Involvement of geranylgeranylation of Rho and Rac GTPases in adipogenic and RANKL expression, which was inhibited by simvastatin

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Short Title: Distinct Rho family is used in adipogenesis and RANKL expression

ABSTRACT

Simvastatin suppresses myoblast differentiation via inhibition of Rac GTPase, which is involved in the mevalonic acid pathway that produces cholesterol. Statins also inhibit adipogenic differentiation and receptor activator of NFkB ligand (RANKL) expression, possibly through the mevalonic acid pathway, although the involvement of that pathway and effector proteins in these cellular events has not been fully clarified. In the present study, we aimed to elucidate the mechanism of the effects of simvastatin on adipogenic differentiation and calcitriol-induced RANKL expression in bone marrow stromal ST2 cells. Adipogenesis and mRNA up-regulation of peroxisome proliferator–activated receptor g and adipocyte fatty acid–binding protein were induced by troglitazone, and those events were efficiently inhibited by simvastatin. In addition, RANKL expression induced by calcitriol was abrogated by simvastatin in ST2 cells. The inhibitory effects of simvastatin were adequately compensated by the addition of either mevalonic acid or an intermediate of the mevalonic acid pathway, geranylgeranyl pyrophosphate, but not by another intermediate, farnesyl pyrophosphate. These findings suggest that protein geranylgeranylation is related to cellular differentiation in those two directions. Furthermore, inhibitor analysis demonstrated that Rac GTPase is involved in adipogenic differentiation, whereas Rho GTPase was found to be involved in RANKL expression. Taken together, the present findings suggest that geranylgeranylation of Rho family GTPase is involved in both adipogenesis and RANKL expression of stromal cells, while Rac GTPase is involved in adipogenesis and Rho GTPase in RANKL expression.

KEY WORDS— adipogenesis; farnesyl pyrophosphate; geranylgeranyl pyrophosphate; mevalonic acid pathway; RANKL; Rho family; statin

INTRODUCTION

Statins inhibit the activity of 3-hydroxy-3-methyl-glutaryl (HMG)–CoA reductase, a key enzyme of the mevalonic acid pathway that produces cholesterol; thus, they are widely used for lowering blood cholesterol levels to prevent cardiovascular diseases. In addition to their effect on cholesterol level, enhancement of bone formation by statins has been demonstrated.[1–4] As for the effects on osteoblasts, it has been reported that atorvastatin enhanced osteoblastic differentiation and osteoprotegerin production[5] and that lovastatin suppressed adipocyte differentiation of D1 cells established from bone marrow stromal cells and differentiated them into osteoblastic cells.[6] Moreover, statins assist bone formation through the suppression of bone resorption.[7] For instance, simvastatin suppresses osteoclast differentiation through down-regulation of receptor activator of NFkB ligand (RANKL).[8] Furthermore, Woo et al.[9] reported that mevastatin inhibited multinuclear osteoclast formation induced by RANKL.

Based on these findings, statins seem to stimulate bone formation via alteration of cell lineage and suppression of osteoclast differentiation. For stimulation of bone formation, statins suppress Rho GTPase activity and restore the function of bone morphogenetic protein-2.[10] Regarding its alter- ation of cell lineage, we previously reported that simvastatin suppressed myoblast differentiation into myotubes by acting on the downstream effector of small G protein, Rac GTPase, but not Rho GTPase, via the mevalonic acid pathway.[11] In studies of bone resorption, it has been reported that statin signalling passes through geranylgeranyl pyrophosphate (GGPP), an intermediate of the mevalonic acid pathway, whereas its downstream effectors remain unknown.[5,9]

The stromal cell line ST2 that originated from mouse bone marrow[12] develops into an osteoblastic phenotype when cultured in osteogenic media supplemented with ascorbic acid and b-glycerophosphate.[13] ST2 cells also express RANKL in the presence of 1α, 25-$(OH)_2D_3$,[14] and RANKL-expressing cells support the differentiation of splenic cells into osteoclasts.[15,16] In contrast, ST2 cells are capable of differentiating into adipocytes when incubated with insulin or corticoid,[17,18] and that adipocyte differentiation is inhibited by simvastatin. Therefore, at least a part of the mevalonic acid pathway and its intermediates are expected to be involved in both adipogenic differentiation and RANKL expression in mouse stromal cells.

