Lanthanum Chloride Bidirectionally Influences Calcification in Bovine Vascular Smooth Muscle Cells

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ABSTRACT

Vascular calcification (VC) is frequent prevalence in patients with chronic kidney disease (CKD) and atherosclerosis. Lanthanum carbonate is used as an orally administered phosphate-binding agent to reduce the gastrointestinal absorption of phosphate and ameliorate VC in advanced CKD. In this study, we used bovine vascular smooth muscle cells as a model VC in vitro and studied the effects of lanthanum chloride on calcium deposition. Exposure of cells to LaCl₃ at the concentration of 0.1 μM suppressed the β-glycerophosphate-induced alkaline phosphatase activity and calcium deposition. Furthermore, LaCl₃ upregulated the β-glycerophosphate-suppressed expression of calcium-sensing receptor. In contrast to the inhibitory effect of LaCl₃ on calcium deposition, higher level lanthanum (50 μM) was found to promote immediately precipitation of calcium phosphate in cell culture medium. At this concentration, LaCl₃ was found to induce cell apoptosis which involves caspases-9 and -3. These data indicate that the promotory effect of LaCl₃ on calcium deposition is likely mediated by induction of apoptosis. Our in vitro findings do suggest that, in the context of raised lanthanum, greater attention should be paid to potential toxic effects associated to the use of lanthanide-based drugs. J. Cell. Biochem. 113: 1776–1786, 2012. C2011 Wiley Periodicals, Inc.

KEY WORDS: VASCULAR SMOOTH MUSCLE CELLS; LANTHANUM CHLORIDE; CALCIUM PHOSPHATE DEPOSITION; CALCIUM-SENSING RECEPTOR; APOPTOSIS

Vascular calcification (VC) is frequent prevalence in patients with chronic kidney disease (CKD) and atherosclerosis [Doherty, 2004]. It is a pathological process involving medial and intimal calcification, and causes myocardial ischemia and contributes to the morbidity and mortality [Moe and Chen, 2008]. Growing evidence suggests that VC, like bone formation, is an actively regulated process involving both inductive and inhibitory factors. In the advanced CKD population, elevated serum phosphate and increased parathyroid hormone (PTH) levels are likely to be major contributors to VC [Giachelli, 2009]. Thus, phosphate control has become a therapeutic target with phosphate binders [Hutchison, 2009] and calcimimetics [Saidak et al., 2009; Silver and Naveh–Many, 2009].

Cell apoptosis plays an important role in many pathophysiological conditions and has been recognized as an important mode of cell death in response to cytotoxic treatments. Some studies have shown that vascular smooth muscle cells (VMSCs) are directly responsible for VC [Bennett and Boyle, 1998; Trion and Laar, 2004; Clarke et al., 2008]. Apoptotic bodies derived from VSMCs may act as nucleating structures for calcium phosphate deposition in the form of hydroxyapatite, so apoptosis is sufficient to precede calcification process [Proudfoot et al., 2000]. Due to the cytotoxic potential of calcium phosphate particles, the crystals have been proved to induce apoptosis in VSMCs [Ewence et al., 2008].

The extracellular calcium-sensing receptor (CaR) belongs to family C of the G-protein-coupled receptors. Extracellular calcium can elicit complex intracellular signals, which plays an important role in regulating mineral ion homeostasis [Hofer and Brown, 2003; Hofer and Lefkimmiatis, 2007; Smajilovic and Tielt-Hansen, 2007]. Nowadays, it is well-known fact that the CaR is expressed in all the organs and cells that maintain systemic Ca²⁺. Noteworthy studies have demonstrated that the expression of CaR is reduced in human calcified arteries and in mineralized VSMCs, CaR present in VMSCs may directly prevent mineral deposition [Alam et al., 2009]. Due to its low specificity, cationic lanthanum (La³⁺), primarily based on its
similarity to Ca$^{2+}$, also can activate CaR at nanomolar concentration [Carrillo-Lo´pez et al., 2010].

