Simvastatin Suppresses the Differentiation of C2C12 Myoblast Cells via a Rac Pathway

Tomomi T. Baba,[1]* Takayuki K. Nemoto,[1] Toshihiro Miyazaki,[2] and Shinichiro Oida[3]

[1]Department of Oral Molecular Biology, Unit of Basic Medical Sciences, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan
[2]Department of Cell Biology, Unit of Basic Medical Sciences, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan
[3]Department of Oral Biochemistry, School of Dentistry, Tsurumi University, Yokohama 230-8501, Japan

*Correspondence to: Tomomi T. Baba, DDS, PhD, Department of Oral Molecular Biology, Unit of Basic Medical Sciences, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan.
TEL: +81-95-819-7641, FAX: +81-95-819-7642, E-mail: baba@nagasaki-u.ac.jp

Abstract

Statins, which are known as cholesterol-lowering drugs, have several additional effects including the enhancement of bone formation and the stimulation of smooth muscle cell proliferation. In this study, we investigated the signal pathway of simvastatin operating in C2C12 myoblast cells. Myotube formation of C2C12 cells was efficiently blocked by 1 μM simvastatin, and mevalonic acid was able to cancel this effect. Geranylgeranyl pyrophosphate restored the myotube formation, whereas farnesyl pyrophosphate did not. These findings demonstrate that the Rho family, such as Rho, Rac and Cdc42, occurring downstream of geranylgeranyl pyrophosphate in the mevalonic acid pathway, was involved in the simvastatin-mediated blockage of myotube formation. An inhibitor of Rho kinase did not influence the myotube formation; whereas an inhibitor of Rac blocked this process. Taken together, we conclude that the differentiation of C2C12 cells into myotubes was blocked by simvastatin through the pathway mediated by Rac, not by Rho.

Introduction

Statins function as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, one of the enzymes involved in the synthesis of cholesterol. They are commonly prescribed for hypercholesterolemia, and greatly reduce cardiovascular-related morbidity and mortality (Hebert et al. 1997; Pedersen 1999; Maron et al. 2000; Vaughan et al. 2000). Moreover, statins have multipotent effects beyond their cholesterol-lowering properties, such as the decrease in platelet aggregation, thrombus deposition (Lacoste et al. 1995), promotion of angiogenesis (Kureishi et al. 2000), decrease in b-amyloid peptide related to Alzheimer's disease (Fassbender et al. 2001; Kojro et al. 2001; Sidera et al. 2005), suppression of T lymphocyte activation (Kwak et al. 2000), induction of apoptosis (Guijarro et al. 1998; Li et al. 2002), inhibition of valve calcification (Osman et al. 2006a, b), and enhancement of bone formation (Mundy et al. 1999; Maeda et al. 2004).

It had been suspected that statins have a positive effect on bone formation, because statin-administered senior patients tended to have a reduced incidence of osteoporosis. Later, this observation was confirmed by in vitro studies demonstrating that statins enhanced bone formation by osteogenic cells (Mundy et al. 1999). This effect was mainly attributed to the increased expression of bone morphogenetic protein 2 (BMP2) (Mundy et al. 1999; Sugiyama et al. 2000; Ruiz-Gaspa et al. 2007). Based on the results on osteogenic cells, it became clear that the inactivation of Rho is involved in statin-induced BMP2 expression. Because Rho suppresses the BMP2 expression, statins are likely to increase the expression of BMP2 as a result of the inactivation of Rho (Ohnaka et al. 2001).

BMP2 is one of the transforming growth factor-β superfamily members and strongly induces osteogenic differentiation in several types of cells. In osteogenic cells, BMP2 increases an alkaline phosphatase (ALP) activity, stimulates osteocalcin production, and finally enhances mineralization. Moreover, osseous tissue is induced by BMP implanted under rat skin or in the muscle (Wozney et al. 1988). Furthermore, BMP2 increases ALP activity in non-osteogenic cells, such as C2C12 and C3H10T1/2 cells (Katagiri et al. 1990, 1994), and causes them to change from myoblastic and fibroblastic cells, respectively, to osteogenic cells. Osteogenic differentiation in bone marrow stromal cells is also enhanced by BMP. BMP leads bone marrow stromal cells to differentiate into osteoblastic cells and blocks the adipocyte differentiation of ST2 cells induced by hydrocortisone and indomethacin (Song et al. 2003). Statins also possess this osteoblastic differentiationpromoting effect on bone marrow stromal cells. For instance, the adipocyte differentiation of D1, an established bone marrow stromal cell, has been reported to be suppressed by lovastatin (Li et al. 2003).