In the present study, we used troglitazone[19,20] to induce adipogenic differentiation and calcitriol for RANKL expression in ST2 cells, and then examined the inhibitory effects of simvastatin on these cellular processes in order to elucidate the relationship between the mevalonic acid pathway and protein prenylation of Rho family GTPases. The inhibitory effect of simvastatin on the mevalonic acid pathway was also examined using mouse embryo fibroblast C3H10T1/2 cells, which differentiated into adipocytes following treatment with troglitazone.[21,22] Our results demonstrated that depletion of geranylgeranyl pyrophosphoric acid impaired adipogenesis of these cells and RANKL expression in ST2 cells, and that adipogenic differentiation is mediated by geranylgeranylated active Rac, while RANKL expression is possibly mediated by Rho activation.

MATERIALS AND METHODS

Reagents

Simvastatin was purchased from Calbiochem (San Diego, CA); troglitazone and calcitriol from Cayman Chemistry (Ann Arbor, MI); and mevalonic acid, GGPP and farnesyl pyrophosphate (FPP) from Sigma Chemical Co. (St. Louis, MO). Y27632 came from Wako Pure Chemical Industries (Osaka, Japan) and NSC23766 from Tocris Bioscience (Bristol, UK).

Cell cultures

The ST2 cell line, derived from mouse bone marrow stromal cells, and the C3H10T1/2 cell line, from mouse fibroblast cells, were obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were plated in six-well plates at a concentration of $1x10^4$ cells·cm^{-2} and cultured in minimum essential medium α modification (Sigma Chemical Co.) supplemented with 10% foetal bovine serum, 50 units·ml^{-1} penicillin, 50 μg·ml^{-1} streptomycin (Life Technology, Inc., Grand Island, NY), 80 μg·ml^{-1} L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries) and 5 mM β-glycerophosphate (Kanto Chemical Co., Tokyo, Japan). The medium was changed every 2 or 3 days. When the cells reached confluence (defined as day 0), simvastatin (1 μM) was added with either troglitazone (5 μM), calcitriol (20 nM), mevalonic acid (500 μM), GGPP (10 and 20 μM), FPP (10 and 20 μM), NSC23766 (100 and 200 μM) or Y27632 (30 and 60 μM), or combinations of those reagents. The vehicle (dimethyl sulfoxide) was added to control cultures.

Staining of adipocytes with oil red O

ST2 cells were cultured with 5 μM troglitazone for 6 days and then fixed with 4% formaldehyde containing 85 mM $CaCl_2$ for 15 min and washed with distilled water. The fixed specimens were soaked in 99% propanol for 1 min and stained with oil red O for 15 min at 37˚C. Thereafter, the samples were washed with 99% propanol for 2 min and then distilled water for 5 min. Aliquots of samples were mounted and observed with a microscope. For quantification, stained cells were further washed with 99% propanol for 5 min and then dried and extracted with 60% propanol for 30 min, after which absorbance at 450 nm was measured.

Quantitative and conventional RT-PCR

Total RNA was isolated using Isogen reagent (Nippon Gene Co., Tokyo, Japan). cDNA was prepared from 1 μg of RNA with reverse transcriptase (TAKARA BIO Inc., Otsu, Japan) in a reaction mixture (20 μl) containing 0.5 mM dNTPs and 0.2 μg of oligo dT (Life Technology Inc.). Conventional PCR was performed for 30 cycles according to a previously reported method.[23] The primer sets used were as follows: for peroxisome proliferator–activated receptor γ (PPARγ), 5'-TGAAACTCTGGGAGATTCTCCTG-3' and 5'-CCATGGTAATTTCTTGTGAAGTGC-3'; for adipocyte fatty acid–binding protein (aP2), 5'-TCGTCTGCGGTGATTTCATC-3' and 5'-CAGCACTCACTGCTTTTATAG-3'; for RANKL, 5'-TCACCATCCGGTCAGAGAGTA-3' and 5'- TGAAGATAGTCTGTAGGTACG-3'; and for alkaline phosphatase (ALP), 5'-TCCTTAGGGCTGCCGCT-3' and 5'-TGTACCCTGAGATTCGTCC-3'. PCR products were separated on 1.8% agarose gels. mRNA expression levels were quantified by qPCR and a delta–delta–CT method using the level of GAPDH mRNA as the standard.[11] The reaction mixture

(25 µl, n=3) carrying 0.5 µl of cDNA and 0.4 µM primers containing Full Velocity SYBR Green QPCR reagent (Stratagene, Amsterdam, The Netherlands) was subjected to the reaction using an Mx3005P real-time PCR system (Stratagene, La Jolla, CA).

