Lanthanum Acetate Inhibits Vascular Calcification Induced by Vitamin D3 Plus Nicotine in Rats

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Lanthanum, a rare earth element, has been used to decrease serum phosphorus level in patients with chronic renal disease and hyperphosphatemia. We aimed to observe the effect and mechanism of two doses of lanthanum acetate (375 and 750 mg/kg/day) on vascular calcification induced by vitamin D3 plus nicotine treatment in rats for 4 weeks. As compared with control rats, rats with calcification showed widespread calcified nodules and irregular elastic fibers in calcified aorta on von Kossa calcium staining and increased aortic calcium and phosphorus contents, alkaline phosphatase (ALP) activity and bone-related protein expressions for osteopontin (OPN) and type III sodium dependent phosphate cotransporter Pit-1 (Pit-1). After treatment with either dose of lanthanum acetate, the calcified nodules and degree of irregular elastic fibers decreased in aortas. Lanthanum acetate at 750 mg/kg/day was more effective than 375 mg/kg/day in lessening vascular calcification by significantly reducing plasma phosphorus level, calcium × phosphorus product and ALP activity, by 30.3%, 28.6%, and 68.6%, respectively; reducing aortic phosphorus and calcium contents and ALP activity, by 48%, 53.1%, and 63.5% (all $P < 0.01$), respectively; reducing aortic mRNA level of OPN and Pit-1, by 55.8% ($P < 0.01$) and 38.8% ($P < 0.05$) and protein level of OPN and Pit-1, by 37.2% and 27.2% (both $P < 0.01$), respectively; and increasing carboxylated matrix Gla-protein (MGP) protein expression by 33.7% ($P < 0.05$), as compared with rats treated with vitamin D3 and nicotine alone. Lanthanum acetate could effectively inhibit the pathogenesis of vascular calcification.
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Key words: vascular calcification; lanthanum acetate; type III sodium dependent phosphate cotransporter Pit-1; matrix Gla-protein

Introduction

Vascular calcification is a common pathological phenomenon in atherosclerosis, diabetes mellitus, chronic renal failure and aging and is believed to be an important risk factor of cardiovascular diseases (1). In recent years, studies of vascular imaging and cellular molecular biology demonstrated that vascular calcification is an actively regulated, preventable and reversible cell-mediated process and shares several features with osteogenesis at the cellular and molecular levels (2–3). Under certain stimulating conditions, vascular cells, especially vascular smooth muscle cells (VSMCs), could transform into an osteoblast-like phenotype characterized by increased expression of bone-related proteins such as alkaline phosphatase, osteopontin (OPN), and osteocalcin and form calcifying nodules in the extracellular matrix or cytoplasm (4–5).

At the present, the prevention of and therapy for vascular calcification is formidable; some agents such as estrogen, statins, and antioxidants can dissolve hydroxyapatite deposited in tissues, but none of these agents can heal vascular calcification completely (6–8). For instance, bisphosphonates are effective for patients with osteoporosis accompanied by vascular calcification, but bisphosphonates induced aortic root inflammation and plaque rupture in apoE-knockout mice (9–10). According to the pathogenesis of vascular calcification, a key point is to prevent or reverse

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vascular calcification by inhibiting the deposition of calcium phosphate in extracellular and intracellular spaces and inhibit the phenotype transformation of VSMCs into osteoblast-like cells, both complex and multifactor-affected processes, with little known about their exact mechanisms. To date, no effective or satisfactory clinical strategies exist for limiting the development of vascular calcification.

Rare-earth elements include the 15 elements of lanthanides and the elements Sc and Y. Lanthanum (139 kDa), a basic element of lanthanides, belongs to the trivalent rare-earth metals and has higher binding affinity than calcium for binding sites such as receptors and other proteins. Therefore, lanthanum can be used extensively as an antagonist to explain many of the known biological effects of calcium (11–12) or calcium channel blockers, which prevent calcium from entering into the intracellular space (13–14). Lanthanum is a well-known non-calcium, non-aluminum phosphate-binding agent. Lanthanum carbonate (Fosrenol) is a safe phosphate binder for treating patients with end-stage renal disease with hyperphosphatemia; it is effective, well tolerated and has positive effects in relieving hypercalcemia or worse metabolic acidosis in patients with chronic renal failure (15–17).

In addition, lanthanum enhanced the contractile force of isolated perfused rat hearts and slowed the progression of established atherosclerotic lesions in cholesterol-fed rabbits (18–19). Furthermore, a recent study showed that lanthanum chloride could counteract H_2O_2-enhanced calcification in rat calcified vascular cells by suppressing the activation of the JNK (JNK2 but not JNK1) and p38 MAPK signaling pathway (20). All of these findings indicate that lanthanum may have a role in inhibiting vascular calcification *in vivo*.

Since the effects of lanthanum on vascular calcification have not been well documented, in this study we observed the effects of lanthanum acetate on aortic calcium and phosphorus contents, alkaline phosphatase (ALP) activity, and the mRNA and protein expression of OPN, matrix Gla-protein (MGP) and type III sodium-dependent phosphate cotransporter (Pit-1) in rats with vascular calcification induced by vitamin D3 plus nicotine (VDN). We aimed to investigate the therapeutic mechanism of lanthanum acetate in vascular calcification.

