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Journal of Cellular Biochemistry 108:1184–1191 (2009)

Journal of Cellular Biochemistry

Lanthanum Suppresses Osteoblastic Differentiation Via Pertussis Toxin-Sensitive G Protein Signaling in Rat Vascular Smooth Muscle Cells

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ABSTRACT

A major cellular event in vascular calcification is the phenotypic transformation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells. After demonstrating that lanthanum chloride ($LaCl_3$) suppresses hydrogen peroxide-enhanced calcification in rat calcifying vascular cells (CVCs), here we report its effect on the osteoblastic differentiation of rat VSMCs, a process leading to the formation of CVCs. Cells were isolated from aortic media of male SD rats, and passages between three and eight were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 10 mM β-glycerophosphate (β-GP) in the presence or absence of $LaCl_3$. Exposure of cells to $LaCl_3$ suppressed the β-GP-induced elevations in calcium deposition, alkaline phosphatase (ALP) activity, and Cbfa1/Runx2 expression, as well as the concomitant loss of SM α-actin. Furthermore, $LaCl_3$ activated the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), and the blockage of either pathway with a specific inhibitor abolished the effects of $LaCl_3$. In addition, pretreatment of the cells with pertussis toxin (PTx), an inhibitor of G protein-mediated signaling pathway, repealed all the changes induced by $LaCl_3$. These findings demonstrate that $LaCl_3$ suppresses the β-GP-induced osteoblastic differentiation and calcification in rat VSMCs, and its effect is mediated by the activation of both ERK and JNK MAPK pathways via PTx-sensitive G proteins. J. Cell. Biochem. 108: 1184–1191, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: VASCULAR SMOOTH MUSCLE CELL; OSTEOBLASTIC DIFFERENTIATION; LANTHANUM; G PROTEIN; MAPK PATHWAYS

Vascular calcification is frequently seen in atherosclerosis, diabetes, and chronic kidney disease [Touyz and Montezano, 2009]. Depending on the sites being the media or intima, it may cause either vascular stiffening or plaque formation and rupture [Schoppet et al., 2008]. In the active cell-mediated processes, a major cellular event is the phenotypic transformation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells, which is indicated by the loss of the lineage markers such as SM22α and smooth muscle α-actin, as well as the expression of osteogenic markers such as core binding factor a1 (Cbfa1/Runx2), osteocalcin, osteopontin, alkaline phosphatase (ALP), and the presence of matrix vesicles and the bone mineral hydroxyapatite [Steitz et al., 2001]. Various factors may elicit or influence the phenotypic transformation, such as hypertension, reactive oxygen species, and some bone morphogenic proteins [Guzman, 2007; Schoppet et al., 2008]. Besides, in in vitro studies, β-glycerophosphate (β-GP) is frequently used as an inducer to trigger the osteoblastic differentiation in primary cultures of VSMCs of human [Alam et al., 2009], bovine [Shioi et al., 1995; Steitz et al., 2001; Bear et al., 2008], and rat [Li et al., 2003; Shi et al., 2009] through a mechanism possibly involving the production of inorganic phosphate and activation of Smad 1/5/8 signaling. The β-GP-induced differentiation in VSMCs is enhanced by certain extracellular matrix proteins, such as fibronectin [Hedin et al., 1988; Ding et al., 2006] and oxidized low-density lipoprotein [Bear et al., 2008], although neither of the two proteins alone is capable of inducing the phenotypic transformation.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 20571006, 20637010; Grant sponsor: National Basic Research Program of China; Grant number: 2007CB516806.

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Received 13 May 2009; Accepted 13 August 2009 • DOI 10.1002/jcb.22348 • © 2009 Wiley-Liss, Inc.
Published online 23 September 2009 in Wiley InterScience (www.interscience.wiley.com).

In addition, high level of extracellular calcium also leads to the phenotypic change of VSMCs, possibly via a mechanism involving calcium-sensing receptor (CaR) [Alam et al., 2009]. The role of CaR is to sense alterations in the level of extracellular calcium and induce a cellular response directed at normalizing the blood calcium concentration [Touyz and Montezano, 2009]. CaR is coupled with a subfamily of guanine nucleotide regulatory proteins (G proteins), which functions to inhibit adenylate cyclase activity and to decrease cAMP level [Malbon, 1997]. The G protein-mediated mitogen-activated protein kinase (MAPK) signaling pathways have been reported in rat VSMCs [Jing et al., 1999; Verdeguer et al., 2007]. It has been shown that, among the three major subfamilies of MAPK in mammalian cells, the extracellular signal-regulated kinase (ERK) is most frequently activated by G proteins [Molostvov et al., 2007; Alam et al., 2009].

