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Lanthanum chloride suppresses hydrogen peroxide-enhanced calcification in rat calcifying vascular cells

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Abstract Lanthanum chloride ($LaCl_3$) has been shown to retard the progression of established atherosclerotic lesions in animal models, and used as a calcium channel blocker in various cellular experiments. In this study, we assessed the role of lanthanum chloride ($LaCl_3$) in H_2O_2-enhanced calcification in rat calcifying vascular cells (CVCs) and examined the involvement of MAPK signaling pathways. H_2O_2 induced growth inhibition of CVCs, as well as increases in intracellular levels of calcium and reactive oxygen species, ALP activity, apoptosis and calcium deposition. These effects of H_2O_2 were suppressed by pretreatment of the cells with 1 μM of $LaCl_3$ for 2 h. In addition, H_2O_2 activated the phosphorylation of ERK1/2, JNK and p38 MAPK, but only the last two were associated with the ALP activity. Our findings demonstrate that H_2O_2-enhanced osteoblastic differentiation and apoptosis are responsible for the increased calcification in rat CVCs, and $LaCl_3$ can counteract these effects by suppressing the activation of JNK (JNK2, but not JNK1) and p38 MAPK signaling pathway.

Keywords Lanthanum · Hydrogen peroxide · Calcifying vascular cells · Calcification · MAPK signaling

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Introduction

Lanthanum chloride ($LaCl_3$) has been shown to retard the progression of established atherosclerotic lesions in animal models (Gillies et al. 1989; Kramsch et al. 1980). Atherosclerosis is frequently associated with arterial calcification that has been recognized as an active, cell-controlled event (Doherty 2004; Shao et al. 2006). Intracellular calcium ions ($[Ca^{2+}]_i$) may act as a second messenger. Since lanthanum cation (La^{3+}) is similar to Ca^{2+} in ionic radius, it has a high affinity for Ca^{2+} sites on some proteins and hence can act as either a calcium channel blocker or a probe (Fricker 2006). Therefore, La^{3+}-modulated cell proliferation or apoptosis in vitro may be derived from its effect on the inhibition of calcium fluxes which are required for cell cycle regulation (Dai et al. 2002; Heffeter et al. 2006; Sato et al. 1998; Shi and Huang 2005).

Oxidative stress is involved in the development of atherosclerosis (Rojas et al. 2006; Vokurkova et al.

2007). In vascular smooth muscle cells (VSMCs), the increased reactive oxygen species (ROS) is mostly produced by NADPH oxidase (Mody et al. 2001). ROS released from other cells, e.g. macrophages and endothelial cells within the atherosclerotic lesion, can also enters VSMCs. ROS signaling can induce VSMCs to undergo osteoblastic differentiation (Parhami et al. 1997; Tabet et al. 2005; Touyz 2003). Increasing evidence indicates that this event may occur through the activation of mitogen-activated protein kinases (MAPKs) (Su et al. 2001; Tabet et al. 2005; Touyz and Schiffrin 2004; Ungvari et al. 2006). Among MAPKs, JNK and p38 MAPK are mostly associated with cellular responses to diverse stresses including oxidative stress (Humara et al. 2007), whereas extracellular signal-regulated kinase 1/2 (ERK1/2) has been known to plays a major role in regulating cell survival and proliferation (Meloche and Pouysségur 2007).

Vascular smooth muscle cells, along with macrophages and mast cells, are believed to be the primary cells involved in atherosclerotic intimal calcification (Johnson et al. 2006). Under the induction of β-glycerophosphate, VSMCs from normal vessels may express osteoblast-specific genes, such as alkaline phosphatase (ALP), collagen types I and II, osteocalcin and osteopontin. This subpopulation of smooth muscle cells is known as "calcifying vascular cells" (CVCs) that exhibit osteoblastic characteristics and form calcified nodules in vitro (Wallin et al. 2001; Watson et al. 1994), providing a suitable model for the study of vascular calcification mechanism (Shioi et al. 1995).

Lanthanum cation has been used as a calcium channel blocker in various cellular experiments, and shown to retard the progression of established atherosclerotic lesions in animal models (Gillies et al. 1989; Kramsch et al. 1980). As a "super-calcium" ion, La^{3+} is expected to affect CVCs via calcium homeostasis. The aim of the present study was to assess the role of lanthanum chloride ($LaCl_3$) in H_2O_2-enhanced calcification in rat CVCs and to examine the involvement of MAPK signaling pathways.

Materials and methods

Cell culture

VSMCs were isolated from rat aortic media, and cultured in DMEM (Gibco) containing 15% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO_2 atmosphere according to the method described previously (Ray et al. 2002). After confluence, the VSMCs were switched to culture media with 10 mM sodium β-glycerophosphate (Sigma–Aldrich) for 12 days, followed by von Kossa staining to characterize the calcifying matrix. These cells were used as calcifying vascular cells (CVCs) for the following experiments (Shioi et al. 1995). In the time course experiments, the beginning day of CVCs culture was defined as day 0.