Materials and Methods

Materials. Male Sprague-Dawley rats (weighing 180–200 g) were purchased from the Animal Center, Health Science Center of Peking University, and were housed under standard conditions (room temperature 20 ± 8°C, humidity 60 ± 10%, 12-hrs light/12-hrs dark) and given standard rodent chow and water freely. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998). Vitamin D3 and nicotine were purchased from Sigma (St. Louis, MO, USA); lanthanum acetate was obtained from Beijing Zhongke Huanfa Science and Technology; the calcium assay kit was purchased from Beijing Zhongsheng Biosino Biotechnology and Science; kits for ALP and inorganic phosphorus assay were obtained from Nanjing Jiancheng Bioengineering (China); Trizol was from GIBCO BRL (Gaithersburg, MD, USA); dNTP was from Clontech Laboratories (Palo Alto, CA, USA); and Moloney murine leukemia virus transcriptase (MMLV), Taq, RNAsin and Oligo (dT)15 primer were from Promega (Madison, WI, USA). Oligonucleotides were synthesized by Sai Baisheng Biotechnology (Beijing, China). The sequences of oligonucleotide primers were as follows: OPN-S, 5′-CTCGCGGTGAAAGTGGCTGA-3′ and OPN-A, 3′-GACCTCAGAAGATGAACTCT-5′; MGP-S, 5′-AATCT-CACGAAAGCATGGAATC-3′ and MGP-A, 3′-GCA-GGCTTGTTGAGTTCCC-5′; Pit-1-S, 5′-TGGCTT-CTGCTTTATGGTGGT-3′ and Pit-1-A, 3′-CCAGTCAA-CAGCCTTCTTGGA-5′, and beta-actin-S, 5′-ATCTGG-CACCACACCTTC-3′ and beta-actin-A, 3′-AGCC-AGGTCCAGACGCA-5′ for calibration of sample loading. Anti-rat polyclonal antibodies of goat against OPN (SC-10593, Santa Cruz, CA, USA), rabbit against Pit-1 (SC-442, Santa Cruz, CA, USA) and mouse against carboxylated MGP (Lot L18770, Lausen, Switzerland) were used to analyze the level of protein expression. Nitrocellulose membrane was from Hybond-C (Amersham Life Science, England). Super ECL Plus Detection Reagent (P-1010) was from Applygen Technology (Beijing, China). Other chemicals and reagents were of analytical grade.

Preparation of Vascular Calcification Model. Rats were randomly divided into the following groups for treatment (n = 10 rats each): control group, calcified group (VDN group); calcified group with lanthanum acetate, 375 mg/kg/day (VDN+Lan [L] group); and calcified group with lanthanum acetate, 750 mg/kg/day (VDN+Lan [H] group). The preparation for the vascular calcification model (VDN groups) was as previously described (21); in brief, rats were intramuscularly injected with 300,000 IU/kg vitamin D3 plus nicotine intragastric administered (25 mg/kg in 5 ml peanut oil) at 9 a.m. on day 1. The nicotine administration was repeated at 5 p.m. The experiment continued for 4 weeks. Rats in the VDN+Lan groups were treated as for the VDN-alone group, with the exception of oral gavage of lanthanum acetate (375 or 750 mg/kg/day). Rats in the control group received normal saline injection and oral gavage of peanut oil.

Measurement of Blood Pressure and Cardiac Function. At the end of the experiment, rats were anesthetized with ethylcarbamate (10 mg/kg, i.p.). Aortic blood pressure was measured by use of a pressure transducer after intubation of a polyethylene catheter (PE-50) filled with heparin through the right carotid artery for 30 mins. After measurement of blood pressure, the catheter was maneuvered from the right carotid artery into the left ventriculum (LV), and heart rate (HR), left ventricular end-systolic pressure (LVSP) and end-diastolic pressure

(LVEDP), left ventricular peak rate of contraction (+LV dp/dtmax) and peak rate of relaxation (-LV dp/dtmax) were measured with use of a PowerLab of BL-420F instrument (TaiMeng, Chengdu, China). A blood sample was drawn and mixed with 1 mg/ml ethylene diamine tetraacetic acid-Na2 and 500 KIU/ml of aprotinin. Plasma was obtained by centrifugation at 3000 × *g* for 15 mins at 4°C and stored at –70°C. The heart and intact aorta were then removed to cold (4°C) PBS. Hearts were weighted and aortas were stripped of adventitia with sterile forceps. The samples of tissues were stored at –70°C until use.

Von Kossa Staining. As described previously by our lab (22), a 1-cm segment of aorta was placed in 4% phosphate buffered neutral formalin (pH 7.4, 0.1 *M*) for 12 hrs, immersed in 20% sucrose solution for storage, then embedded in optimal cutting temperature compound before use. Six-micrometer-thick frozen sections of aorta tissue were cut. The slides were dehydrated before being immersed in a light-protected, silver nitrate solution (1 g in 100 ml distilled water) for 30 mins under an intense sunbeam, then immersed in a solution of 5% sodium thiosulfate in distilled water for 2 mins. Finally, slides were counterstained with aldehyde-fuchsin and imaged under an Olympus BX 50 microscope (Olympus Optical Tokyo, Japan).

Measurement of Calcium and Inorganic Phosphorus Levels in Plasma. Plasma calcium concentration was measured by the o-cresolphthalein colorimetric (OCPC) method of color development to quantify calcium concentration. Plasma samples, 0.02 ml, were added into the mixed working reagent solution, including the ethanolamine buffer solution and OCPC and 8-hydroxyquinoline solution, and the volume ratio to sample volume was 1:25. The solutions were left to stand for 5 mins at 30°C. The red absorbance of the compound solution was measured at 600 nm. The detection of inorganic phosphorus in plasma involved the malachite green direct development process. A 1.0-ml carbamide reagent was added into a 0.02-ml plasma sample with 3.0 ml malachite green molybdic acid reagent and then mixed thoroughly. After 15 mins of color development at room temperature (23 ± 1°C), the absorbance was read at 640 nm. The calculation of plasma calcium and phosphorus concentration followed the kit manufacturer's instructions.

Measurement of Calcium and Phosphorus Contents in Aorta. Abdominal aortas (10 mg) were directly dissolved in HNO_3, then dried in an oven and redissolved with the blank solution (27 n*M* KCl and 27 μ*M* $LaCl_3$). The calcium content was measured by use of an atomic absorption spectrophotometer (novAA 300, Analytik Jena AG, Germany) at 422.7 nm as described previously by our lab (23). Calcium content of the aorta was normalized to tissue dry weight. For phosphorus assay, the samples of aorta measuring 1 cm were incubated in 150 m*M* HCl for 24 hrs with gentle agitation. Phosphorous content was measured by the ammonium molybdate method (24).

Measurement of Lanthanum Content in Plasma and Aorta. An amount of 0.5 ml serum and media-denuded thoracic aortas, about 10 mg tissue, were separately placed into the crucible, the electron heat board was heated until the sample was fully dissolved in concentrated nitric acid, and the sample was further diluted with high purity deionized water and adjusted to desired pH for analysis. Rhodium was used as internal standard, added to all samples and standard solutions. The acid concentration was kept as close as possible in standards and samples. The content of lanthanum was calculated and expressed as nanograms per milliliter for plasma and nanograms per gram wet weight for tissues and measured by inductively coupled plasma-mass spectroscopy (ICP-MS) (ELAN DRC II PE, New York, USA).

