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Statins Inhibit In Vitro Calcification of Human Vascular Smooth Muscle Cells Induced by Inflammatory Mediators

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Abstract Although lipid-lowering therapy with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) decreases the progression of coronary artery and aortic valve calcification, the mechanism of action of these drugs to inhibit the calcification process remains unclear. In this study, we investigated the effect of statins such as cerivastatin and atorvastatin on vascular calcification by utilizing an in vitro model of inflammatory vascular calcification. Cerivastatin and atorvastatin dose-dependently inhibited in vitro calcification of human vascular smooth muscle cells (HVSMCs) induced by the following inflammatory mediators (IM): interferon-γ, 1α,25-dihydroxyvitamin D_3, tumor necrosis factor-α, and oncostatin M. These statins also depressed expression of alkaline phosphatase (ALP) in HVSMCs induced by these factors. Mevalonate and geranylgeranylpyrophosphate reversed the inhibitory effect of cerivastatin on ALP expression in HVSMCs, while farnesylpyrophosphate showed no effect on the ALP activities inhibited by this drug, suggesting that inhibition of Rho and its downstream target, Rho kinase may mediate the inhibitory effect of cerivastatin. Cerivastatin prevented RhoA activation in HVSMCs induced by the IM. A specific inhibitor of Rho kinase (Y-27632) inhibited in vitro calcification and induction of ALP in HVSMCs. These findings provide a possible mechanism of statins to prevent the progression of calcification in inflammatory vascular diseases such as atherosclerosis and cardiac valvular calcification. J. Cell. Biochem. 93: 1011–1019, 2004.

Key words: atherosclerosis; calcium; inflammation; lipids; smooth muscle cell

Vascular calcification including coronary artery calcification and aortic valve calcification is a common and clinically significant component of atherosclerosis and cardiac valvular disease. The amount of coronary calcification correlates with the overall coronary plaque burden and an increased risk of myocardial infarction [Frink et al., 1970; Stanford et al., 1993; Wayhs et al., 2002]. The degree of aortic valve calcification is a strong predictor both for the progression and outcome of aortic stenosis [Rosenhek et al., 2000]. These calcified lesions contain not only various components associated with bone mineralization, including osteoblast-like vascular cells, matrix vesicles, alkaline phosphatase (ALP), bone morphogenetic proteins, osteocalcin (OC), and osteopontin (OPN), but also inflammatory cells such as macrophages and lymphocytes [Jeziorska et al., 1997; Mohler et al., 2001; Shioi, 2002]. Therefore, atherosclerotic and cardiac valve calcification may be an osteogenic process associated with inflammatory reactions.

Vascular cells such as vascular smooth muscle cells (VSMCs), pericyte-like cells, and valvular cells play an important role in vascular calcification. VSMCs and pericyte-like cells derived from bovine aorta have calcifying capacity and express noncollagenous matrix proteins such as OPN, matrix gla protein (MGP), and OC [Bostrom et al., 1993; Shioi et al., 1995; Doherty et al., 1998; Mori et al., 1999]. Valvular cells derived from aortic valves can also calcify their extracellular matrix [Mohler et al., 1999]. In vitro calcification by

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vascular cells can be modulated by calciotropic hormones, steroid hormones, transforming growth factor-β, and 25-hydroxycholesterol [Bostrom et al., 1993; Balica et al., 1997; Jono et al., 1998; Mohler et al., 1999]. These in vitro evidences suggest that phenotypic changes of vascular cells in mesenchymal origin, especially acquisition of calcifying phenotype under various pathological conditions may contribute to the development of vascular calcification. However, the precise roles of osteogenic differentiation of vascular mesenchymal cells especially in human atherosclerotic calcification remain to be clarified.

The roles of inflammation in vascular calcification have been investigated in vitro. Monocytes/macrophages and proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) can enhance in vitro calcification of vascular cells [Tintut et al., 2000, 2002; Parhami et al., 2002a]. We have also demonstrated the important roles of macrophages in vascular calcification by using a coculture of human VSMCs (HVSMCs) with THP-1 macrophages and developed an in vitro model of vascular calcification induced by inflammatory mediators (IM) such as interferon-γ (IFN-γ), 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), TNF-α, and oncostatin M (OSM) [Shioi et al., 2002].