MTT assay

VSMCs (2×10^4 cells/well in 96-well plates) were cultured with 10 mM sodium β-glycerophosphate for 12 days, then the obtained CVCs were incubated with H_2O_2. Some of the CVCs were pretreated with 1 μM of $LaCl_3$ for 2 h before exposure to H_2O_2. Cells were treated with the MTT solution (1 mg/ml, Sigma–Aldrich) for 4 h. The dark-blue formazan crystals formed in intact cells were dissolved in 150 μl DMSO, and the OD value at 540 nm was measured with a microplate reader. Results are expressed as the percentage of MTT reduction relative to the control cells.

Alkaline phosphatase assay

Cells were washed 3 times with PBS, lysed with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for ALP activity (Shioi et al. 1995) with or without pre-treatment with the specific inhibitors (Sigma–Aldrich) of ERK1/2 (PD98059), JNK (SP600125) and p38 (SB202190). Briefly, 160 μl of substrate mixture consisting of 16 mM *p*-nitrophenyl phosphate sodium in 350 mM 2-amino-2-methyl-L-propanol (AMP) (Sigma–Aldrich) and 2 mM $MgCl_2$ at pH 10.5 was added to 40 μl of each thawed lysate in 96-well plates. The plates were incubated at 37°C for 1 h and the reaction was terminated by adding 12 μl of 1 M NaOH. The ALP activity was assayed by conversion of a colorless *p*-nitrophenyl phosphate (Sigma–Aldrich) to a colored *p*-nitrophenol. The color change was measured spectrophotometrically at 490 nm. ALP levels were normalized to the total protein content of cells at the end of the experiment.

Quantization of calcium deposition

Calcification was examined according to the published procedure (Jono et al. 1997). Briefly, CVCs were rinsed with phosphate buffered saline (PBS) and decalcified with 0.60 mM HCl at 4°C for 24 h. The calcium content of HCl supernatant was determined colorimetrically by measuring the *o*-cresolphthalein complexone. After decalcification, the cells were washed three times with PBS and solubilized with 0.10 M NaOH/0.10% SDS, and the total protein content was determined with Lowry method. The calcium content of the cell layer was normalized to cellular protein of the culture.

Measurement of intracellular Ca^{2+}

The Ca^{2+} responses in CVCs were assessed using fluo-3-AM (Sigma, Aldrich) in conjunction with a fluorometric imaging plate reader (Leica, TCS SP2). Cells were loaded with 5 μM fluo-3-AM in the presence of 0.02% pluronic F-127 (Sigma–Aldrich) in loading-buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM glucose, 1 mM $CaCl_2$, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); pH 7.5) for 20 min at room temperature. Add 5 volumes of HBSS containing 1% fetal calf serum, and continue the incubation for another 20 min. The plate was immediately placed on the plate reader and 50 μM of H_2O_2 was added at 1 min. The relative fluorescence change of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

ROS generation analysis

CVCs with or without pre-treatment with 1 μM of $LaCl_3$ for 2 h were stimulated with 50 μM H_2O_2 for 1 h. Intracellular levels of ROS were assessed using the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes). After membrane diffusion, DCFH-DA is deacetylated by intracellular esterases to DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Cells were incubated with 5 μM DCFH-DA in the dark at 37°C for 20 min. Images were captured on a confocal fluorescence microscope (Leica, TCS SP2).

Annexin-V-FITC/propidium iodide staining

Apoptotic cells were detected using an annexin-V-FITC Apoptosis Detection Kit (BioVision) according to the manufacturer's instructions. Phosphatidylserine (PS) is exposed during early apoptosis by flipping from the inner to the outer plasma membrane leaflet, and annexin V-FITC can bind to PS with high affinity. Propidium iodide (PI) conjugates to necrotic cells. A double staining with annexin V-FITC and PI was detected to identify the apoptotic cells. After treatment with 50 μM H_2O_2 for 1 day in the absence or presence of 1 μM $LaCl_3$, cells were incubated with FITC-labeled annexin-V and propidium iodide, washed, and analyzed on a confocal fluorescence microscope (Leica, TCS SP2).