We have previously reported that LaCl$_3$ suppresses the β-glycerophosphate-induced calcification [Shi et al., 2009]. However, other study found that 50 µM GdCl$_3$ can increase the deposition of a mineralized matrix [Alam et al., 2009]. As yet, the exact mechanism of calcification in response to lanthanides is not clearly elucidated. We set out to determine the effects of increased LaCl$_3$ concentrations on in vitro calcification of bovine VSMCs (BVSMCs).

**MATERIALS AND METHODS**

**MATERIALS**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were obtained from Gibco BRL (Rockville, MD). Anti-CaR monoclonal antibody was from Affinity Bioreagents (Cambridge, UK). β-actin and the corresponding secondary antibodies were from Cell Signaling (Beverly, MA). NPS2143 was from Tocris Bioscience (Ellisville, MS). Annexin V-FITC Apoptosis detection kit I was obtained from BD Pharmingen (San Diego, CA). Z-VAD-FMK, caspases-3 and -9 activity kit were from Beyotime Institute of Biotechnology (Haimen, China). Unless otherwise indicated, all other reagents, including LaCl$_3$, β-glycerophosphate, Alizarin Red S, MTT, and Rhodamine123 (Rh123) were from Sigma (St. Louis, MO).

**VASCULAR SMOOTH MUSCLE CELL CULTURES AND TREATMENT**

Bovine vascular smooth muscle cells were isolated from aortic media of bovine by enzymatic isolation according to the method described previously, and cultured in growth medium (DMEM containing 10% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin) at 37°C in a humidified atmosphere with 5% CO$_2$. Cells were maintained in growth medium and were used between passages 3 and 8.

After confluence, the cells were then incubated in growth medium or calcification medium (growth medium plus 10 mM β-glycerophosphate) in the presence or absence of LaCl$_3$. The media were replaced every 3 days with fresh one. Full details of culture conditions used in each experiment were specified in the relevant figure legends. Experiments were performed at least three times with triplicate cultures used in every experiment, unless stated otherwise.

**ALKALINE PHOSPHATASE ASSAY**

Bovine VMSCs were plated in 24-well plates at a density of 5,000 cells/well in growth medium or calcification medium. At the end of the incubation for 9 days, cells were washed three times with PBS, lysed with 1% Triton X-100 in 0.9% NaCl, and then centrifuged. Supernatants were assayed for ALP activity as described previously. ALP values (U/mg) were normalized to protein content that was measured with Bradford assay.

**QUANTIFICATION OF CALCIUM DEPOSITION**

Bovine VMSCs were plated in 24-well plates at a density of 5,000 cells/well in growth medium or calcification medium. At the end of the incubation for 12 days, calcium deposition was examined according to the published procedure. Briefly, the cells were decalcified with 0.6 M HCl for 24 h. The calcium content of HCl supernatant was checked by measuring the cell media via inductively coupled plasma–mass spectrometry (HP 7500c, Agilent Technologies, Santa Clara, CA). After decalcification, the cells were washed three times with PBS and solubilized with 0.1 M NaOH/0.1% SDS, and the total protein content was measured with Bradford assay. The calcium content of the cell layer was normalized to cellular protein content.

To visualize the calcified nodules, cells in 12-well plates were washed three times with PBS at day 12, and exposed to Alizarin Red S (2% aqueous) for 5 min at room temperature. After rinsed with PBS, the mineralized nodules were photographed.

**WESTERN BLOT ANALYSIS**

Western blot analysis was examined according to the published procedure. Briefly, After being washed twice with cold PBS, cells were treated in 250 μl of ice-cold lysis buffer (1 mM EGTA, 1 mM dithiothreitol, 300 mM aprotinin, 50 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 10 mM Tris–HCl pH 7.4) on ice for 10 min, scraped into lysis buffer, and sonicated for 20 s on ice, then were centrifuged at 2,000 rpm at 4°C for 10 min. The supernatant were harvested to determine the protein expression of CaR.