Lipids may contribute to cardiac valve calcification as well as atherosclerotic calcification. A significant influence of serum low density lipoprotein (LDL) cholesterol levels on the progression of both aortic valve and coronary calcification has been documented [Pohle et al., 2001]. In vitro studies have demonstrated the stimulatory effects of oxidized lipids including oxidized LDL on vascular calcification [Parhami et al., 1997]. Lipid-lowering therapy with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) decreases the progression of coronary calcification and may also prevent aortic valve [Callister et al., 1998; Achenbach et al., 2002; Shavelle et al., 2002]. However, the mechanism of action of these drugs to inhibit coronary and aortic valve calcification remains unclear. Beyond their potent lipid-lowering effect, statins exert pleiotropic effects on vascular wall cells including improvement of endothelial function, stabilization of atherosclerotic plaque, and decrease of oxidative stress and vascular inflammation [Takemoto and Liao, 2001]. Many of these pleiotropic effects of statins are mediated by their ability to block the production of isoprenoid intermediates such as farnesyl- or geranylgeranyl-pyrophosphates, which are important in activation of small GTP-binding proteins including Rho, Ras, and Rac through their isoprenylation [Takemoto and Liao, 2001; Yoshida et al., 2001; Park et al., 2002].

In this study, we have investigated the effect of statins such as cerivastatin and atorvastatin on vascular calcification by utilizing an in vitro calcification model of inflammatory vascular diseases and demonstrated that statins inhibit HVSMC calcification through inhibiting RhoA/Rho kinase pathway.

MATERIALS AND METHODS

Reagents

Media and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). $1,25(OH)_2D_3$ was obtained from Solvay Duphar BV (The Netherlands). β-Glycerophosphate (β-GP), mevalonate (MVA), farnesylpyrophosphate (FPP), and geranylgeranylpyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO). Recombinant IFN-γ, TNF-α, and OSM were obtained from Genzyme/Techne (Cambridge, MA). A specific Rho kinase inhibitor (Y27632) was purchased from Calbiochem (La Jolla, CA). Mouse anti-RhoA monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Unless otherwise mentioned, all other reagents were obtained from Wako (Osaka, Japan). Cerivastatin and atorvastatin were kindly provided from Bayer Yakuhin Ltd. (Osaka, Japan) and Pfizer, Inc. (New York, NY), respectively.

Cell Culture

Primary HVSMCs derived from neonatal umbilical arteries were obtained from Cell Systems (Kirkland, WA), maintained in DMEM supplemented with 10% FBS containing 100 U/ml of penicillin and 100 μg/ml of streptomycin, and passaged weekly using trypsin-EDTA (Sigma).

Induction of HVSMC Calcification by IM

HVSMCs were cultured for the indicated period of time with 10 mmol/L β-GP in the presence of IFN-γ (100 ng/ml), $1,25(OH)_2D_3$ (10^{-7} mol/L), TNF-α (1.0 ng/ml), and OSM (10 ng/ml) as previously described [Shioi et al.,

2002]. Calcium deposits of cell layer were assessed by *o*-cresolphthalein complexone method (Calcium C-test Wako) and normalized by protein content as previously described [Shioi et al., 2002].

ALP Assay

After the cells were washed twice with PBS, the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were assayed for ALP activity as described previously [Shioi et al., 1995]. One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Northern Blot Analysis

Total RNA isolated from HVSMCs was used for Northern blot analysis and a cDNA probe for human tissue-nonspecific ALP was labeled with [α-^{32}P]dCTP as described [Shioi et al., 1995].