Western blot analysis

CVCs were cultured in serum-free medium for 4 h. And then CVCs with or without pretreatment with 1 μM of $LaCl_3$ for 2 h were incubated with H_2O_2. After being washed with ice-cold PBS and TSE, cells were scraped in 300 μl ice-cold lysis buffer (1 mM DTT, 300 nM aprotinin, 50 μM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF). After centrifugation at 1,000×*g* for 15 min, the supernatant was separated and stored at −70°C. The total protein concentration was determined using Bradford method. Extracts were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel, and subsequently transferred to polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA, USA) that was blocked with TTBS (0.5 mM Tris–HCl, pH 7.5 and 0.2% Tween-20) containing 5% BSA for 1 h at room temperature. Western blot analysis was performed by incubating the membranes with specific antibodies against phosphorylated and non-phosphorylated ERK1/2, JNK and p38 (1:1,000, Cell Signalling, Beverly, MA, USA). After three washes with Tris-buffered saline with 0.1% Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibodies (Cell Signalling) in TBST with 5% BSA at a 1:2,000 dilution for 1 h at room temperature. After being washed three times in TBST buffer, the blots were developed using the enhanced chemiluminescence (ECL) detection method by immersing them for 5 min in a mixture of ECL reagents A and B at the ratio 1:1

and exposing them to photographic film for a few minutes. Densitometric analysis of bands was done by Scion Image software, and the results are presented as percentage of phosphorylation compared to the phosphorylation induced by H_2O_2.

Statistics

Each experiment was repeated at least three times. Data were expressed as means ± standard deviations. Comparisons were performed through One-way ANOVA. A value of $P < 0.05$ was considered significant.

Results

Lanthanum chloride abrogates H_2O_2-induced growth inhibition

We examined the effect of $LaCl_3$ on H_2O_2-induced growth inhibition in CVCs using MTT assay. Cell viability decreased with increasing concentration of exogenous H_2O_2 (Fig. 1a). The inhibitory effect of H_2O_2 was suppressed by pretreatment of the cells with 1 μM of $LaCl_3$ for 2 h (Fig. 1b), although $LaCl_3$ alone did not affect cell growth significantly (data not shown).

Lanthanum chloride counteracts H_2O_2-induced increase in ALP activity

ALP is a marker of the osteoblastic differentiation of CVCs. H_2O_2 at concentrations ranging from 20 to 100 μM significantly increased ALP activity. A bell-shaped dose-dependence was observed with a maximum at 50 μM H_2O_2 (Fig. 2a). The H_2O_2-induced increase at this concentration was abolished or even over-counteracted by pretreating the cells with $LaCl_3$ for 2 h, although $LaCl_3$ alone did not affect the ALP activity significantly at the indicated concentrations (Fig. 2b).

Lanthanum chloride inhibits H_2O_2-enhanced calcium deposition

Addition of H_2O_2 at concentrations ranging from 20 to 100 μM significantly increased calcium deposition in extracellular matrix. A bell-shaped dose-dependence was observed with a maximum at 50 μM H_2O_2 (Fig. 3a). The enhancive effect of H_2O_2 at this concentration was abolished by pretreatment of the cells with $LaCl_3$ for 2 h, although $LaCl_3$ alone did not affect calcium deposition significantly at the indicated concentrations (Fig. 3b).

Lanthanum chloride blocks H_2O_2-induced increase in $[Ca^{2+}]_i$

The effect of exogenous H_2O_2 on calcium mobilization was examined in fluo3-AM-loaded CVCs. In the presence of extracellular Ca^{2+}, a transient increase in $[Ca^{2+}]_i$ was observed upon addition of 50 μM H_2O_2 (Fig. 4a). Pretreatment of CVCs with 1 μM $LaCl_3$ blocked the H_2O_2-induced calcium signal as shown in (Fig. 4b).

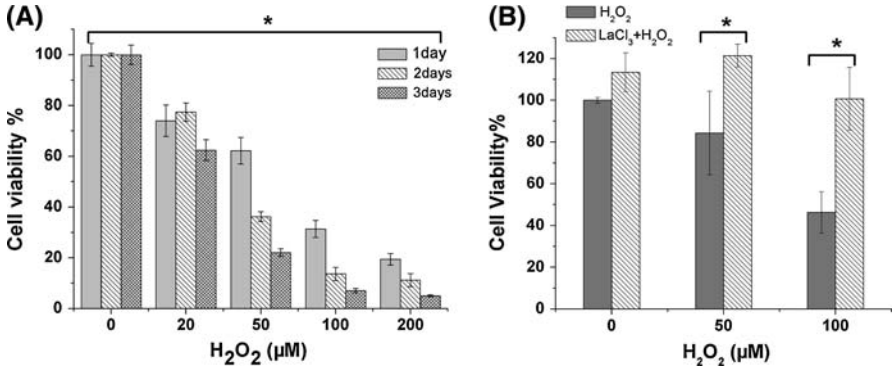


Fig. 1 Growth inhibition of CVCs induced by H_2O_2 **a** Concentration dependence. *$P < 0.05$ versus control; **b** Effect of $LaCl_3$ on H_2O_2-induced growth inhibition for 1 day. Cells were pretreated with 1 μM of $LaCl_3$ for 2 h before the addition of H_2O_2. *$P < 0.05$ versus lanthanum-free control. Cell viability was examined with MTT assay and expressed as a percentage of the control. Experiments were performed at least three times. Data are mean ± standard deviations of triplicate determinations from one experiment