ALP activity

Alkaline phosphatase activity was determined according to the protocol previously described.[24] In brief, cells were sonicated in solubilization buffer (10 mM Tris–HCl, pH 7.4, containing 0.1% Triton X-100) and centrifuged at 12000 xg for 20 min. 2 ml of supernatant and the reaction mixture (0.15 ml) comprising 4 mM p-nitrophenyl phosphate and 5 mM $MgCl_2$ in 50 mM carbonate buffer (pH 10) were incubated at room temperature for 6 min, mixed with 50 µl of 2 M NaOH to stop the reaction, and then, absorbance at 405 nm was determined. One unit was defined as the quantity of ALP that produced 1mg of p-nitrophenol in 1 h.

Statistical analysis

All values are presented as the mean ± SE. Differences between the two groups were assessed using Student's t-test.

RESULTS

Relationship between mevalonic acid pathway and adipocyte differentiation

Bone marrow stromal ST2 and fibroblast C3H10T1/2 cells were cultured in the presence of troglitazone. Numerous lipid droplets stained by oil red O were found in ST2 cells 6 days after reaching confluence (Figure 1), whereas the number of droplets was severely reduced with co-administration of simvastatin. Induction of adipogenesis by troglitazone and the reverse effect of simvastatin were also demonstrated in C3H10T1/2 cells. In addition to the increase in oil red O–stained cells, the expression levels of the adipocyte marker genes PPARγ and aP2 were also increased. Again, co-administration of simvastatin significantly suppressed the troglitazone-induced expression of the two genes. These results clearly indicate that simvastatin potently suppresses troglitazone-induced adipocyte differentiation of both bone marrow stromal- and fibroblast-derived cells. Since simvastatin also inhibited the activity of 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase (see Figure 2), it is considered that intermediates derived from HMG–CoA in the mevalonic acid pathway may be involved in adipocyte differentiation of these cells. On the other hand, simvastatin arrested the adipogenesis in ST2 cells, but it could not upregulate the expression of ALP gene, indicating that statin could not affect an osteogenesis (Figure 1J).

Simvastatin stimulates osteogenic differentiation not only in osteoblastic cells[25] but also in non-osteogenic cells, namely ES cells[26] and bone marrow stromal cells.[27] Then, we tried to evaluate the osteogenic differentiation of ST2 cells. Figure 3 shows the result of osteoblast induction. ALPase activity in ST2 cells increased; however, simvastatin could not enhance it significantly.

To investigate this possibility of mevalonic acid involvement in adipogenesis differentiation, mevalonic acid and its downstream intermediates, GGPP and FPP, were added to ST2 and C3H10T1/2 cells in the presence of troglitazone and simvastatin, and then the mRNA levels of PPARg and aP2 were quantified by qPCR. In ST2 cells, the suppression of PPARγ expression by simvastatin was largely restored by 500 μM of mevalonic acid and completely cancelled by 10 and 20 μM of GGPP (Figure 4A). The expression of aP2 suppressed by simvastatin was also markedly relieved by addition of either mevalonic acid or GGPP. In contrast, FPP did not restore the suppressed expression of either PPARg or aP2 genes by simvastatin. Similarly, in C3H10T1/2 cells, the reduced expressions of PPARγ and aP2 by simvastatin were significantly recovered by GGPP, while FPP had no effect on the inhibition by simvastatin (Figure 4B). Additional increases in these expression levels were observed following the addition of 500 μM of mevalonic acid. These results suggest that the inhibitory effect of simvastatin on adipocyte differentiation is mediated through reduced production of mevalonic acid and, therefore, its downstream intermediate in the mevalonic acid pathway, GGPP. Since only GGPP restored the inhibitory effect of simvastatin, geranylgeranylation of some effector proteins seems to directly participate in adipocyte differentiation.

We previously demonstrated that simvastatin suppresses myoblast differentiation via inhibition of the Rac GTPase protein, which was connected to the mevalonic acid pathway.[11] Rho family GTPases, including Rho, Rac and Cdc42, are known to be posttranslationally prenylated with GGPP and FPP,[25,28,29] and thus act as effector molecules in transducing cellular differentiation

signalling.[30] Because geranylgeranylation of small G-proteins is also concerned in the suppression by simvastatin as shown in Figure 4, we further conducted an inhibition analysis to elucidate which of the Rho family proteins were involved in this event. When the Rho kinase inhibitor Y27632 was added to ST2 cells in the presence of troglitazone, the expression level of PPARγ showed an increasing tendency (Figure 5A), while aP2 expression did not change. In contrast, addition of the Rac inhibitor NSC23766 completely suppressed troglitazone-induced expression of both the PPARγ and aP2 genes, indicating that activation of Rac was involved in the observed adipogenic differentiation. A similar tendency was seen in C3H10T1/2 cells, as Y27632 slightly raised the expression of both, whereas NSC23766 completely suppressed the up-regulation of PPARγ mRNA by troglitazone and significantly suppressed that of aP2 mRNA (Figure 5B). Taken together, the results presented in Figures 1, 3, 4 and 5 suggest that adipogenic differentiation induced by troglitazone is related to the geranylgeranylation of Rac, whereas farnesylation of the Rac super family and geranylgeranylation of the Rho molecule are not involved.