Cell Viability Assay

For the determination of HVSMC viability, we estimated bioreduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide (MTT). HVSMCs were cultured for 14 days in 96-well plates in the presence of the indicated concentrations of cerivastatin with or without the IM as described. The cells were incubated with MTT (0.5 mg/ml) for 2 h at 37°C. After aspiration of medium, formazan precipitate was extracted from cells with 200 μl isopropyl alcohol containing 0.04 N HCl. Absorbance of the resulting solution was measured in a microplate reader (BioRad) at 570 nm with a reference wavelength of 630 nm.

RhoA Translocation Assay

A RhoA translocation assay was performed as previously described with a slight modification [Yoshida et al., 2001]. Briefly, HVSMCs were incubated in a lysis buffer containing 50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 1 mmol/L $MgCl_2$, 2 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/ml leupeptin, 1 mmol/L $NaVO_4$, and 0.1% Triton X-100 for 5 min on ice. The cell lysate was passed through a 27-G needle five times to disrupt cell membrane for the release of cytosol protein. The lysate was centrifuged at 15,000 rpm for 15 minutes. After the supernatant was collected as the cytosol fraction, the pellet was resuspended in 1% Triton X-100 in the lysis buffer and centrifuged at 15,000 rpm for 15 min. The supernatant was collected as the membrane fraction. Equal amounts of protein from each fraction were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, followed by immunobloting with anti-RhoA monoclonal antibody. The blots were visualized utilizing ECL detection reagents (Amersham) according to the manufacturer's instructions. The staining intensities were quantified by densitometric scanning.

Statistics

In certain experiments, data were analyzed for statistical significance by ANOVA with post-hoc analysis (Fisher's protected least significant test). These analyses were performed with the assistance of a computer program (StatView version 5.0, Abacus Concepts, Berkeley, CA).

RESULTS

As previously demonstrated, HVSMCs have acquired a calcifying capacity by the treatment with the following IM: IFN-γ, 1,25$(OH)_2D_3$, TNF-α, and OSM [Shioi et al., 2002]. A significant increase of calcium deposition in the cultures with these factors was observed at 11 days and thereafter as compared with the control cultures [Shioi et al., 2002]. Therefore, we examined the effect of cerivastatin on in vitro calcification on day 14. Cerivastatin dose-dependently inhibited HVSMC calcification and calcium deposition was almost completely blocked at the doses of 5 and 10 nmol/L (Fig. 1a). To exclude the possibility that the toxic effects of cerivastatin on HVSMCs may mediate the inhibitory action on HVSMC calcification, we examined the effect of cerivastatin on HVSMC viability on day 14 by MTT assay. Cerivastatin did not exert any significant effect on HVSMC viability at the doses between 1 and 10 nmol/L in the absence and presence of IM (Fig. 1b). However, the higher doses of this drug significantly reduced the cell viability under both conditions.

Since ALP is functionally important in this calcification model [Shioi et al., 2002], we examined whether cerivastatin may affect the expression of ALP in HVSMCs induced by the IM. Cerivastatin inhibited the induction of ALP activities in HVSMCs in a dose-dependent manner on day 4 (Fig. 2a). The inhibitory effect