The protein concentration of the supernatant was determined using the Bradford protein assay with BSA as standard. Equal amounts of the proteins were subjected to a 10% SDS–PAGE and then the resolved proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). The membranes were washed with TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20), blocked with 5% BSA solution in TBST for 1 h at room temperature, and then incubated with anti-CaR monoclonal antibody (1:2,500) in 5% BSA solution at 4°C overnight. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:2,000 in TBS-T for 1 h at room temperature. After extensive washing, bands were visualized by chemiluminescent method according to the manufacturer’s instructions. The optical densities of bands were quantified by Bio-Rad Quantity One 4.6.2 software. β-actin was used at a dilution of 1:400 as a loading control.

**MEASUREMENT OF PARTICLE SIZE DISTRIBUTION**

The size of the particles in complete cell culture medium and conditional medium with or without various concentrations of LaCl$_3$ was determined with a Zetasizer Nano ZS90 (Malvern Instruments, UK) based on dynamic light scattering at 37°C. Multiple experimental runs were performed to ensure the result. The result was given graphically as a histogram showing particle size distribution.

**SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSES**

Bovine VMSCs were plated in 24-well plates with glass cover slip 4 piece/well at a density of 5,000 cells/well in growth medium. At the end of the incubation for 12 days, cells were washed three times with
PBS and were fixed with step ethanol and osmic acid and then air-dried. The samples were fixed on a polymeric support, and then a thin layer of carbon was sputtered to increase the particles' electrical conductivity, and was submitted to scanning electron microscopy studies and to energy dispersive X-ray microanalysis (Lexica S440i and Supra 55). Microphotographs were recorded, and ratios of Ca/P and La/Ca were checked with the energy X-ray dispersive detector.

CELL VIABILITY ASSAY
BVMSCs were plated in 24-well plates at a density of 5,000 cells/well in growth medium or calcification medium. At the end of the incubation for 12 days, cell viability was measured by assessing mitochondrial activity using MTT assay. MTT in PBS (5 mg/ml) was added to reach a final concentration of 0.5 mg/ml. Further incubation at 37°C for 4 h, the supernatant was removed and the formazan dye dissolved in 150 μl dimethyl sulfoxide. Absorbance in each well was measured at 570 nm on a microplate reader (Multiskan Spectrum, Thermo Electron Corporation). Results are expressed as the percentage of MTT reduction relative to the control cells.

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL
Mitochondrial membrane potential (ΔΨₘ) was monitored using flow cytometry analysis with rhodamine 123 (Rh123) staining. Briefly, BVMSCs were grown in six-well plates, at the end of the incubation for 12 days, the medium was removed and the treated cells were washed three times in PBS. The cells were stained with Rh123 (10 μM) for 30 min at room temperature in the dark before cell harvesting. The washed cells were resuspended in PBS and the uptake of Rh123 was analyzed using a flow cytometer FACSVantage (SE, Becton Dickinson) with the excitation setting at 488 nm, and signals were acquired at the FL-2 channel. At least 10,000 cells per sample were acquired in histograms and data analyzed by CellQuest software.

Confocal microscope was also used to detect the change of mitochondrial membrane potential in multicellular nodules.

DETECTION OF APOPTOSIS
Apoptosis was evaluated using a flow cytometer with Annexin V-FITC and propidium iodide (PI) staining. Briefly, BVMSCs were grown in six-well plates, at the end of the incubation for 12 days, cells from each sample were suspended in a mixture of 5 μl Annexin V-FITC and 195 μl Annexin V-FITC binding buffer and were then incubated at room temperature for 10 min. The cells were centrifuged at 1,000g for 5 min, suspended again in 190 μl of binding buffer, and then added to with 10 μl of PI working solution. After being filtrated, the samples were analyzed using a flow cytometer FACSVantage. At least 10,000 events were analyzed.

In addition, to get rid of the lysed influence by trypsin, confocal microscope was also used to find out the apoptotic cells morphology in multicellular nodules.

