3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitors Attenuate Vascular Smooth Muscle Proliferation by Preventing Rho GTPase-induced Down-regulation of p27^Kip1^*†

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The mechanism by which platelet-derived growth factor (PDGF) regulates vascular smooth muscle cell (SMC) DNA synthesis is unknown, but may involve isoprenoid intermediates of the cholesterol biosynthetic pathway. Inhibition of isoprenoid synthesis with the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, simvastatin (Sim, 1–10 μM), inhibited PDGF-induced SMC DNA synthesis by >95%, retinoblastoma gene product hyperphosphorylation by 90%, and cyclin-dependent kinases (Cdks)-2, -4, and -6 activity by 80 ± 5, 50 ± 3, and 48 ± 3%, respectively. This correlated with a 20-fold increase in p27^Kip1^ without changes in p16, p21^Waf1^, or p53 levels compared with PDGF alone. Since Ras and Rho require isoprenoid modification for membrane localization and are implicated in cell cycle regulation, we investigated the effects of Sim on Ras and Rho. Up-regulation of p27^Kip1^ and inhibition of Rho but not Ras membrane translocation by Sim were reversed by geranylgeranylpyrophosphate, but not farnesylpyrophosphate. Indeed, inhibition of Rho by Clostridium botulinum C3 transferase or overexpression of dominant-negative N19RhoA mutant increased p27^Kip1^ and inhibited retinoblastoma hyperphosphorylation. In contrast, activation of Rho by Escherichia coli cytotoxid necrotizing factor-1 decreased p27^Kip1^ and increased SMC DNA synthesis. These findings indicate that the down-regulation of p27^Kip1^ by Rho GTPase mediates PDGF-induced SMC DNA synthesis, and suggest a novel direct effect of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors on the vascular wall.

Vascular proliferative diseases such as atherosclerosis, postangioplasty re-stenosis, and transplant arteriosclerosis are characterized by vascular smooth muscle cell (SMC) DNA synthesis (1). The entry and progression of SMC into the cell cycle is stimulated by growth factors derived from inflammatory cells, platelets, and the vascular wall (2). Although these growth factors which include basic fibroblast growth factor, platelet-derived growth factor (PDGF), transforming growth factor-β1, angiotensin II, and insulin-like growth factor utilize distinct signaling pathways to promote SMC DNA synthesis, these signaling pathways, however, must converge upon common regulators of the cell cycle (3). These regulators include the cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors. Indeed, gene therapy with Cdk inhibitors or treatment with a neutralizing antibody to PDGF inhibits neointimal smooth muscle DNA synthesis after balloon angioplasty (1, 4).

The transition through the cell cycle is regulated by the expression and activity of cell cycle checkpoint proteins comprising of cyclins and Cdks (5). These in turn are regulated by the family of Cdk inhibitor proteins, such as p16, p21^Waf1^, and p27^Kip1^. The final common pathway leading to G1/S transition is the hyperphosphorylation of the retinoblastoma gene product (Rb), which functions as a molecular switch dedicating the cell to DNA replication. Hyperphosphorylation of Rb results in the release of the transcription factor E2F, which induces the expression of genes required for the progression through S, G2, and M phases (5). Despite recent advances in the understanding of cell cycle regulation in proliferative vascular diseases such as atherosclerosis and post-angioplasty restenosis, therapy is still lacking which can effectively prevent SMC DNA synthesis.

Large clinical trials have shown that inhibition of cholesterol biosynthesis by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins improve clinical outcomes in patients with atherosclerosis. For example, treatment with HMG-CoA reductase inhibitors reduces post-angioplasty re-stenosis, coronary bypass occlusions (6, 7), and transplant arteriosclerosis (8). Although HMG-CoA reductase inhibitors have been shown to inhibit SMC proliferation in vitro (9), the mechanism(s) by which they inhibit cell growth is not known. The HMG-CoA reductase inhibitors not only inhibit cholesterol synthesis, but also, inhibit the synthesis of important isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). Both FPP and GGPP are important lipid attachments for the post-translational modification of a variety of proteins, including Ras and Rho GTP-binding proteins (10, 11). We and others (12, 13) have recently shown that some of the direct effects of HMG-CoA reductase inhibitors on the vascular wall are mediated by inhibition of isoprenoid but not cholesterol synthesis. The purpose of this study, therefore, is to determine the role and mechanism of isoprenoid intermediates in regulating cell cycle progression in human vascular SMC.
Regulation of PDGF-induced Cell Cycle Progression by Rho GTPase

