was only weakly expressed and cbfa-1 was not induced (3B).

**Bone histomorphometry**

Bone histomorphometric data are shown in Table 3. Induction of CRF with an adenine-enriched diet for 4 weeks significantly increased the osteoid area, perimeter and width as well as the osteoblast perimeter. Both the eroded perimeter and osteoclast perimeter tended to be elevated in CRF rats compared to in-house reference values of animals with normal renal function. These data reflect an increased bone turnover, the hallmark of secondary hyperparathyroidism.

Treatment with 1% La resulted in a significantly higher bone area and osteoid width compared to untreated CRF animals, whereas all other bone parameters did not change significantly. In 2% La-treated CRF rats neither osteoid
parameters (osteoid area, perimeter and width) nor any of the other bone parameters showed significant differences in comparison with the untreated CRF rats.

**Discussion**

In order to reduce hyperphosphataemia and secondary hyperparathyroidism, most patients with end-stage renal disease receive calcium-containing phosphate binders. Recent concern exists about the contribution of excess exogenous calcium in accelerating vessel calcification. Therefore, we investigated the effect of two lanthanum carbonate doses, a non-calcium phosphate binder, on the development of media calcification in an adenine-induced CRF rat model.

The present study demonstrates that treatment of adenine-induced CRF rats with 2% La inhibits the development of uraemia-related media calcification. *Ex vivo* micro-CT measurements showed that aortic media calcification in rats treated with 2% La was significantly reduced.
Lanthanum and vascular calcification

Table 3. Bone parameters of untreated and lanthanum-treated CRF rats

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>CRF</th>
<th>1% La</th>
<th>2% La</th>
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</thead>
<tbody>
<tr>
<td>Bone area (%)</td>
<td>27.6 ± 8.0</td>
<td>25.7 ± 16.5</td>
<td>37.0 ± 12.7a</td>
<td>29.2 ± 17.1</td>
</tr>
<tr>
<td>Osteoid area (%)</td>
<td>1.5 ± 1.1a</td>
<td>10.1 ± 4.0</td>
<td>11.7 ± 4.1</td>
<td>16.7 ± 11.3</td>
</tr>
<tr>
<td>Osteoid width (µm)</td>
<td>4.2 ± 0.5a</td>
<td>9.0 ± 3.8</td>
<td>13.1 ± 5.7a</td>
<td>15.1 ± 14.9</td>
</tr>
<tr>
<td>Osteoid perimeter (%)</td>
<td>9.7 ± 6.1a</td>
<td>49.3 ± 15.5</td>
<td>49.0 ± 11.6</td>
<td>55.0 ± 17.6</td>
</tr>
<tr>
<td>Eroded perimeter (%)</td>
<td>10.4 ± 3.9</td>
<td>17.0 ± 10.3</td>
<td>17.8 ± 9.4</td>
<td>13.8 ± 7.0</td>
</tr>
<tr>
<td>Osteoblast perimeter (%)</td>
<td>2.8 ± 3.1a</td>
<td>29.5 ± 14.4</td>
<td>28.2 ± 11.8</td>
<td>27.5 ± 13.7</td>
</tr>
<tr>
<td>Osteoclast perimeter (%)</td>
<td>2.0 ± 1.1</td>
<td>7.0 ± 6.0</td>
<td>4.4 ± 3.1</td>
<td>5.0 ± 3.3</td>
</tr>
</tbody>
</table>

La = lanthanum, CRF = chronic renal failure.
The first column represents reference values of control rats (CTL) with normal renal function.
Osteoblast and osteoclast perimeter are both expressed as % of total bone perimeter.

$^a$P < 0.05 versus CRF group.

compared to untreated CRF rats. Vascular calcium content and area% media calcification showed a similar trend in animals receiving the highest lanthanum dose. Moreover, the number of animals that developed vessel calcification was significantly lower in the group treated with 2% La when compared to the untreated CRF group. In this context, it is worth mentioning that the ratio of daily phosphorus diet (450 mg/kg/day) and the highest dose of phosphate binder intake (900 mg/kg/day) in our rat study are in the same range as the phosphorus intake (1200 mg/day) over lanthanum carbonate dose (1500–3000 mg/day) ratio in humans, supporting the clinical relevance of our experimental findings. The effect of this non-calcium phosphate binder on the development of uremia-induced media calcifications is in line with the data recently reported by Mathew et al [24]. Indeed, this group found that lanthanum carbonate treatment attenuated atherosclerotic plaque calcification in a LDL receptor knockout mouse model with induced chronic kidney disease.

