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RhoA/Rho kinase (ROCK) alters fetuin-A uptake and regulates calcification in bovine vascular smooth muscle cells (BVSMC)

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Abstract

RhoA/Rho kinases (ROCK) play a critical role in vascular smooth muscle cell (VSMC) actin cytoskeleton organization, differentiation, and function and are implicated in the pathogenesis of cardiovascular disease. We have previously determined that an important step in the regulation of calcification is fetuin-A endocytosis, a process that is dependent on changes in the cytoskeleton, which, in turn, is known to be affected by the RhoA/ROCK signaling pathway. In the present study, bovine VSMC (BVSMC) were treated with the ROCK inhibitor Y-27632 or transfected with ROCK small interfering (si) RNA to knock down ROCK expression. Both conditions resulted in reduced actin stress fibers and increased Cy5-labeled fetuin-A uptake. Inhibition of ROCK by Y-27632 or siRNA also significantly increased BVSMC alkaline phosphatase (ALP) activity and calcification of BVSMC and rat aorta organ cultures. Cells were then incubated in calcification media in the presence or absence of Y-27632 and matrix vesicles (MV) isolated by collagenase digestion. These MV, isolated from BVSMC incubated with Y-27632, had increased ALP activity and increased ability of MV to subsequently calcify collagen by 66%. In contrast, activation of RhoA, which is upstream of ROCK, by transfecting plasmids encoding the dominant active Rho GTPase mutant (Rho-L63) led to decreased fetuin-A uptake and

OCK signaling pathway is an important negative regulator of vascular calcification.

Keywords: vascular calcification, endocytosis, cytoskeleton, Rho GTPase

VASCULAR CALCIFICATION IS a prominent finding in aging, diabetes, chronic kidney disease (CKD), and inflammatory diseases. Both coronary artery and peripheral artery calcification are associated with increased morbidity and mortality in the general population (1), diabetes (18), and in patients with CKD (2, 20). The pathophysiology of vascular calcification is complex but appears to be similar to normal bone osteogenesis where osteoblast/chondrocytes [or osteoblast-like vascular smooth muscle cells (VSMC)] produce cell outpockets that contain calcium and phosphorus called matrix vesicles (MV). MV contain various proteins, including alkaline phosphatase (ALP), annexins, and fetuin-A, a circulating inhibitor of mineralization (6, 25). These MVs bind to extracellular matrix proteins to initiate mineralization.

The Rho families of GTPases are regulatory molecules that link surface receptors to organization of the

actin cytoskeleton and regulate fundamental cellular processes ([26]). RhoA signaling is regulated by growth factors, hormones, and cytokines, all of which have been shown to be critical in calcification in vitro ([22]). The biological effects of RhoA are mediated by a number of downstream effector proteins; the best characterized is Rho kinase (ROCK) ([10]). ROCK has been shown to be critical in VSMC differentiation and function. A recent study demonstrated that activation of RhoA with the bacterial toxin *Pasteurella multocida* toxin (PMT) alters actin cytoskeleton organization and inhibits the differentiation of osteoblasts as assessed by decreased expression of Cbfa1, osteocalcin, and ALP, and decreases mineralization. In contrast, inhibition of ROCK stimulates osteoblast differentiation and mineralization ([12]). Dynamic rearrangement of actin is associated with endocytosis, exocytosis, phagocytosis, and migration, processes that would be critical for a MV to "pinch" off from a cell membrane. We have previously demonstrated that fetuin-A, a circulating inhibitor of mineralization, is taken up by bovine VSMC (BVSMC) cells in a fluid-phase endocytic pathway that is calcium dependent. Furthermore, the content of fetuin-A in MV determines their ability to take up ^{45}Ca and mineralize extracellular collagen ([8], [25]). The objective of this study is to determine the effect of RhoA signaling in fetuin-A uptake, calcification, and MV activity in BVSMC.

MATERIALS AND METHODS

Cell culture. BVSMC were isolated from the descending thoracic aorta by the explant method as previously described ([9]). The BVSMC were grown in DMEM (Sigma, St. Louis, MO) with 10% FBS until confluent, at which time they were replated for specific experiments. Only cells between *passages 2* and *8* were used in the experiments. To induce calcification, BVSMC were treated with 10 mmol/l β-glycerophosphate, 10^{-7} mol/l insulin, and 50 μg/ml ascorbic acid in the presence of 15% serum ([9]). Control or noncalcifying BVSMC were cultured in identical conditions but without the β-glycerophosphate. In some experiments, BVSMC were also treated with Rho kinase inhibitor Y-27632 (Calbiochem).

Fluorescent labeling of fetuin and live cell imaging. Fetuin-A uptake was determined using live cell imaging as previously described ([6]). Briefly, fetuin-A from fetal calf serum (Sigma) was labeled with FluoroLink Cy5 monofunctional dye (Amersham Biosciences, Piscataway, NJ). It was separated on a column of Bio-Gel P-30 Gel (Bio-Rad Laboratories, Hercules, CA). BVSMC were seeded on glass bottom Microwell dishes (MatTek, Ashland, MA) in 10% FBS DMEM for 72 h. Ten minutes before addition of Cy5-fetuin, the media was replaced with M2 media (150 mM NaCl, 20 mM HEPES, 0 or 1.3 mM CaCl$_2$, 5 mM KCl, 1 mM MgCl$_2$, 50 mM glucose, at pH 7.4) at 37°C. Labeled fetuin-A was then added, and MRC-1024 laser-scanning confocal microscopy (Bio-Rad) was used to capture images. To quantify the uptake of fluorescently labeled fetuin-A, image processing was conducted using Metamorph software (Universal Imaging, West Chester, PA). Six images with four to six cells per image were quantified to allow for a representative assessment of the uptake. The actin cytoskeleton was examined after fixation, permeabilization, blocking, and staining with FITC-labeled phalloidin (Invitrogen, Carlsbad, CA) as previously described ([4]).