MATERIALS AND METHODS

Reagents—All standard culture reagents were obtained from JRH Bioscience (Lenexa, KS). Mevastatin (compactin), farnesylpyrophosphate, geranylgeranylpolyphosphate, and t-leucovorone were purchased from Sigma. Low density lipoprotein, FTT-276, and GGTI-286 were obtained from Calbiochem (San Diego, CA). Simvastatin was obtained from Merck Sharp and Dohme, Inc. Mevastatin and simvas-
tation (Arlington Heights, IL). The cDNA for Waf1 was obtained from List Biological Laboratories, Inc. (Campbell, CA). Recombinant Escherichia coli tyrosine nitrating factor (CNF)-1 and RhoA mutants were kindly provided by K. Aktories (University of Freiberg, Germany) and W. Moelaena (Netherlands Cancer Institute, Netherlands), respectively.

Cell Culture—Human vascular SMC were isolated from outgrowths of tunica media explants derived from human aortic and saphenous vein tissues as described previously (14). Cells of two to four passages were grown in culture medium containing Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT), and antibiotic mixture of penicillin (100 units/ml) streptomycin (100 μg/ml) and fungizone (1 mg/ml). The cultures were characterized pictorially using phase-contrast microscopy and staining for SMC-specific α-actin. Confluent SMC were rendered quiescent by incubation in 0.4% FCS for 48 h before experiments. Cellular viability was determined by cell counting and morphology. By trypan blue exclusion.

Protein Isoprenylation—For measurement of isoprenoid incorporation, SMC were treated with simvastatin (10 μM) for 24 h before addition of 50 μCi/ml [3H]GGPP or [3H]FPP, respectively. After 24 h, cells were washed four times with PBS. Aliquots of the cell lysates were counted in a liquid scintillation counter (Beckman LS1800) and once with kinase buffer (without ATP). The purified enzyme was then incubated with a full-length pRb-3-transferase fusion protein (2 μg, Santa Cruz Biotech) as a substrate in 15 μl of kinase buffer containing Hesperes (20 mM, pH 7.7), MgCl2 (10 mM), ATP (10 μM), and MgATP (10 μM) for 30 min at 30 °C. Inorganic phosphate was removed and the samples were transferred to 300 μl Na2VO4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM dithiothreitol and once with kinase buffer (without ATP). The purified enzyme was then incubated with a full-length pRb-glutathione S-transferase fusion protein (2 mg, Santa Cruz Biotech) as a substrate in 15 μl of kinase buffer containing Hesperes (20 mM, pH 7.7), MgCl2 (10 mM), ATP (10 μM), and MgATP (10 μM) for 30 min at 30 °C. Inorganic phosphate was removed and the samples were transferred to 300 μl Na2VO4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM dithiothreitol and once with kinase buffer (without ATP). The purified enzyme was then incubated with a full-length pRb-glutathione S-transferase fusion protein (2 μg, Santa Cruz Biotech) as a substrate in 15 μl of kinase buffer containing Hesperes (20 mM, pH 7.7), MgCl2 (10 mM), ATP (10 μM), and MgATP (10 μM) for 30 min at 30 °C. 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24 h. By [3H]thymidine incorporation, Sim inhibited SMC DNA synthesis by 94 ± 0.3% which was completely reversed by co-treatment with mevalonate (200 μM) (Fig. 1A). Mevalonate alone, however, had no effect on [3H]thymidine uptake (not shown). This decrease in [3H]thymidine incorporation by Sim correlated with a concentration-dependent decrease in Rb hyperphosphorylation by 98 ± 4% (Fig. 1B). Similar effects were observed with another HMG-CoA reductase inhibitor, mevastatin (data not shown). Inhibition of SMC DNA synthesis was independent of extracellular cholesterol concentration since all cells were cultured in 10% fetal calf serum under cholesterol-clamped conditions. Furthermore, addition of low density lipoprotein-cholesterol (1 mg/ml) did not reverse the effects of Sim on [3H]thymidine incorporation (not shown). These findings suggest that isoprenoid synthesis is important in PDGF-induced SMC DNA synthesis.