In osteoblastic cells, statins have been reported to block mevalonic acid production by inhibiting HMG-CoA reductase activity, leading to inactivation of Rho, which is located downstream of mevalonic acid, and a subsequent increase in BMP2 production (Mundy et al. 1999; Sugiyama et al. 2000; Ruiz-Gaspa et al. 2007). In addition to osteoblastic cells, the stimulation of BMP2 expression by statins has been reported on bone marrow stromal cells (Song et al. 2003) and ES cells (Phillips et al. 2001). However, there have so far been few reports describing the signals of statins in myoblast cells (Matzno et al. 2005; Madonna et al. 2005; Ogura et al. 2007). Rhabdomyolysis, which is muscular cell collapse accompanied by the release of myoglobin, is a serious side effect of statins (Sica and Gehr 2002; Jamal et al. 2004). In addition, an inhibitory effect of statins on proliferation of smooth muscle cell has been reported (Corsini et al. 1998). Hence, it is of importance to evaluate the effect of statins on muscle cells and elucidate the mechanism of their action.

The goal of the present study was to elucidate the signal pathway, by which statins inhibit myotube formation in C2C12 myoblast cells. We examined the effect of simvastatin on C2C12 cells, which develop myotubes by cell fusion during cultivation after confluence (Yaffe and Saxel 1977; Yamasaki et al. 1999). We demonstrate that simvastatin suppressed the myotube formation of C2C12 cells by acting through the Rac pathway.

Materials and methods

Reagents

Simvastatin was purchased from Calbiochem (San Diego, CA, USA). Mevalonic acid (MVA), geranylgeranyl pyrophosphoric acid (GGPP), and feranyl pyrophosphoric acid (FPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human BMP2 and Y27632 were obtained from Wako Pure Chemical Industry (Osaka, Japan). NSC23766 came from Tocris Bioscience (Bristol, UK).

Cell culture

C2C12 mouse myoblast cells (obtained from RIKEN cell bank, Tsukuba, Japan) were plated in 12-well plates at a concentration of 1 x 10^4 cells/cm^2 and cultured in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co.) supplied with 10% fetal bovine serum (FBS: Biological Industries, Kibbutz bet Haemek, Israel), 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technology, Inc., Grand Island, NY, USA). After reaching confluence (defined as day 0), the cells were cultured in Minimum Essential Medium alpha modification (Sigma Chemical Co.) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, 80 μg/ml L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industry), and 5 mM b-glycerophosphate (Kanto Chemical Co., Tokyo, Japan). The medium was changed every 2–3 days. On day 0, simvastatin (1 μM), MVA (1 mM), GGPP (8.9 μM), FPP (9.2 μM), NSC23766 (100 μM) or Y27632 (60 μM) or their combinations were added. The vehicle (dimethyl sulfoxide) was added as a control.

Evaluation of myotube formation

C2C12 cells cultured in 12-well plates in the absence or presence of chemical reagents for up to 11 days were washed three times with Dulbecco's phosphate-buffered saline (Sigma Chemical Co.) at 4°C, fixed with 4% formaldehyde containing 85 mM $CaCl_2$ for 15 min, and then stained with 0.05% toluidine blue for 30 min. The myotubes in nine fields of vision (1.05 x 0.71 mm) were counted and expressed as the number ± SEM per 1 mm^2.