Assay of Alkaline Phosphatase (ALP) Activity in Plasma and Aorta. Homogenates of endothelium-denuded thoracic aortas (homogenized buffer: 20 m*M* HEPES containing 0.2% NP-40 and 20 m*M* $MgCl_2$) were prepared by use of a Polytron. After centrifugation at 8000 × *g* for 10 mins, the protein content of the supernatant was determined by the Bradford method (25). ALP activity was measured by use of the ALP assay kit (alkaline buffer: stock substrate solution 1:1). The samples of plasma and supernatant for tissue homogenate of thoracic aorta were mixed with 1 ml reaction mixture, respectively. The mixture was then incubated for 15 mins at 37°C. Then the developer (1.5 ml) was added to each well; the red color indicated ALP activity, and the absorbance was determined at 520 nm. ALP activity was calculated according to the manufacturer's instructions. One unit was defined as 1 g tissue protein producing 1 mg phenol for 15 mins.

Measurement of OPN, Pit-1 and MGP mRNA. RT-PCR assay was used to assess the expression of OPN, Pit-1 and MGP mRNA as described (26). Total RNA of aortic tissues was extracted by standard techniques. Isolated total-tissue RNA was then quantified by use of an ultraviolet (UV) spectrophotometer (Eppendorf AG 22331, Hamburg, Germany). An amount of 2 μg of total RNA samples was primed with MMLV and oligo (dT) 15 primer. The PCR reaction mixture was in a 25-μl volume containing 2.5 m*M* dNTP 1 μl, 10 × PCR buffer (20 m*M* $MgCl_2$, 500 m*M* KCl, 1.5 *M* Tris–HCl, pH 8.7) 2.5 μl, cDNA 2 μl, 200 n*M* of the appropriate OPN, Pit-1 or MGP paired primers 1 μl and 1.25 U Taq DNA polymerase 1 μl. The following PCR cycles were used: 94°C for 30 secs, 57°C for 30 secs and 72°C for 40 secs, for 30 cycles, then 74°C for 5 mins. As an internal control for each PCR reaction, beta-actin mRNA was also amplified with each sample. A total of 200 n*M* of actin primers and 2 μl cDNA was amplified under the same reaction conditions. All PCR products were loaded onto a 1.5% agarose–Tris–acetate–EDTA gel before electrophoresis, and products were visualized on ethidium bromide staining. The UV illumination photos then underwent computerized densitometric analysis. The final results are expressed as the ratio of OPN (871 bp), MGP (131 bp) or

Table 1. General Characteristics of Rats with Vascular Calcification[a]

	Control	VDN	VDN+Lan [L]	VDN+Lan [H]
Body weight (g)	356 ± 13	288 ± 7**	302 ± 7	329 ± 12$^{\#}$
LVW/BW (mg/g)	2.54 ± 0.10	3.58 ± 0.10**	3.14 ± 0.12$^{\#\#}$	2.71 ± 0.10$^{\#\#,\Delta\Delta}$
HR (beats/min)	349 ± 14	412 ± 10*	386 ± 18	378 ± 11
SAP (mmHg)	113 ± 5	141 ± 6*	137 ± 6	116 ± 6$^{\#,\Delta}$
DAP (mmHg)	68 ± 2.9	71 ± 1.8	70 ± 3.6	73 ± 4.7
PP (mmHg)	45 ± 1.0	70 ± 3.6**	66 ± 3.9	44 ± 1.2$^{\#\#,\Delta\Delta}$
LVSP (mmHg)	121 ± 2.8	148 ± 5.6**	135 ± 2.3$^{\#}$	124 ± 4.4$^{\#\#}$
LVEDP (mmHg)	−3.7 ± 0.22	−3.8 ± 0.17	−3.7 ± 0.11	−3.8 ± 0.11
+LV dp/dt_{max} (mmHg/s)	5453 ± 207	5853 ± 268	5747 ± 265	5358 ± 167
-LV dp/dt_{max} (mmHg/s)	4309 ± 105	4765 ± 142	4516 ± 120	4372 ± 145

[a] BW, body weight; LVW, left ventricular weight; HR, heart rate; SAP, systolic aortic pressure; DAP, diastolic aortic pressure; PP, pulse pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; ±LV dp/dt_{max}, left ventricular peak rate of contraction and peak rate of relaxation were measured *in vivo*. VDN, rats given vitamin D3 plus nicotine for 4 weeks; Lan, lanthanum acetate; VDN+Lan [L], calcified aortas treated with lanthanum acetate at 375 mg/kg/day for 4 weeks; VDN+Lan [H], calcified aortas treated with lanthanum acetate at 750 mg/kg/day for 4 weeks. Values are means ± SEM. $n = 6$ in each group. * $P < 0.05$, ** $P < 0.01$ compared with control; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ compared with VDN; $^{\Delta}$ $P < 0.05$, $^{\Delta\Delta}$ $P < 0.01$ compared with VDN+Lan [L].

Pit-1 (121 bp) PCR product to the beta-actin PCR product (291 bp) for each sample. All experiments were repeated three times.

Measurement of OPN, Pit-1 and MGP Protein Contents. The rat vessel tissues were prepared by adding a membrane lysis buffer containing a detergent (16 m*M* CHAPS, 20 m*M* Tris–HCl, pH 7.5, 1 m*M* Na_2EDTA, and 1 m*M* DTT) with protease inhibitors (1 m*M* benzamidine, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 0.5 m*M* PMSF). The samples were then centrifuged at 15,000 × *g* for 30 mins, and the pellets were suspended in lysis buffer. The protein concentration was estimated by the Bradford method (25). The amounts of proteins used for Western blot analysis were 60 μg, 40 μg and 20 μg to detect OPN, Pit-1 and carboxylated MGP, respectively, in rat aortas. The proteins were loaded onto 10% SDS-polyacrylamide gels and underwent electrophoresis and blotting onto nitrocellulose membranes. The membranes were saturated for 1 hr in TBS containing 0.5% Tween and 5% non-fat milk. We used anti-rat polyclonal antibodies of goat against OPN, rabbit against Pit-1, and mouse against carboxylated MGP at 1:2000 dilution. The peroxidase-conjugated rabbit anti-goat, goat anti-rabbit and rabbit anti-mouse antibodies were used at 1:4000 dilution for OPN, Pit-1 and carboxylated MGP as secondary antibodies, respectively. The membrane was immediately visualized on a phosphor imager after the addition of the ECL substrate, and the OPN, Pit-1 or carboxylated MGP contents were normalized to that of beta-actin.