Cationic lanthanum (La^{3+}) also binds to the G protein-coupled CaR and may affect the osteoblast-like differentiation in VSMCs. In recent years, lanthanide compounds have attracted intensive research interest in medical applications [Fricker, 2006; Thompson and Orvig, 2006]. Lanthanum carbonate is currently used in the treatment of hyperphosphatemia of chronic renal failure [Berns and Korbin, 2006]. In addition, lanthanum chloride ($LaCl_3$) has been shown to retard the progression of established atherosclerotic lesions in animal models [Kramsch et al., 1980; Gillies et al., 1989], and our previous findings have demonstrated that $LaCl_3$ suppresses hydrogen peroxide-enhanced calcification of rat calcifying vascular cells (CVCs) [Shi et al., 2009] but enhances differentiation in rat osteoblasts [Wang et al., 2008]. Given that La^{3+} is an agonist of CaR in VSMCs [Smajilovic and Tfelt-Hansen, 2007; Alam et al., 2009] and a calcium channel blocker [Sato et al., 1998; Dai et al., 2002; Shi and Huang, 2005; Heffeter et al., 2006], its role in the osteoblastic differentiation of VSMCs is worthy of evaluation.

The aims of the present paper were to assess (i) the effect of La^{3+} on the β-GP-induced osteoblastic differentiation in rat VSMCs, and (ii) the involvement of pertussis toxin (PTx)-sensitive G proteins and MAPK signaling pathways in the phenotypic change.

MATERIALS AND METHODS

MATERIALS

Dulbeccol's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and trypsin were obtained from Gibco BRL (Rockville, MD). Anti-ERK 1/2, anti-phosphorylated ERK 1/2 (p-ERK 1/2), anti-c-Jun N-terminal kinase (JNK) 1/2, anti-phosphorylated JNK 1/2 (p-JNK 1/2), actin, and the corresponding secondary antibodies were from Cell Signaling (Beverly, MA). Cbfa1 and SM α-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PTx was from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Unless otherwise indicated, all other reagents, including β-GP, PD98059, SP600125, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin, and streptomycin, were from Sigma (St. Louis, MO). $LaCl_3$ solution was prepared from lanthanum oxide (purity >99.99%).

VASCULAR SMOOTH MUSCLE CELL CULTURES AND TREATMENT

VSMCs were isolated from aortic media of male SD rats (140–160 g) by enzymatic isolation, and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (growing medium) at 37°C in a 5% CO_2 atmosphere according to the method described previously [Ray et al., 2002]. The cells between passages three and eight were used for all experiments.

After confluence, VSMCs were pretreated with various concentrations of $LaCl_3$ and specific inhibitors, including PTx (20 ng/ml) to inhibit PTx-sensitive G proteins, PD98059 (20 μM) to inhibit ERK pathway, and SP600125 (10 μM) to inhibit JNK pathway for various times in growing medium. The cells were then incubated in DMEM containing 10% FBS and 10 mM β-GP (differentiation medium) 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.

CELL VIABILITY ASSAY

At ~70% confluence, VSMCs were pretreated with or without 10 μM $LaCl_3$ for 2 h, followed by incubation in the differentiation or growing medium for 12 days. At the end of the incubation, MTT in phosphate buffered saline (PBS) (5 mg/ml) was added to reach a final concentration of 0.5 mg/ml. After the cells were left at 37°C for 4 h, the supernatant was removed and the formazan dye dissolved in 150 μl DMSO. Absorbance 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.

ALKALINE PHOSPHATASE ASSAY

Calcifying VSMCs were washed three times with PBS, lysed with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for ALP activity as described previously [Shioi et al., 1995]. ALP values (U/mg) were normalized to protein content that was measured with Bradford assay.

QUANTIFICATION OF CALCIUM DEPOSITION

After pretreatment with various concentrations of $LaCl_3$ for 2 h, cells were incubated in growing and differentiation medium, respectively. Calcification was examined at day 12 according to the published procedure [Wada et al., 1999]. Briefly, the cells were decalcified with 0.6 M HCl 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 0.1 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 day16, followed by fixation in 75% ethanol for 1 h and staining with 0.1% Alizarin Red S for 5 min. After rinsed with PBS, the mineralized nodules were photographed.

WESTERN BLOT ANALYSIS

After being washed twice with cold PBS, cells were treated in 250 μl of ice-cold lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM

dithiothreitol, 300 nM aprotinin, 50 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate) on ice for 10 min, then scraped into lysis buffer and sonicated for 20 s on ice. After centrifugation at 12,000 rpm at 4°C for 10 min, the cytosolic extracts were harvested to determine the protein expression of SM α-actin, p-ERK, and p-JNK. The nuclear pellets were further lysed with buffer containing 40 mM Tris–HCl pH 7.9, 350 mM NaCl, 2.0 mM $MgCl_2$, 1.0 mM EDTA, 0.2 mM EGTA, and 20% glycerol on ice for 20 min, and then harvested to determine Cbfa1 activation by centrifugation at 12,000 rpm at 4°C for 10 min. Protein contents in cytosolic and nuclear extracts were determined with Bradford assay.