Quantitative RT-PCR analysis

CellularRNAwas isolated from C2C12 cells by using Isogen reagent (Nippon Gene Co., Tokyo, Japan), and cDNA was prepared from 2.5 μg of RNA with reverse transcriptase (Takara, Shiga, Japan) in a reaction mixture (20 μl) containing 0.5 mM deoxynucleotide triphosphates and 0.2 μg of oligo dT (Life Technology Inc.). The expression level of myogenin mRNA was quantified by real-time RT-PCR (Mx3005P Real-time PCR system, Stratagene, La Jolla, CA, USA). The reaction mixture (25 μl) contained 0.5 μl of cDNA and 0.4 μM primers, and the reaction was performedwith the Full Velocity SYBR Green QPCR system. The primer set used for the amplification of mouse myogenin was 5'-CATCCAGTACATTGAGCGCCTA-3' (sense) and 5'-GAGCAAATGATCTCCTGGGTTG-3' (antisense) (Wedhas et al. 2005). The reaction was performed as follows: incubation at 94°C for 10 min to activate the polymerase followed by 40 cycles of amplification. Each cycle consisted of denaturation at 94°C for 20 s and an annealing/extension at 60°C for 1 min. Reactions were carried out in triplicate. The relative expression of myogenin mRNA was normalized by the level of mouse GAPDH mRNA.

Alkaline phosphatase activity

Alkaline phosphatase activity was determined according to the protocol described previously (Baba 2000). In brief, the cells were sonicated in solubilization buffer (10 mM Tris–HCl, pH 7.4, containing 0.1% Triton X-100) and centrifuged at 12,000g for 20 min. The supernatant was used for the measurement of the ALP activity. The reaction mixture (0.15 ml) comprising 4 mM p-nitrophenyl phosphate and 5 mM $MgCl_2$ in 50 mM carbonate buffer (pH 10) was incubated at room temperature for 6 min, mixed with 50

μl of 2 M NaOH, and then, absorbance at 405 nm was determined. One unit was defined as the quantity of ALP that produced 1 mg of p-nitrophenol in 1 h.

Protein concentration

Protein concentrations were determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instruction. Bovine serum albumin was used as a reference.

Statistical analysis

All values were presented as means ± SEM. The difference between 2 groups was assessed by Student's t-test.

Results

Effects of BMP2 and simvastatin on differentiation of C2C12 cells

It has been reported that statins enhance the expression of BMP2 in osteoblast cells. Because BMP2 strongly induces the differentiation of several types of cells including C2C12 myoblastic cells into osteoblastic cells, it seemed reasonable to speculate that statins would also induce the differentiation of C2C12 cells into osteoblastic cells. When C2C12 cells were successively cultured after confluence, myotube formation was recognized on day 2, and the number of myotubes formed consistently increased until day 11 (Fig. 1A). In contrast, myotubes were not observed in the cultures treated with 3 nM BMP2 or 1 μM simvastatin even on day 11 (Fig. 1A).

The ALP activity was constant for 11 days in the control. In contrast, the activity significantly increased in the BMP2 group until day 8, on which it was 14-fold higher than that of the control group. The ALP activity of the simvastatin group increased 2.7-fold on day 8 in comparison with the control level, i.e., only one-fifth of that of the BMP group (Fig. 1B).

The expression level of the mRNA of myogenin, a marker protein of myoblasts, was quantified by real-time RT-PCR (Fig. 1C). On day 3, 38.5% suppression of the myogenin expression was achieved by the addition of BMP2. In contrast, simvastatin did not affect the mRNA level. Taken together, these data indicated that BMP2 blocked the myotube formation, increased the ALP activity, and reduced the myogenin mRNA expression, thus demonstrating that BMP2 modified the lineage of C2C12 cells from the myogenic to the osteoblastic cells as reported previously (Katagiri et al. 1997). However, simvastatin seemed to block the myotube formation in a manner distinct from that of BMP2, because the expression of MyoD mRNA (data not shown) and myogenin mRNA was not reduced by simvastatin and the ALP activity did not significantly increased.

Mechanism on differentiation inhibition of C2C12 cells by simvastatin

It has been reported that osteoblast differentiation induced by statins is tightly associated with the inhibition of Rho (Ohnaka et al. 2001). In contrast, the functional mechanism of statins on myoblastic cells remains to be elucidated. As shown in Fig. 1, the effect of simvastatin on C2C12 cells appeared to be distinct from that of BMP2. This raised a question on the pathway that BMP2 mediates the inhibition of Rho in C2C12 cells. To address this issue, we first examined whether simvastatin inhibited myotube formation via the mevalonic acid pathway. That is, 1 mM mevalonic acid was added to the C2C12 culture in the presence or absence of 1 μM simvastatin (Fig. 2). Myotube formation efficiently inhibited by simvastatin (2.2% of the control) was reversed by the addition of mevalonic acid, resulting in the increase up to 89.1% of the control.