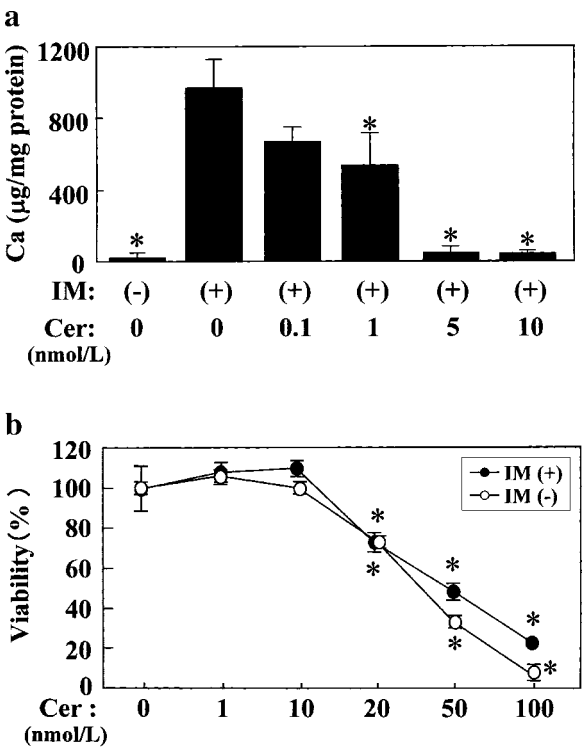

Fig. 1. **a**: Dose-dependent effects of cerivastatin (Cer) on human vascular smooth muscle cell (HVSMC) calcification. HVSMCs were treated for 14 days with Cer at the indicated concentrations. HVSMCs were cultured in the media containing 10 mmol/L β-glycerophosphate (β-GP) in the presence or absence of the inflammatory mediators (IM, 100 ng/ml of IFN-γ, 10^{-7} mol/L of $1,25(OH)_2D_3$, 1.0 ng/ml of TNF-α, and 10 ng/ml OSM) (IM) as described in Materials and Methods. Calcium deposition was quantified at the indicated time point by *o*-cresolphthalein complexone methods. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures with IM. **b**: The effects of Cer on HVSMC viability. HVSMCs were cultured for 14 days in 96-well plates with Cer at the indicated concentrations in the absence (open circle) or presence (closed circle) of IM. The viability of HVSMCs was determined by MTT assay. The data are presented as mean ± SEM (n = 6). $^*P < 0.05$ versus each control without Cer.

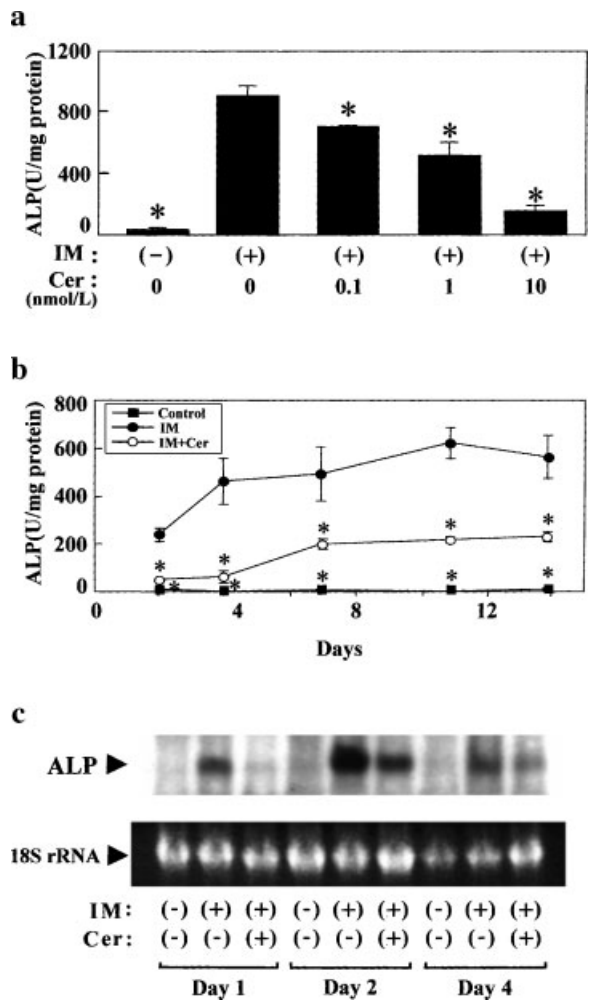

Fig. 2. Cer inhibited the induction of alkaline phosphatase (ALP) in HVSMCs. HVSMCs were incubated in the presence or absence of the IM with or without Cer. ALP activities were measured, normalized to cellular protein contents, and are presented as mean ± SEM (n = 3). **a**: Dose-dependent effects of Cer on ALP activities in HVSMCs at day 4. HVSMCs were treated with Cer at the indicated concentrations. $^*P < 0.05$ versus the cultures with IM. **b**: Time course of the inhibitory effect of Cer (10 nmol/L) on ALP activities in HVSMCs stimulated with IM. Control, the cultures without reagents. $^*P < 0.05$ versus the cultures with IM. **c**: Time course of the inhibitory effect of Cer on expression of ALP mRNA in HVSMCs. HVSMCs were cultured for the indicated period of time in the presence or absence of the IM with or without 5 nmol/L Cer. 18S rRNA was utilized for assessing equal RNA loading.