Statistical Analysis. Data are shown as mean ± SEM. Differences among groups were analyzed by one-way ANOVA, then Student-Newman-Keuls test. Comparisons between two groups involved use of unpaired Student's *t* test. A $P < 0.05$ was considered statistically significant.

Results

General Characteristics of Vascular Calcification *In Vivo*. Treatment with VDN induced vascular calcification in rats. Compared with the control group, rats with vascular calcification showed decreased body weight, by 19% ($P < 0.05$) and increased ratio of LV weight to body weight by 41% ($P < 0.05$) (Table 1). HR, systolic aortic pressure, pulse pressure, and LV systolic pressure were significantly increased, by 18%, 25%, 57% and 22% (all $P < 0.01$ or 0.05), respectively, in the VDN group (Table 1), with no change in diastolic aortic pressure, LVEDP and +/-LV dP/dt_{max} (all $P > 0.05$) (Table 1).

Lanthanum Levels in Plasma and Aorta. At the end of the experiment, the plasma lanthanum concentration in lanthanum-treated groups did not differ from that of control or VDN group, did not depend on the dose administered and reached a plateau after 4 weeks of treatment. However, the aortic lanthanum level was higher in the VDN-treated group than in controls (294.1 ± 22.02 vs. 121.3 ± 12.18 ng/g wet weight, $P < 0.05$), moreover, VDN+Lan [H] rats showed elevated aortic lanthanum content (1746 ± 135.3 ng/g wet weight, $P < 0.01$) compared with control and VDN-alone groups.

Lanthanum Acetate Ameliorated VDN-Induced Vascular Calcification in Rats. Compared with the control group, the VDN group showed strong positive von Kossa staining for calcification in aortic tissues among irregular elastic fibers of the medial layer (Fig. 1B); the aortic calcium and phosphorus contents and ALP activity were significantly increased, by 2.52-, 4.92- and 2.26-fold (all $P < 0.01$), respectively, and plasma ALP activity increased by 4.08-fold ($P < 0.01$); however, the plasma calcium and phosphorus levels and calcium × phosphorus (Ca × P) product did not significantly differ between the control and VDN groups ($P > 0.05$) (Table 2).

Compared with the VDN-alone group, the lanthanum acetate-treated groups showed lower positive staining for calcium deposition and irregular elastic fibers in the aortic medial layer (Fig. 1C, D). Lanthanum acetate at 375 or 750 mg/kg/day decreased plasma phosphorus level by 12.5% (*P*

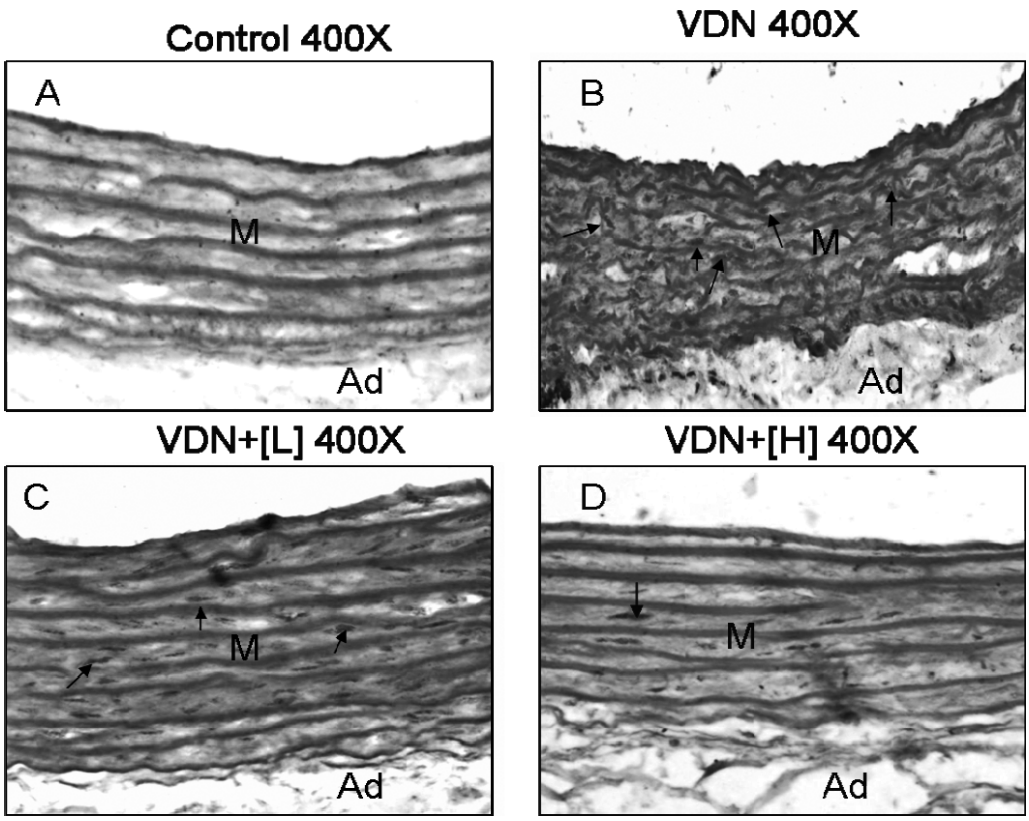

Figure 1. Effects of lanthanum acetate on calcific nodules and elastic fibers in thoracic aorta of calcified rats ($n = 6$/ group, original magnification ×400 of all von Kossa staining sections). Rats were bred for 4 weeks. VDN, rats given vitamin D3 plus nicotine for 4 weeks; Lan, lanthanum acetate; VDN+Lan [L], calcified aortas treated with lanthanum 375 mg/kg/day for 4 weeks; VDN+Lan [H], calcified aortas treated with lanthanum acetate 750 mg/kg/day for 4 weeks; M, media layer in aorta; Ad, adventitia layer in aorta. The arrows indicated calcium nodules on the irregular elastic fibers. Von Kossa staining for calcium nodules showed A) none of the positive staining for calcium nodules and none of the irregular elastic fibers in the aortic medial layer in control rats; B) a deal of positive staining for calcium nodules and irregular elastic fibers in the aortic medial layer in VDN rats; C) dispersed calcific nodules and relative regular elastic fibers in the aortic medial layer in VDN+Lan [L] rats; D) less positive staining for calcium nodules and more regular elastic fibers in the aortic medial layer in calcific nodules in VDN+Lan [H] rats.