Not all animals develop vascular calcification in the adenine-induced CRF rat model as illustrated by the results obtained in the untreated control group in which only 7 out of 19 animals calcified. At present, there is no satisfactory explanation for this important biological variability. This variable tendency to develop vascular calcifications is also well known in humans [25–27] and has been noticed by others using the same experimental model [28].

Sevelamer suppresses aortic calcification in both adenine-induced CRF [18] and 5/6 nephrectomized rats [29]. Clinical studies provide supporting evidence for these experimental findings. Both the Treat-to Goal . . . trials [11] and Renagel in New Dialysis trial [12] found that sevelamer resulted in attenuated progression of artery calcification as compared to calcium-containing phosphate binders in prevalent and incident haemodialysis patients, respectively. As phosphate and calcium are well-known mediators involved in the calcification process, it seems probable that the mechanism by which both sevelamer and lanthanum carbonate reduce vessel calcification consists of a strong phosphate-binding capacity in the intestine without excessive calcium loading. At the end of our study, urinary phosphate excretion was significantly lower in animals treated with 2% La compared to untreated rats and rats treated with 1% La. These results indicate that administration of 2% La causes a clinically relevant amount of dietary phosphate to be bound, which can explain why this lanthanum dose was able to reduce the development of artery calcification, while administration of half of the dose had no significant effect. Lanthanum carbonate also significantly reduced serum phosphate levels at Week 5, after 3 weeks of treatment. The fact that at the end of the study, serum phosphate concentrations of lanthanum-treated CRF rats were not reduced compared to untreated CRF rats was due to the withdrawal of lanthanum carbonate in the powdered diet 24 h before sacrifice to avoid lanthanum contamination of serum and tissue samples during sacrifice. Importantly, considering the rats treated with 1% or 2% La, serum phosphate concentrations were significantly lower in non-calcified animals compared to animals with aortic calcifications at Week 5, confirming that strict serum phosphate control within narrow limits is critical for the prevention of arterial calcification.

Arterial calcification involves the conversion of medial smooth muscle cells into chondrocyte- and osteoblast-like cells with the expression of various bone-related proteins [13,14,25]. Our previous study revealed that, in this adenine-induced CRF rat model, the development of media calcification is associated with the transdifferentiation of arterial smooth muscle cells into bone/cartilage-like cells [19]. Since cell culture experiments showed that phosphate is a major inducer of this transdifferentiation process [5, 30], the present study investigated whether strong phosphate binding can modulate this mechanism, besides exerting a direct physicochemical effect on vascular calcification. Interestingly, the chondrocyte transcription factor sox-9 was expressed to the same degree in 2% La-treated rats, whereas the expression of cbfa-1 and collagen II was less pronounced compared to the untreated control group. The significantly lower serum phosphate levels together with the absence of cbfa-1 expression in lanthanum-treated rats without vascular calcification as compared to calcified lanthanum-treated rats are in agreement with the molecular mechanisms underlying the process of calcification. Indeed, phosphate can enter vascular smooth muscle cells through the sodium-dependent phosphate co-transporter Pit-1, thereby inducing the expression of the osteoblast transcription factor cbfa-1 [31]. Adequate phosphate binding with 2% La reduced media calcification, however, could not completely block the initial cellular transdifferentiation mechanism underlying the onset of the calcification process. These results suggest that the highest lanthanum dose reduced media calcification by decreasing the availability of phosphate as a substrate for hydroxyapatite formation in the arterial wall and that, in addition to phosphate, other uraemic factors can modulate the transdifferentiation mechanism of vascular smooth muscle cells.