Knockdown of ROCK expression by small interfering RNA transfection. To further confirm the involvement of Rho kinase, the expression of ROCK was knocked down using small interfering (si) RNAs. Briefly, BVSMC were seeded in six-well culture plates until they were 60−70% confluent. Ten micromolar siRNA specifically targeted to ROCK-1 (Santa Cruz Biotechnology, Santa Cruz, CA) as well as scrambled siRNA (transfection control) were used to transfect BVSMC using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's instructions. The knockdown of ROCK was confirmed by Western blotting of cell lysate from transfected BVSMC with equal protein loading of all lanes, confirmed by striping the blot and reprobing with β-actin as a loading control.

Organ culture. Segments of rat thoracic aorta measuring 3 cm were harvested from male Sprague-Dawley rats and gently cleared of surrounding tissues. Studies were approved by the Indiana University

Institutional Animal Care and Use Committee. Aortic segments were incubated in DMEM (GIBCO, Long Island, NY) containing 1× penicillin/streptomycin and without serum at 37°C in a humidified 5% CO_2-95% air incubator. To induce calcification, 7.5 U/ml of calf intestinal ALP was added to DMEM, and the phosphate ion concentration was increased to 3.8 mM by addition of sodium phosphate (= calcification medium) according to the methods of O'Neil and colleagues ([19]). ROCK inhibitor Y-27632 (10 µM) was preequilibrated in medium and then added to the culture medium. The aorta ring cultures were also treated with doxycycline (50 µg/ml) to serve as a treatment control, as doxycycline has been shown to inhibit rat aorta calcification ([24]). The culture medium was changed every 2–3 days. After 7 days, aortic specimens were rinsed in normal saline, minced, and decalcified in 300 µl of 0.6 N HCl for 72 h with gentle agitation. The calcium content of HCl supernatants was determined colorimetrically as described below and normalized by tissue weight. Viability of aorta organ cultures was determined by lactic dehydrogenase secretion into the medium using a CytoTox-One Homogeneous Membrane Integrity Assay (Promega).

Transient transfection of plasmid vector with Rho-N19 or Rho-L63. To determine the involvement of the RhoA signaling pathway, BVSMC cells were transfected with plasmids with Rho-N19 or Rho-L63 cDNA expressed from SV40 promoters ([11]) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were incubated with the transfection mixtures for 5 h, and then this mixture was replaced with normal growth media. Cells were analyzed at various time points.

MV isolation. MV were isolated by collagenase digestion as previously described ([8]). Cells were incubated with crude collagenase (500 U/ml, type IA, Sigma) in a solution of 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl, and 0.02 M Tris buffer, pH 7.45, at 37°C for 3 h. The digests were centrifuged at 800 and 30,000 g to remove cell debris and microsomes, respectively. The supernatant was centrifuged at 250,000 g to pellet the MV, followed by resuspension in TBS (pH 7.6) with 0.25 M sucrose. The MV amount was determined by protein concentration (Bio-Rad).

Calcium deposition. BVSMC were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (calcium kit; Pointe Scientific) and normalized to protein content as previously described ([9])

ALP activity. ALP activity was measured using a *p*-nitrophenyl substrate supplied in an ALP assay kit (Pointe Scientific) and normalized by protein content ([9]).

MV-collagen calcification assay. MV-collagen calcification was determined as we previously described ([8]). Briefly, glass coverslips were coated with type I collagen (Sigma) in a 0.01% solution in 0.1 M acetic acid at room temperature for 4 h, which yields an ∼8–10 µg/cm² coating. MV isolated as above were added in equal concentrations (10 µg/dish) to type I collagen-coated coverslips in the presence of calcification media (DMEM with 15% FBS and 10 mM β-glycerophosphate) to yield an acellular MV-collagen culture and incubated at 37°C for 72 h. To determine the magnitude of calcification of this MV-collagen culture, the media was removed and the MV-ECM complex on the coverslips was incubated in 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific).

Statistical analysis. Statistical analysis was conducted using ANOVA with Fisher's post hoc analysis. The results are expressed as means ± SD, with $P < 0.05$ considered significant (StatView, SAS Institute, Cary, NC).

RESULTS

ROCK inhibition induced actin cytoskeleton reorganization and enhanced fetuin-A uptake in BVSMC. To determine the effect of ROCK on actin cytoskeleton reorganization and fetuin-A uptake, BVSMC were incubated with 0, 5, 10, or 20 µM ROCK inhibitor (Y-27632) for 24 h. Actin cytoskeleton reorganization was examined by staining with FITC-phalloidin, and fetuin-A uptake examined by confocal microscopy

with Cy5-labeled fetuin-A (100 µg/ml). The results demonstrated that treatment of BVSMC with Y-27632 caused dramatic cytoskeletal reorganization and reduced stress fibers (Fig. 1A, *top*) as expected. Furthermore, live cell imaging showed that fetuin-A uptake (Fig. 1A, *bottom*) was dose dependently increased in the presence of increasing concentration of ROCK inhibitor in BVSMC (Fig. 1B, $P < 0.05$). However, inhibition of ROCK with Y-27632 had no effect on the uptake of the fluid-phase endocytosis marker dextran (Texas red-labeled, 70 kDa, similar molecular weight to fetuin-A) in BVSMC (data not shown). These results indicate that ROCK is involved in actin cytoskeleton reorganization and fetuin-A uptake in BVSMC and that the mechanism of action is not by disruption of fluid-phase endocytosis.

Inhibition of ROCK increased ALP activity and enhanced calcification in BVSMC. To determine the effect of ROCK on ALP activity and calcification in BVSMC, cells were incubated with control (no β-glycerophosphate) or calcification media (with β-glycerophosphate) in the presence or absence of 10 µM Y-27632 (ROCK inhibitor) for 7 days, and calcification and ALP activity were determined. The results demonstrated that BVSMC treated with calcification media had elevated ALP activity compared with cells treated with controls. However, the inhibition of ROCK significantly increased ALP activity in calcified BVSMC but had no effect on control BVSMC (374 ± 13 vs. 284 ± 9.7 U/g protein, $P < 0.01$, Fig. 2A). Furthermore, ROCK inhibition significantly enhanced the calcification of BVSMC (Fig. 2B).