> 0.05) and 30.3% ($P < 0.01$); calcium × phosphorus (Ca × P) product by 9.3% ($P > 0.05$) and 28.6% ($P < 0.01$); and ALP activity by 11.5% and 68.6%, respectively (both $P < 0.01$) (Table 2). Lanthanum acetate at both doses also decreased the aortic phosphorus content by 28% and 48%; calcium content by 29% and 53.1%; and ALP activity by 19.6% and 63.5%, respectively (all $P < 0.01$) (Table 2). Compared with 375 mg/kg/day treatment, 750 mg/kg/day lanthanum acetate was more effective in decreasing plasma phosphorus level, calcium × phosphorus (Ca × P) product, plasma and aortic ALP activity and aortic calcium and phosphorus contents (all $P < 0.05$ or $P < 0.01$) (Table 2).

Table 2. Calcium-Phosphorus Related Parameters of Plasma and Aortic Tissues in Experimental Rats[a]

	Control	VDN	VDN+Lan [L]	VDN+Lan [H]
Plasma ionized calcium level (mg/dl)	9.25 ± 0.21	9.63 ± 0.42	9.53 ± 0.22	9.36 ± 0.42
Plasma phosphorus level (mg/dl)	5.05 ± 0.23	5.22 ± 0.22	4.57 ± 0.23	$3.64 \pm 0.13^{\#\#,\Delta\Delta}$
Plasma Ca × P product (mg^2/dl^2)	46.44 ± 1.90	48.88 ± 1.34	44.34 ± 1.88	$34.92 \pm 0.74^{\#\#,\Delta\Delta}$
Aortic calcium content (μmol/g)	22.28 ± 2.56	78.51 ± 5.05**	$55.75 \pm 2.20^{\#\#}$	$36.82 \pm 2.64^{\#\#,\Delta}$
Aortic phosphorus content (μmol/g)	7.63 ± 0.20	44.58 ± 1.29**	$32.30 \pm 0.86^{\#\#}$	$23.48 \pm 0.72^{\#\#,\Delta\Delta}$
Plasma ALP activity (U/l)	66.90 ± 2.43	340.0 ± 5.73**	$301.0 \pm 5.92^{\#\#}$	$106.8 \pm 4.66^{\#\#,\Delta\Delta}$
Aortic ALP activity (U/g Pro)	59.2 ± 2.62	193.0 ± 6.30**	$155.2 \pm 6.89^{\#\#}$	$70.4 \pm 2.90^{\#\#,\Delta\Delta}$

[a] The plasma ionized calcium and inorganic phosphorus levels and plasma and aortic ALP activities were determined by assay kits; aortic calcium content was detected by atomic absorption spectrophotometer. Rats were bred for 4 weeks. Ca × P product indicated the multiplication for calcium and phosphorus levels; ALP, alkaline phosphatase; VDN, rats given vitamin D3 plus nicotine for 4 weeks; Lan, indicates lanthanum acetate; VDN+Lan [L], calcified aortas treated with lanthanum 375 mg/ kg/day for 4 weeks; VDN+Lan [H], calcified aortas treated with lanthanum acetate 750 mg/kg/day for 4 weeks. Values are means ± SEM. $n = 6$ in each group.
** $P < 0.01$ compared with control; $^{\#\#}$ $P < 0.01$ compared with VDN; $^{\Delta}$ $P < 0.05$, $^{\Delta\Delta}$ $P < 0.01$ compared with VDN+Lan [L].