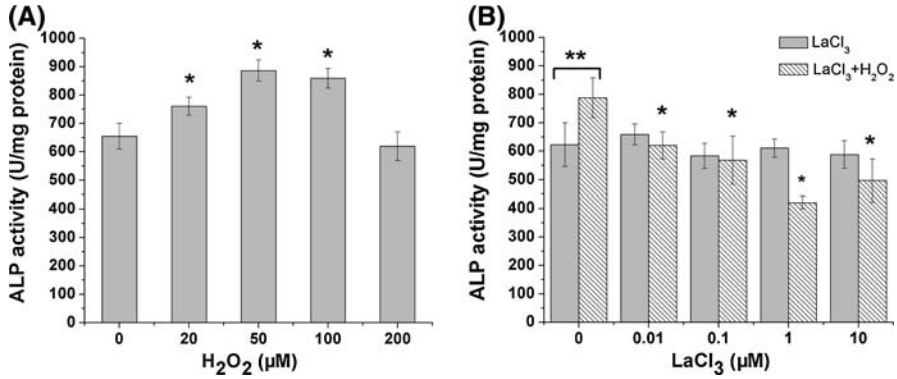


Fig. 2 The activity of ALP after treatment of CVCs with H_2O_2 for 4 day. **a** Concentration dependence. $*P < 0.05$ versus control; **b** Inhibition of 50 μM·H_2O_2-enhanced ALP activity by $LaCl_3$. $**P < 0.05$ versus control, $*P < 0.05$ versus 50 μM H_2O_2 control. Experiments were performed at least three times. Data are mean ± standard deviations of triplicate determinations from one experiment

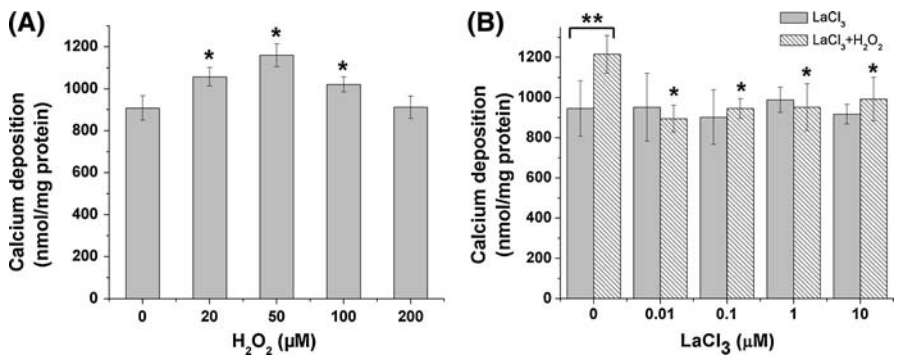


Fig. 3 Calcium deposition in CVCs after treatment with H_2O_2 for 4 day. **a** Concentration dependence. $*P < 0.05$ versus control; **b** Inhibition of 50 μM H_2O_2-enhanced calcium deposition by $LaCl_3$. $**P < 0.05$ versus control, $*P < 0.05$ versus 50 μM H_2O_2 control. Experiments were performed at least three times. Data are mean ± standard deviations of triplicate determinations from one experiment

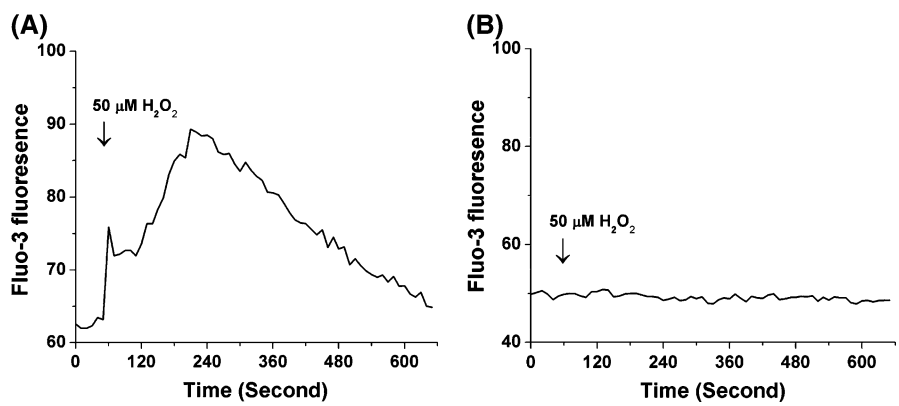


Fig. 4 Effect of H_2O_2 on intracellular calcium concentration in CVCs. **a** control, without $LaCl_3$. **b** Pretreatment with 1 μM lanthanum chloride for 2 h. The time of addition of H_2O_2 is indicated. Results shown are representative tracings of $n = 12$