To further confirm the specificity of ROCK inhibition by Y-27632, the expression of ROCK was knocked down using ROCK1 siRNAs. As shown in Fig. 3A, ROCK1 siRNA significantly reduced ROCK expression in BVSMC by 70, 60, and 30% at *days 2, 3*, and *6*, respectively. However, control siRNA had no effect. ROCK inhibition with siRNA also had no effect on annexin II expression in BVSMC [934 ± 26 optical density units (ODU), nontransfected; 915 ± 57 ODU, ROCK siRNA; 888 ± 49 ODU, control siRNA, $P =$ not significant]. Similar to ROCK inhibition with Y-27632, confocal microscopy showed reduced stress fibers and increased fetuin-A uptake in ROCK siRNA-transfected BVSMC compared with control siRNA-transfected BVSMC (data not shown). These ROCK siRNA- or control siRNA-transfected BVSMC were then treated with calcification media, and ALP activity and calcification were determined at *days 3* and *6*. The results demonstrated that compared with control siRNA, the knockdown of ROCK expression by siRNA significantly increased ALP activity at *days 3* and *6* (Fig. 3B, $P < 0.05$) and calcification at *day 6* (Fig. 3C, $P < 0.05$). These results demonstrated that ROCK maintains the integrity of the cytoskeleton, and without that, fetuin-A uptake/endocytosis is impaired and calcification is inhibited.

Ex vivo effect of ROCK inhibition on rat aorta calcification. We next investigated whether the observed in vitro effect of ROCK inhibition on BVSMC calcification could be reproduced ex vivo using an organ culture system. The aorta organ cultures have the benefit of including both endothelial and vascular smooth muscle cells as well as extracellular matrix proteins which contribute to aorta calcification. Aorta rings were calcified in an organ culture system by addition of calf intestinal ALP and sodium phosphate to DMEM without serum (calcification media) in the presence or absence of ROCK inhibitor Y-27632. The results demonstrated that inhibition of ROCK increases calcification in rat aorta ring cultures whereas doxycycline inhibits aorta calcification (Fig. 4). These results suggest that similar to the in vitro cell study, ROCK inhibition significantly enhanced the calcification in rat aorta calcification ex vivo.

Inhibition of ROCK increased ALP activity in MV from calcified BVSMC and increased ability of MV to calcify collagen.
To determine whether inhibition of ROCK also affects MV ALP activity and its ability to calcify type I collagen, BVSMC were incubated in DMEM in calcification media (with 10 mM β-glycerophosphate) or noncalcifying media (no β-glycerophosphate) for 7 days in the presence or absence of ROCK inhibitor Y-27632 and MV isolated by collagenase digestion from these cell cultures. As we have previously shown (8), MV isolated from calcified BVMSC had increased ALP activity compared with that from control BVSMC (Fig. 5A). However, blockade of ROCK by Y-27632 during MV synthesis significantly

increased ALP activity in MV isolated from calcified BVSMC (Fig. 5A; calcified = 2,548 ± 484 U/g protein; calcified+Y-27632 = 3,348 ± 325 U/g protein, $P < 0.01$) but had no effect on ALP activity in MV from control (noncalcified) BVSMC (control = 607 ± 231 U/g protein; control+Y-27632 = 804 ± 347 U/g protein).

To determine the role of ROCK in MV calcification in extracellular matrix, BVSMC were incubated in calcifying or noncalcifying conditions in the presence or absence of Y-27632 for 7 days, and MV were isolated, placed on type I collagen-coated dishes, and incubated with calcification media (10 mM β-glycerophosphate) but no cells for 3 days for the MV-collagen calcification assay. The results demonstrated that MV isolated from BVSMC incubated with Y-27632 increased the ability of the MV to subsequently calcify collagen (Fig. 5B, *$P < 0.01$, calcified vs. control; #$P < 0.005$, Y-27632 vs. no Y-27632). These results demonstrated that inhibition of ROCK increased MV ALP activity and enhanced the ability of MV to mineralize. These data demonstrated that ROCK is an important negative regulator of MV activity and calcification in BVSMC.

RhoA activation leads to increased actin stress fibers, reduced fetuin-A uptake, and decreased calcification in BVSMC.

RhoA is an upstream regulator of ROCK; therefore, to determine whether activation of RhoA results in the opposite effect of inhibition of ROCK, transient transfection was performed with plasmids encoding dominant active Rho GTPase mutant (Rho-L63) and dominant negative Rho GTPase mutant (Rho-N19) proteins. Both of these constructs contain a Myc epitope tag engineered at the amino terminus that allowed detection of the exogenous proteins and identification of transfected cells using a monoclonal antibody against c-myc. As shown in Fig. 6A, 48 h after transfection, both Rho-N19- and Rho-L63-transfected BVSMC expressed myc-tagged mutant RhoA, whereas there was no detectable mutant RhoA expression in nontransfected BVSMC. Confocal microscopy demonstrated that there were increased stress fibers and decreased fetuin-A uptake in dominant active Rho GTPase mutant (Rho-L63)-transfected BVSMC (data not shown). However, similar to inhibition of ROCK, dominant negative Rho GTPase mutant (Rho-N19)-transfected BVSMC had reduced stress fibers but increased fetuin-A uptake (data not shown). To further assess the role of RhoA activation in BVSMC calcification, Rho-L63- and Rho-N19-transfected BVSMC were incubated with calcification media for 6 days and calcification was determined. The results demonstrated that compared with Rho-N19, Rho-L63-transfected BVSMC decreased calcification (Fig. 6B). These data are consistent with our results with ROCK inhibition and ROCK siRNA.