of cerivastatin at 10 nmol/L was sustained until 14 days (Fig. 2b). Furthermore, Northern blot analysis showed that the tissue non-specific ALP mRNA was induced in HVSMCs by these factors and that cerivastatin (5 nmol/L) inhibited the induction of ALP mRNA until 4 days (Fig. 2c). To further confirm the inhibitory effect of statins on vascular calcification, we investigated the effects of atorvastatin in this calcification model. Atorvastatin also inhibited in vitro calcification and ALP activities in HVSMCs induced by IM (Fig. 3a,b, respectively). These data suggest that statins inhibit in vitro calcification by depressing the expression of ALP in HVSMCs.

To evaluate whether cerivastatin may block ALP induction in HVSMCs by the IM through inhibiting the biosynthesis of isoprenoids, we examined the effects of MVA, FPP, and GGPP on the inhibition of ALP activities by cerivastatin. MVA and GGPP almost completely reversed the inhibitory effect of cerivastatin at 100 and 10 μmol/L, respectively (Fig. 4). On the other

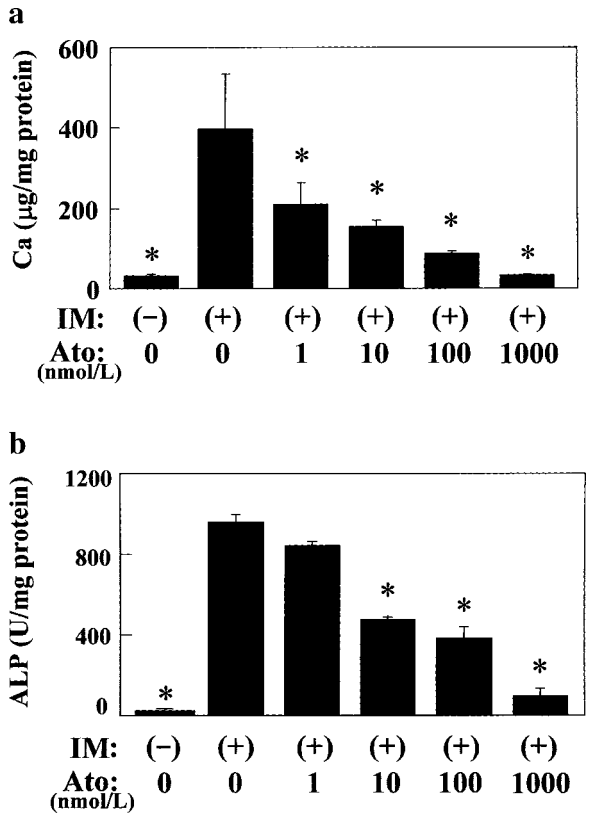

Fig. 3. **a**: Effects of atorvastatin (Ato) on HVSMC calcification. HVSMCs were cultured for 14 days in the presence or absence of the IM with the indicated concentrations of atorvastatin (Ato). Calcium deposition was quantified at the indicated time point by *o*-cresolphthalein complexone methods. The data are presented as mean ± SEM (n = 3). $*P < 0.05$ versus the cultures with IM. **b**: Effects of atorvastatin on ALP activities in HVSMCs. HVSMCs were treated for 4 days with Ato at the indicated concentrations. ALP activities were measured, normalized to cellular protein contents, and are presented as mean ± SEM (n = 3). $*P < 0.05$ versus the cultures with IM.