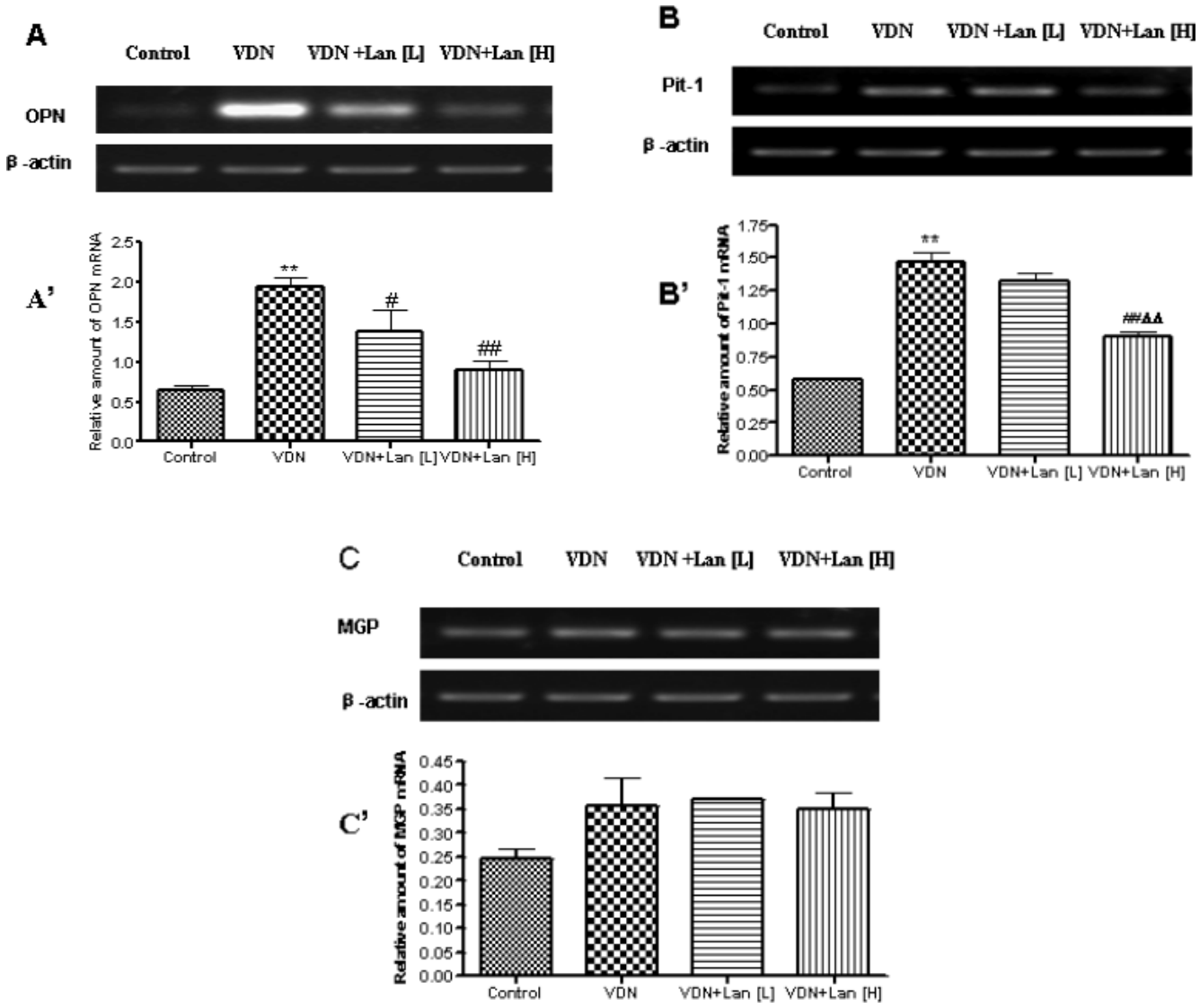

Figure 2. Effects of lanthanum acetate on the mRNA expressions of osteopontin (OPN) (A), type III sodium dependent phosphate cotransporter Pit-1(Pit-1) (B), matrix Gla-protein (MGP) (C) and β-actin in aortas of rats as assessed by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. Rats were bred for 4 weeks. VDN, rats given vitamin D3 plus nicotine for 4 weeks; Lan, indicates lanthanum acetate; VDN+Lan [L], calcified aortas treated with lanthanum 375 mg/kg/day for 4 weeks; VDN+Lan [H], calcified aortas treated with lanthanum acetate 750 mg/kg/day for 4 weeks. A, B and C photographs of the RT-PCR analysis from a representative experiment. A', B' and C' quantification of these mRNA expressions normalized against the β-actin mRNA levels. Data were obtained from three independent experiments and are presented as mean ± SEM. ** $P < 0.01$ compared with control; # $P < 0.05$, ## $P < 0.01$ compared with VDN; $^{\Delta\Delta}$ $P < 0.01$ compared with VDN+Lan [L].

Lanthanum Acetate Decreased the Aortic mRNA and Protein Levels of OPN and Pit-1 in Rats Treated with VDN. The VDN group showed 213.0% and 152.2% higher OPN and Pit-1 mRNA levels in aortic tissue than the control group (both $P < 0.01$; Fig. 2A, A', B, B'). Groups treated with lanthanum acetate (375 or 750 mg/kg/day) showed down-regulated mRNA expression of OPN, by 32.2% ($P < 0.05$) and 55.8% ($P < 0.01$), and Pit-1, by 9.7% ($P > 0.05$) and 38.8% ($P < 0.05$), respectively, compared with the VDN-alone group. The VDN group showed 208.6% and 57.4% higher OPN and Pit-1 aortic protein expression than the control group (both $P < 0.01$; Fig. 3A, A', B, B'). Treatment with lanthanum acetate (375 or 750 mg/kg/day) down-regulated the protein expression of OPN, by 11.8% ($P > 0.05$) and 37.2% ($P < 0.01$), and Pit-1 by 14.4% ($P < 0.05$) and 27.2% ($P < 0.01$), respectively, as compared with the VDN-alone group. Lanthanum treatment at 750 mg/kg/day was more potent in decreasing Pit-1 mRNA expression and OPN protein expression than 375 mg/kg/day treatment in the aorta ($P < 0.01$).

Lanthanum Acetate Increased Carboxylated MGP Protein Expression in Rats Treated with VDN. The mRNA level of MGP did not differ in the VDN and control groups (Fig. 2C, C'). The VDN group showed 32.4% lower aortic carboxylated MGP protein expression than the control group ($P < 0.01$; Fig. 3C, C').

