Statins prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells via inhibition of Rho/Rho-kinase pathway

Toshiyuki Kozai [a,b,d], Masato Eto [a,b], Zhihong Yang [a,b,c], Hiroaki Shimokawa [d], Thomas F. Lüscher [a,b,*]

[a] *Cardiology, Cardiovascular Center, University Hospital, CH-8091 Zürich, Switzerland*
[b] *Cardiovascular Research, Institute of Physiology, University of Zürich, Zürich, Switzerland*
[c] *Vascular Biology, Institute of Physiology, University of Fribourg, Fribourg, Switzerland*
[d] *Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan*

Abstract

Objective: Pulsatile forces regulate vascular remodeling and trigger vascular diseases such as saphenous vein graft disease. The saphenous vein is exposed to high pressure and pulsatility only after implantation. Statins have been proved to reduce the incidence of vein graft failure. Thus, we investigated the molecular mechanisms of pulsatile stretch-induced saphenous vein smooth muscle cell (SMC) proliferation and potential beneficial effects of statins.

Methods and results: Human saphenous vein SMCs were subjected to cyclic stretch (60 cycles/min) in Flex I plates. Cerivastatin and simvastatin significantly prevented stretch-induced increase in SMC proliferation. Stretch induced the membrane accumulation of Rho A and Rho kinase inhibitors (Y-27632 and hydroxyfasudil) and dominant negative Rho A mutant significantly prevented stretch-induced SMC proliferation. In addition, stretch increased the levels of both p44/42 mitogen-activated protein (MAP) kinase and Akt phosphorylation. MAP kinase kinase (MEK)1/2 inhibitor U0126, phosphatidylinositol (PI) 3-kinase inhibitors (wortmaninn and LY294002), and dominant negative Akt mutant significantly prevented stretch-induced SMC proliferation. Cerivastatin significantly prevented stretch-induced membrane accumulation of Rho A. On the other hand, stretch-induced phosphorylation of p44/42 MAP kinase and Akt was not prevented by cerivastatin. Mevalonate restored the preventive effect of cerivasatain on stretch-induced Rho A membrane accumulation. Stretch induced hyperphosphorylation of retinoblastoma protein (pRb), which was prevented by cerivastatin and the Rho kinase inhibitors.

Conclusion: Statins prevent stretch-induced saphenous vein SMC proliferation via inhibition of the Rho/Rho-kinase pathway. This may explain the beneficial effects of this class of drug, especially for patients after coronary artery bypass grafting.

Keywords: Stretch; SMC proliferation; Stains

1. Introduction

Vascular remodeling is an important feature of atherosclerosis, restenosis and vein graft failure. Mechanical factors such as shear stress and pulsatile stretch importantly contribute to these events. Up to 50% of implanted saphenous veins occlude within 10 years after implantation [1]. Saphenous vein graft disease is composed of three different processes: thrombosis, intimal hyperplasia, and atherosclerosis [2]. Mechanical factors appear closely related to graft failure due to smooth muscle cell (SMC) proliferation, which plays an important role in the pathogenesis of intimal hyperplasia [2]. Indeed, the saphenous vein is exposed to both high pressure and pulsatile

* Corresponding author. Cardiology, Cardiovascular Center, University Hospital, CH-8091 Zürich, Switzerland. Tel.: +41 1 255 21 21; fax: +41 1 255 42 51.
E-mail address: cardiotfl@gmx.ch (T.F. Lüscher).

stretch after implantation in the coronary circulation, while other factors remain unchanged.

Elevated pulsatile stretch is an atherogenic stimulus [3]. Interestingly, pulsatile stretch induces proliferation of SMCs obtained from the saphenous vein but not in internal mammary artery [4]. Stretch-induced SMC proliferation involves PDGF expression [5], protein kinase C activation [6], and superoxide production [7]. In SMCs, stretch also induces expression and translocation of early growth response gene-1 and c-*jun* [8] and fibronectin gene expression [9]. However, the precise molecular mechanisms of the cellular responses to pulsatile stretch remain to be elucidated.

Rho A is a member of a family of small G proteins, which plays a central role in the organization of the actin cytoskeleton. Activation of Rho A and its downstream effector, Rho kinase may also be involved in stretch-induced SMC proliferation [10]. The Rho/Rho-kinase pathway also contributes to growth factor-induced SMC proliferation [11,12] and neointimal formation after balloon injury [13].

Phosphatidylinositol (PI) 3-kinase is activated by growth factors and is involved in cell cycle progression [14]. PI 3-kinase mediates SMC proliferation after arterial injury [15] and is required for insulin-like growth factor 1-induced vascular SMC proliferation [16]. The serine/threonine kinase Akt is the downstream signaling enzyme of PI 3-kinase; in vascular SMCs it is involved in cell survival and replication [17,18]. Thus, the PI 3-kinase/Akt pathway may play an important role in vascular remodeling.