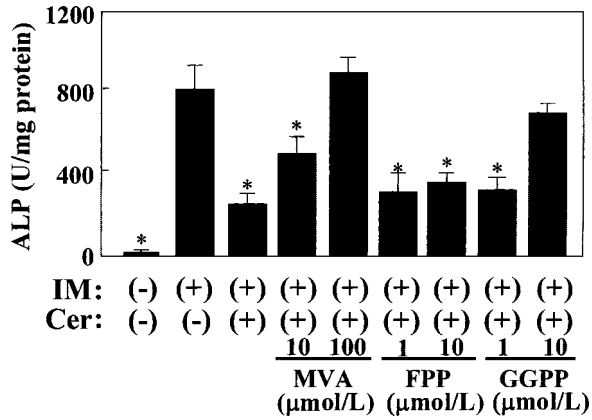

Fig. 4. Effects of mevalonate (MVA), geranylgeranylpyrophosphate (GGPP), and farnesylpyrophosphate (FPP) on the ALP activities in HVSMCs inhibited by Cer. HVSMCs were incubated with the IM and 10 nmol/L Cer in the presence or absence of MVA (100 μmol/L) or GGPP (10 μmol/L) or FPP (10 μmol/L). ALP activities were measured, normalized to cellular protein contents, and are presented as mean ± SEM (n = 3). $*P < 0.05$ versus the control.

hand, FPP had no effect on the ALP activities inhibited by cerivastatin even at 10 μmol/L (Fig. 4). These data suggest that the suppression of geranylgeranylpyrophophate production by cerivastatin may be involved in its inhibitory action on ALP activities in HVSMCs.

Because Rho proteins are mainly geranylgeranylated, while Ras proteins are predominantly farnesylated, inhibition of Rho and its downstream target, Rho kinase is a likely mechanism mediating the inhibitory effect of cerivastatin on ALP induction in HVSMCs. Since cerivastatin may exert its pleiotropic effects through inhibiting RhoA activation [Yoshida et al., 2001], we examined the effect of cerivastatin on membrane translocation of RhoA in HVSMCs. Immunoblot analysis of cytosol and membrane fractions prepared from HVSMCs revealed that the membrane-associated RhoA proteins were increased by treatment with the IM at 18 h and that cerivastatin (10 nmol/L) inhibited the membrane translocation of RhoA stimulated by these factors at the same time point (Fig. 5a). To confirm the involvement of RhoA/Rho kinase pathway in the induction of ALP, we examined the effect of Y27632, a specific inhibitor of Rho kinase, a downstream target of RhoA on ALP activities in HVSMCs. Y27632 dose-dependently inhibited ALP activities induced by IM (Fig. 5b). Furthermore, Y27632 almost completely depressed HVSMC calcification at the doses of 10 and 20 μmol/L (Fig. 5c). These data suggest that cerivastain may inhibit in vitro calcification and ALP expression in HVSMCs by blocking RhoA/Rho kinase pathway.

DISCUSSION

In this study, we have investigated the effects of statins such as cerivastatin and atorvastatin on vascular calcification induced by IM [Shioi et al., 2002]. Statins inhibited in vitro calcification of HVSMCs and their expression of ALP, which may play an important role in vascular calcification such as atherosclerotic and cardiac valve calcification [Proudfoot et al., 2002; Rajamannan et al., 2002]. The inhibitory action of cerivastatin was mediated through blocking RhoA/Rho kinase pathway. Furthermore, the