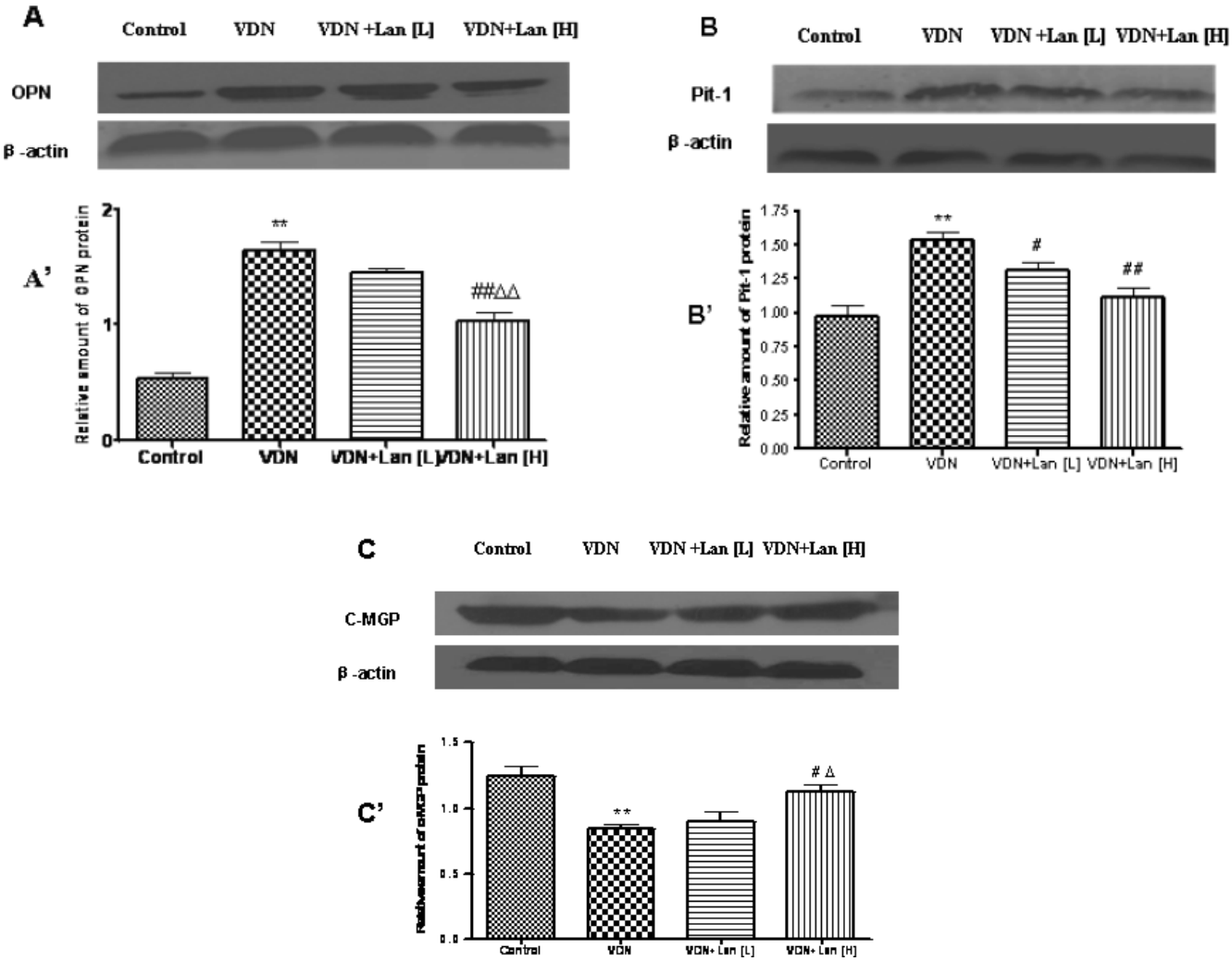

Figure 3. Effects of lanthanum acetate on the protein expressions of osteopontin (OPN) (A), type III sodium dependent phosphate cotransporter Pit-1(Pit-1) (B), carboxylated matrix Gla-protein (C-MGP) (C) and β-actin in aortas of rats as assessed by Western blotting. Rats were bred for 4 weeks. VDN, rats given vitamin D3 and nicotine for 4 weeks; Lan, indicates lanthanum acetate; VDN+Lan [L], calcified aortas treated with lanthanum 375 mg/kg/day for 4 weeks; VDN+Lan [H], calcified aortas treated with lanthanum acetate 750 mg/kg/day for 4 weeks. A, B and C photographs of the Western blotting analysis from a representative experiment. A', B', and C' quantification of these protein expressions normalized against the β-actin protein levels. Data were obtained from three independent experiments and are presented as mean ± SEM. ** $P < 0.01$ compared with control; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ compared with VDN; $^{\Delta}$ $P < 0.05$, $^{\Delta\Delta}$ $P < 0.01$ compared with VDN+Lan [L].

Groups treated with lanthanum acetate (375 or 750 mg/kg/day) showed an increase in carboxylated MGP protein expression, by 6.9% ($P > 0.05$) and 33.7% ($P < 0.05$), respectively, compared with the VDN-alone group. The carboxylated MGP expression with high-dose lanthanum acetate was higher than that with low-dose treatment ($P < 0.05$; Fig. 3C, C').

Discussion

Administration of vitamin D3 and nicotine to rats resulted in serious vascular calcification, with systolic hypertension and LV concentric hypertrophy, which suggests the reduced arterial compliance and the compensatory potentiation of cardiac contractile function. Aortic calcium content and ALP activity were significantly increased in VDN-treated rats, and von Kossa staining showed positive staining for calcium accumulation and irregular elastic fibers in vascular media as was shown in previous reports (27).

Hypervitaminosis D is well known to promote degradation of elastic fibers and deposition of the calcium and phosphate in cardiovascular tissues through excess absorption of calcium and phosphorus in the intestinal tract. Vitamin D could activate vitamin D receptors (VDRs), abundant in VSMCs; the activation of VDRs promotes the uptake of calcium into the intracellular space, which leads to calcium overload and induces expression of genes for calcification (28). Nicotine increases the fragmentation of collagen, which amplifies the calcifying effects of vitamin D (29–30). Both vitamin D and nicotine together exert their effects on promoting vascular calcification pathogenesis.

In our study, compared with the VDN-alone group, rats

treated with lanthanum acetate showed ameliorated VDN-induced aortic calcification, as shown by von Kossa staining for calcification, decreased aortic calcium deposition and regular elastic fibers in the medial layer of the vessel wall (Fig. 1C, D). Moreover, plasma phosphorus level, Ca × P product and ALP activity; aortic calcium and phosphorus contents and ALP activity; and mRNA and protein levels of aortic OPN and pit-1 were significantly lower than that in the VDN-alone group. In addition, the level of aortic carboxylated MGP protein expression was increased in lanthanum-treated groups. Furthermore, the anti-calcification effect with high-dose lanthanum (750 mg/kg/day) was stronger than with low-dose treatment (375 mg/kg/day).

Lanthanum belongs to the group of elements known as "lanthanons". It is the most electropositive rare earth element and is uniformly trivalent. It can be used as a substitute or antagonist for calcium or as a calcium channel blocker in studying a variety of cellular reactions. Lanthanum is also being used extensively to study anatomical barriers, membrane structure, and subcellular transport systems, particularly the calcium-involved pathway (11–14). La3+ may produce marked effects on cardiovascular function. For instance, in isolated perfused rat hearts, at different extracellular calcium concentrations, high extracellular Ca2+ reduced the La3+-induced initial decrease in force of contraction but potentiated the late increase in contractile force by La3+; the cardioprotective mechanism of lanthanum could be involved in modulating Na+–Ca2+ exchange (18, 31). La3+ also strengthened the contraction responsibility of blood vessel rings in spontaneously hypertensive rats (32). *In vivo* studies showed that $LaCl_3$, as a Ca2+ antagonist, could slow the progression of established atherosclerotic lesions by decreasing serum cholesterol level and atherosclerotic plaque area in cholesterol-fed rabbits (19). Furthermore, lanthanum carbonate, as a new non-calcium, non-aluminum phosphate binder, has been studied extensively in reducing serum phosphorus levels in patients with end-stage renal disease (15–17). Especially, $LaCl_3$ could counteract H_2O_2-enhanced calcification in rat calcifying vascular cells (20). Since lanthanum targets the heart and blood vessels and has direct actions on calcium–phosphate regulation, lanthanum could reasonably affect vascular calcification *in vivo*. Lanthanum acetate is easier to be dissolved in water with a high affinity for phosphate that is pH-independent, and ionic lanthanum would exert its effects immediately after lanthanum acetate gets into the stomach. It is easier and more active to be utilized by rats because lanthanum acetate belongs to organic salts, and it will not produce obvious side effects for body utilization compared with inorganic salt. Moreover, lanthanum acetate was found to suppress the development of ischemic neuron damage in the rat's cerebral cortex (33). Therefore, in this study, we applied lanthanum acetate to observe its effects on vascular calcification in rats induced by vitamin D plus nicotine.