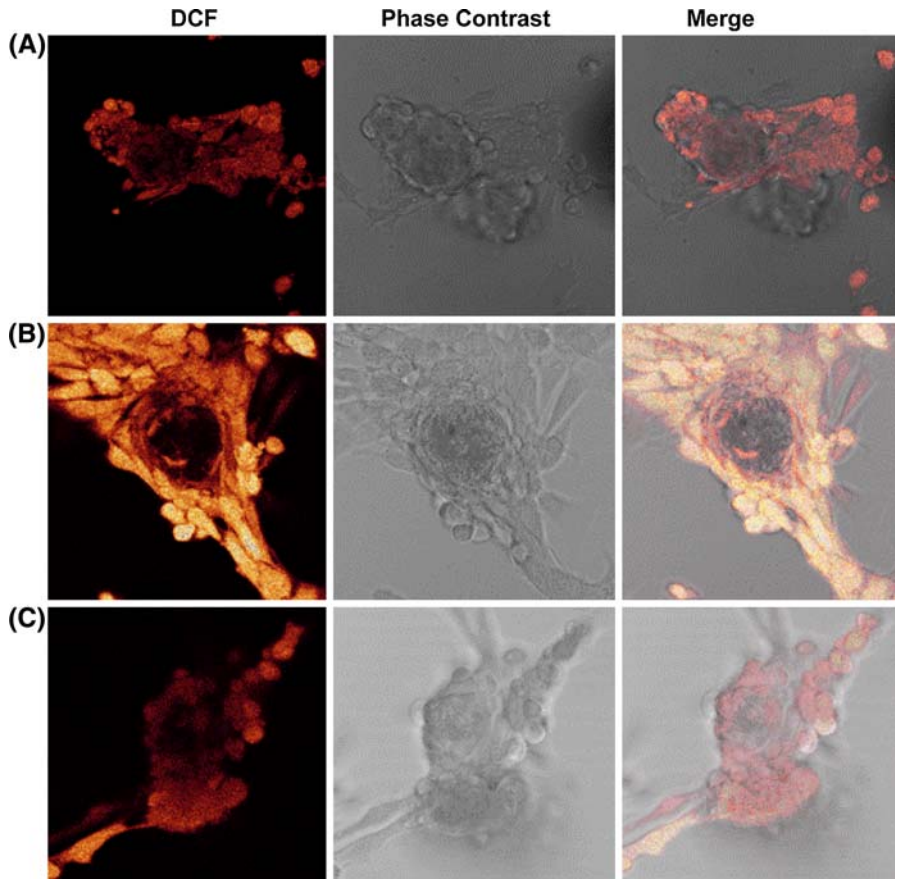


Fig. 5 In situ ROS generation staining with CM-H_2DCFDA in CVCs. **a** Untreated control cells; **b** Cells treated with 50 μM H_2O_2 for 1 h; **c** Cells pretreated with 1 μM $LaCl_3$ for 2 h, and then exposed to 50 μM H_2O_2 for 1 h

Lanthanum chloride inhibits H_2O_2-induced elevation in ROS level

In CVCs around a calcified nodule, the base level of intracellular ROS was markedly elevated upon treatment with 50 μM of H_2O_2 for 1 h (Fig. 5a, b). And the elevation was inhibited by pretreatment of the cells with 1 μM of $LaCl_3$ for 2 h before the exposure to H_2O_2 (Fig. 5c).

Lanthanum chloride reduces H_2O_2-promoted apoptosis

Apoptosis of CVCs around calcified nodules was visualized by using confocal microscopy after double staining with PI and FITC-labeled annexin V. The green and red fluorescence in Fig. 6 indicate early apoptotic cells and late apoptotic/necrotic cells respectively. Pretreatment of the cells with 1 μM of $LaCl_3$ for 2 h efficiently reduced H_2O_2-promoted apoptosis (Fig. 6c).

Lanthanum chloride affects H_2O_2-activated MAPK signaling pathways

No matter whether H_2O_2 presented, ALP activity was markedly reduced by pretreatment of CVCs with the specific inhibitors of JNK (SP600125) and p38 (SB202190) MAPK signaling pathways respectively (Fig. 7a), but no significant change was caused by the ERK1/2-specific inhibitor (PD98059). In the absence of H_2O_2, La(III) activated ERK1/2 and JNK but not p38 (Fig. 7d, f, h). Exposure of CVCs to 50 μM of H_2O_2 induced a transient activation of JNK within 15 min, while the activations ERK1/2 and p38 MAPK sustained for up to 60 min (Fig. 7c, e, g). However, pretreatment of the cells with 1 μM of $LaCl_3$ reduced the effect of H_2O_2 on the phosphorylation of JNK (JNK2, not JNK1) and p38 MAPK (Fig. 7f, h). The effect of $LaCl_3$ on the H_2O_2-enhanced activation of ERK1/2 MAPK was not statistically significant (Fig. 7d).