Western blot analysis was performed by the following procedure. Briefly, cell-free lysates were prepared as described above. Equal amounts of the proteins were run on 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, 150 mM NaCl, pH 7.5, and 0.1% Tween-20), blocked with 5% BSA solution in TBST for 1 h at room temperature, and then blotted with appropriate antibodies in 5% BSA/TBST at 4°C overnight. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody 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. Here, β-actin was used as a loading control.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard deviation (SD) from two to four separate experiments each performed in quadruplicate or triplicate. Significance was determined using one-way analysis of variance. $P < 0.05$ was considered significant.

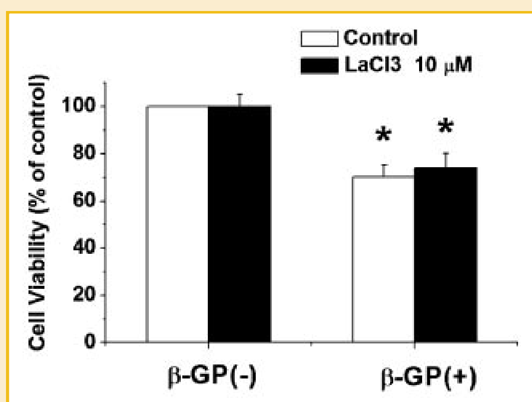

Fig. 1. Effect of $LaCl_3$ on the viability of rat vascular smooth muscle cells. After pretreatment with or without 10 μM $LaCl_3$ for 2 h, cells were incubated in growing and differentiation medium, respectively. Cell viability was examined at day 12 by MTT assay. Data are mean ± SD of two separate experiments performed in quadruplicate. $^{*}P < 0.05$ versus β-GP-free control.

RESULTS

LANTHANUM DOES NOT AFFECT VIABILITY OF RAT VSMCS

Since there is a possibility that the effect of $LaCl_3$ results from its stimulatory action on cell proliferation, we assessed the cell viability by MTT assay. Compared with the control, cell viability decreased to

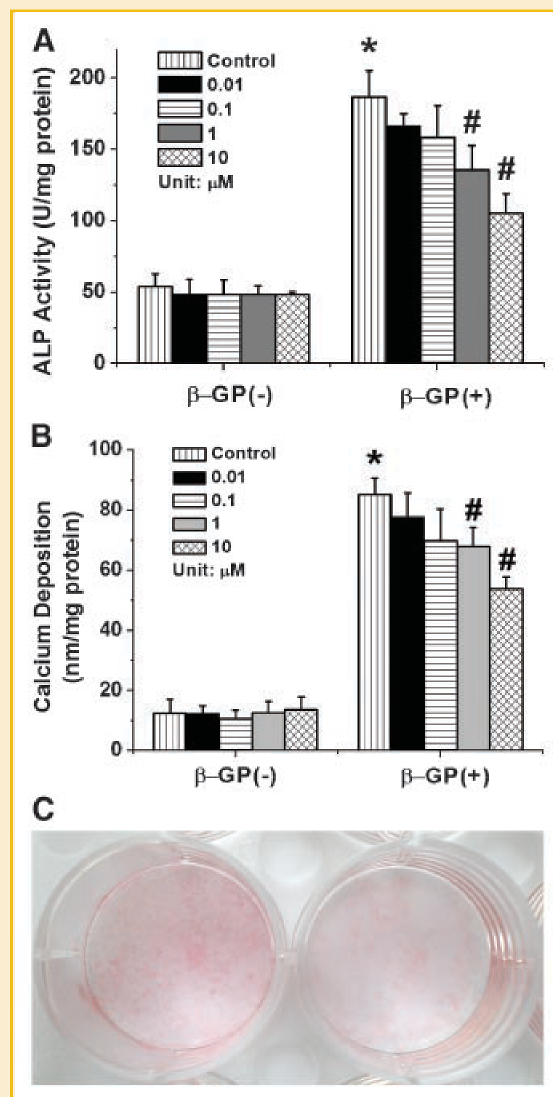

Fig. 2. Effect of $LaCl_3$ on ALP activity and calcium deposition in rat vascular smooth muscle cells. A: Concentration dependence of ALP activity on $LaCl_3$. After pretreatment with various concentrations of $LaCl_3$ for 2 h, cells were cultured in growing and differentiation medium, respectively. ALP activity was determined at day 9 and normalized to cellular protein content. B: Concentration dependence of calcium deposition on $LaCl_3$. After pretreatment with various concentrations of $LaCl_3$ for 2 h, cells were cultured in growing and differentiation medium, respectively. The calcium deposit was measured at day 12 and normalized to cellular protein content. Data are mean ± SD from a representative of three separate experiments performed in triplicate. $^{*}P < 0.05$ versus non-differentiation control; $^{\#}P < 0.05$ versus $LaCl_3$-free differentiation control. C: Photograph of mineralized nodules at day 16 stained with Alizarin Red S. Left: 10 mM β-GP; Right: 10 μM $LaCl_3$ + 10 mM β-GP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

70% after treatment with 10 mM β-GP for 12 days (Fig. 1). However, regardless of the presence or absence of β-GP, no significant difference was observed in cells treated with 10 μM $LaCl_3$.