Rho GTPase activation via the mevalonic acid pathway in RANKL expression in ST2 cells

Under the normal culture condition, ST2 cells scarcely expressed the RANKL gene, while the mRNA level of RANKL was markedly enhanced by addition of calcitriol (Figure 6). Again, co-administration of simvastatin nearly completely abolished the calcitriol-induced RANKL expression in ST2 cells. This inhibitory effect of simvastatin suggests involvement of the mevalonic acid pathway, the same as that seen with troglitazone-induced adipogenesis. As expected, the inhibitory effect of simvastatin was significantly restored by addition of either mevalonic acid or GGPP, whereas FPP did not restore the level of RANKL mRNA. Furthermore, results of our inhibitor analysis demonstrated that Y27632 reduced the calcitriol-induced up-regulation of RANKL mRNA to 40%, while NSC23766 did not have any effects on that enhanced expression. Thus, in contrast to the adipocyte differentiation findings, these results suggest that geranylgeranylation of Rho GTPase, but not Rac GTPase, is involved in differentiation of ST2 cells into osteoclast-supporting cells expressing RANKL

DISCUSSION

Our results demonstrated that mouse bone marrow stromal ST2 cells have an ability to differentiate into both adipocytes and RANKL-expressing, osteoclast-supporting cells following the addition of troglitazone and calcitriol, respectively. Furthermore, mevalonic acid and GGPP were found to be closely related to both processes. These results are consistent with findings presented in previous reports[31,32] in which statin-induced inhibition was not restored by FPP. In addition, we clearly demonstrated that Rho GTPase family proteins were alternatively employed in the two processes, as adipogenesis was mediated by Rac GTPase and the induction of RANKL expression was mediated by Rho GTPase (Figure 2).

Small GTPases are important modulators involved in various cellular events, such as proliferation, differentiation and survival of cells.[33,34] In mouse embryo–derived fibro- blast 3T3-L1 cells, activation of Rho GTPase suppressed adipogenesis and changed the cell lineage to myoblasts.[35,36] Similar to those previous reports, we demonstrated that inhibition of Rho kinase by Y27632 induced expression of the adipogenic marker genes PPARγ and aP2 (Figure 5). Furthermore, since Rho inhibition by Y27632 caused marked enhancement of PPARγ expression compared to that in the troglitazone-treated cells. This is coincident with the report by Noguchi et al.,[35] where Y27632 enhanced the adipogenesis in 3T3-L1 stimulated by 3-isobutyl-1-methylxanthine and dexamethasone. Although they suggested that Y27632 has the ability to enhance p38MAPK and Ark, the acceptable pathway is still unclear, because the enhancement of p38MAPK was slight and the phosphorylation of Ark occurred only when insulin was present.

As shown in Figure 6, mevalonic acid and GGPP restored the expression of RANKL mRNA suppressed by simvastatin, while that expression was suppressed by inhibition of Rho kinase, indicating that activation and geranylgeranylation of Rho are closely related to calcitriol-induced RANKL expression in ST2 cells. These observations are in good agreement with a previous study in which the expression of RANKL in primary osteoblasts was decreased by inhibition of Rho.[37] In contrast, it has also been reported that RANKL expression in osteosarcoma UMR-106 cells was increased by suppression of Rho activity.[38] The suppression of RANKL expression by Y27632 in calcitriol-induced ST2 showed 60% inhibition, suggesting that an additional pathway may exist. Recently, Tsubaki et al.[39] have reported that simvastatin inhibited RANKL mRNA expression via suppression of Ras/MEK/ERK pathway not via Rho/ROCK inhibition. They showed that Y27632 did not inhibit the RANKL expression but stimulated the p38MAPK phosphorylation. The reason that they could not suppress the RANKL expression by Y27632 may be due to the concentration of Y27632, which might be lower than the threshold to exert the inhibitory effect, or co-stimulation with dexamethasone. Together with our data and Tsubakis' data, we suppose that statin may inhibit the RANKL expression via both Rho/ROCK inhibition and suppression of Ras/MEK/ERK pathway.

