Mevastatin Can Cause G₁ Arrest and Induce Apoptosis in Pulmonary Artery Smooth Muscle Cells Through a \(p27^{\text{Kip1}}\)-Independent Pathway

Brian W. Fouty, David M. Rodman

Abstract—Advanced pulmonary arterial hypertension is characterized by extensive vascular remodeling that is usually resistant to vasodilator therapy. Mevastatin is an inhibitor of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase, the rate-limiting step for cholesterol synthesis. HMG-CoA reductase inhibitors have been shown to upregulate the cyclin-dependent kinase inhibitor \(p27^{\text{Kip1}}\) and to block cell proliferation through cholesterol-independent pathways. The aim of this study was to determine the effect of mevastatin on DNA synthesis, cell cycle progression, and cell proliferation in rat pulmonary artery smooth muscle cells (PASMCs). We found that mevastatin induced G₁ arrest and decreased DNA synthesis in rat PASMCs and did so in association with an increase in both total and cyclin E–bound \(p27^{\text{Kip1}}\). This caused a marked decrease in cyclin E kinase activity, which suggests an important role for \(p27^{\text{Kip1}}\) in the ability of mevastatin to induce G₁ arrest. However, in PASMCs lacking functional \(p27^{\text{Kip1}}\), mevastatin still decreased cyclin E kinase activity, caused G₁ arrest, and decreased DNA synthesis. In \(p27^{\text{Kip1}}\)-deficient PASMCs, mevastatin induced a greater reduction of cyclin E protein levels (to 35% of control) than in wild-type cells (to 70% of control) and also reduced the phosphorylation of cdk2 on threonine 160. Mevastatin also caused apoptosis in both wild-type and \(p27^{\text{Kip1}}\)-deficient PASMCs and was able to do so at a dose that did not induce cell cycle arrest. These data suggest that HMG-CoA reductase inhibitors can both inhibit cell proliferation and induce apoptosis in PASMCs through \(p27^{\text{Kip1}}\)-independent pathways and may be important therapeutic agents in pulmonary arterial hypertension. *(Circ Res. 2003;92:1111-1121)*

Key Words: vascular smooth muscle ■ pulmonary hypertension ■ \(p27^{\text{Kip1}}\) ■ HMG-CoA reductase

Pulmonary hypertension is a progressive and often fatal disease for which there is little effective treatment at present. Artery wall remodeling is a hallmark of severe, advanced pulmonary hypertension, presenting histologically as neointimal proliferation, medial and adventitial hyperplasia and hypertrophy, muscularization of peripheral vessels, and vaso-occlusive plexiform lesions. Pulmonary vascular remodeling can be initiated by a variety of stimuli, including chronic hypoxia, increased pulmonary blood flow, collagen vascular disease, anorectic and other drugs, and idiopathic causes.

The progression to neointimal, medial, and adventitial hyperplasia requires that normally quiescent pulmonary artery smooth muscle cells (PASMCs) enter and progress through the cell cycle. Holoenzymes known as cyclin-dependent kinases (cdk) and their regulatory subunits, the cyclins, are required to shepherd cells through G₁ and past the G₁/S transition, at which time the cell is committed to cell replication. Binding of the cdk to its specific cyclin leads to partial kinase activity, but full activity occurs only after phosphorylation by the cdk activating kinase (CAK). Although cyclin-cdk complexes are required throughout the entire cell cycle, they are under mitogenic control only through early and late G₁. Once a cell passes the restriction point, usually marked by the presence of active cyclin E complexes and hyperphosphorylated retinoblastoma (Rb), it no longer requires external stimuli to complete cell replication. Cdk activity is opposed by cdk inhibitors from either the INK4 (inhibitors of cdk4) or the Cip (cdk inhibitory proteins) family, each of which binds cyclin-cdk complexes and inhibits their activity.

3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, the statins, are important cholesterol-lowering agents that are effective in decreasing cardiac events. Recent studies demonstrate a direct inhibitory effect of these agents on vascular smooth muscle cell proliferation. The ability of statins to induce G₁ arrest and decrease cell growth is thought to be mediated partly through their upregulation of the cdk inhibitor \(p27^{\text{Kip1}}\). To further understand the antiproliferative effects of HMG-CoA reduc-
tase inhibitors in PASMCs, we studied the effect of mevastatin on the following two important parameters of PASMC response to serum stimulation: progression through the cell cycle (as determined by DNA synthesis and percentage of cells entering S phase) at 24 hours and proliferation (as determined by cell number) at 5 days. We also examined expression of cell cycle proteins critical for G1/S transition. We found that mevastatin caused G1 arrest and inhibited DNA synthesis through inhibition of cyclin E kinase activity. In wild-type cells, this appeared to be due to an increase in p27\(^{kip1}\)-cyclin E binding, whereas in p27\(^{kip1}\)-deficient cells, it was associated with a decrease in cyclin E protein levels and a decrease in activated cdk2. Mevastatin also induced apoptosis in both wild-type and p27\(^{kip1}\)-deficient cells. Induction of apoptosis occurred at a dose of mevastatin that minimally inhibited cyclin E kinase activity and did not cause G1 arrest, which suggests that mevastatin-induced cell cycle arrest and apoptosis were not linked. These studies indicate that al-