To further elucidate its downstream events, the effects of GGPP and FPP, i.e., alternative metabolites of mevalonic acid in the pathway, were examined. Myotube formation was restored up to 54.1% of the control by the addition of 4.4 μM GGPP (data not shown) and 85.1% by 8.9 μM GGPP, although GGPP itself did not further enhance the myotube formation (Fig. 3A). In contrast, 9.2 μM FPP could not restore the formation (Fig. 3B). This finding indicated that the effect of simvastatin was mediated by GGPP.

Members of the Rho family are prenylated by GGPP. In order to compare with simvastatin signaling in osteoblastic cells where Rho-kinase activity was suppressed by statins, we examined the effects of Rho kinase inhibitor, Y27632. Sixty μM Y27632, which was sufficient for kinase inhibition (Li et al. 2002; Tuner et al. 2007), had no inhibitory effect on myotube formation (Fig. 4A). This finding further suggested that the signal pathway in myoblastic cells was distinct from that in osteoblastic cells.

To evaluate the signal pathway downstream of GGPP, NSC23766, an inhibitor of the pathway mediated by Rac (another member of Rho family) was tested. As a result, myotube formation was 67% suppressed by the addition of 50 μM NSC23766 (data not shown), and 100 μM NSC23766 shut down the myotube formation to 3.1% of the control (Fig. 4B). These findings demonstrated that the simvastatin signaling downstream of GGPP was mediated by Rac, not by Rho.

Discussion

Statins are HMG-CoA reductase inhibitors, which strongly block the conversion of HMG-CoA to mevalonic acid. In osteoblastic cells, statins block mevalonic acid production and reduce the activity of Rho located downstream of the mevalonic acid of this pathway, thus inducing BMP2 production (Mundy et al. 1999; Sugiyama et al. 2000; Ruiz-Gaspa et al. 2007). In addition to osteoblastic cells, bone marrow stromal cells (Song et al. 2003) and ES cells (Phillips et al. 2001) have been reported to be stimulated by statins to express BMP2. However, there have so far been few reports describing the signals of statins in myoblast cells.

The present study confirmed that BMP2 diverted the lineage of C2C12 cells from myoblastic cells into osteoblastic cells. In contrast, simvastatin neither significantly increased the ALP activity nor suppressed the myogenin mRNA expression, although it did inhibit the myotube formation of the cells. These findings suggested that the signal pathway of simvastatin in C2C12 cells was not associated with the BMP2 expression. We further demonstrated that simvastatin utilized the mevalonic acid pathway to block myotube formation. Moreover, GGPP, one of the intermediate metabolites of the pathway, selectively restored the myotube formation. The events further downstream of GGPP are known to be mediated by Rho family proteins, which are small G proteins, such as Rho, Rac and Cdc42 (Zhang and Casey 1996). Finally, NSC23766 specifically interfered with myotube formation, but Y27632 could not, which showed that the simvastatin signal was passed along by Rac, not Rho. This finding convinced that the functional mechanism of statins in C2C12 cells was not associated with BMP2, of which expression was suppressed by Rho (Fig. 5).

There might be a possibility that the signal of simvastatin in C2C12 is mediated by Cdc42, the third major member of Rho-family. Because the present study focused on the discrimination of the effects and the pathways between simvastatin and BMP2, we did not investigate the role of Cdc42 in the simvastatin pathway. The role of Cdc42 and the downstream of Rac should be evaluated on C2C12 in future studies.

Figure 5 summarizes the mevalonic acid pathway in consideration with the simvastatin signal in C2C12 cells. GGPP can be converted from FPP by trans-prenyl transferase in the mevalonic acid pathway (broken arrow), whereas this reaction seemed not to occur or proceeded very slowly in C2C12 cells, because the effects of GGPP and FPP were different. Similarly, it was reported that PDGF-stimulated migration of osteoblasts was blocked by simvastatin and that the blockage was reversed by GGPP, but not by FPP (Fukuyama et al. 2004). The suppression of smooth muscle-cell proliferation by simvastatin was blocked by GGPP, but not by FPP (Takeda et al. 2006).