DISCUSSION

In the present study, we have demonstrated that inhibition of ROCK with Y-27632 or ROCK siRNA altered actin cytoskeletal arrangement with reduced actin stress fibers and increased fetuin-A uptake in BVSMC. Inhibition of ROCK also significantly increased BVSMC ALP activity and calcification, and MV ALP activity and ability to subsequently calcify on collagen. Furthermore, ex vivo studies demonstrated that inhibition of ROCK enhanced rat aorta calcification in organ culture system. We further substantiated our findings by activating RhoA, an upstream regulator of ROCK, finding opposite effects of ROCK inhibition on ALP activity and calcification. Thus inhibition of RhoA or its downstream effector, ROCK, led to similar results, demonstrating the RhoA/ROCK signaling pathway as an important negative regulator of vascular calcification.

The Rho/ROCK pathway plays an important role in various cellular functions that are involved in the pathogenesis of cardiovascular disease (29). Accumulating evidence has indicated that the Rho-kinase-mediated pathway is involved in the pathogenesis of atherosclerosis (26). Inhibition of ROCK has been shown to reduce plaque inflammation, increase endothelial eNOS activity, and exert cardiovascular protection in human endothelial cells and mouse animal models (35). On the other hand, activation of ROCK is associated with macrophage accumulation, collagen deposition, and transforming growth

factor (TGF)-β1 expression in the intima of porcine coronary arteries (21) and is involved in increased endothelial permeability, therefore enhancing atherosclerosis (33). Thus the final effect on atherosclerosis is a balance of these effects. In addition, the RhoA/ROCK signaling pathway is regulated by inflammatory cytokines, multiple hormones, bisphosphonates, and statins (26), all factors that have been associated with both atherosclerosis and arterial calcification in humans or rodent models. These data and the results of our current study support that the RhoA/ROCK pathway may be a common signaling pathway for both atherosclerosis and nonatherosclerotic arterial calcification. The mechanism by which inhibition of RhoA/ROCK signaling may increase calcification is likely multifactorial and includes effects on apoptosis, metalloproteinase production, phenotypic alterations, and endocytosis (3, 26).

We have previously demonstrated that fetuin-A is endocytosed in BVSMC in a calcium-dependent manner (6). Endocytosis requires remodeling of the cytoskeleton (13), and the present study confirmed that RhoA/ROCK inhibition resulted in an alteration of the actin cytoskeleton, leading to in increased fetuin-A uptake in BVSMC. Fetuin-A (α-2-Heremens-Schmidt glycoprotein) is a circulating reverse acute-phase protein made predominately in the liver, but found in the circulation and in bone (15, 16, 32). Fetuin-A knockout animals have diffuse extraosseous calcification (27), indicating that fetuin-A is a naturally occurring inhibitor of unwanted mineralization. In vitro, fetuin-A inhibits calcification of both VSMC and osteoblasts (23, 25, 28) and when present in secreted matrix vesicles, renders them incapable of ^{45}Ca uptake (25). Our previous studies have shown that cell-derived MV must have low fetuin-A and increased annexin content to calcify on an extracellular matrix of type I collagen (8). We have also previously demonstrated that inhibition of the annexin calcium channel by K201 (8), or verapamil (but not nifedipine) (5), significantly decreased fetuin-A uptake, reduced calcification in BVSMC, and decreased MV activity and the ability to subsequently calcify. These studies have led to our working hypothesis that the regulation of MV content is at the cell membrane: if fetuin-A stays at the membrane and is not endocytosed, it becomes incorporated into MV that cannot induce calcification and are released from the cells. In contrast, if the fetuin-A is endocytosed into the cell, then the MV derived from the membrane would have low fetuin-A content and are procalcifying. Stimuli that decrease endocytosis of fetuin-A, such as blockade of calcium channel activity by K201 (8) or verapamil (5), or alteration of the actin cytoskeleton by RhoA/ROCK activation, decrease calcification in VSMC. We have additionally found that inhibition of ROCK did not affect annexin II expression. Thus, although the exact mechanism is not known, RhoA/ROCK is involved in regulating fetuin-A uptake and calcification in BVSMC. The mechanism appears specific for fetuin-A and not a general effect on fluid-phase endocytosis.

Interestingly, a recent study demonstrated that RhoA/ROCK is also an important negative regulator of osteoblast differentiation and bone formation (12). Activation of RhoA by PMT caused dramatic cytoskeletal rearrangements, with highly organized actin stress fibers and inhibited osteoblast differentiation and mineralization (12). However, inhibition of ROCK by Y-27632 stimulated the expression of ALP and osteocalcin in osteoblasts and enhanced bone nodule calcification (12). Our current studies and these data indicate that vascular calcification is a regulated process similar to bone mineralization, and the RhoA/ROCK signaling pathway is as an important negative regulator of mineralization.

The present study is also interesting in view of the observed stimulation of bone formation by statins (36). Statins inhibit geranylgeranylpyrophosphate, which prenylates RhoA. Thus statins inhibit RhoA and might be expected to increase calcification based on our results (30). Supporting this, a study in osteoblasts demonstrated that statins stimulate alkaline phosphates and bone formation through increased production of BMP, factors known to induce calcification (31). In contrast, statins decreased atherosclerotic (intimal) calcification in the ApoE null mouse given chronic kidney disease (14). However, we have previously found no effect of statins on vascular calcification in our BVSMC model,

and no effect of RhoA/ROCK inhibition on BMP-2 secretion or expression (data not shown). Our results differ somewhat from a study by Kizu et al. (17) showing that statins inhibit in vitro calcification of human vascular smooth muscle cells induced by inflammatory mediators. However, in their study, they utilized human neonatal VSMC which expressed low ALP and did not calcify even in the presence of β-glycerophosphate, whereas BVSMC used in our current study are differentiated VSMC that express high levels of ALP and calcify extracellular matrix in the presence of β-glycerophosphate (34). Thus the role of statins in calcification, and perhaps RhoA/ROCK, may be variable, depending on the differentiation of the cells and the calcification induction model.