Although lanthanum carbonate is an efficacious phosphate binder, neither lanthanum dose reduced the high serum PTH concentrations induced by the relatively severe CRF in adenine rats, which can be explained by two
mechanisms. First, lanthanum, when added as the chloride compound, has been shown to block epithelial Ca\(^{2+}\) channels \textit{in vitro} [32], which are also expressed in the duodenum and jejunum. Hence, as lanthanum carbonate administered via the diet dissociates in the gut, it may inhibit intestinal calcium absorption by blocking intestinal Ca\(^{2+}\) channels in a similar way, resulting in decreased serum ionized calcium levels abolishing the potential lowering effect of decreased serum phosphate levels on PTH secretion. Second, the installation of CRF with 0.75% adenine causes a dramatic increase in serum PTH concentrations up to 925 ± 516 pg/ml at the end of the study. Possibly, this excessive induction of PTH secretion in a short time frame may result in an autonomous production of the PTH that cannot be reversed by phosphate binder administration. However, further evidence is required to support these hypotheses.

Lanthanum carbonate, administered in a dose that could reduce the development of artery calcification, did not significantly affect bone histomorphometric parameters. The excessive secondary hyperparathyroid bone disease induced by the adenine-induced renal insufficiency remained unchanged in CRF rats treated with lanthanum carbonate. Only a slight trend was noted towards an increased osteoid area and osteoid width under lanthanum carbonate treatment that can be ascribed to a reduced mineralization caused by the compound’s efficacious phosphate binding capacity as reflected by the decreased urinary phosphate excretion in rats treated with 2% La. Furthermore, the number of osteoblasts was not altered, confirming previous findings in our laboratory that a decreased mineralization of the osteoid, which also appears in rats treated with the other non-calcium phosphate binder sevelamer, is not due to a direct toxic effect of lanthanum on bone cells [21,33].

The present study indicates that, if phosphorus is well controlled, lanthanum carbonate can inhibit the development of uraemia-related media calcification without affecting the bone. These results need to be confirmed in humans. Although a tremendous discussion regarding the impact of calcium-containing phosphate binders on the progression of arterial calcification is still going on, it is yet advisable to consider non-calcium alternatives in haemodialysis patients with hypercalcaemia or in patients with manifested vessel calcification.

In summary, we found that efficient phosphate binding can reduce excessive calcification in the tunica media of adenine-induced CRF rats. Since patients with end-stage renal disease have a high cardiovascular mortality risk, lanthanum carbonate can be a promising alternative for the calcium-containing phosphate binders.

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References

Heat shock protects HPMCs

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Abstract

Background. Chronic peritoneal dialysis (PD) is one of the major therapies for uremic patients. However, the peritoneal mesothelial cells (PMCs) are subject to the injury by bioincompatible dialysates. The aim of this study is to investigate the protective roles and mechanisms of heat shock response in PMCs.

Methods. Primary cultured human PMCs (HPMCs) were subjected to commercial peritoneal dialytes. The cell viability was assayed by MTT test and Annexin V assay. The expression of HSPs was detected by Western blots analysis. Intracellular hydrogen peroxide and superoxide anion were detected using H2DCFDA and dHE probe, respectively, with flow cytometry. The mitochondrial membrane potential (ΔΨm) of HPMCs was evaluated using JC1 probe with flow-cytometry.

Results. Exposure of HPMCs to 1.5%, 2.5%, and 4.25% dextrose, and 7.5% icodextrin dialysates, respectively, for 60 min resulted in significantly accumulation of intracellular reactive oxygen species (ROS), ΔΨm loss, and cell death in HPMCs. Amino acid dialysates exhibited no significant cytotoxicity. Adjusting the acidity in 1.5% dextrose and icodextrin dialysate significantly attenuated the dialysate-induced ROS generation and cell death in HPMCs. Heat pretreatment (41°C, 30 minutes), which induced HSP 27 and 72 syntheses, significantly attenuated the dialysate-induced intracellular ROS accumulation, Dym loss, and cell death in HPMCs.

Conclusions. In conclusion, the acidic bioincompatible dialysates induce oxidative stress, ΔΨm loss, and subsequent cell death in HPMCs. Amino acid dialysates is more biocompatible than glucose and icodextrin dialysates to...