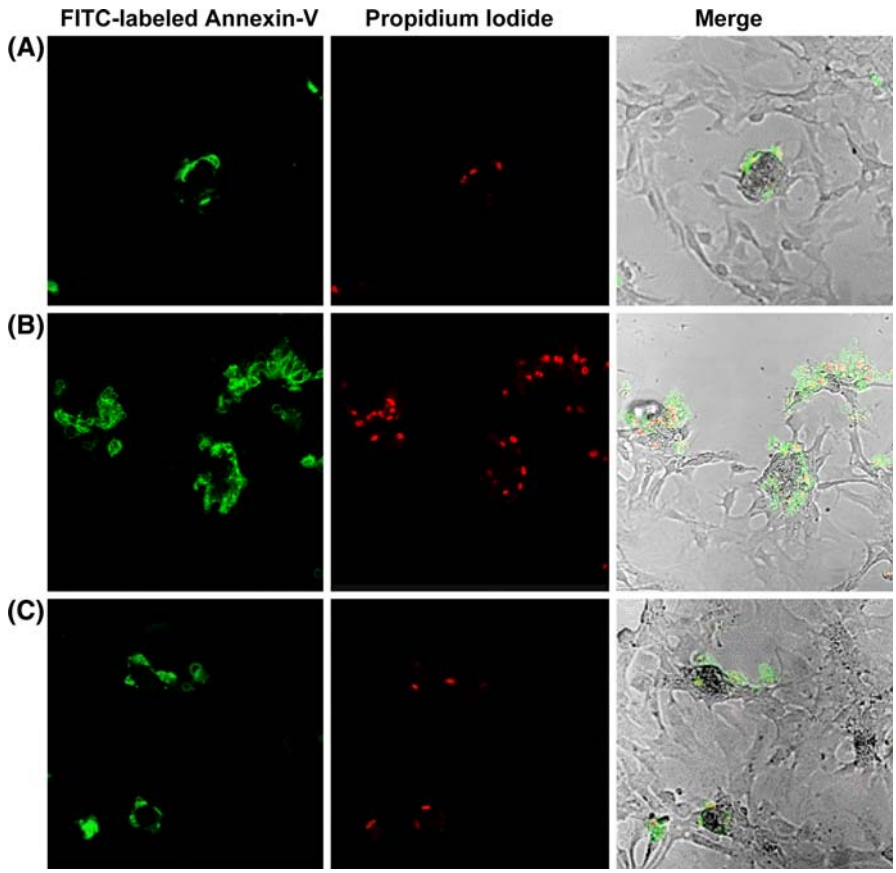


Fig. 6 Photomicrographs of annexin V-FITC/propidium iodide staining. The left and middle panels show the fluorescence from FITC-labeled annexin-V and propidium iodide, respectively. **a** Untreated control cells; **b** Cells treated with 50 μM of H_2O_2 for 1 day; **c** Cells pre-treated with 1 μM of $LaCl_3$ for 2 h, and then exposed to 50 μM of H_2O_2 for 1 day

Discussion

Vascular calcification shares several features with mineralization in skeletal tissue (Doherty 2004; Johnson et al. 2006), and one of them at cellular level is the phenotypic transformation from VSMCs into CVCs. CVCs resemble osteoblasts as indicated by the expression of bone matrix proteins (Doherty 2003; Lee et al. 2006; Wada 1999; Wallin et al. 2001) and the formation of calcified nodules. By means of the rat CVC model (Boström 2001), we have examined the role of $LaCl_3$ in H_2O_2-enhanced calcification and assessed the involvement of MAPK signaling pathways.

The H_2O_2-enhanced calcification in rat CVCs can be inhibited by pretreating the cells with $LaCl_3$. Addition of H_2O_2 elevated the intracellular levels of Ca^{2+} (Fig. 4) and ROS (Fig. 5), and increased the activity of ALP, a marker of osteoblastic differentiation (Fig. 2). The H_2O_2-promoted osteoblastic differentiation has previously been reported for bovine CVCs (Mody et al. 2001). In addition, H_2O_2 also promoted apoptosis in CVCs (Fig. 6). A relatively high rate of apoptosis can occur in nodules and the alteration of the apoptotic rate influences calcification (Proudfoot et al. 2000). By producing matrix vesicles (Reynolds 2004) and apoptotic bodies (Proudfoot et al. 2000), respectively, both the differentiated and apoptotic CVCs may contribute to the increased calcium deposition (Fig. 3). Nevertheless, all these effects of H_2O_2 were suppressed by pretreatment of CVCs with 1 μM of $LaCl_3$. ROS may lead to an increase of $[Ca^{2+}]_i$ (Bejarano et al. 2007; Mata et al. 2008; Pariente et al. 2001; Wilkinson et al. 2008), and a role for calcium signaling in apoptosis is well documented (Hajnóczky et al. 2003; Krebs 1998; McConkey and Orrenius 1997). Since La^{3+} is known to be a calcium channel blocker, its role in suppressing H_2O_2's effects might be played through calcium signaling in CVCs. The counteraction of H_2O_2-induced changes by $LaCl_3$, such as increases in intracellular

calcium concentration and myosin light chain phosphorylation, has been documented for bovine aortic endothelial cells (López-Ongil et al. 1999). To our knowledge, the data from the present study provide the first case on the protective role of $LaCl_3$ against H_2O_2-enhanced calcification in rat CVCs.