Recently, Ogura et al. (2007) reported the simvastatin signaling in C2C12 cells. They cultured the cells under three conditions; proliferating, differentiating, and differentiated, and insisted that the simvastatin functioned in anHMG-CoA reductase inhibition-independent manner, because, under the differentiating condition, the effect of simvastatin was not restored by mevalonic acid and GGPP. However, under the differentiated condition, which resembled the culture condition employed in our study, the cell viability was dependent on HMG-CoA reductase inhibition. This indicated that simvastatin signal was mediated through mevalonic acid and GGPP, although they did not further study on the subsequent pathway. Accordingly, their report could be compatible with our result that simvastatin signal passed through GGPP in C2C12 cells.

In this report, we found that the simvastatin signaling in myoblasts is different from that in osteoblasts. This finding may shed light on myopathy, side-effect of statin treatment. The suppression of myotube formation is concerned with Rac, whereas stimulation of bone formation is concerned with Rho. If Rac activity can be specifically restored by a reagent, bone formation could be stimulated by statins avoiding any muscle problem.

In conclusion, the inhibition of C2C12 cell differentiation into myotube by simvastatin was mediated through the mevalonic acid-GGPP-Rac pathway, distinct from the pathway mediated by mevalonic acid-GGPP-Rho-BMP2 operated in osteoblastic cells.

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Legends

Figure 1

Inhibition of myotube formation of C2C12 cells by BMP2 and simvastatin. (A) Phase-contrast microscopic images of C2C12 cells cultured for 11 days in the absence (control) or presence of 3 nM BMP2 or 1 □M simvastatin (SMV). (B) ALP activity in C2C12 cells cultured for 2, 5, 8 or 10 days in the absence (control) and presence of 3 nM BMP2 or 1 □M simvastatin (SMV). (C) Myogenin expression in C2C12 on day 3 in the absence (control) and presence of 3 nM BMP2 or 1 □M simvastatin (SMV) was measured by real-time RT-PCR as described in "Materials and methods."

Figure 2.

Mevalonic acid canceled the inhibitory effect of simvastatin. (A) Phase-contrast microscopic images of C2C12 cells cultured for 4 days in the absence (control) and presence of 1 □M simvastatin (SMV) or 1 □M simvastatin plus 1 mM mevalonic acid (SMV+MVA). Arrows show myotubes. (B) Number of myotubes formed by C2C12 cells cultured for 4 days in the absence of additives (control), presence of 1 □M simvastatin (SMV), 1 mM mevalonic acid (MVA) or their combination (SMV+MVA). Bar= 0.2 mm.

Figure 3.

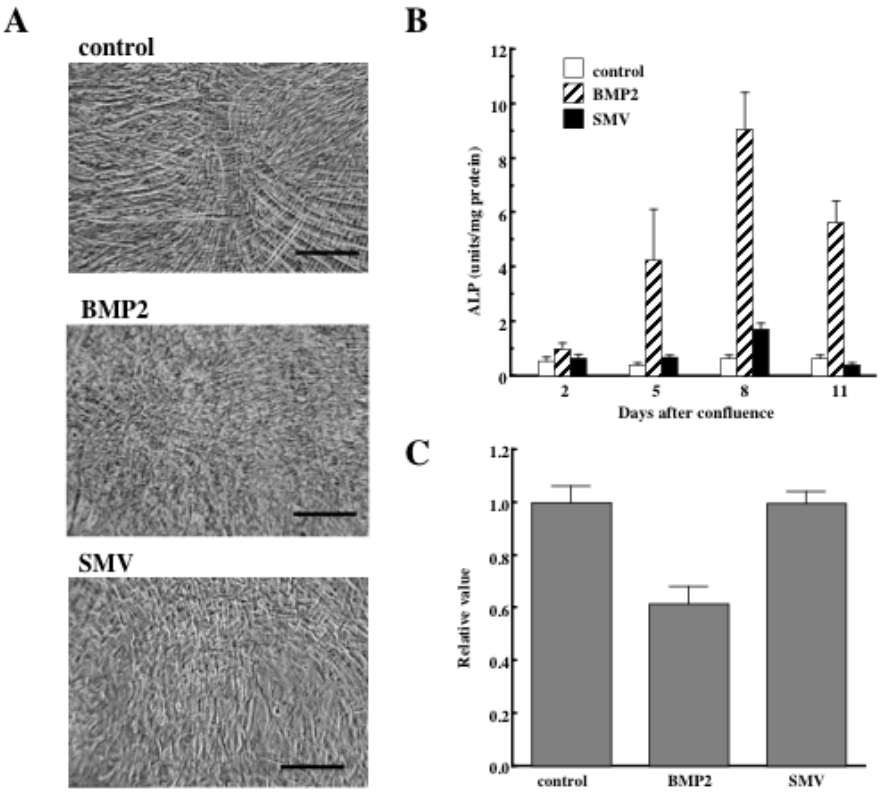
Differential effect of GGPP and FPP on simvastatin-induced myotube formation by C2C12 cells. (A) C2C12 cells were cultured in the absence of additives (control), presence of 1 □M simvastatin (SMV), 8.9 μM GGPP or their combination (SMV+GGPP). Histological images on day 4 are shown (left), and the number of myotubes was evaluated (right). (B) C2C12 cells were cultured in the absence of additives (control), presence of 1 □M simvastatin (SMV), 9.2 μM FPP or their combination (SMV+FPP). Histological images on day 4 are shown (left), and the number of myotubes was evaluated (right). Bar= 0.2 mm.