Statins are widely used for the treatment of hypercholesterolemia. They reduce the incidence of ischemic stroke and myocardial infarction [19,20] as well as that of bypass graft failure [21,22]. Some benefits of statins might be related to direct vascular effects [23].

This study was designed to elucidate the intracellular signaling mechanisms of stretch-induced saphenous vein SMC proliferation as well as the beneficial effects of statins. Specifically, we addressed (1) what signaling pathways contribute to pulsatile stretch-induced SMC proliferation and (2) whether statins prevent stretch-induced SMC proliferation and if so, what signaling pathway is involved in this context.

2. Methods

2.1. Materials

Mevalonate was obtained from Sigma (Buchs, Switzerland), ^{3}H-methyl-thymidine was from Amersham (Zurich, Switzerland), wortmannin and LY294002 were from Calbiochem (Lucerne, Switzerland), U0126 was from Cell Signaling (Schwalbach/Taunus, Germany). Dominant negative Rho A mutant (T19N) and Akt1 mutant (K179M) were obtained from Upstate Biotechnology (Lake Placid, NY) and Superfect reagent was from Qiagen (Basel, Switzerland). Cerivastatin was supplied by Bayer AG (Leverkusen, Germany), Simvastatin was by MSD (Glattburg, Switzerland), Y-27632 ws by Welfide Corp. (Osaka, Japan) and Hydroxyfasudil was by Asahi Chemical Industry Co. Ltd. (Shizuoka, Japan).

Rabbit polyclonal anti-human Rho A and p27 (C19) antibodies were obtained from Santa Cruz Biotechnology (Basel, Switzerland), rabbit polyclonal anti-human phospho-pRb (Ser807/811), phospho-p44/42 MAP kinase (Thr202/Tyr204), phospho-Akt (Ser473), p44/42 MAP kinase and Akt antibodies were from Cell Signaling, mouse monoclonal anti-human p21^{WAF1} (Ab-1) antibody was from Calbiochem.

2.2. Cell culture

SMCs were isolated by a modified explant method from human saphenous veins obtained from patients undergoing coronary bypass surgery and cultured in DMEM containing 10% FCS in a humidified atmosphere (95% air/5% CO_2). Culture medium was replaced every 3 days. Experiments were done between passages 5 and 8. The patients gave informed consent in accordance with the Declaration of Helsinki.

2.3. Stretch device for cultured cells

Cells were cultured onto Flex I plates coated with type-I collagen (density, 4×10^4/ml) with medium containing 0.2% FCS. Flex I plates were placed on a computerized Flexercell strain unit gasketed baseplate and were subjected to cyclic stretch (60 cycles/min; 25% elongation at the periphery of the culture plate bottom). Flex I culture plates not subjected to pulsatile stretch served as controls.

2.4. Proliferation assays

To study cell growth, DNA synthesis by ^{3}H-thymidine (0.5 μCi/ml; 70 to 85 Ci/mol) incorporation and cell number were measured. After each stimulation, cells were pulsed with ^{3}H-thymidine for 4 h and then washed with PBS before 10% trichloroacetic acid was added at 4 °C for 30 min. Incorporated radioactivity was measured. Cell number was determined by an electronic counter (Coulter, Instrumenten-Gesellschaft AG, Basel, Switzerland).

2.5. MAP kinase and Akt phosphorylation

We analyzed p44/42 MAP kinase and Akt phosphorylation by Western blotting using the phospho-specific antibodies. Stimulated cells were harvested with cold extraction buffer (120 mmol/l sodium chloride, 20 mmol/l sodium fluoride, 1 mmol/l benzamidine, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 6 mmol/l EGTA, 15 mmol/l sodium pyrophosphate, 0.8 μg/ml leupeptin, 30 mmol/l *p*-nitrophenyl phosphate, 0.1 mmol/l phenylmethylsulfonyl

fluoride and 1% NP-40, 50 mmol/l Tris, pH 8.0). Samples were treated with 5× Laemmli SDS-PAGE sample buffer and Western blotting with 10% SDS-PAGE gels was performed using phospho-p44/42 MAP kinase or phospho-Akt antibody.