MAPKs are differentially phosphorylated according to the intra- or extracellular origin of the Ca^{2+} source. Extracellular Ca^{2+} can promote ERK phosphorylation through calcium-sensing receptor (CaR) in rat aortic VSMCs (Smajilovic et al. 2006). We found that $LaCl_3$ induced the phosphorylation of ERK1/2 and JNK (JNK2, but not JNK1) MAPK in CVCs (Fig. 7d, f). Since La^{3+} may act as a calcium channel blocker and a CaR agonist (Hofer and Brown 2003; Smajilovic and Tfelt-Hansen 2007), its effect on the MAPK signalling could be executed through binding to CaR (Shorte and Schofield 1996).

The protective role of La^{3+} might be associated with MAPK signaling pathways. It has been reported that ROS signaling modulates the recapitulation of osteogenesis in CVCs (Parhami et al. 1997). ROS can lead to activation of the pathways that control cell differentiation and apoptosis, including MAPKs (Kefaloyianni et al. 2006; Taniyama and Griendling 2003). H_2O_2 has been shown to activate JNK and p38 MAPK, but its effect on ERK1/2 is controversial, with some reports showing inhibition and others demonstrating stimulation (Clempus and Griendling 2006; Griendling et al. 2000). We confirmed that although all the three subfamilies of MAPKs were stimulated by H_2O_2 (Fig. 7c, e, g), only the JNK and

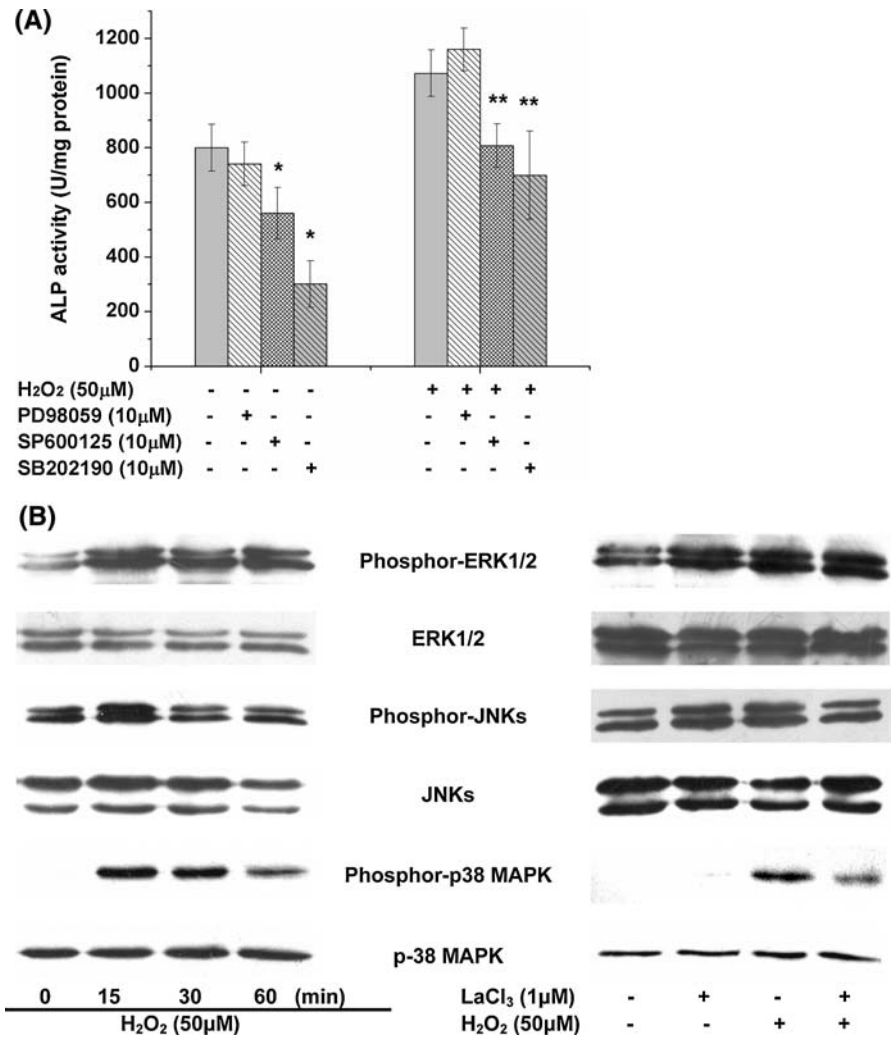
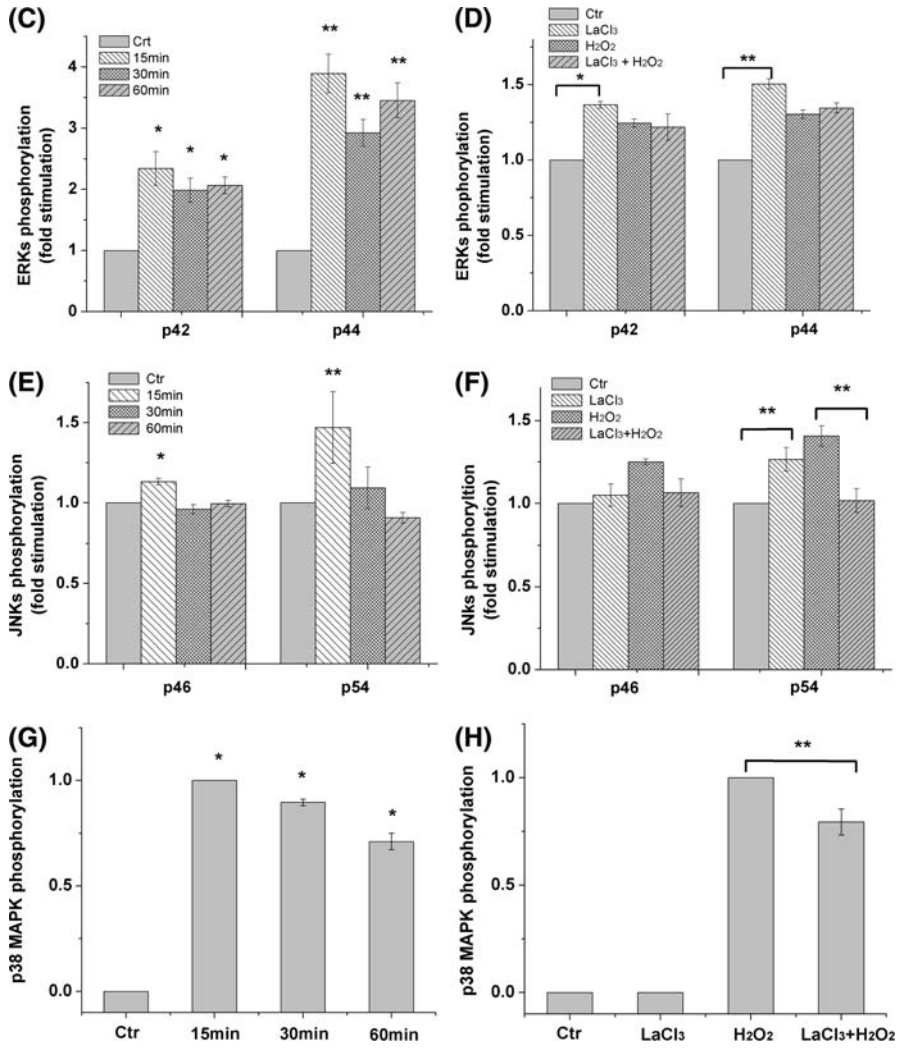