Rac suppression by NSC23766 did not alter calcitriol- induced RANKL expression. Recently, Croke et al.[40] reported that loss of Rac in knockout mice resulted in osteopetrosis development, which was caused by osteoclast dysfunction, but not arrest of osteoclast differentiation. The loss of

Rac resulted in a failure of the cytoskeleton network, as evidenced by reduced cell motility and lack of ruffled border formation, whereas it did not affect osteoclast differentiation. Their results are in accord with our experimental data, in which a Rac inhibitor, NSC23766, did not have effects on the expression of RANKL.

It is noteworthy that simvastatin blocked the adipogenesis of ST2 but did not alter the direction of differentiation into osteoblasts (Figures 1J and 3), unlike the results with D1 cells reported by Li et al.[6] This difference may be attributed to the intrinsic properties of these cells, because ST2 cells primarily possess an adipogenic tendency even in the absence of ascorbic acid,[13] and accordingly, an alteration of cell lineage into osteoblasts is more difficult for ST2 cells than D1 cells. In conclusion, the present findings demonstrated that Rho, which is one of the members of the Rho family, was geranylgeranylated in adipogenesis, whilst Rac, which is another member of the Rho family, was geranylgeranylated in RANKL expression in mouse stromal cells. This may be the main mechanism of statin-induced inhibition of cell differentiation that results in bone formation enhancement.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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FIGURE LEGENDS

FIGURE 1. Troglitazone-induced adipogenesis and its suppression by simvastatin in ST2 and C3H10T1/2 cells. ST2 (A-C) and C3H10T1/2 (D-F) cells were cultured without (A, D), or with 5 μM troglitazone (B, E), or 5 μM troglitazone and 1 μM simvastatin (C, F). After 6 days, cells were stained with Oil Red O. Bar represents 100 μm. Oil Red O stained ST2 (G) and C3H10T1/2 (H) cells was solubilized in 99% isopropyl alcohol, then absorbance at 450 nm was measured. *P<0.01. mRNA levels of PPARγ, aP2 and alikaline phosphatase (ALP) in ST2 cells were assessed by RT-PCR (I, J). TGZ: troglitazone, SMV: simvastatin.

FIGURE 2. Mevalonic acid pathway and protein geranylgeranylation are involved in adipocyte differentiation and RANKL expression. The mevalonic acid pathway is schematically illustrated, as reported previously.[41] Dotted arrows represent routes proposed from results of the present study.

FIGURE 3. ST2 cells were cultured in the presence of 0.5 or 1.0 μM simvastatin or DMSO (vehicle). ALP activity was determined on 7, 11, and 16 days after confluent. Simvastatin could not enhance it significantly.

FIGURE 4. Involvement of mevalonic acid pathway in adipogenesis. ST2 (A) and C3H 10T1/2 (B) cells were cultured with or without 5 μM troglitazone, 1 μM simvastatin, 500 μM mevalonic acid, 10 or 20 μM GGPP, and 10 or 20 μM FPP. After 7 days, mRNA levels of PPARγ (open bars) and aP2 (closed bars) were measured using RT-qPCR. Statistical significance was determined by comparing with the TGZ plus SMV group. *P<0.01, ^{+}P<0.05. TGZ: troglitazone, SMV: simvastatin, MVA: mevalonic acid.

FIGURE 5. Suppression of adipogenic marker expression by Rac inhibition. mRNA levels of PPARγ (open bars) and aP2 (closed bars) were measured using RT-qPCR in ST2 cells (A) and C3H10T1/2 cells (B) cultured with or without 5 μM troglitazone, 30 or 60 μM Y27632 (Rho kinase inhibitor), and 100 or 200 μM NSC23766 (Rac inhibitor). Statistical significance was determined by comparing with the TGZ group. *P<0.01, ^{+}P<0.05. TGZ: troglitazone.

FIGURE 6. Effects of Rac and Rho family GTPase inhibitors on calcitriol-induced RANKL expression in ST2 cells. ST2 cells were cultured with various combinations of 20 μM calcitriol, 1 μM simvastatin, 500 μM mevalonic acid, 20 μM GGPP, and 20 μM FPP. After 5 days, RANKL mRNA levels were measured using RT-qPCR (A). ST2 cells were cultured with or without calcitriol and 60 μM Y27632 or 200 μM NSC23766 (B). Statistical significance was determined by comparing with the calcitriol group; *P<0.01. SMV: simvastatin, MVA: mevalonic acid.