2.6. Subcellular fractionation and Rho A membrane accumulation

Subcellular fractionation was performed as described [24]. Briefly, cells were harvested with hypotonic lysis buffer (3 mmol/l $MgCl_2$, 1 mmol/l benzamidine, 1 mmol/l dithiothreitol, 0.8 μg/ml leupeptin, 0.1 mmol/l phenylmethylsulfonyl fluoride and 20 mmol/l Tris, pH 8.0). After three cycles of freeze and thaw, the samples were ultracentrifuged at 100,000×*g* at 4 °C for 1 h. The supernatant was saved as a cytosol fraction. The pellets were resuspended in lysis buffer supplemented with 1% Triton X-100 and 0.1% SDS and saved as a membrane fraction. Rho A accumulation in each fraction was determined by Western blotting with 15% SDS-PAGE gels using Rho A antibody.

2.6.1. Cell cycle regulatory proteins

To study the role of cell cycle regulatory proteins, cells were harvested after 24 h stretching. Western blotting with 15% SDS-PAGE gels was performed using specific antibodies against phospho-pRb, p21 and p27.

2.7. Transient transfer of dominant negative constructs

Transient transfer of dominant negative Rho A mutant (T19N) and Akt1 mutant (K179M) were performed with Superfect reagent [25]. Briefly, 1 μg of dominant negative construct was mixed with 5 μl of Superfect for 10 min in room temperature for the complex formation. After complex formation, the complex was given to SMCs for 3 h, and then removed. After transfer, SMCs were cultured for 24 h to obtain the gene expression and served for the experiments. Transfer rate was approximately 50%.

2.8. Statistical analysis

Data are expressed as the means±S.E.M. In all experiments, *n* equals the number of patients from which vessels were obtained. Multiple comparisons were made by ANOVA followed by Fisher's test. A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of statins on stretch-induced SMC proliferation

In human saphenous vein SMCs, pulsatile stretch significantly enhanced ^{3}H-thymidine incorporation (Fig. 1A). Pretreatment with cerivastatin for 1 h significantly

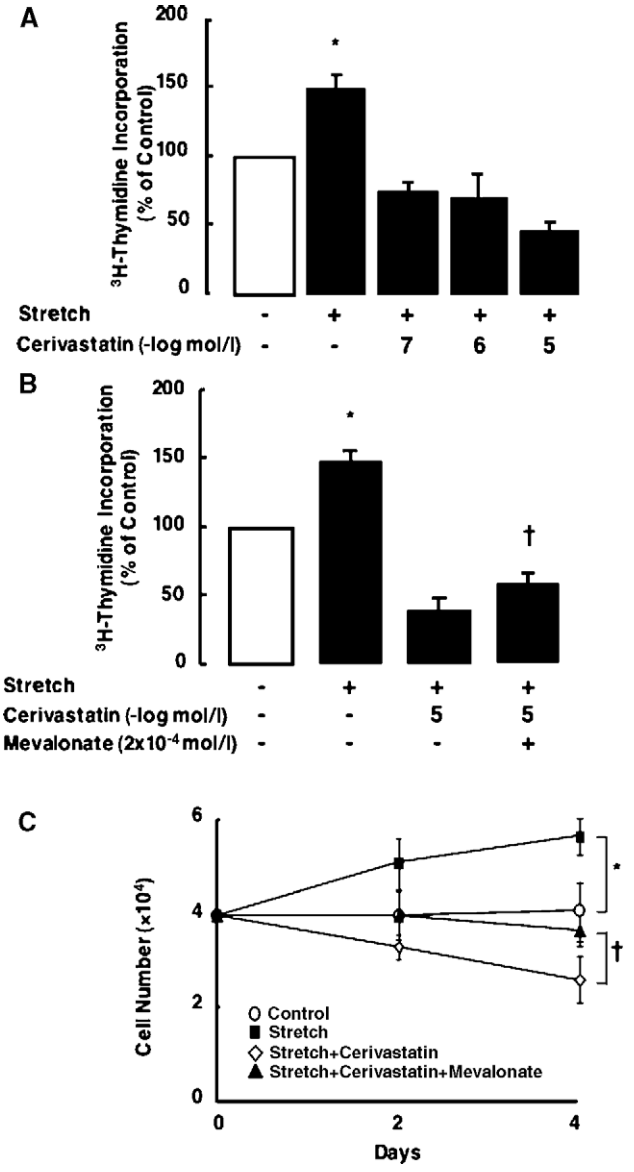

Fig. 1. Effects of cerivastatin on pulsatile stretch (24 h) savenous vein SMC proliferation. (A) Pretreatment with cerivastatin prevented stretch-induced increase in ^{3}H-thymidine incorporation ($n=3$). *=$p<0.01$ vs. control and stretch plus cerivastatin. (B) Inhibitory effects of cerivastatin were only partially restored by mevalonate ($n=4$). *=$p<0.01$ vs. control and stretch plus cerivastatin. †=$p<0.05$ vs. stretch plus cerivastatin. (C) Increase in cell number to stretch was prevented by cerivastatin and cotreatment with mevalonate only partially restored the effect of statin ($n=3$). *=$p<0.01$ vs. control and stretch plus cerivastatin. †=$p<0.05$ vs. stretch plus cerivastatin. Results are expressed as mean±S.E.M.