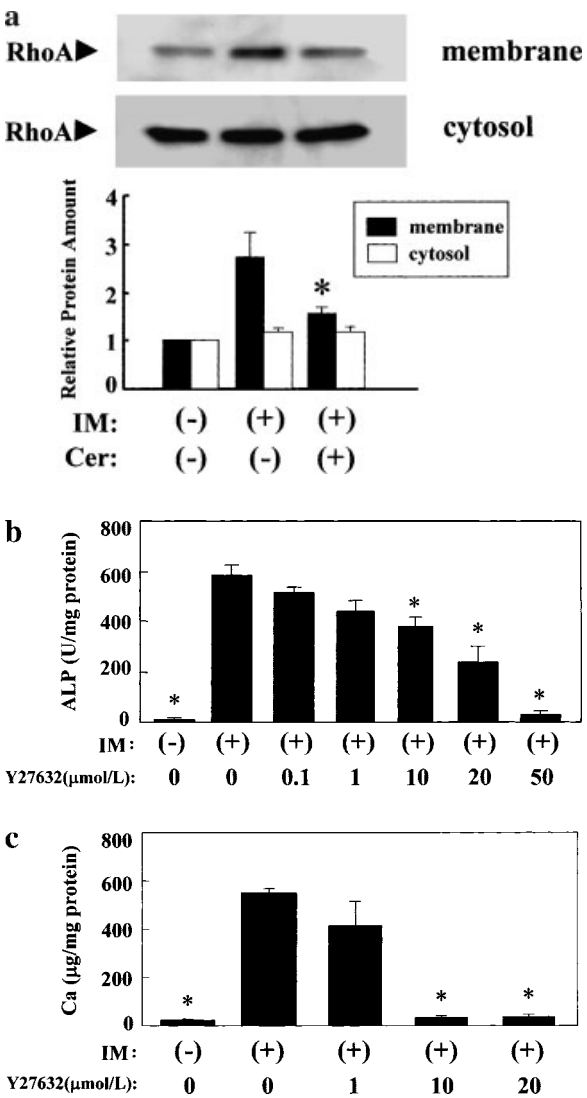

Fig. 5. **a**: Immunoblot analysis of cytosol and membrane fractions prepared from HVSMCs. HVSMCs were incubated with the IM in the presence or absence of 10 nmol/L Cer for 18 h. The total cell lysate was separated into membrane and cytosol fractions as described in Materials and Methods. Equal amounts of proteins of each fraction were analyzed by Western blotting with anti-RhoA monoclonal antibody. The staining intensities were quantified by densitometric scanning and the data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures with IM. **b**: Effects of a Rho kinase inhibitor (Y27632) on ALP activities in HVSMCs. HVSMCs were cultured for 4 days with the IM in the presence of the indicated concentrations of Y27632. ALP activities were measured, normalized to cellular protein contents, and are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures with IM. **c**: Effects of Y27632 on in vitro calcification of HVSMCs. HVSMCs were cultured for 14 days as described in Materials and Methods in the presence of the indicated concentrations of Y27632. Calcium deposition was quantified by *o*-cresolphthalein complexone methods. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures with IM.

effect of cerivastatin was observed at the concentrations from 0.1 to 10 nmol/L. These concentrations can be achieved in human plasma after an oral administration of this drug [Stein et al., 1999]. These data suggest that statins may inhibit vascular calcification associated with inflammatory diseases.

Statins exert their pleiotropic effects including anti-inflammatory effect mainly through inhibiting the isoprenylation of small G-proteins, such as Ras, Rho, Rac, Ral, and Rap [Takemoto and Liao, 2001]. Among these small G-protein-coupled signaling pathways, Rho-dependent pathway controls VSMC functions such as contraction, migration, apoptosis, and proliferation and is also involved in the development of atherosclerosis as well as in-stent neointimal formation [Shibata et al., 2001; Shimokawa et al., 2001]. The present study has demonstrated that RhoA/Rho kinase pathway is involved in acquisition of the calcifying phenotype of HVSMC by IM and that cerivastatin inhibits HVSMC calcification through blocking this signaling pathway. Therefore, RhoA/Rho kinase pathway may contribute to the progression of inflammatory vascular calcification and may be a possible target to prevent this pathological condition.

Recently, several studies have demonstrated that statins stimulate bone formation through increasing the production of bone morphogenetic protein-2 (BMP-2) by osteoblasts [Mundy et al., 1999; Sugiyama et al., 2000]. Simvastatin, one of these compounds, stimulates ALP activity in murine nontransformed osteoblast-like cells (MC3T3-E1) and their mineralization [Maeda et al., 2001]. However, statins such as mevastatin and mevinolin suppress the induction of ALP in pluripotent M2-10B4 mouse marrow stromal cells undergoing osteoblastic differentiation [Parhami et al., 2002b], suggesting that the effects of these compounds on osteoblastic differentiation may vary with target cell types and culture conditions. Since osteogenic differentiation of vascular cells plays an important role in the development of vascular calcification, it is important to clarify whether statins may stimulate vascular calcification through promoting osteogenic differentiation of vascular cells. The present study demonstrated that cerivastatin and atorvastatin inhibit in vitro calcification of HVSMCs induced by IM through suppressing their expression of ALP. Therefore, these compounds may exert

their inhibitory effects on vascular calcification, especially associated with inflammatory vascular diseases including atherosclerosis.