In conclusion, we have demonstrated that RhoA/ROCK may be a common signaling pathway by which multiple calcification stimulators induce vascular calcification by regulating the cytoskeleton, endocytosis of fetuin-A, and subsequent MV activity, providing potential therapeutic targets for vascular calcification.

GRANTS

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figures and Tables

Fig. 1.
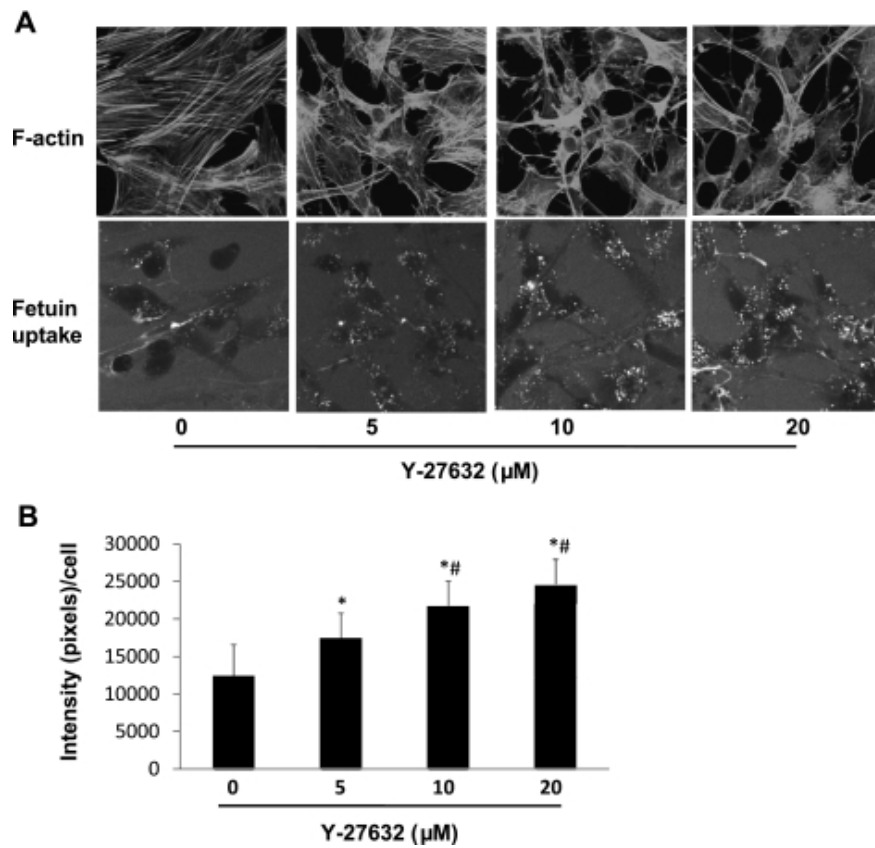
Effect of RhoA/Rho kinase (ROCK) inhibitor (Y-27632) on actin cytoskeleton reorganization and fetuin-A uptake in bovine vascular smooth muscle cells (BVSMC). BVSMC were incubated with 0, 5, 10, or 20 μM ROCK inhibitor (Y-27632) for 24 h. Actin cytoskeleton reorganization was examined by staining with FITC-phalloidin (*A*, *top*), and fetuin-A uptake was examined by confocal microscopy with Cy5-labeled fetuin-A (100 μg/ml; *A*, *bottom*). The magnitude of fetuin-A uptake was quantified by MetaMorph software (intensity/cell; *B*). The results demonstrated ROCK inhibitor altered actin cytoskeleton with reduced stress fibers and dose dependently increased fetuin-A uptake in BVSMC. Values are means ± SD from 3 experiments (*n* = 9 individual samples). *$P < 0.05$, different from untreated control (no Y-27632). #$P < 0.05$, different from 5 μM Y-27632.

Fig. 2.
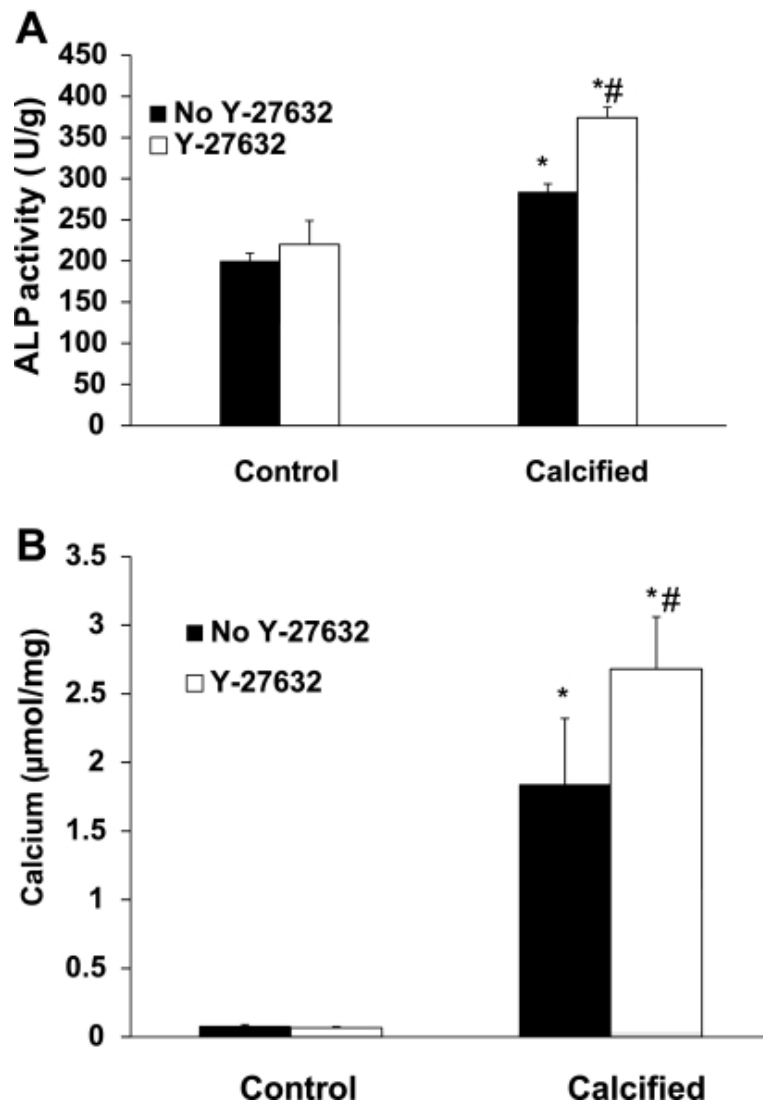
Effect of ROCK inhibitor (Y-27632) on alkaline phosphatase (ALP) activity and calcification in BVSMC. BVSMC were treated with control (no β-glycerophosphate) or calcification media (with 10 mM β-glycerophosphate = calcified) in the presence or absence of 10 μM Y-27632 for 7 days, and ALP activity and calcification were determined. The results demonstrated ROCK inhibition increased ALP activity (*A*) and calcification (*B*) in calcified BVSMC but had no effect on control BVSMC. Values are means ± SD from 4 separate experiments (final $n = 12$ individual samples). *$P < 0.01$, calcified vs. control, no Y-27632 or Y-27632. #$P < 0.01$, Y-27632 vs. no Y-27632, calcified or control.