inhibited the stretch-induced enhancement of ^{3}H-thymidine incorporation in a concentration-dependent manner (10^{-7}–10^{-5} mol/l, 24 h; Fig. 1A). The inhibitory effect of cerivastatin (10^{-5} mol/l) was only partially restored by mevalonate (Fig. 1B). Simvastatin (10^{-5} mol/l) exerted similar inhibitory effect on stretch-induced SMC proliferation as did cerivastatin and again mevalonate only partially restored the proliferation which was inhibited by the statin (stretch; 178±23%, stretch plus simvastatin; 89±15%,

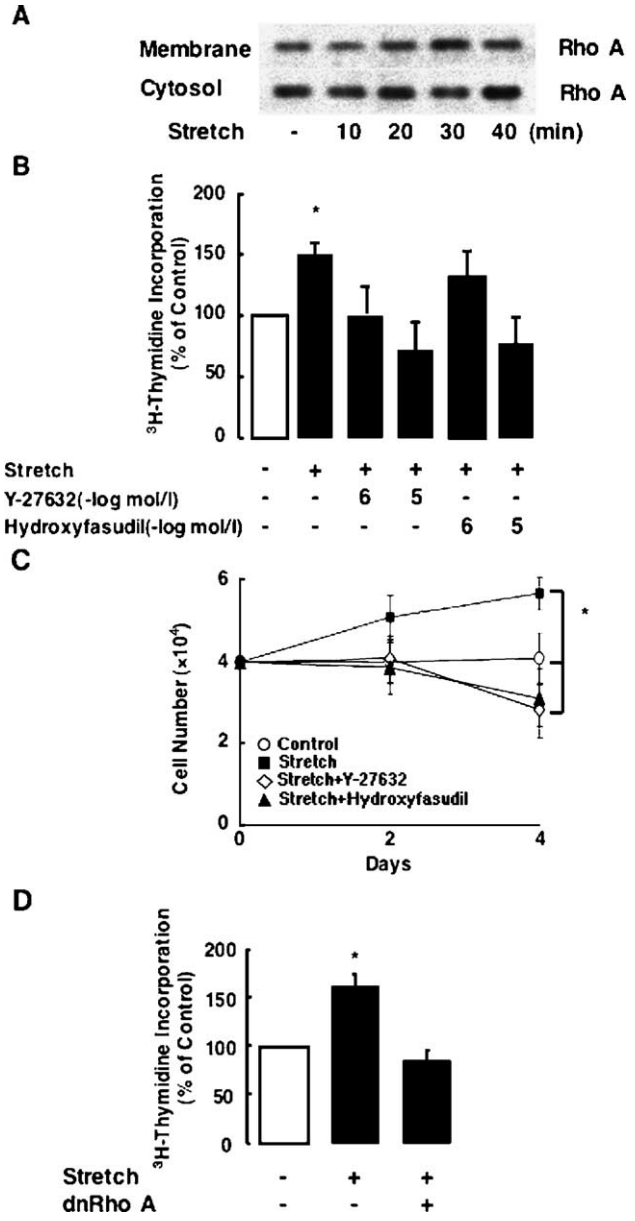

Fig. 2. Role of Rho/Rho-kinase pathway in stretch-induced SMC proliferation. (A) Stretch induced membrane accumulation of Rho A with maximal translocation at 30 min. The results of Western blot were repeated in three independent experiments. (B) Pretreatment with Rho kinase inhibitors prevented stretch-induced increase in ^{3}H-thymidine incorporation ($n=3$). *=$p<0.01$ vs. control and stretch plus Y-27632 or hydroxyfasudil. (C) Increase in cell number to stretch was inhibited by Rho kinase inhibitors ($n=3$). *=$p<0.01$ vs. control and stretch plus Y-27632 or hydroxyfasudil. (D) Transient transfer of dominant negative Rho A mutant (dnRho A) prevented stretch-induced increase in ^{3}H-thymidine incorporation ($n=3$). *=$p<0.01$. Results are expressed as mean±S.E.M.

stretch plus simvastatin and mevalonate; 97±12% above control, $n=3$). Similarly, the increase in cell number in response to stretch was significantly inhibited by cerivastatin and this inhibitory effect of cerivastatin was only partially restored by mevalonate (Fig. 1C). Neither cerivastatin nor mevalonate had any effects on basal level of ^{3}H-thymidine incorporation or cell number (data not shown).