MEASUREMENT OF CASPASE-3 AND CASPASE-9 ACTIVITY
Caspase activities were determined by a colorimetric assay based on the ability of caspases-3, and -9 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and acetyl-Leu-Glu-His-Asp p-nitroanilide (Ac-LEHD-pNA) into a yellow formazan product, respectively. The absorbance was measured at 405 nm as directed by the caspases-3 and -9 activity assay kit. At the end of the incubation for 12 days, samples were washed three times with PBS, lysed with 1% Triton X-100, and then centrifuged. Sample supernatants containing 50 μg of total protein were added to a reaction buffer containing Ac-DEVD-pNA (2 mM) or Ac-LEHD-pNA (2 mM), incubated for 4 h at 37°C, and then the absorbance of yellow pNA was measured by a spectrometer at 405 nm. The specific caspase-3 or -9 activity was normalized for total protein measured according to Bradford method.

CASPASE INHIBITION STUDIES
Bovine VSMCs were plated at a density of 5,000 cells/well in growth medium or calcification medium in 24-well plates. With or without pretreatment with 20 μM Z-VAD-FMK for 1 h as described in the figure legends, cells were incubated in the absence or presence of LaCl₃. At the end of the incubation for 12 days, cells were collected and washed twice with chilled PBS, calcium deposition was examined according to the quantification of calcium deposition procedure.

STATISTICAL ANALYSIS
Data shown are mean ± standard deviation (SD) from 2 to 4 separate experiments each performed in quadruplicate or triplicate. Significance was determined using one-way analysis of variance. P < 0.05 was considered significant.

RESULTS
EFFECT OF LaCl₃ ON β-GLYCEROPHOSPHATE-INDUCED CALCIFICATION IN BVMSCs
The addition of β-glycerophosphate to the culture medium induces osteoblastic differentiation and mineral deposition in BVMSCs. In this study, cells showed a marked increase in ALP activity and calcium content when incubated with calcification medium (Fig. 1A,B). Calcification was assessed by alizarin red staining, cells did not calcify when cultured in growth medium without β-glycerophosphate (Fig. 1C). Interestingly, we observed that LaCl₃ played a dual effect on β-glycerophosphate-induced calcification: lower concentrations of lanthanum (0.1 μM) led to a significant reduction of ALP activity and calcium content compared to β-glycerophosphate alone, but higher concentration of lanthanum (50 μM) showed a stimulative role (Fig. 1).

EFFECT OF LaCl₃ ON EXPRESSION OF CaR IN BVMSCs
CaR downregulation has been shown to be involved in the process of calcification of BVMSCs. We observed that β-glycerophosphate could lead to an ~5-fold downregulation of CaR expression relative to the non-calcification control, and this decrease was blocked with 0.1 μM LaCl₃ (Fig. 2A,B). Upon pretreating cells with NPS2143, a CaR antagonist, the inhibitory effect of 0.1 μM LaCl₃ on β-glycerophosphate-induced calcification was abolished (Fig. 2C). These results imply that the significance of CaR in mediating the effect of LaCl₃. Although 50 μM LaCl₃ increased β-glycerophosphate-induced calcification, a further β-glycerophosphate-induced decrease in CaR expression was not observed.
EFFECT OF LaCl₃ ON MORPHOLOGY AND COMPOSITION OF PRECIPITATES IN BVMSCs

Some population of BVMSCs grow into multicellular nodules, the matrix of which became calcified. Under calcification conditions, cells grouped into multicellular aggregates or nodules (Fig. 3).

**Fig. 1.** Effect of LaCl₃ on β-glycerophosphate-induced ALP activity and calcium deposition in bovine VMSCs. Cells were incubated in growth medium and calcification medium in the absence or presence of the indicated concentration of LaCl₃. ALP activity (A) and calcium deposition (B) were determined with cells after treatment for 9 and 12 days, respectively. Both are normalized to cellular protein content. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate.