Fig. 3.

B

C
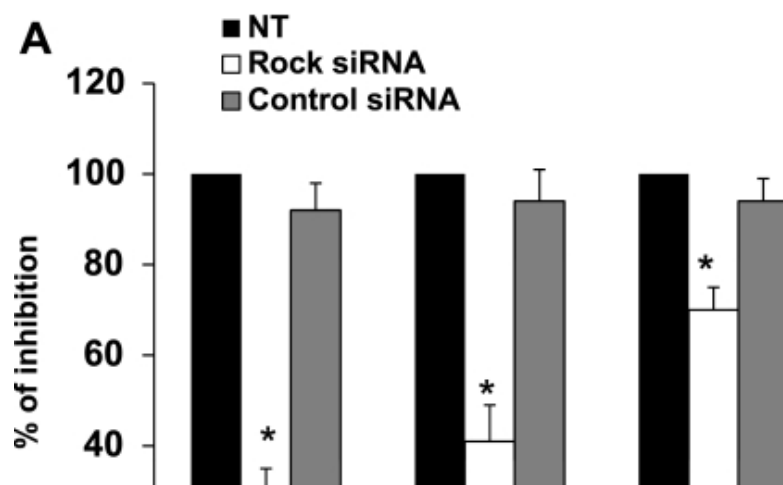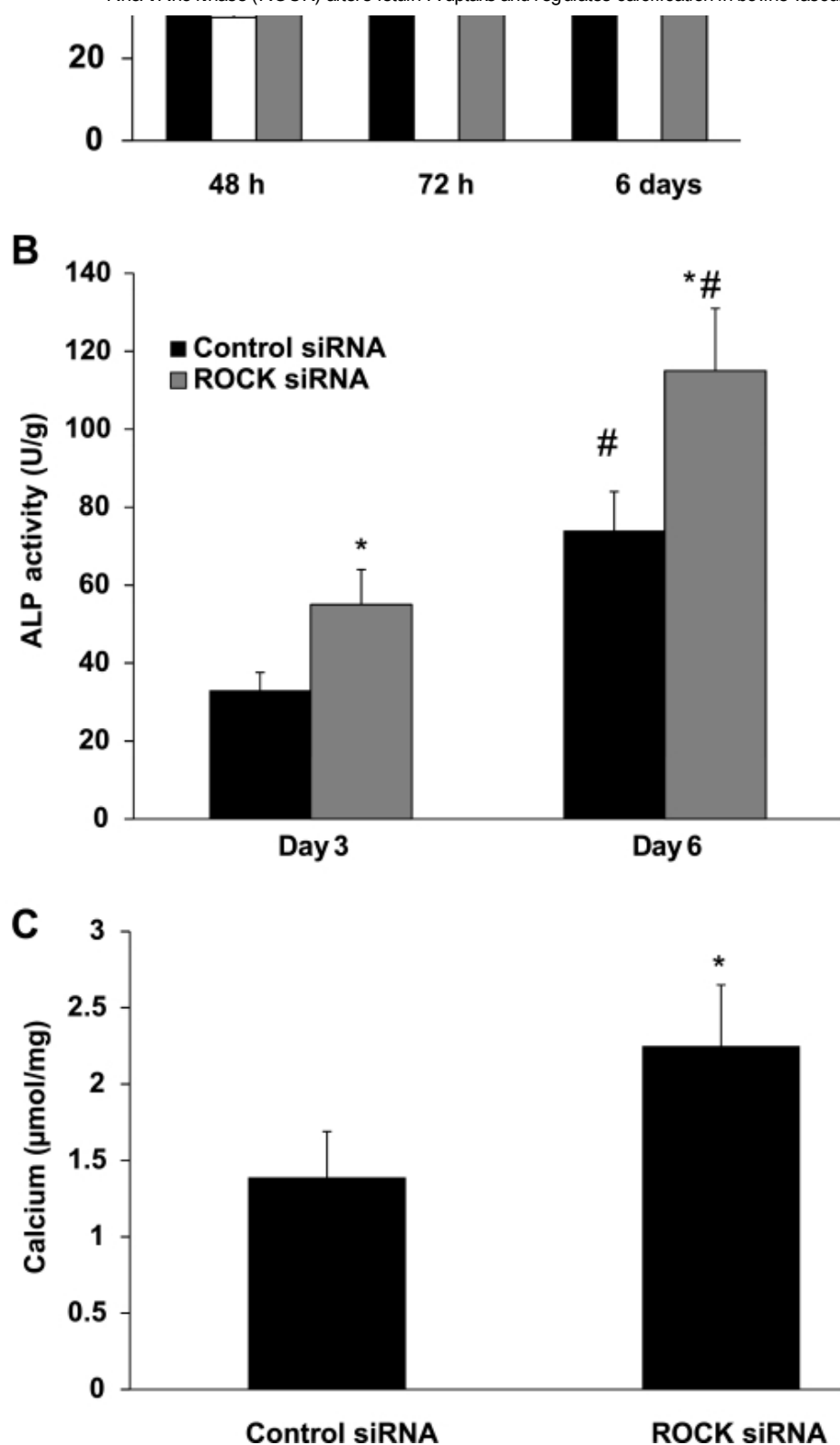
Knockdown of ROCK expression by ROCK small interference (si) RNA transfection significantly increased alkaline phosphate activity and calcification in BVSMC. To further confirm the involvement of ROCK, the expression of ROCK were knocked down using 10 μM siRNA specifically targeted to ROCK-1 as well as scrambled siRNA (transfection control). The nontransfected BVSMC (NT) were also used as ROCK expression controls. The knockdown of ROCK was confirmed by Western blotting for cell lysate from transfected BVSMC (*A*). The results demonstrated that ROCK siRNA dose dependently decreased ROCK expression, whereas control siRNA had no effect on ROCK expression. These ROCK siRNA- or control siRNA-transfected BVSMC were then treated with calcification media, and ALP activity and calcification were determined at *days 3* and *6*. The results demonstrated that compared with control siRNA, knockdown ROCK expression by ROCK siRNA significantly