LANTHANUM SUPPRESSES OSTEOBLASTIC DIFFERENTIATION IN RAT VSMCS

The osteoblastic differentiation were induced by β-GP in the primary cultures of rat VSMCs, as indicated by the marked increases in ALP activity (Fig. 2A), calcium deposition (Fig. 2B), the protein expression of Cbfa1/runx2, and the concomitant decrease in SM α-actin (Fig. 3C–E). Treatment with $LaCl_3$ at the concentrations of 1 and 10 μM attenuated the increases in ALP activity (Fig. 2A) and calcium deposition (Fig. 2B and C), although it alone caused no significant change. Moreover, treatment with $LaCl_3$ alleviated the β-GP-induced loss of SM α-actin and increase in Cbfa1/runx2 (Fig. 3C–E). These results confirmed the effectiveness of β-GP in inducing differentiation in the primary culture of VSMCs and the inhibitory effect of $LaCl_3$.

ERK AND JNK MAPK PATHWAYS ARE INVOLVED IN MEDIATING THE INHIBITORY EFFECT OF LANTHANUM ON OSTEOBLASTIC DIFFERENTIATION IN RAT VSMCS

Pretreatment of the cells with either the ERK inhibitor (PD98059) or the JNK inhibitor (SP600125) for 1 h abolished all the inhibitory