We utilized human neonatal SMCs derived from umbilical artery in the present study. The neonatal SMCs cultured alone expressed low levels of ALP and did not calcify spontaneously nor even in the presence of β-GP, while bovine vascular cells including mature VSMCs express high levels of ALP and can calcify extracellular matrix spontaneously or in the presence of β-GP [Bostrom et al., 1993; Shioi et al., 1995; Wada et al., 1999]. As shown in the previous study, the neonatal SMCs acquired calcifying capacities based on high levels of ALP expression when incubated with TNF-α and OSM in the presence of IFN-γ and $1,25(OH)_2D_3$ [Shioi et al., 2002]. Since ALP can degrade β-GP, releasing inorganic phosphate, ALP-dependent calcification in the presence of β-GP may be mediated through increased concentrations of phosphorus in culture media [Shioi et al., 1995; Wada et al., 1999]. Moreover, inorganic phosphate itself induced in vitro calcification of human fetal and mature SMCs [Jono et al., 2000]. Therefore, the findings of this study with neonatal SMCs expressing high levels of ALP are comparable to those with mature SMCs. Furthermore, it has been well recognized that neointimal and neonatal SMCs share similarities in morphology and expression of genes for matrix proteins and some differentiation factors [Majesky et al., 1992; Shanahan and Weissberg, 1998]. Since neointima is formed in various vascular inflammatory diseases, the cultures of neonatal SMCs in the presence of IM utilized in this study may be a useful tool to analyze the mechanism of atherosclerotic calcification.

Both in vivo and in vitro studies suggest that lipids may be involved in the progression of atherosclerotic and valvular calcification [Parhami et al., 1997; Mohler et al., 1999; Demer, 2001; Proudfoot et al., 2002; Rajamannan et al., 2002; Sun et al., 2002]. A possible role of lipids, especially oxidized LDL in atherosclerotic and valvular calcification is thought to stimulate inflammatory responses [Libby, 2002]. These modified lipids can induce the expression of adhesion molecules, chemokines, proinflammatory cytokines, and other mediators of inflammation in macrophages and vascular wall cells and the immune responses through activation of T lymphocytes [Libby, 2002]. Clinically, the serum levels of high sensitive C-reactive protein (hsCRP), a serum marker of inflammation, are associated with increased coronary calcification [Wang et al., 2002]. In vitro studies demonstrate that monocytes/macrophages and proinflammatory cytokines can enhance in vitro calcification of vascular cells [Tintut et al., 2000; Parhami et al., 2002a; Shioi et al., 2002; Tintut et al., 2002]. Therefore, inflammatory responses may play an important role in the development of vascular calcification.

Statins not only decrease the progression of coronary artery calcification, but also reduce hsCRP levels in a lipid-independent manner in hypercholesterolemic patients [Callister et al., 1998; Ridker et al., 2001; Achenbach et al., 2002; Plenge et al., 2002], suggesting the possible contribution of anti-inflammatory effects of statins to clinical benefits. Various studies have demonstrated anti-inflammatory effects of statins including the decreased expression of adhesion molecules, chemokines, proinflammatory cytokines, major histocompatibility complex II molecules, tissue factor, and matrix-degrading enzymes and the increased expression of nitric oxide [Takemoto and Liao, 2001]. In this study, we have demonstrated for the first time the inhibitory effect of cerivastatin and atorvastatin on in vitro calcification and ALP expression in HVSMCs induced by IM, providing a possible mechanism of statins to reduce the progression of coronary calcification as evidenced by clinical studies.

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