increased ALP activity at *days 3* and *6* (*B*) and calcification at *day 6* (*C*). Values are means ± SD from 3 separate experiments (*n* = 9 individual samples). *$P < 0.05$, ROCK siRNA vs. control or control siRNA. #$P < 0.05$, *day 6* vs. *day 3*, control or Rock siRNA.

Fig. 4.
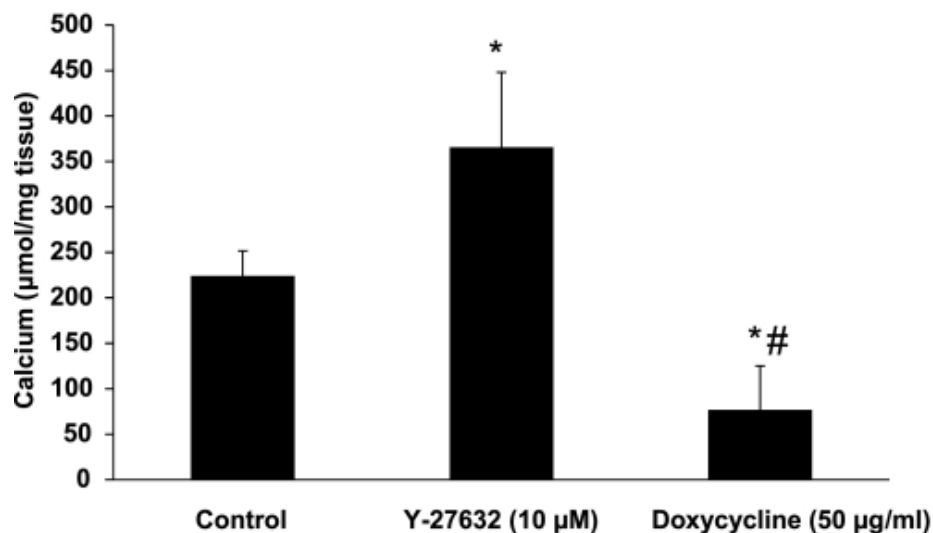
Effect of ROCK inhibition on ex vivo rat aorta calcification. Rat aortic rings were incubated immediately after harvest in DMEM with 7.5 U/ml of calf intestinal ALP and 3.8 mM sodium phosphate (calcification medium) in the presence of ROCK inhibitor Y-27632 (10 μM) or doxycycline (50 μg/ml) for 7 days. The calcium content were determined by HCl extraction and normalized by tissue weight. The results demonstrated that ROCK inhibition increased rat aortic calcification whereas doxycycline inhibited aorta calcification. Values are means ± SD (*n* = 3 from a total of 3 rats). *$P < 0.01$ vs. control. #$P < 0.01$, doxycycline vs. Y-27632.