**Fig. 2.** Effect of LaCl₃ on β-glycerophosphate-induced expression of CaR in bovine VSMCs. Cells were incubated for 12 days under growing and calcifying conditions in the absence or presence of the indicated concentration of LaCl₃. CaR protein was detected by Western blotting in total lysates after 12 days. A: CaR protein was detected by Western blotting in total lysates after 12 days. B: the data on O.D. (% of control) are presented as mean ± SD (n = 3). Typical Western blot detections are shown at the bottoms of bar graphics. *P < 0.05 versus non-calcification control; #P < 0.05 versus LaCl₃-free calcification control. C: After pretreatment with 0.1 ng/ml NPS-2143 for 1 day, cells were incubated in the calcification medium in the absence or presence of the indicated concentration of LaCl₃ for 12 days. Calcium deposition was measured, and data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. #P < 0.05 versus LaCl₃-free calcification control; &P < 0.05 versus LaCl₃-containing calcification control.

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Fig. 3. Scanning electron micrographs of precipitates formed in multicellular nodules under calcifying conditions. A: LaCl₃-free calcification control; B: 0.1 μM LaCl₃ + 10 mM β-GP; C: 50 μM LaCl₃ + 10 mM β-GP. Scale bars are noted in each panel, and elemental compositions of precipitates are analyzed by energy dispersive X-ray analysis, respectively.
Electron microscopic and energy dispersive X-ray analysis of nodules showed that they consisted of mostly calcium, phosphorous, and oxygen, consistent with an apatite-like material. In the presence of 50 μM LaCl3, VMSCs formed more and bigger nodules in which were confirmed the coexistence of lanthanum, calcium, and phosphorus by elemental analysis (Fig. 3C).

**EFFECT OF LaCl3 ON SIZE DISTRIBUTION OF PRECIPITATES IN CULTURE MEDIUM**

Culture medium is a metastable calcium phosphate system. In this study, dynamic light scattering experiments were carried out for detection of a scattered intensity of particles in the solutions. Figure 4A shows typical profiles of particle size distribution in culture medium. The new peak on the right side is caused by addition of LaCl3, showing larger size and higher intensity with increasing concentration of LaCl3. Moreover, the size distributions of precipitates increase with the supply amount of Ca2⁺ (Fig. 4B). In addition, the size distribution of these particles ranges from 100 to 400 nm in cell culture medium. These results confirmed the promotory effect of La3⁺ on calcium precipitation, and the majority of the observed precipitates was calcium phosphate.

**EFFECT OF LaCl3 ON CELL VIABILITY IN BVMSCs**

Calcium phosphate particles exhibit a cytotoxic potential. To determine the promotion in calcification was due to the reduction in cell viability, the effects of LaCl3 on cytotoxicity were investigated with an MTT assay. As shown in Figure 5, lower concentration of lanthanum (0.001–1 μM) did not reduce cell viability in the presence of β-glycerophosphate, but the exposure to higher LaCl3 (10–200 μM) caused a significant decrease in VMSC viability in a concentration-dependent way.

**EFFECT OF LaCl3 ON MITOCHONDRIAL MEMBRANE POTENTIAL IN BVMSCs**

Mitochondrial depolarization is considered as an irreversible step in the apoptosis process. Consequently, we have made an attempt to measure the loss of mitochondrial membrane potential (ΔΨm) using...
a fluorogenic probe Rh123. As shown in confocal imaging, lower fluorescence in the presence 50 µM LaCl₃ indicates significantly greater loss of mitochondrial membrane potential in multicellular nodules compared to the LaCl₃-free calcified individuals (Fig. 6C). A concentration-dependent decrease in $\Delta \Psi_m$ after exposure to higher concentration of LaCl₃ (50–200 µM) was also observed (Fig. 6D). In addition, 0.1 µM of LaCl₃ did not affect the level of fluorescence compared to the LaCl₃-free calcified individuals (Fig. 6B).

**EFFECT OF LaCl₃ ON CELL APOPTOSIS IN BVMSCs**

Apoptosis precedes VMSCs calcification and apoptotic bodies may act as nucleating structures for calcium crystal formation. To find out whether apoptosis occurred in BVMSCs nodules, Annexin V-FITC and PI were used to visualize apoptotic cells. As shown in Figure 7, a 12-day incubation with higher concentration of LaCl₃ (50 µM) led to an increase in both apoptosis (Annexin V-FITC positive) and necrosis (PI positive). Flow cytometer analysis also showed that LaCl₃ (50–200 µM) markedly increase apoptotic index (Fig. 7D). However, after 0.1 µM of LaCl₃ treatment, apoptotic and necrotic cells in nodules were decreased compared to the LaCl₃-free calcified individuals (Fig. 7B).