Fig. 7 **a** Effects of MAPKs inhibitors on ALP activity in CVCs after treatment with H_2O_2 for 4 day; **b** Phosphorylation of MAPKs in CVCs after exposure to 50 μM H_2O_2 without (*left*) and with pretreatment with 1 μM $LaCl_3$ for 2 h (*right*, 15 min). The protein levels were detected by immunoblotting, using specific antibodies against p-ERK1/2 and ERK1/2 (**c**, **d**), p-JNKs and JNKs (**e, f**), p-p38 and p38 (**g**, **h**, the values are normalized with the signal for p-p38 MAPK). These results are representative of three independent experiments. There was a significant difference $^*P < 0.05$ and $^{**}P < 0.05$ versus the control, respectively

Fig. 7 continued



p38 MAPK were associated with the modulation of ALP activity. Pretreatment with 1 μM of $LaCl_3$ significantly suppressed H_2O_2-induced activation of JNK (JNK2, but not JNK1) and p38 MAPK (Fig. 7f, d, h). Since some events of the H_2O_2-involved signalings are mediated by Ca^{2+} (Qin et al. 2000), La^{3+} may play a role in them as an analogue of Ca^{2+}. Actually, La^{3+} has been shown to bind to calmodulin (Mills and Johnson 1985). Because Ca^{2+}/calmodulin pathway plays a key role in H_2O_2-induced activation of MAPK in VMSCs (Blanc et al. 2004; Zhang et al. 1998), $LaCl_3$ may counteract H_2O_2-activated JNK (JNK2, but not JNK1) and p38 MAPK by interfering with Ca^{2+} signaling. Besides, La^{3+}-catalysed disproportionation of H_2O_2 may also contribute to its protective effect (Nardello et al. 2003).

In summary, lanthanum chloride inhibits H_2O_2-promoted intracellular ROS elevation, growth inhibition, osteoblastic differentiation, apoptosis and calcification in rat calcifying vascular cells, and its role is associated, at least partly, with JNK (JNK2, but not JNK1) and p38 MAPK signaling pathways.

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