Figure 4

Blockage of myotube formation by NSC23766, but not by Y27632. (A) C2C12 cells were cultured in the absence (control) or presence of 60 μM Y27632. Histological images on day 4 are shown (left), and the number of myotubes was evaluated (right). (B) C2C12 cells were cultured in the absence (control) or presence of 100 μM NSC23766. Histological images on day 3 are shown (left), and the number of myotubes was evaluated (right). Bar= 0.2 mm.

Figure 5.

Metabolism of mevalonic acid pathway in C2C12 cells. The metabolic pathway of mevalonic acid is schematically illustrated, as shown previously [Horiuchi and Maeda 2006]. The major metabolic route in C2C12 cells revealed in the present study is represented by the bold arrows. A broken arrow shows a minor or negligible pathway (see text).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control
BMP2
SMV

Fig. 1

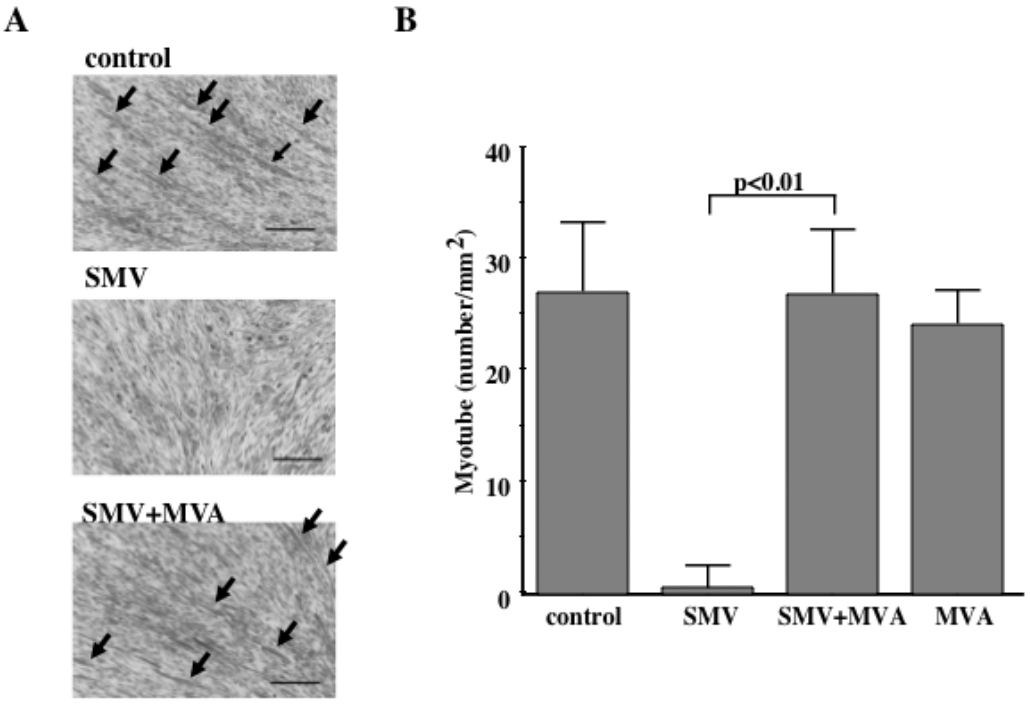
A
control
SMV
SMV+MVA
B
Myotube (number/mm^2)
40
30
20
10
0
p<0.01
control
SMV
SMV+MVA
MVA