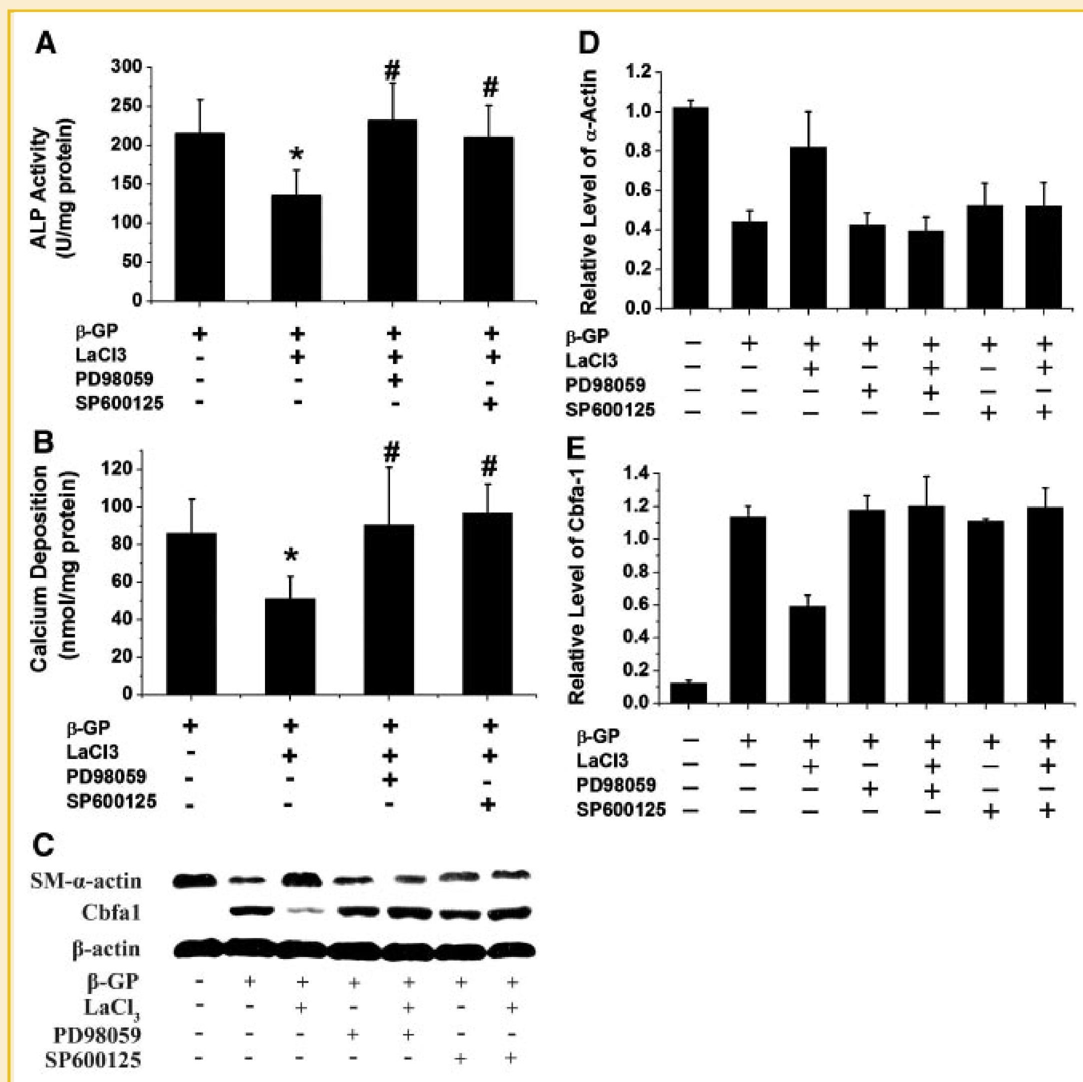

Fig. 3. Effects of ERK1/2 and JNK1/2 inhibitors on the $LaCl_3$-suppressed osteoblastic differentiation in rat vascular smooth muscle cells. A: ALP activity. After pretreatments with ERK1/2 or JNK1/2 inhibitors (20 μM PD98059 and 10 μM SP600125, respectively) for 1 h and 10 μM $LaCl_3$ for 2 h successively, cells were cultured in differentiation medium. ALP activity was determined at day 9 and normalized to cellular protein content. B: Calcium deposition at day 12. Data in (A) and (B) are mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus $LaCl_3$-free control; $^{\#}P < 0.05$ versus $LaCl_3$-containing, inhibitor-free sample. C: Protein expressions of SM α-actin and Cbfa1. After pretreatments with ERK1/2 or JNK1/2 inhibitors (20 μM PD98059 and 10 μM SP600125, respectively) for 1 h and 10 μM $LaCl_3$ for 2 h successively, cells were cultured in differentiation medium. Protein expressions of SM α-actin and Cbfa-1 were determined at day 6. D,E: Quantification of the blots for SM α-actin and Cbfa-1. Data are mean ± SD of two separate experiments.

effects of $LaCl_3$ on the β-GP-induced changes, including ALP activity (Fig. 3A), calcium deposition (Fig. 3B) and the protein expressions of SM α-actin and Cbfa1 (Fig. 3C–E). These findings prompted us to examine the effects of $LaCl_3$ on the activation of ERK 1/2 and JNK 1/2 in growing medium. As demonstrated in Figure 4A, $LaCl_3$ stimulated the phosphorylation of a specific ERK 1/2 in the VSMCs. The stimulation reached a maximum at 5 min and sustained up to 15 min before returning to the control level. The maximal activation of JNK 1/2 was also observed at 5 min, but it sustained a shorter period (Fig. 4B). The phosphorylation of both JNK 1/2 and ERK 1/2 at 5 min showed a similar concentration dependence on $LaCl_3$ (Fig. 3C–F). No phosphorylated p38 MAPK was observed under the same condition (data not shown).

PERTUSSIS TOXIN ABOLISHES THE EFFECTS OF LANTHANUM ON OSTEOBLASTIC DIFFERENTIATION OF RAT VSMCS AND ACTIVATIONS OF ERK AND JNK

Since cationic lanthanum is a known agonist of CaR that is coupled to G proteins, we examined the role of CaR in mediating the effects of $LaCl_3$. After pretreatment with PTx for 24 h, cells were exposed to 10 μM $LaCl_3$ for 2 h, followed by incubation in the differentiation medium for 9 days. PTx treatment abolished the inhibitory effect of $LaCl_3$ on the elevations of both ALP activity (Fig. 5A) and Cbfa1 protein expression (Fig. 5B and C) at day 9, though causing no significant change in the absence of $LaCl_3$. In addition, PTx completely abolished the $LaCl_3$-activated phosphorylation of ERK1/2 and JNK1/2, while it alone (without $LaCl_3$) failed to cause any significant change (Fig. 6). Taken together, these results indicate that PTx-sensitive G proteins were involved in mediating the effect of $LaCl_3$ on the activations of ERK 1/2 and JNK 1/2 and on the suppression of the osteoblastic differentiation induced by β-GP in rat VSMCs.

DISCUSSION

Vascular calcification is closely associated with the osteoblastic differentiation of VSMCs. The subpopulation of differentiated cells that express osteoblastic markers and form calcified nodules in vitro is known as CVCs. We have recently demonstrated the suppressive effect of $LaCl_3$ on H_2O_2-enhanced CVC calcification [Shi et al., 2009]. In the present study, we further investigate how $LaCl_3$ affects the osteoblastic differentiation through which VSMCs transform into CVCs. By means of an in vitro model with rat VSMCs, we examined the effect of $LaCl_3$ on the β-GP-induced phenotypic transformation and the roles of G proteins and MAPK signaling pathways in the process.