**Effects of HMG-CoA Reductase Inhibitors on Cdk Activity**—The entry into and progression of the cell cycle is regulated by cyclin complexes of cyclins and Cdkks (5). To determine whether isoprenoids can regulate Cdk activity, we measured the effect of Sim on PDGF-induced Cdk-2, Cdk-4, and Cdk-6 activity. Using glutathione S-transferase-Rb fusion protein as substrate for Cdk, we found that quiescent SMC have little or no Cdk-2, -4, and -6 activity (Fig. 2). Stimulation with PDGF (6 ng/ml, 24 h) increased Cdk-2, -4, and -6 activity. Co-treatment with Sim (5 μM) inhibited PDGF-induced Cdk-2, -4, and -6 kinase activity by 80 ± 5, 60 ± 3, and 48 ± 3%, respectively. Thus, inhibition of isoprenoid synthesis down-regulated Cdk activity with the greatest decrease observed with Cdk-2.

**Effects of HMG-CoA Reductase Inhibitors on Cdk Inhibitors**—The Cdk inhibitors such as p16, p21<sup>Waf1</sup>, and p27<sup>Kip1</sup> bind to and inhibit the activation of Cdk-cyclin complexes. The tumor suppressor gene p53 regulates cell cycle progression, in part, by up-regulating the expression of p21<sup>Waf1</sup> (1). To determine whether inhibition of isoprenoid synthesis can affect the expression of Cdk inhibitors, we investigated the effects of Sim (5 μM) on p16, p21<sup>Waf1</sup>, p27<sup>Kip1</sup>, and p53 levels in SMC stimulated with PDGF (6 ng/ml) for 12, 24, and 36 h. Compared with PDGF alone, we find that Sim did not affect the levels of p16 and p21<sup>Waf1</sup> (Fig. 3). In contrast, stimulation with PDGF decreased p27<sup>Kip1</sup> by 80 ± 7, 95 ± 3, and 75 ± 8% after 12, 24, and 36 h, respectively. Co-treatment with Sim (5 μM) reversed the down-regulation of p27<sup>Kip1</sup> by PDGF resulting in 20-fold increase in p27<sup>Kip1</sup> after 24 h compared to that of PDGF. Although p53 levels decline in a time-dependent manner after PDGF stimulation, the levels were not different in the presence or absence of Sim. These findings indicate that inhibition of isoprenoid synthesis by Sim preferentially up-regulates p27<sup>Kip1</sup> levels and suggest that p27<sup>Kip1</sup> may be involved in the inhibition of Cdk-2 activity.

**Effects of Isoprenoids on Rho and Ras Membrane Localization**—The small GTPases of the Ras and Rho family have been shown to regulate entry into the cell cycle (17, 18). Ras and Rho are post-translationally modified by isoprenylation which is necessary for their membrane localization and function (11). We have previously shown that Ras farnesylation and Rho geranylgeranylation are required for their membrane-associated GTPase activity (13). Stimulation with PDGF (6 ng/ml, 24 h) increased the level of membrane-bound RhoA by 420 ± 20% without significantly affecting the total amount of RhoA in smooth muscle cells (Fig. 4). Inhibition of isoprenoid synthesis by Sim (5 μM) completely reversed and prevented PDGF-induced RhoA membrane localization. Interestingly, in the presence of PDGF, Sim increased the total cellular expression of RhoA possibly to compensate for decreases in membrane-associated RhoA activity. Indeed, co-treatment with GGPP, but not FPP reversed the effect of Sim on RhoA membrane localization. Interestingly, the expression of Ras in SMC membranes was not significantly altered by treatment with PDGF or Sim after 24 h (Fig. 4). In total cell lysates, however, treatment with Sim resulted in the appearance of a higher molecular weight non-farnesylated Ras. Previous studies have demonstrated that farnesylated Ras migrates slightly faster on SDS-PAGE than unmodified Ras (19, 13). Indeed, the shifted band corresponding to non-farnesylated Ras in SMC treated with PDGF and Sim is absent in the presence of FPP, but not GGPP. These findings confirm that inhibition of geranylgeranylation by Sim specifically prevents PDGF-induced membrane localization of Rho, but not Ras.