3.2. Rho/Rho-kinase pathway and SMC proliferation

Pulsatile stretch induced membrane accumulation of Rho A as early as 20 min after stretching with maximal response at 30 min (Fig. 2A). We tested two different Rho kinase inhibitors, Y-27632 [26] and hydroxyfasudil [27]. In SMCs pretreated with either one of the Rho kinase inhibitors for 1 h, stretch-induced increase in ^{3}H-thymidine incorporation was significantly inhibited in a concentration-dependent manner (10^{-6}–10^{-5} mol/l; Fig. 2B). Similarly, both Rho kinase inhibitors (10^{-5} mol/l) also inhibited the increase in cell number in response to stretch (Fig. 2C). Transient transfer of dominant negative Rho A mutant also significantly prevented stretch-induced increase in ^{3}H-thymidine incorporation (Fig. 2D). Rho kinase inhibitors did not affect the basal level of ^{3}H-thymidine incorporation (data not shown).

3.3. MAP kinase pathway and SMC proliferation

Stretch induced the increase in p44/42 MAP kinase phosphorylation level as early as 10 min after stretching with maximal response at 30 min (Fig. 3A). The MEK1/2 inhibitor U0126 significantly prevented the increase in ^{3}H-thymidine incorporation induced by pulsatile stretch (Fig. 3B).

3.4. PI 3-kinase/Akt pathway and SMC proliferation

Pulsatile stretch induced the increase in Akt phosphorylation level as early as 10 min after stretching with

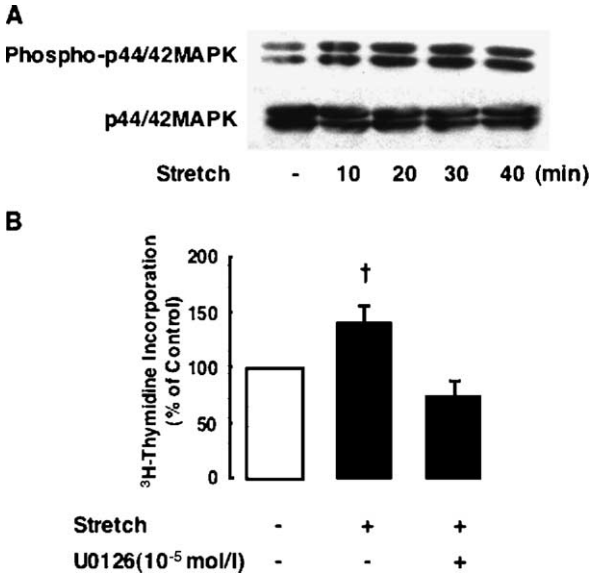

Fig. 3. Role of p44/42 MAP kinase pathway in stretch-induced SMC proliferation. (A) Stretch induced p44/42 MAP kinase phosphorylation with maximal phosphorylation at 30 min. The results of Western blot were repeated in three independent experiments. (B) MEK1/2 inhibitor U0126 prevented stretch-induced increase in ^{3}H-thymidine incorporation induced by ($n=3$). †=$p<0.01$ vs. control and stretch plus U0126. Results are expressed as mean±S.E.M.

maximal response at 30 min (Fig. 4A). The PI 3-kinase inhibitors, wortmannin and LY294002 significantly prevented the increase in ^{3}H-thymidine incorporation induced by stretch (Fig. 4B). Additionally, transient transfer of dominant negative Akt mutant also significantly prevented the increase in ^{3}H-thymidine incorporation induced by stretch (Fig. 4C).

3.5. Effects of statins on signaling pathways

To clarify the responsible signaling pathway for the growth inhibitory effect of statins, we examined the effects of cerivastatin and mevalonate on the signaling pathways. Cerivastatin significantly inhibited the membrane accumulation of Rho A induced by stretch (Fig. 5A). In contrast to ^{3}H-thymidine incorporation and cell number experiments (Fig. 1B, C), the inhibitory effect of cerivastatin on the membrane accumulation of Rho A was fully restored by mevalonate (Fig. 5A).

Pretreatment with cerivastatin did not affect p44/42 MAP kinase phosphorylation, while U0126 inhibited it (Fig. 5B).

Pretreatment with cerivastatin had no effect on Akt phosphorylation at the time point of 30 min in the cells receiving pulsatile stretch while wortmaninn inhibited it (Fig. 5B). However, cotreatment with mevalonate reduced the level of Akt phosphorylation (Fig. 5B). Interestingly, cerivastatin alone increased Akt phosphorylation level at 10 min after treatment under the condition without stretch (Fig. 5C). In addition, mevalonate reduced the level of Akt phosphorylation increased by cerivastatin (Fig. 5D). To explore the interaction between the Rho/Rhokinase pathway and the Akt pathway, we tested the effects of the Rho kinase inhibitors on Akt phosphorylation. Both Rho kinase inhibitors also increased Akt phosphorylation at 10 min after treatment under the condition without stretch (Fig. 5D).