$LaCl_3$ inhibits the calcification of rat VSMCs by suppressing the β-GP-induced osteoblastic differentiation. The examined indices for the phenotypic transformation include a smooth muscle marker (SM α-actin), an osteoblastic marker (Cbfa1), ALP activity, and calcium deposition. Treatment with $LaCl_3$ suppressed the β-GP-induced elevation of ALP activity and calcium deposition in extracellular matrix, and the effect increased with higher concentration (Fig. 2). In addition, in parallel with the loss of a smooth muscle lineage marker, the expression of Cbfa1 increased dramatically (Fig. 3). Cbfa1 is a key regulatory transcription factor in osteoblastic

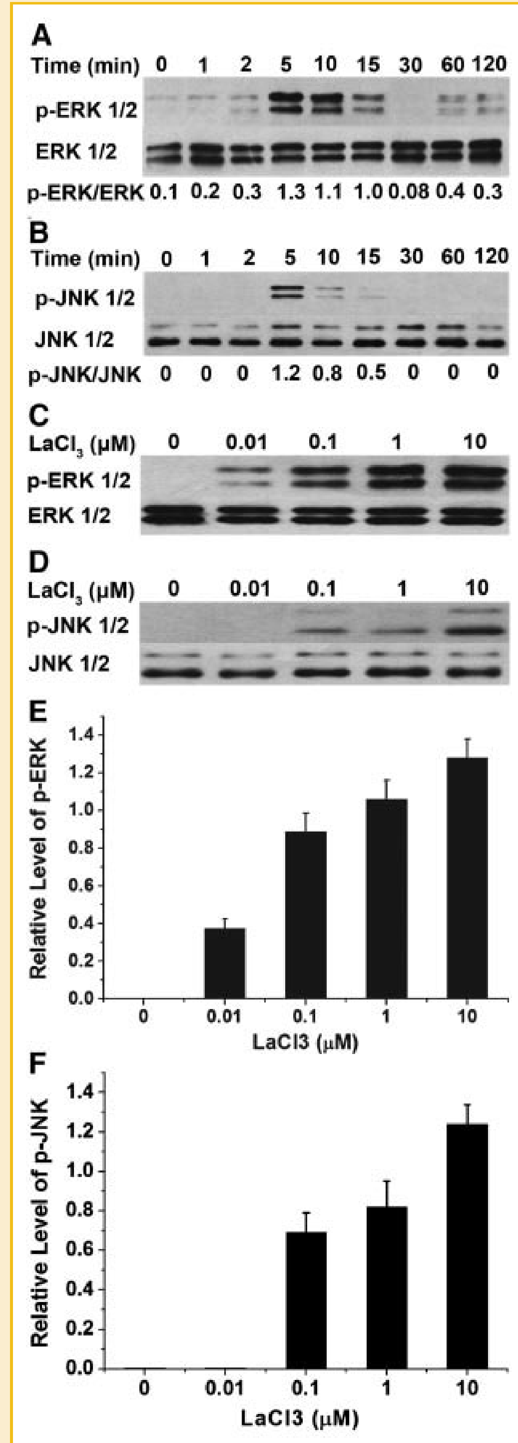

Fig. 4. Effect of $LaCl_3$ on the phosphorylation of ERK1/2 and JNK1/2 in rat vascular smooth muscle cells. A,B: Time dependence of ERK1/2 and JNK1/2 phosphorylation induced by 10 μM $LaCl_3$. After incubation with 10 μM $LaCl_3$ in growing medium for the indicated times, cells were lysed and the phosphorylated ERK1/2 and JNK1/2 determined. C,D: Concentration dependence of $LaCl_3$ on the phosphorylation of ERK1/2 and JNK1/2, respectively. After incubation in growing medium with various concentrations of $LaCl_3$ for 5 min, cells were lysed and the phosphorylated ERK1/2 and JNK1/2 determined. E,F: Quantification of the blots for p-ERK/ERK and p-JNK/JNK. Data are mean ± SD of two separate experiments.