**Effects of Rho on p27<sup>Kip1</sup> Expression**—To determine which isoprenoid intermediate down-regulates p27<sup>Kip1</sup>, PDGF (6 ng/ml)-induced SMC were treated with Sim (5 μM) in the presence or absence of GGPP (5 μM) and FPP (5 μM). Co-treatment with GGPP, but not FPP, completely reversed the up-regulation of...
To determine whether changes in p27Kip1 are specific to inhibition of Rho, we transfected SMC with wild-type RhoA (wtRhoA) and a dominant-negative RhoA (N19RhoA). Both of these RhoA constructs contain a N-terminal c-myc tag. The transfection efficiency as measured by β-galactosidase fluorescence and c-myc immunofluorescence is approximately 8–10%. Using dual immunofluorescent microscopy, overexpression of wtRhoA in PDGF-induced SMC did not change p27Kip1 expression relative to non-transfected cells (i.e. cells without c-myc immunofluorescence) (Fig. 6). In contrast, SMC overexpressing the dominant-negative N19RhoA mutant, as identified by co-staining for c-myc, showed increased p27Kip1 expression. Taken together, these findings indicate that RhoA negatively regulates p27Kip1 expression and suggest that Sim up-regulates p27Kip1 by inhibiting Rho membrane localization and activity.

Effects of Rho on Smooth Muscle Cell Cycle Progression—To determine the effects of Rho on cell cycle progression, we investigated the effects of PDGF (6 ng/ml), Sim (5 μM), GGPP (5 μM), GGPP (5 μM, 5 μM), C3 transferase (0.001) (Fig. 7A). Direct inactivation of Rho by C3 transferase decreased Rb hyperphosphorylation by 80 ± 7%. This decrease in Rb hyperphosphorylation by C3 transferase was not reversed by GGPP or FPP (data not shown). When SMC was treated with E. coli CNF-1 (200 ng/ml) which is known to directly activate Rho by blocking Rho GTP hydrolysis (21, 22), Rb became hyperphosphorylated in the absence of fetal calf serum (Fig. 7B). Furthermore, in the presence of 10% fetal calf serum in the culture medium, CNF-1 augmented Rb hyperphosphorylation by approximately 2-fold.

Compared with treatment with PDGF (6 ng/ml), thymidine incorporation was inhibited in the presence of Sim (6 ± 0.3% incorporation, p < 0.001) (Fig. 8A). Co-treatment with GGPP (5 μM) reversed the inhibitory effect of Sim (88 ± 6% incorporation, p > 0.05 compared with PDGF), whereas FPP (5 μM) only partially reversed the effects of Sim (48 ± 4% incorporation, p < 0.01 compared with PDGF). Co-treatment with low density lipoprotein-cholesterol (1 mg/ml) did not reverse the inhibitory effects of simvastatin (p > 0.05). Co-treatment with CNF-1 (200 ng/ml) increased thymidine incorporation by 42 ± 2% while co-treatment with C3 transferase (50 μg/ml) reduced thymidine incorporation by 56 ± 1% (p < 0.05 for both compared with PDGF). To determine the degree that geranylgeranylation and farnesylation contribute to PDGF-induced SMC DNA synthesis, we investigated the effects of inhibitors of geranylgeranyltransferase and farnesyltransferase on PDGF-induced thymidine incorporation. The geranylgeranyltransferase inhibitor (GGTI-286, 30 μM) decreased PDGF (6 ng/ml)-induced thymidine incorporation by 91 ± 5% (p < 0.01) while the farnesyltransferase inhibitor (FTI-276, 10 nM) decreased...
PDGF (6 ng/ml)-induced thymidine incorporation by 37 ± 4% (p < 0.05) (Fig. 8B).