Figure 1

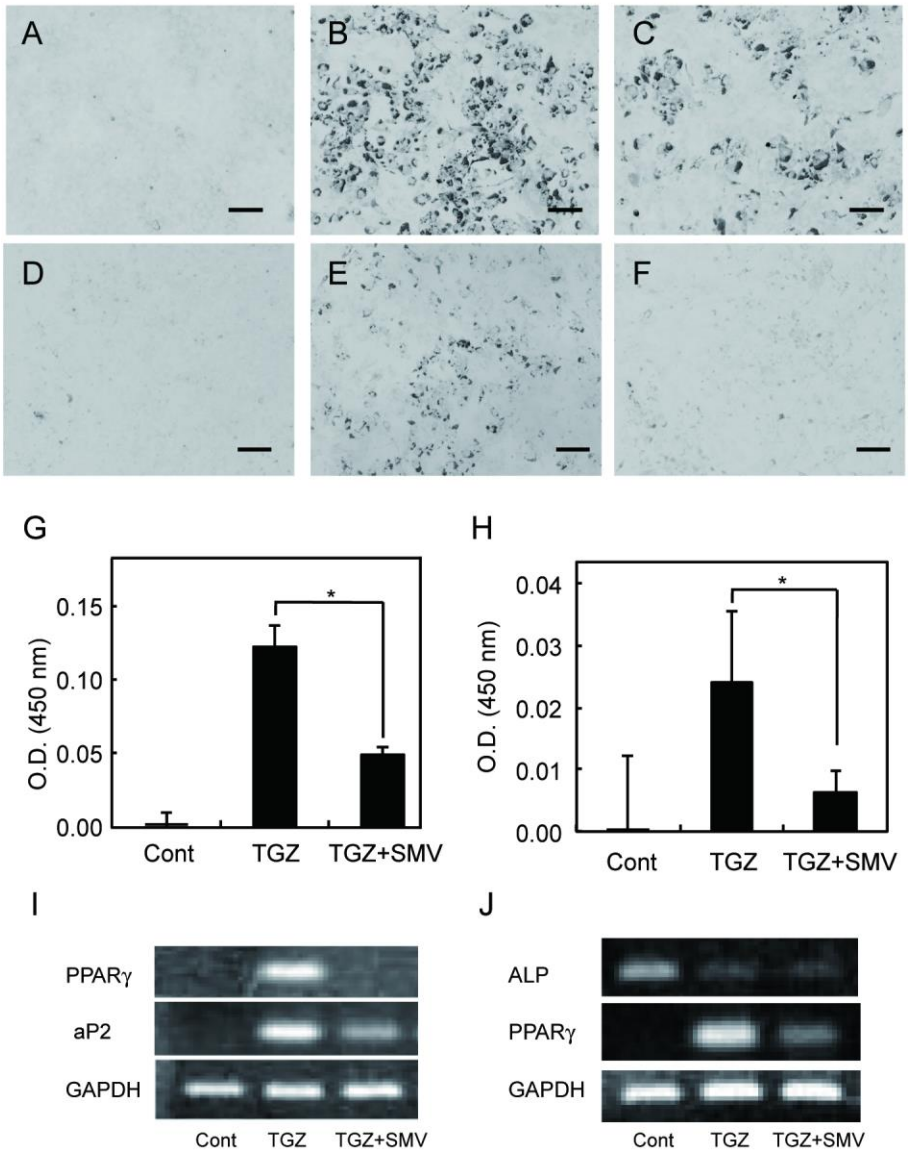

A
B
C
D
E
F
G
O.D. (450 nm)
0.15
0.10
0.05
0.00
*
Cont
TGZ
TGZ+SMV
H
O.D. (450 nm)
0.04
0.03
0.02
0.01
0.00
*
Cont
TGZ
TGZ+SMV
I
PPARγ
aP2
GAPDH
Cont
TGZ
TGZ+SMV
J
ALP
PPARγ
GAPDH
Cont
TGZ
TGZ+SMV