Fig. 5.
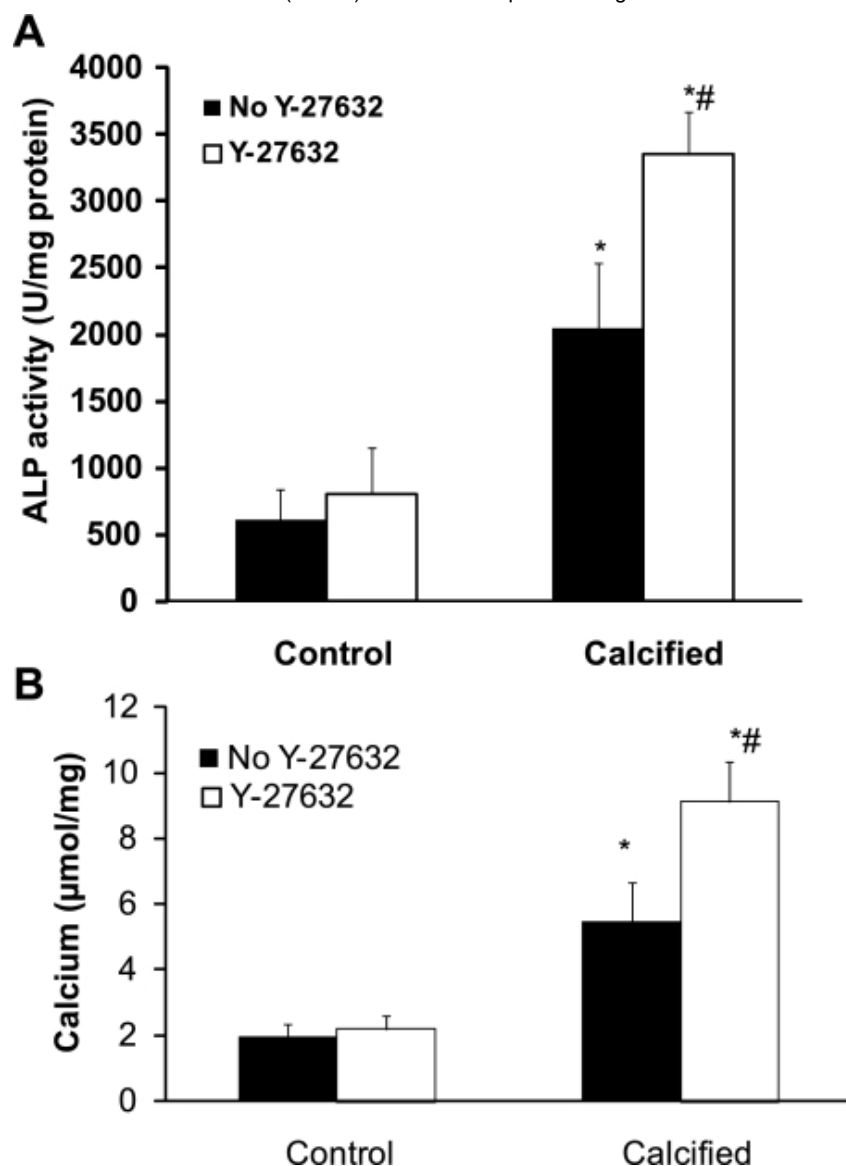
Effect of ROCK inhibition on matrix vesicle (MV) activity. BVSMC were incubated in control (no β-glycerophosphate) or calcification media (with 10 mM β-glycerophosphate, calcified) for 7 days in the presence or absence of 10 μM Y-27632, and MV were isolated by collagenase digestion. MV ALP activity was determined (*A*). These MV were then added to type I collagen-coated coverslips in the presence of calcification media (with 10 mM β-glycerophosphate) but no cells, and incubated for 3 days. The calcium content was determined by HCl extraction (*B*). These results demonstrated that inhibition of Rho kinase decreased MV ALP activity and inhibited the ability of MV to mineralize. Values are means ± SD from 4 separate experiments (*n* = 12 individual samples). *$P < 0.02$, calcified vs. control, no Y-27632 or Y-27632. #$P < 0.05$, Y-27632 vs. Y-27632, calcified or control.

Fig. 6.
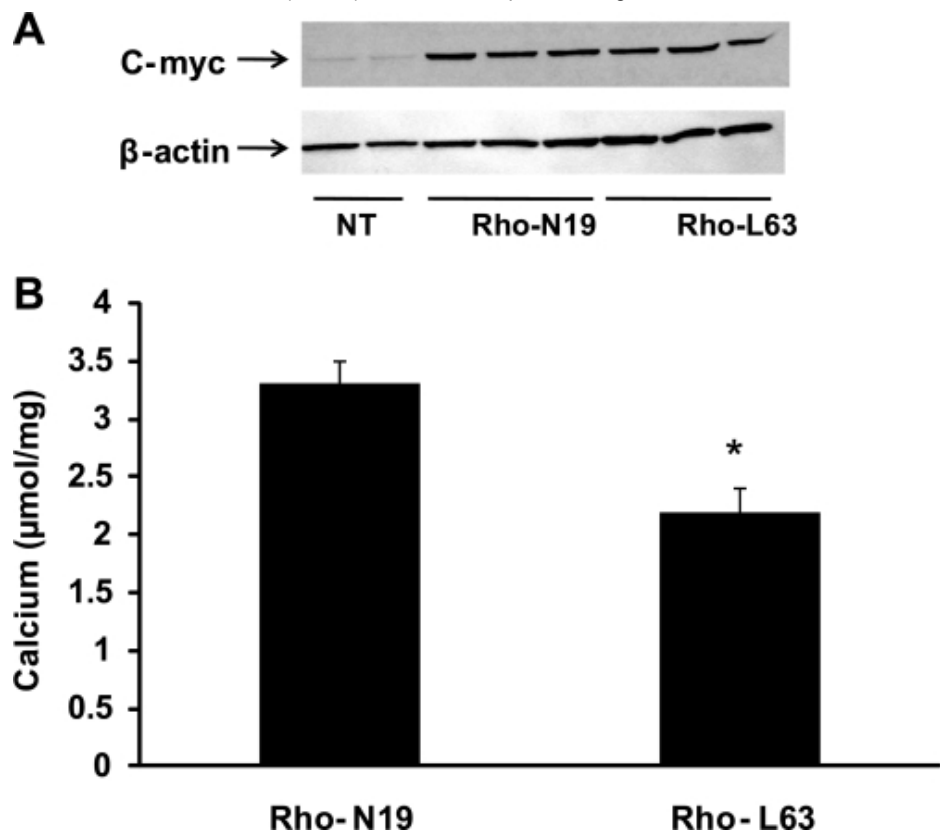
Effect of RhoA activation on calcification in BVSMC. BVSMC were transfected with Myc-tagged plasmids encoding dominant active Rho GTPase mutant (Rho-L63) or dominant negative Rho GTPase mutant (Rho-N19) proteins. The expression of myc-tagged mutant RhoA in transfected BVSMC was confirmed by Western blotting. The blot was stripped and reprobed with β-actin as a loading control (A). Rho-L63- and Rho-N19-transfected BVSMC were incubated with calcification media for 6 days, and calcification was determined. The results demonstrated that compared with Rho-N19, Rho-L63-transfected BVSMC decreased calcification (B). Values are means ± SD from 3 separate experiments (n = 9 individual samples). *P < 0.01, Rho-L63 vs. Rho-N19.