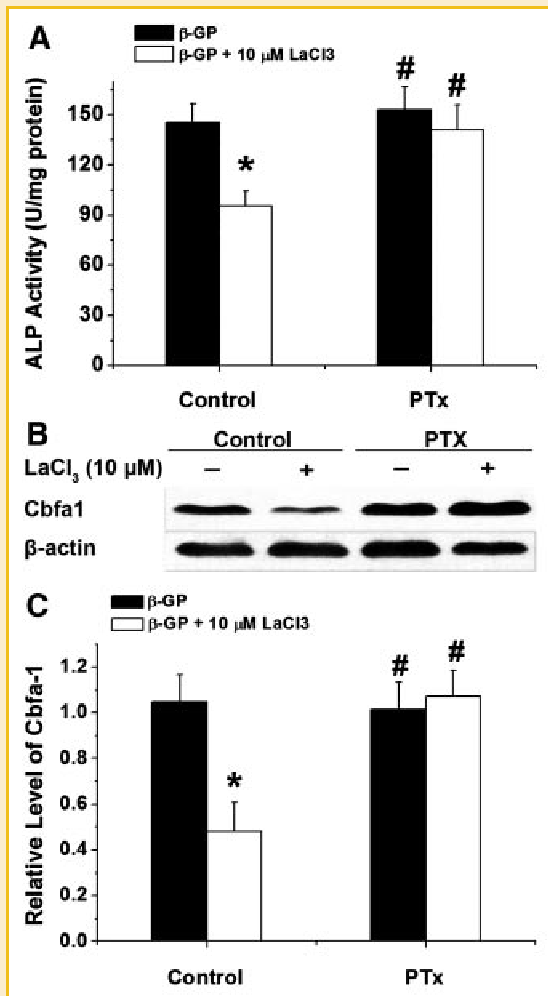

Fig. 5. Effect of PTx on the $LaCl_3$-suppressed osteoblastic differentiation of rat vascular smooth muscle cells. A: ALP activity. After pretreatment with PTx 20 ng/ml for 24 h and 10 μM $LaCl_3$ for 2 h successively, cells were cultured in the differentiation medium. ALP activity was measured at day 9 and normalized to the cellular protein content. Data are mean ± SD from a representative of two separate experiments performed in triplicate. B: Cbfa1 protein expression at day 6 by Western blotting. C: Quantification of the blots. Data are mean ± SD of three separate experiments. $^{*}P < 0.05$ versus $LaCl_3$-free differentiation control; $^{\#}P < 0.05$ versus $LaCl_3$-containing, PTx-free control.

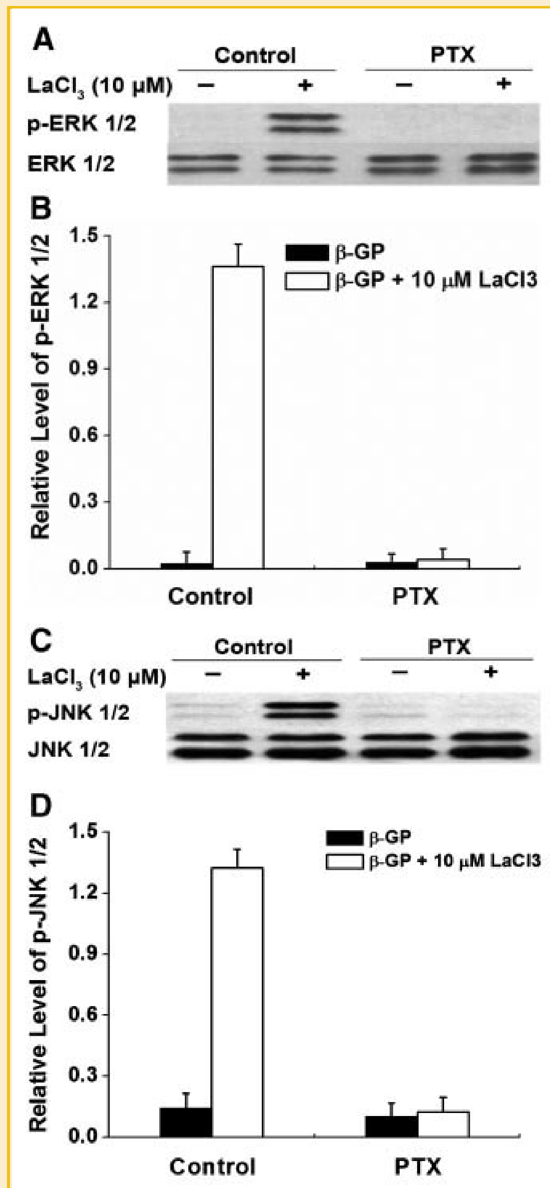

Fig. 6. Effects of PTx on $LaCl_3$-activated ERK1/2 and JNK1/2 phosphorylation in rat vascular smooth muscle cells. After pretreatment with 20 ng/ml PTx for 24 h, cells were cultured with or without 10 μM $LaCl_3$ in the growing medium for 5 min and then lysed. A,C: Phosphorylation of ERK1/2 and JNK 1/2. B,D: Quantification of the blots for p-ERK/ERK and p-JNK/JNK. Data are mean ± SD of two separate experiments.