Fig. 2

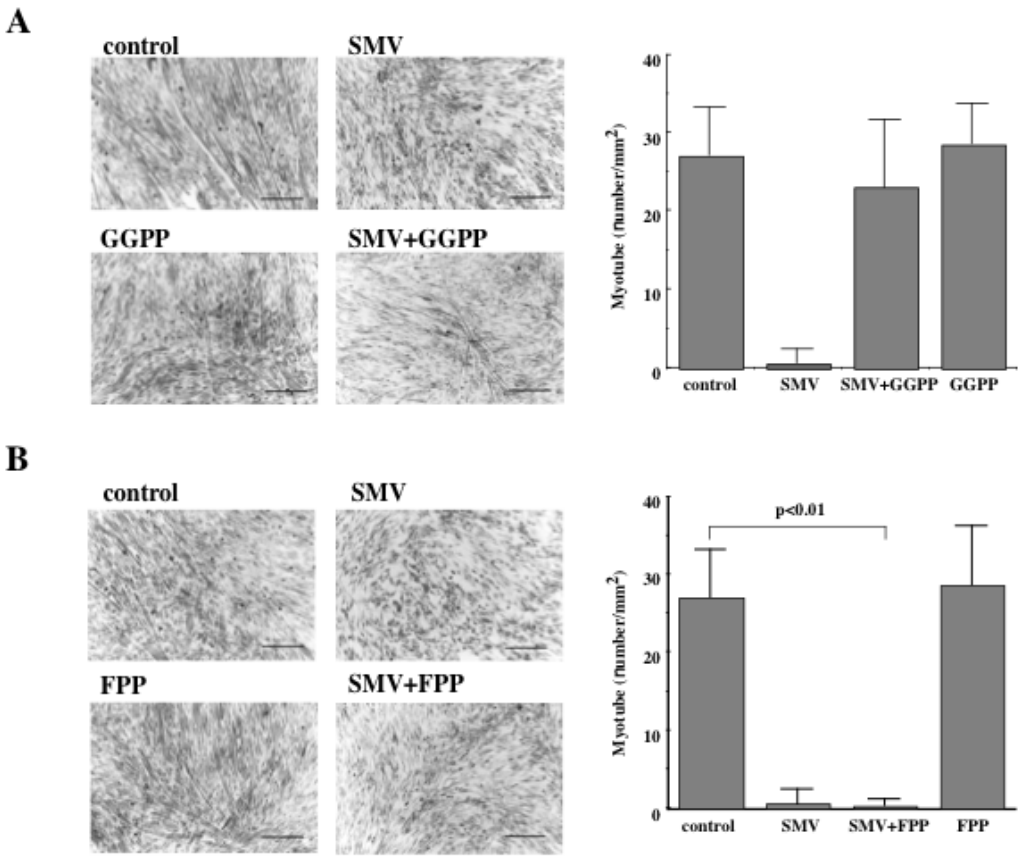
A
control
SMV
GGPP
SMV+GGPP
Myotube (number/mm²)
40
30
20
10
0
control
SMV
SMV+GGPP
GGPP
B
control
SMV
FPP
SMV+FPP
p<0.01
Myotube (number/mm²)
40
30
20
10
0
control
SMV
SMV+FPP
FPP