**EFFECT OF LaCl₃ ON CASPASE ACTIVITIES IN BVMSCs**

The central component of apoptosis is a family of caspses which are responsible for triggering and affecting the apoptotic process. To ascertain whether the effect of LaCl₃ resulted from regulating caspase activity, we measured caspases-9 and -3 catalytic activity using specific chromogenic substrates. Treatment of cells with...
50 μM LaCl₃ caused caspases-9 and -3 activity increase in the presence and absence of β-glycerophosphate (Fig. 8A,B). The effects of LaCl₃ on apoptosis are not only stimulatory but also inhibitory, as shown in Figure 8B, 0.1 μM LaCl₃ decreased significantly β-glycerophosphate-induced caspase-3 activity. In the presence of a pan-caspase inhibitor, Z-VAD-FMK, LaCl₃-promoted calcification was completely blocked through 1 h pretreatment with a (Fig. 8C). These results indicate caspases-9 and -3 were involved in apoptosis induced by higher concentration lanthanum in BVSMCS.

DISCUSSION

Recently, some gadolinium-based contrast agents (GBCAs) has been related with nephrogenic systemic fibrosis [Rofsky et al., 2008; Sanyal et al., 2011]. Lanthanum and gadolinium are close in the periodic table and have analog physiochemical properties. The use of lanthanum carbonate (Fosrenol) as an oral phosphate binder might have some analogies [Aime et al., 2007; Brambilla et al., 2008; Broe, 2008]. Although lanthanum carbonate is apparently safe for patients with advanced CKD, some clinic reports have shown that lanthanum therapy is possibly responsible for acute liver failure [Leeuw et al., 2008] and encephalopathy [Fraile et al., 2011]. Clearly, more work appears necessary to assess potential toxic effects associated to the use of lanthanum-based drugs.

Lower lanthanum level suppresses the calcification via upregulating decreased CaR expression in BVSMCs. The CaR senses alterations in the level of extracellular calcium and responds with changes in function. The study by Alam et al. [2009] strongly suggest that a functional CaR is necessary to maintain a VMSCs
phenotype and to keep calcification suppressed. Moreover, it is shown that the stimulation of CaR with calcimetics attenuates VSMCs calcification [Mizobuchi et al., 2004]. Since lanthanum has higher binding affinity than calcium, it can act as a substitute or antagonist for calcium or as a calcium channel blocker to explain many of the known biological effects of calcium [Kramsch et al., 1980]. The nanomolar LaCl3 has been found to activate the CaR and enhanced CaR sensitivity to calcium [Carrillo-Lo´pez et al., 2010]. In our study, VSMCs treated with 0.1 μM LaCl3 showed ameliorated calcium deposition in extracellular matrix in the presence of β-glycerophosphate. In addition, the level of CaR expression downregulated by β-glycerophosphate was increased. In our previous study, we have found that pretreatment with pertussis toxin (PTx), a Gi protein inhibitor, suppressed the LaCl3-induced osteoblastic differentiation in rat VSMCs [Shi et al., 2009]. Taken together, these results indicate that the significance of CaR in mediating the effect of LaCl3 on inhibiting VSMCs calcification. Lanthanum carbonate is an effective and beneficial phosphate-binding agent [Freitas et al., 2007; Hutchison, 2009]; in addition, other modes of action exist. Supporting this point, a recent animal study has found that lanthanum content was elevated in aortas with VC after administration of lanthanum acetate [Zhou et al., 2009]. Moreover, lanthanum appears to exert its vascular effects via changes in oxidative stress or bone remodeling [Nikolov et al., 2011]. In addition, the level of PTH decreased after the use of lanthanum carbonate compared with sevelamer-HCl [Hutchison and Laville, 2008; Navaneethan et al., 2009]. Due to the low systemic absorption (0.0013%), serum lanthanum levels is <0.2 nM in health human subjects [Pennick et al., 2006]. However, lanthanum exposure could raise the level to 0.1 μM (13.9 ng/ml) at certain circumstances [Hutchison et al., 2008]. Therefore, the roles of lanthanum observed in the present study could be relevant to that happens in vivo.