3.5.1. Cell cycle regulatory proteins and stretch

Pulsatile stretch for 24 h induced pRb hyperphosphorylation and p27 downregulation whereas the expression of p21 was not affected (Fig. 6). Cerivastatin and the Rho kinase inhibitors, Y-27632 and hydroxyfasudil prevented the stretch-induced pRb hyperphosphorylation but had no effects on p27 expression (Fig. 6). Co-treatment with mevalonate only partially reversed the inhibitory effects of cerivastatin on pRb hyperphosphorylation (Fig. 6).

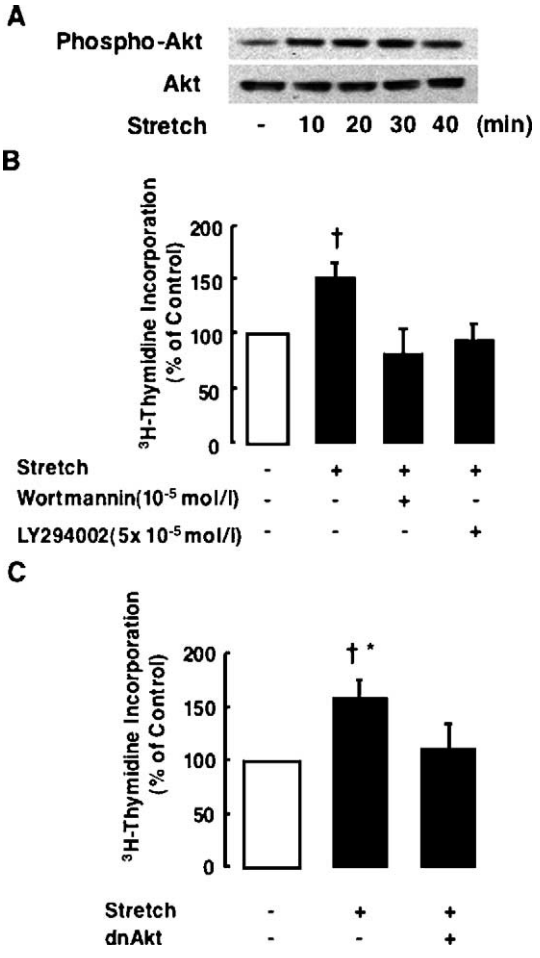

Fig. 4. Role of PI 3-kinase/Akt pathway in stretch-induced SMC proliferation. (A) Stretch induced Akt phosphorylation with maximal phosphorylation at 30 min. The results of Western blot were repeated in three independent experiments. (B) PI 3-kinase inhibitors, wortmannin and LY294002 prevented stretch-induced increase in ^{3}H-thymidine incorporation. †=$p<0.01$ vs. control and stretch plus wortmannin or stretch plus LY294002 ($n=3$). (C) Transient transfer of dominant negative Akt1 mutant (dnAkt) also prevented stretch-induced increase in ^{3}H-thymidine incorporation ($n=3$). †=$p<0.01$ vs. control and *=$p=0.03$ vs. stretch plus dnAkt. Results are expressed as mean±S.E.M.

4. Discussion

Here we show that statins prevented proliferation of human saphenous vein SMCs induced by pulsatile stretch. Moreover, we show that stretch-induced proliferation involved not only activation of Rho/Rho-kinase pathway but also that of p44/42 MAP kinase and PI 3-kinase/Akt pathways. Indeed, all three pathways were activated by stretch and inhibition of any of these pathways completely prevented the pulsatile stretch-induced SMC proliferation. Thus, all three pathways are essential for saphenous vein SMC proliferation. We also show that cerivastatin inhibited the membrane accumulation of Rho A induced by stretch. Statins ihhibit HMG-CoA reductase, which is a rate-limiting enzyme of mevalonate–cholesterol pathway [28]. Activation of this pathway leads to the production of intermediates such as all-trans geranylgeranyl pyrophosphate. This intermediate is essential for Rho activation by posttranslational modification [29]. Inhibition of this mechanism must explain the preventive effect of cerivastatin on Rho A activation. As the inhibition of Rho/Rho-kinase pathway is sufficient for the inhibition of stretch-induced proliferation, the growth inhibitory effect of statins is mediated by the inhibition of this pathway although cerivastatin induced the