Figure 2

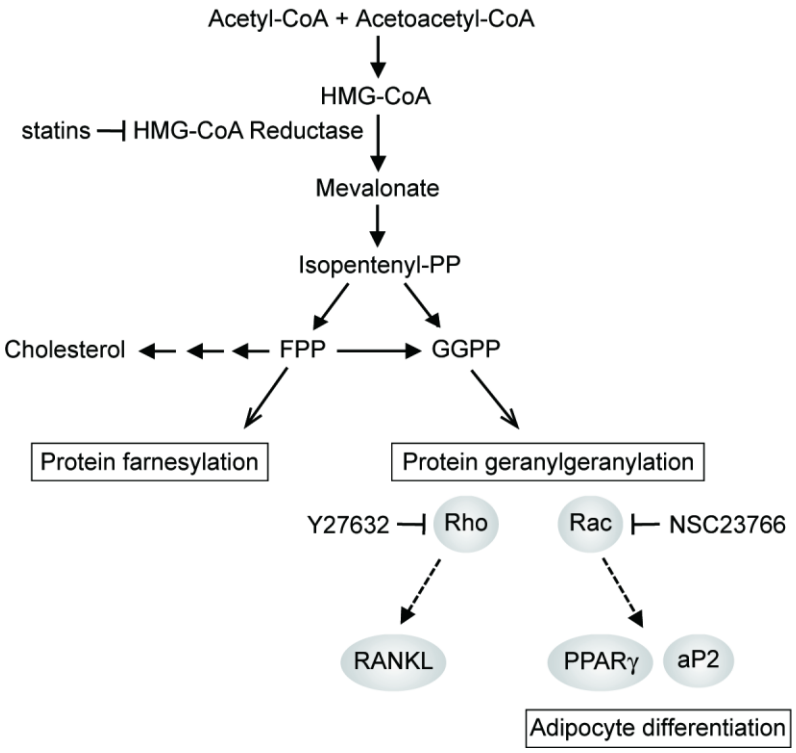


Figure 3

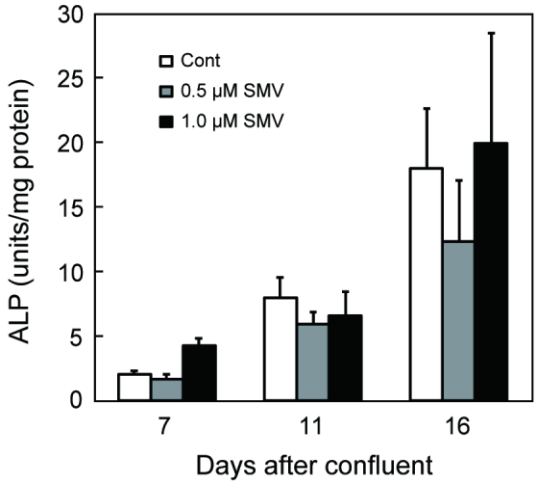


Figure 4

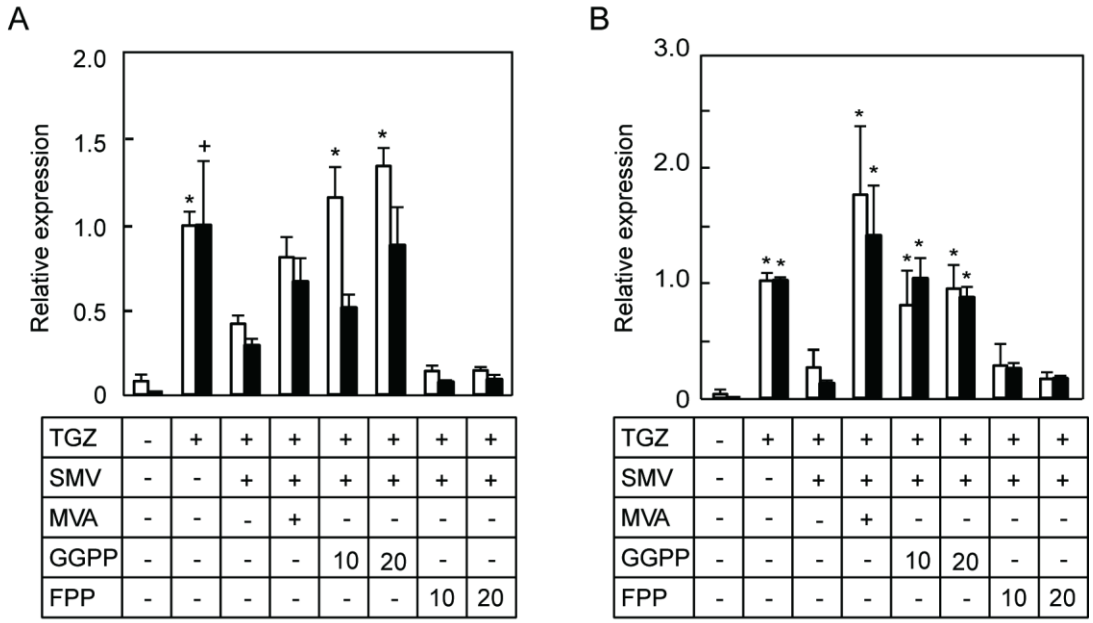


Figure 5

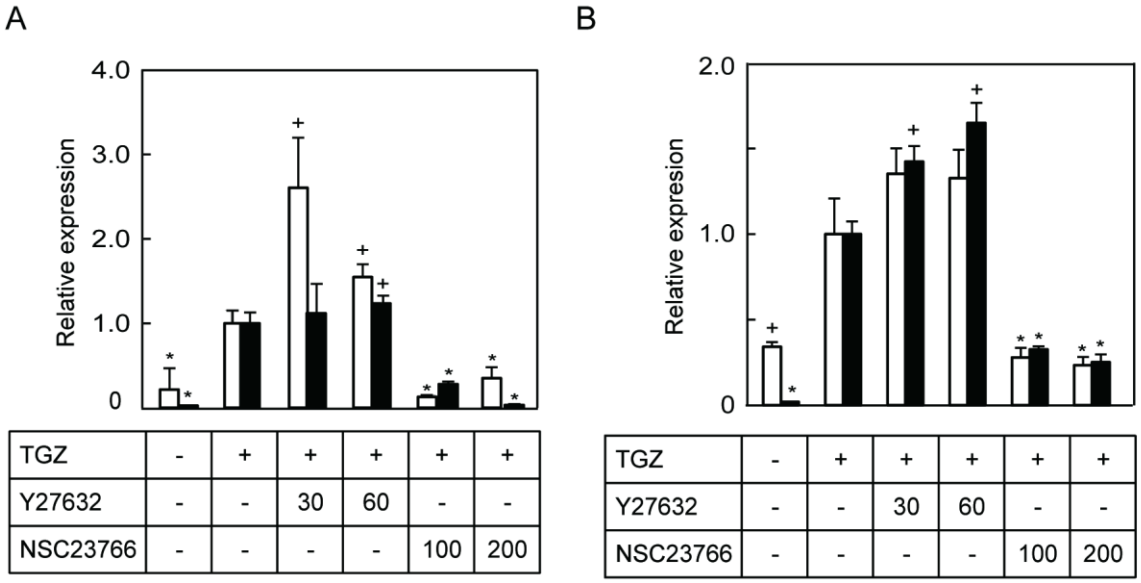