Fig. 3

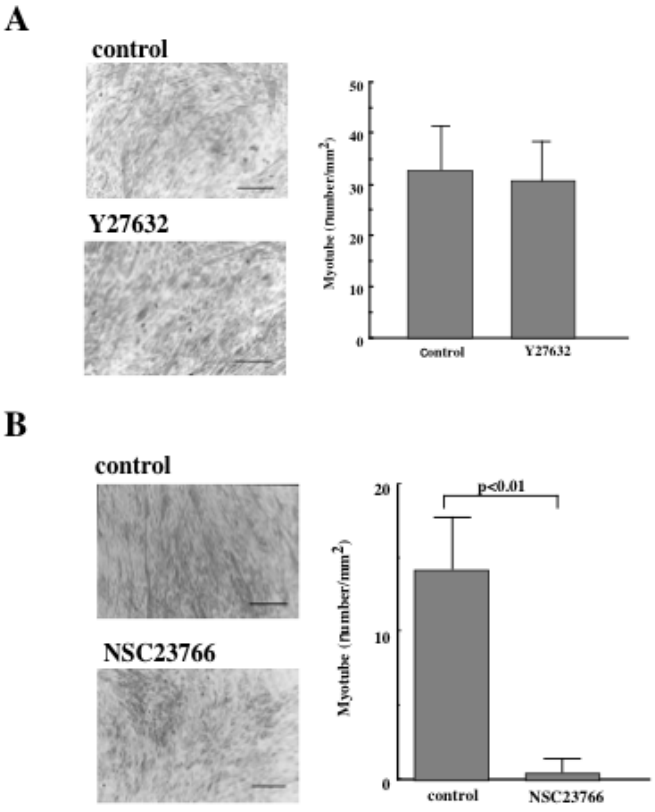
A
control
Y27632
Myotube (number/mm²)
50
40
30
20
10
0
Control
Y27632
B
control
NSC23766
p<0.01
Myotube (number/mm²)
20
10
0
control
NSC23766

Fig. 4

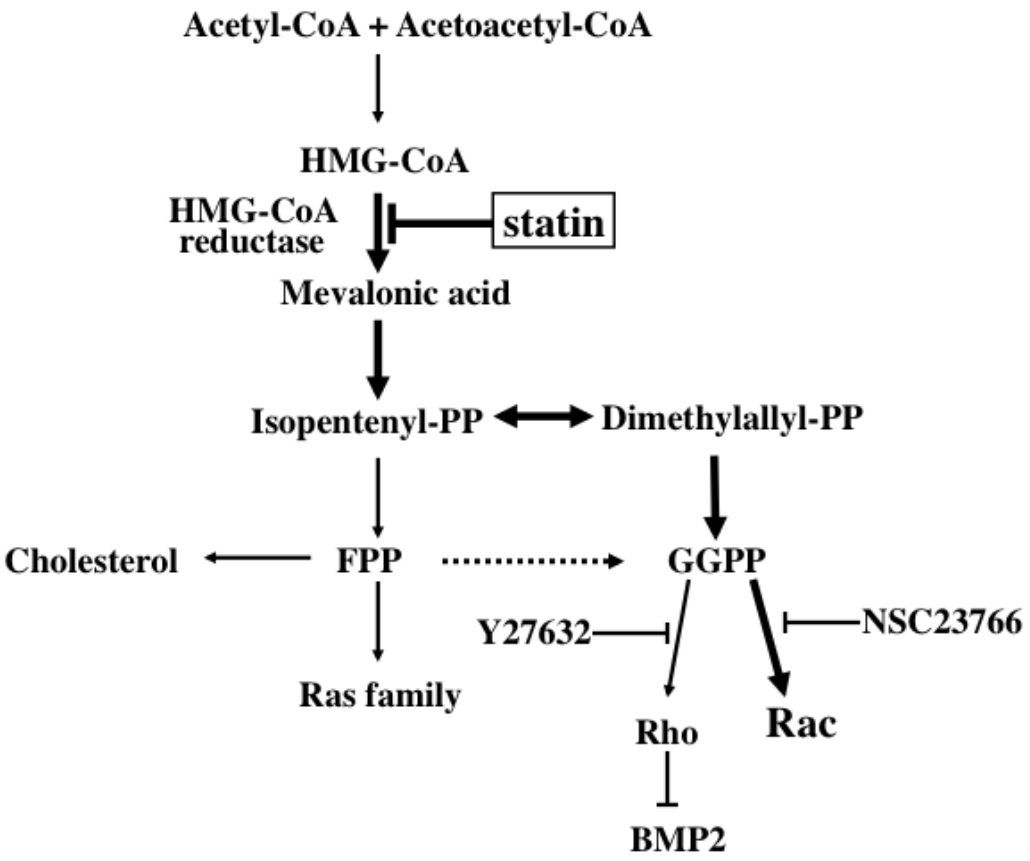
Acetyl-CoA + Acetoacetyl-CoA
HMG-CoA
HMG-CoA reductase
statin
Mevalonic acid
Isopentenyl-PP
Dimethylallyl-PP
Cholesterol
FPP
GGPP
Y27632
NSC23766
Ras family
Rho
Rac
BMP2

Fig. 5