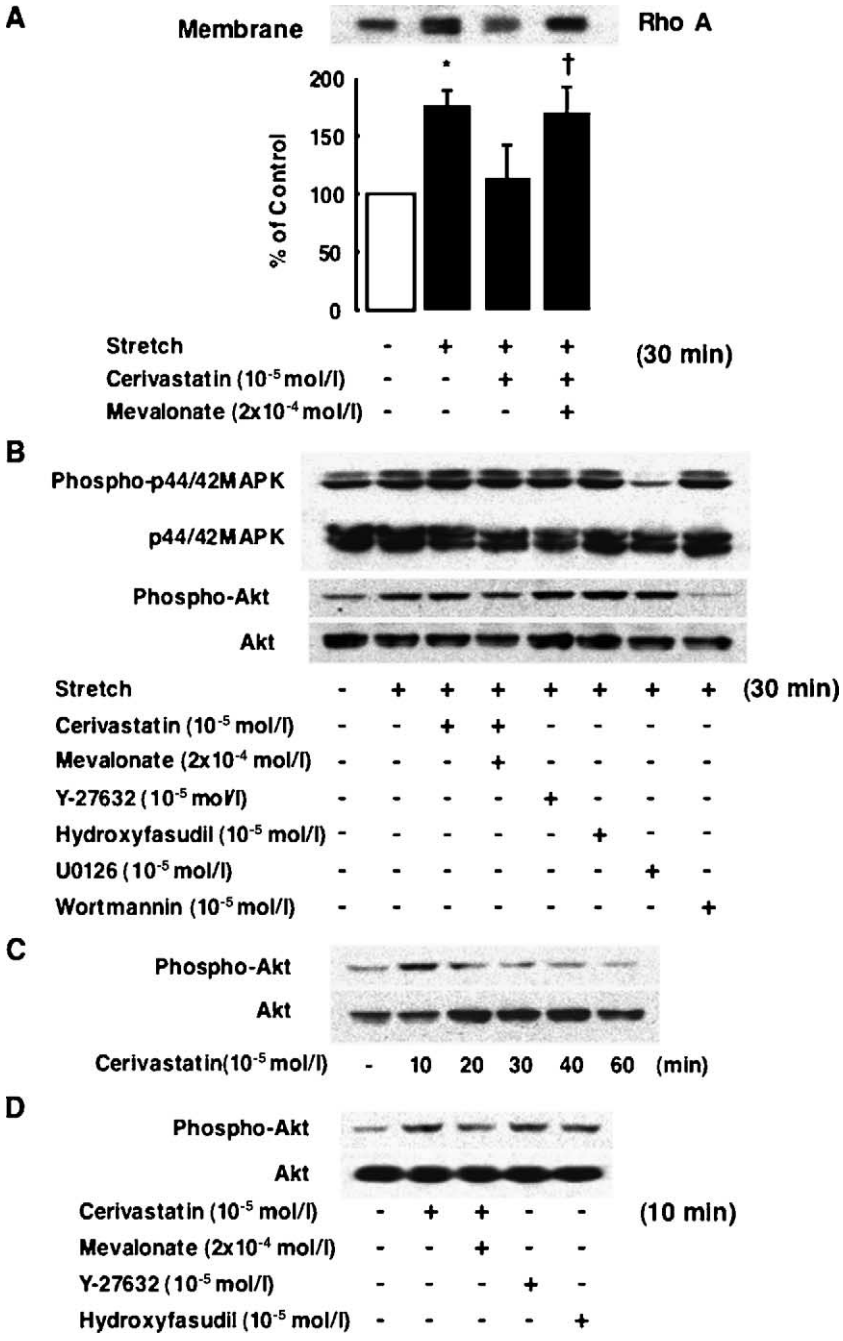

Fig. 5. Effects of cerivastatin on the signaling pathways. (A) Cerivastatin prevented membrane accumulation of Rho A induced by stretch (30 min) and cotreatment with mevalonate restored the effect of cerivastatin ($n=3$). * and †=$p<0.05$ vs. control and stretch plus cerivastatin. (B) Cerivastatin and Rho kinase inhibitors had no significant effects on p44/42 MAP kinase and Akt phosphorylation induced by strech (30 min). Cotreatment with mevalonate reduced phosphorylation of Akt. The results of Western blot were repeated in three independent experiments. (C) Cerivastatin alone induced phosphorylation of Akt under the condition without stretch. The results of Western blot were repeated in three independent experiments. (D) Cotreatment with mevalonate reduced Akt phosphorylation induced by cerivastatin (10 min). Both Rho kinase inhibitors also induced Akt phosphorylation. The results of Western blot were repeated in three independent experiments.

phosphorylation of Akt, which is a positive regulator of stretch-induced SMC proliferation.