differentiation, important for modulating the expression of osteoblast-specific genes such as ALP and OC [Banerjee et al., 1997; Nakashima et al., 2002]. Cbfa1-knockout mice fail to form mineralized bone [Komori et al., 1997]. Furthermore, the expression of Cbfa1 has also been observed in the calcification of atherosclerotic plaques [Tyson et al., 2003]. ALP can raise the local concentration of inorganic phosphate by degrading β-GP [Gronowicz et al., 1989; Shioi et al., 1995], and its role in the activation of Smad 1/5/8 signaling has also been suggested [Bear et al., 2008]. Since 10 μM $LaCl_3$ did not cause any change in cell viability regardless of the presence or absence of β-GP (Fig. 1), the observed effects of $LaCl_3$ should be attributed to its influence on the β-GP-induced osteoblastic differentiation in rat VSMCs. Therefore, in addition to direct binding phosphate in foods, the cellular effect of cationic lanthanum may also contribute to the retardation of the progression of established atherosclerotic lesions in animal models [Kramsch et al., 1980; Gillies et al., 1989].

The effect of $LaCl_3$ on the phenotypic differentiation of rat VSMCs is closely associated with G protein-mediated MAPK signaling. $LaCl_3$ activated the phosphorylation of ERK and JNK in VSMCs (Fig. 4), consistent with that in rat CVCs [Shi et al., 2009]. Either an inhibitory [Radcliff et al., 2005; Liao et al., 2008] or a stimulatory effect [Li et al., 2003; Ding et al., 2006; Bear et al., 2008] has been associated with the activation of the ERK pathway in the osteoblastic

differentiation of VSMCs, and a negative balance between ERK and JNK has been observed in ameliorating the cytotoxicity of H_2O_2 in mouse renal proximal tubule cells [Arany et al., 2004]. Indeed, as recently reviewed by Junttila et al. [2008], the negative regulation of ERK signaling by stress-activated JNK/p38 pathways is not a rarely seen phenomenon. However, data in Figure 3 showed that both ERK and JNK pathways were activated by $LaCl_3$; inhibition of either of the two abrogated the effect of $LaCl_3$ on the β-GP-induced changes in calcium deposition, ALP activity, and the expressions of SM α-actin and Cbfa1. These facts indicate that the activation of both pathways was required for $LaCl_3$ to exert its effect on the β-GP-induced phenotypic transformation of rat VSMCs. Importantly, the $LaCl_3$-activated ERK and JNK pathways, along with the subsequent suppression of Cbfa1 expression and ALP activity, were all completely abolished by the inhibition of PTx-sensitive G proteins (Figs. 5 and 6). The connections between G proteins and the downstream ERK [Verdeguer et al., 2007] and p38 MAPK [Jing et al., 1999; Li et al., 2006] have been demonstrated in VSMCs. Intriguingly, in the absence of $LaCl_3$, the β-GP-induced osteoblastic differentiation in rat VSMCs was not influenced by the inhibition of the two MAPK pathways.

Of note is the significance of CaR in mediating the effect of $LaCl_3$ on the β-GP-induced osteoblastic differentiation in rat VSMCs. After the recognition of the function of CaR in calcium homeostasis, its importance in cardiovascular system is beginning to be appreciated [Smajilovic and Tfelt-Hansen, 2007; Touyz and Montezano, 2009]. CaR is functionally expressed in normal and calcified human arteries [Molostvov et al., 2007], as well as in rat VSMCs [Smajilovic et al., 2006]. Its expression is downregulated in human calcified arteries and in mineralized VSMCs [Alam et al., 2009]. Lanthanum is a known agonist of CaR, and the stimulation of CaR can activate MAPK signaling cascades via G proteins [Hofer and Brown, 2003; Smajilovic and Tfelt-Hansen, 2007]. The blockage of the effect of $LaCl_3$ by PTx (Figs. 5 and 6) might be an indication of the involvement of CaR, though one cannot exclude contributions from other receptor and channel proteins which bind lanthanum cation and are also coupled to PTx-sensitive G proteins. Of note, the contrary effects of $LaCl_3$ on the pathologic differentiation in VSMCs (Figs. 2 and 3) and in the physiologic differentiation in osteoblasts [Wang et al., 2008] of rats may reflect the dependence of the CaR-mediated signaling on the cell type [Smajilovic and Tfelt-Hansen, 2007]. Indeed, beyond triggering the effect of $LaCl_3$ on the β-GP-induced differentiation of rat VSMCs, the role of CaR itself deserves further studies in the phenotypic transformation and vascular calcification.

In summary, $LaCl_3$ suppresses the β-GP-induced osteoblastic differentiation and calcification of rat VSMCs, and its role is mediated by the activation of both ERK and JNK MAPK signaling pathways via PTx-sensitive G proteins.

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