Elevated serum calcium and phosphorus levels and Ca × P product and/or increased vascular calcium and phosphorus contents are recognized risk factors of vascular calcification (34–35). In this study, administration of overdose VDN in rats increased calcium and phosphorus levels slightly in plasma but markedly in aortic tissues. Lanthanum acetate treatment attenuated VDN-induced calcium and phosphorus disturbance in plasma and aorta. The mechanism might be lanthanum acetate inhibiting the uptake of phosphate in the intestinal tract through binding phosphate, which results in a lower plasma phosphorus level and Ca × P product and further decreases vascular calcium and phosphorus deposition.

In the calcified vessel wall, VSMCs transform into osteoblast-like cells and markedly express some bone-related proteins, such as ALP, OPN, and Pit-1 (36), markers of osteogenic differentiation of VSMCs. ALP is an osteogenic enzyme that degrades pyrophosphate, the major initiator of tissue calcification, and generates inorganic phosphate for the deposition of calcium and phosphate and forms calcified nodules. OPN, a multifunctional, acidic and glycosylated phosphoprotein, can be found in abnormal mineralized tissues such as calcified sites in atherosclerotic plaque and calcified aortic valves thought to be involved in regulation of mineralization. The type III Na-Pi transporters Pit-1 and Pit-2 are strongly expressed in rat VSMCs, with Pit-1 expression more abundant than Pit-2. The enhanced expression of Pit-1 mRNA and protein could increase Na-dependent Pi cell uptake, which plays an essential role in calcification of VSMCs. Furthermore, *in vitro* studies showed that ALP, OPN and Pit-1 levels were highly expressed in VSMCs induced by elevated phosphate treatment when VSMCs transformed into osteoblast-like cells (37). In our study, compared with the control group, VDN-treated rats showed increased aortic ALP activity and mRNA and protein level of OPN and Pit-1, which suggests the phenotype transformation from VSMCs into osteoblast-like cells. Lanthanum acetate treatment significantly decreased the aortic ALP activity and the mRNA and protein levels of OPN and Pit-1, which suggests that lanthanum therapy could inhibit phenotype transformation of VSMCs into osteoblasts.

MGP, mineral-binding, gamma-carboxy-glutamate (Gla)-containing protein, has been found in calcified vascular lesions, and genetic studies in mice indicate that MGP is involved in the regulation of vascular calcium deposition and is a major matrix protein regulated by calcium. Inhibition of vascular calcification by MGP mainly depends on γ-glutamyl carboxylation to remove free calcium ions or bind calcium crystals for inhibiting its growth. Carboxylated MGP is the active form that acts as an inhibitor of tissue calcification, and vitamin K is necessary for its γ-glutamyl carboxylation and thus for rendering MGP its biological activity (38). In the present study, VDN treatment decreased the expression of the aortic carboxylated MGP protein. VDN-treated rats administered lanthanum acetate showed significantly increased aortic

carboxylated MGP protein expression, with no significant difference in MGP mRNA expression as compared with the VDN-alone group. From these results, the environment of the calcification may be unfavorable for the efficient vitamin K to exert its roles in γ-glutamyl carboxylation of MGP. Exogenous supplementation with lanthanum could enhance the aortic carboxylated MGP protein expression, which suggests lanthanum fulfills its regulation mainly in MGP protein level but not in mRNA level. The enhanced carboxylated MGP protein expression by lanthanum might be indirectly regulated in aortas, but the mechanism needs to be further explored.

Up to now, the pathogenic mechanism of vascular calcification, especially how the phenotype transformation from VSMCs to osteoblasts, is not clearly elucidated (36). The clinical therapy and prevention of vascular calcification is still unsatisfactory. In this study, administration of lanthanum acetate inhibited the pathogenesis of VDN-induced vascular calcification, and binding phosphorus and inhibiting the phosphorus uptake in the gastrointestinal tract followed by decreased plasma and aortic phosphorus levels could be involved in the main mechanism. Lanthanum acetate could also reduce calcium content in the vascular tissue and inhibit the phenotype transformation of VSMCs with decreased ALP activity and expression of bone-related protein, such as OPN and Pit-1. Furthermore, lanthanum content was elevated in aortas with vascular calcification compared with controls. Importantly, the aortic lanthanum content increased significantly with lanthanum treatment as compared with VDN alone, which indicates that lanthanum may be an important endogenous ion like calcium and involved in the regulation of vascular function to maintain vascular homeostasis. But the role of lanthanum in regulating cardiovascular function is not well known. The increase in lanthanum level may have important compensatory and protective significance in cardiovascular diseases such as vascular calcification. However, the mechanisms of up-regulation and definite protective effects of lanthanum observed in our work need further study. Lanthanum could play a beneficial role in calcification-induced vascular damage. Therefore, we supposed that the increased level of lanthanum during vascular calcification might be an adaptive reaction with compensatory significance. Lanthanum may be a potential therapeutic target for diseases related to vascular calcification.

The degree of absorption and deposition of lanthanum in local tissue and whether it has adverse effects for long-term use still need to be explored. The long-term safety of lanthanum carbonate was examined in studies involving patients with end-stage renal disease exposed for 2 years, with no harmful effects, and similar results were seen in 4-year treatment in such patients (16); in particular, lanthanum was not detected in brains in studying the toxicity of lanthanum and had no other adverse effects on bone metabolism in patients or rats with chronic renal failure (39–40). We still need to further investigate the effective dose, safety of medication duration, and disadvantages of excessive deposition of lanthanum in tissue used as vascular calcification therapy.

In summary, our results showed that application of lanthanum acetate significantly ameliorated VDN-induced vascular calcification in rats, with decreased aortic calcium deposition, inhibition of expression of osteogenic proteins and improvement of vascular function. The value of lanthanum in clinical application deserves further study.

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