Although cerivastatin was taken from the market because of its muscle toxicity, both cerivastatin and simvastatin exhibited similar inhibition of SMC proliferation, suggesting a class effect must be involved. Furthermore, to confirm whether the effects of statins are due to inhibition of the mevalonate pathway, we examined the effect of mevalonate on stretch-induced SMC proliferation. Indeed, mevalonate restored the inhibitory effect of cerivastatin on Rho A membrane translocation. However, mevalonate did not fully restore the inhibitory effect of the statins on SMC proliferation, confirming our previous report using PDGF in human SMCs [23]. It is unlikely that the concentration of

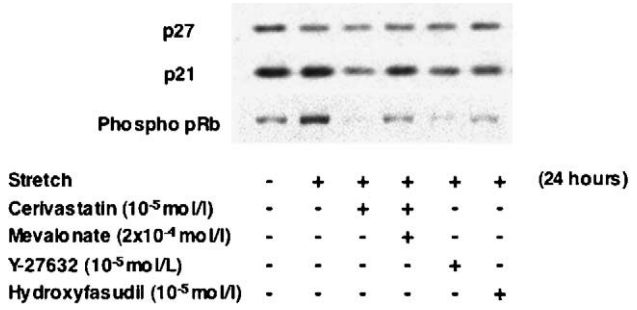

Fig. 6. Effects of stretch, cerivastatin, mevalonate and Rho kinase inhibitors on pRb phosphorylation and p21 and p27 expression. 24 h stretching induced hyperphosphorylation of pRb and downregulation of p27. Cerivastatin and Rho kinase inhibitors prevented pRb hyperphosphorylation but had no effects on p27. Cotreatment with mevalonate partially restored the inhibitory effect of cerivastatin on pRb hyperphosphorylation. Results were repeated in three independent experiments.

mevalonate was not high enough to restore the activity of the mevalonate pathway since mevalonate did restore Rho A membrane accumulation. Interestingly, however, mevalonate simultaneously inhibited Akt phosphorylation, which is essential for stretch-induced SMC proliferation. Thus, although mevalonate fully restores one pathway (Rho A), the simultaneous inhibition of the second pathway (Akt) allows only partial restoration of the biological response, i.e. proliferation induced by stretch. Since both Rho kinase inhibitors increased the levels of Akt phosphorylation 10 min after treatment, we conclude that the Rho/Rho-kinase pathway negatively regulates the Akt pathway in human saphenous vein SMCs. Indeed, consistent with our results in SMCs, Rho kinase also inversely regulates the Akt pathway in endothelial cells [30,31]. This negative interaction between Rho/Rho-kinase pathway and Akt may explain the mechanism of the inhibition of Akt by mevalonate and the activation of this pathway by cerivastatin observed in this study.

Moreover, we investigated the effects of stretch on cell cycle regulatory proteins. Pulsatile stretch induced pRb phosphorylation and downregulated p27. Cerivastatin and Rho kinase inhibitors reduced pRb phosphorylation but did not p27 downregulation. Therefore, it is likely that hyperphosphorylation of pRb by stretch is mainly caused by Rho/Rho-kinase activation.

Although arterial bypass grafting is preferable to venous grafts because of a higher patency rate, saphenous vein grafts are still important for emergency or multivessel grafting. Thus, prevention of vein graft failure is still of clinical relevance. This study demonstrated that statins prevented savenous vein SMC proliferation via Rho/Rho-kinase inhibition. Indeed, statins do reduce the incidence of bypass graft failure [21,22]. Statins not only inhibit SMC proliferation [12,23] but also augment NO release due to phosphorylation of eNOS via activation of the Akt pathway [32] and upregulation of eNOS expression via inhibition of the Rho A pathway [23,33,34]. Therefore, beneficial effects of statins on bypass graft failure must be related at the molecular levels to both Rho/Rho-kinase inhibition and Akt activation. Our study demonstrated that Rho kinase inhibitors also exhibited Akt phosphorylation. Thus, Rho kinase inhibitors also should have beneficial effects on bypass graft failure via inhibition of savenous vein SMC proliferation and improvement of endothelial function.

In conclusion, Rho/Rho-kinase, p44/42 MAP kinase and PI 3-kinase/Akt pathways are all involved in the pulsatile stretch-induced human saphenous vein SMC proliferation and inhibition of either of them prevents stretch-induced SMC proliferation. Statins prevent stretch-induced saphenous vein SMC proliferation via inhibition of Rho/Rho-kinase pathway. This may explain the beneficial effects of this class of drug, especially for patients after coronary artery bypass grafting.

Acknowledgments

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