**DISCUSSION**

We have shown that the isoprenoid, geranylgeranyl, and the protein that it post-translationally modifies, Rho, mediates PDGF-induced cell cycle progression by down-regulating the expression of the Cdk inhibitor p27Kip1 and stimulating the activity of Cdk-2 and hyperphosphorylation of Rb. Treatment with the HMG-CoA reductase inhibitor, simvastatin, decreases Rho geranylgeranylation and membrane localization, inhibits Cdk activity and Rb hyperphosphorylation, and prevents SMC DNA synthesis. Indeed, Rho-induced Rb hyperphosphorylation and DNA synthesis are necessary and sufficient for serum- or PDGF-induced SMC DNA synthesis and are associated with decreases in p27Kip1 expression. These findings indicate an important role of Rho in PDGF-stimulated mitogenesis and are consistent with studies showing that p27Kip1 accumulation mediates cell cycle arrest (23, 24) and that the degradation of p27Kip1 facilitates the growth of rat FRTL-5 cells (25). Our findings, however, are in contrast to previous studies which suggest that PDGF-induced SMC DNA synthesis is mediated predominantly by Ras (17).

The Rho GTPase family which includes RhoA, RhoB, Rac, Cdc42 are major substrates for post-translational modification by geranylgeranylation. Geranylgeranyl modification is important for cellular trafficking and targets these Rho GTPases to the cellular membrane (10, 26). The membrane translocation of inactive or GDP-bound Rho involves the release of its cytoplasmic inhibitor, Rho guanine nucleotide dissociation inhibitor, and activation of Rho through GDP/GTP exchange in the presence of guanine nucleotide exchange factor (27–29). Since most of the cytoplasmic Rho proteins are inactive (GDP-bound state), their GTP binding activity remains invariably low even in the presence of HMG-CoA reductase inhibitor treatment. Thus, inhibition of Rho geranylgeranylation and membrane translocation by HMG-CoA reductase inhibitors leads to a greater accumulation of inactive Rho in the cytoplasm. This is consistent with our finding that in the presence of HMG-CoA reductase inhibitors, addition of GGPP, but not FPP, restores membrane expression of Rho, decreases p27Kip1 levels, and enhances cell cycle progression. Interestingly, in addition to increasing RhoA activity by GTP loading, we find that stimulation with PDGF increases the amount of membrane-associated RhoA. The mechanism by which PDGF increases RhoA in the membrane, however, remains to be determined.

The importance of Rho in SMC DNA synthesis was further confirmed by studies showing that inhibition of Rho by C. FIG. 8. A, effects of simvastatin (Statin, 5 μM), GGPP (5 μM), FPP (5 μM), and C3 transferase (C3, 50 μg/ml) on PDGF (6 ng/ml, 24 h)-induced SMC DNA synthesis. Experiments were performed three times in duplicates. *, p < 0.05 compared with PDGF. B, effects of farnesyltransferase inhibitor (FTI-276, 10 nM) or geranylgeranyltransferase inhibitor (GGTI-286, 30 μM) on PDGF (6 ng/ml, 24 h)-induced SMC DNA synthesis. Experiments were performed two times in quadruplicates. *, p < 0.05 compared with PDGF.
Rho has been shown to decrease p21 effects of treatment with HMG-CoA reductase inhibitors may be needed to determine the precise role of Ras in PDGF-induced progression in NIH 3T3 cells (30). Further studies, therefore, are necessary to determine the precise role of Ras in PDGF-induced SMC cell cycle progression.

Recent clinical data suggest that some of the beneficial effects of treatment with HMG-CoA reductase inhibitors may occur independent of changes in serum cholesterol levels (35). Our data indicate that there is a direct effect of these agents on the vessel wall via inhibition of Rho geranylgeranylation. We propose that Rho is a necessary and sufficient mediator of SMC cell cycle progression and that inhibition of Rho in SMC may have beneficial effects in vascular proliferative diseases such as atherosclerosis and post-angioplasty re-stenosis. The precise mechanism(s) by which Rho up-regulates p27^Kip1 expression, however, remains to be determined.

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