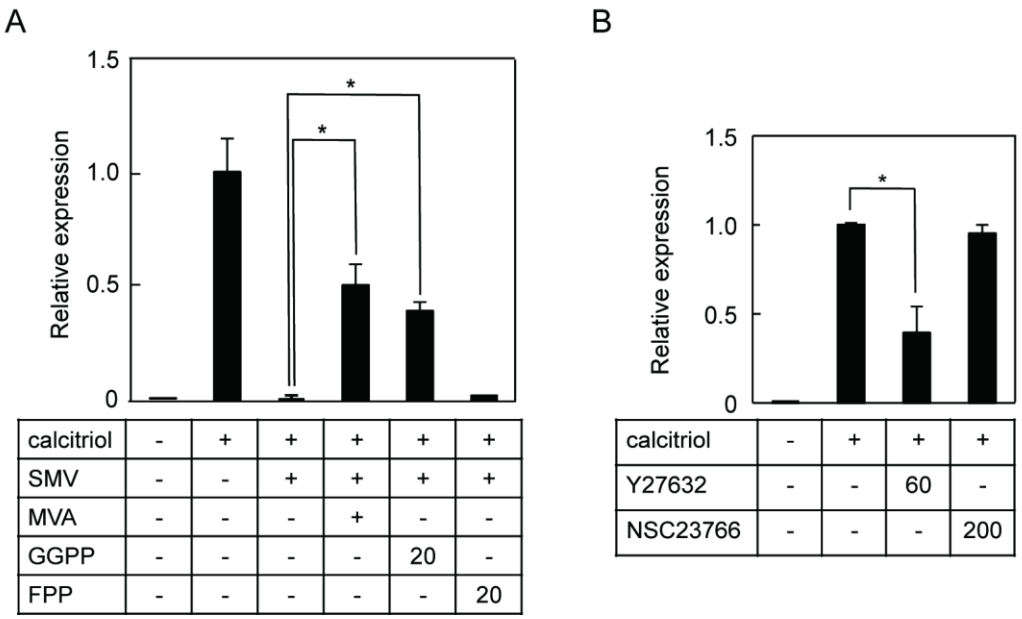


Figure 6



calcitriol	-	+	+	+	+	+
SMV	-	-	+	+	+	+
MVA	-	-	-	+	-	-
GGPP	-	-	-	-	20	-
FPP	-	-	-	-	-	20

calcitriol	-	+	+	+
Y27632	-	-	60	-
NSC23766	-	-	-	200