Higher lanthanum level enhances the calcification via calcium phosphate particles via inducing apoptosis in BVSMCs. Under the same condition, 0.1 μM LaCl3 also upregulated decreased CaR expression similar to 0.1 μM group. However, higher concentration of lanthanum was surprisingly found to promote VSMCs calcification. To explain the reason for the different action pattern, we further found that the lanthanum coexisted with calcium and phosphorus in multicellular nodules. VSMCs were maintained in DMEM medium containing 1.8 mM calcium chloride and 0.9 mM sodium phosphate monobasic, being a metastable calcium phosphate solution [Jonge et al., 2009; Mandel and Tas, 2010]. After adding lanthanum solution into the cell medium at higher concentrations, we observed white cloudy substance on the bottom of culture plates, and identified particulate matter with lanthanum-containing calcium phosphate precipitation. Indeed, calcium phosphate particles are prevailing in humans and have been shown to have biological activities in many cell types [Motskin et al., 2009; Sommer, 2010; Yuan et al., 2010]. In a recent study, this has been shown that synthetic calcium phosphate crystals and human atherosclerosis-derived crystals induced cell apoptosis in human VSMCs with their potency depending on their size [Ewence et al., 2008]. It is well-known that apoptosis precedes VSMC calcification and apoptotic bodies derived from VSMCs may act as nucleating

![Fig. 8. Effect of LaCl3 on enzyme activities of caspase-3 (A) and caspase-9 (B) in bovine VSMCs. Cells were incubated for 12 days under growing and calcifying conditions in the absence or presence of the indicated concentration of LaCl3, caspase activity was determined by a colorimetric assay. Bar graph indicates the mean ± SD (n = 4). *P < 0.05 versus non-calcification control, #P < 0.05 versus LaCl3-free calcification control. C: After pretreatment with 20 μM Z-VAD-FMK for 1 h, cells were incubated in the calcification medium in the absence or presence of the indicated concentration of LaCl3 for 12 days. Calcium deposition was measured, and data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. #P < 0.05 versus LaCl3-free calcification control; &P < 0.05 versus LaCl3-containing calcification control.]
structures for deposition of calcium phosphate mineral [Proudfoot et al., 2000]. The exposure to 50 μM LaCl₃ reduced cell viability, decreased mitochondrial membrane potential, and induced cell apoptosis involving caspases-9 and -3. These facts indicate that higher lanthanum level is likely to have a pro-apoptotic potential and thereby enhance calcification. At the higher concentration of LaCl₃, a part of lanthanum ions are in the “free” form [Kubota et al., 2000], which could upregulate the CaR expression and, at the same time, it is likely to block calcium channels preventing calcium influx. Other studies previously found the role of lanthanum in promoting calcification was also demonstrated in vivo. For example, rats underwent local calcification when injected LaCl₃ into the subcutaneous tissue [Garrett and McClure, 1981], while intravenous injections produced a milieu proven to be conducive to the deposition of calcium phosphate [Johannsson et al., 1968]. Indeed, there is continuing concern regarding the possibility of metal accumulation in tissues over time and possible long-term side effects. In fact, in particular in patients, some reports have suggested this possibility [Lacour et al., 2005; Davis and Abraham, 2009].

In conclusion, our studies have shown for the first time that LaCl₃ bidirectionally influences calcification in BVMSCs: lower LaCl₃ level suppressed the calcification via upregulating decreased CaR expression; higher LaCl₃ level promoted precipitation of calcium phosphate, and these lanthanum-containing calcium phosphate particles induced cell apoptosis and enhanced calcification.

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