### Materials and Methods

#### Materials

- DMEM, Igepal, propidium iodide, RNase, mevastatin, and D-(-)mevalonic acid lactone were obtained from Sigma.
- Trypsin-EDTA and l-glutamine from Gibco; FBS from Gemini; and polyvinylidene difluoride (PVDF) membrane and ECL-Plus from Amersham (Buckinghamshire, England).
- The antibodies used were p21Cip1/Waf1 (c-19), p27Kip1 (c-19), Rb (both mouse monoclonal antibodies) were from BD Transduction Laboratories; cyclin A (c-19), and cyclin E (M-20) (all from Santa Cruz Biotechnology).
- p27Kip1 and Rb (both mouse monoclonal antibodies) were from Pharmingen. Secondary horse radish peroxidase–conjugated antibodies used were donkey-α-mouse, donkey-α-goat, and donkey-α-rabbit (from Jackson Laboratories).

#### Animals

Adult male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind) and housed in the University of Colorado Health Sciences Center animal facility before use. The p27\(^{kip1}\)-deficient mice were bred on a c57B6/sv129 background (the original breeding pair was obtained from Dr Andrew Koff at Memorial Sloan-Kettering Cancer Center, New York, NY). Genotype was confirmed by polymerase chain reaction (PCR) analysis using standard techniques. Animals were fed standard rodent chow and water ad libitum.

#### Cell Culture

Smooth muscle cells were isolated by elastase and collagenase digestion of main (extralobar) pulmonary arteries from adult (24- to 28-week-old) Sprague-Dawley male rats or adult (5- to 8-week-old) mice (see above) and cultured as previously described. Cells were used between passages 2 and 6 (p27\(^{kip1-/-}\) PASMCs were used between passages 3 and 10). Smooth muscle cell identity was verified by positive staining for smooth muscle α-actin (mouse monoclonal antibody, Sigma) at each passage (>95% of cells stained positive for smooth muscle α-actin).

#### [\(^{3}H\)]Thymidine Incorporation

Smooth muscle cells were plated in DMEM–10% FBS at a density of 20 000 cells/well in 24-well plates. After 24 hours, cells were growth-arrested by addition of DMEM with 0.1% FBS. After 72 hours of low serum, cells were labeled with [methyl-\(^{3}H\)]thymidine at 25 μCi/mL after the addition of DMEM with either 0.1% or 10% FBS with or without mevastatin. Mevastatin was reconstituted in DMSO. Cells were harvested 24 hours later in 1% SDS/0.01N NaOH. [\(^{3}H\)]Thymidine incorporation was determined in a Becton Dickinson LS6500 scintillation counter and normalized to cell number (cpm/cell).

#### Cell Proliferation

Smooth muscle cells were plated at 20 000 cells/well, growth-arrested for 72 hours, and then grown in DMEM supplemented with 10% FBS with or without mevastatin. Cells were removed from the wells by 0.05% trypsin/0.53 mmol/L EDTA digestion and counted at days 1, 3, and 5. Cells from 4 wells were counted using a Fischer hemocytometer and the results averaged to obtain a single cell count (±SE) for each time point.

#### Flow Cytometry

Cell cycle was determined by flow cytometry in propidium iodide–stained cells as described.

#### Western Blot Analysis

Protein was harvested from subconfluent cells and Western blots run as previously described. Protein expression was quantified using NIH Image 1.63 and expressed as arbitrary density units relative to control (cells incubated with 10% FBS+DMSO). Results were compared using ANOVA with the Fisher post hoc test, and \(P<0.05\) was considered significant.

#### Immunoprecipitation

Cell lysate (200 μg) was incubated with 2 μg of cyclin E antibody (rabbit polyclonal) and protein A–Sepharose beads (Santa Cruz) overnight at 4°C. Beads were washed twice with RIPA buffer, placed in sample buffer containing β-mercaptoethanol, boiled, separated on a 14% gel, and transferred to PVDF paper as described above. Rabbit nonimmune IgG antibody (2 μg) was used as a negative control.

#### Kinase Activity Assay

Cyclin E was immunoprecipitated as described above. Beads were washed twice with RIPA buffer and twice in cold kinase buffer containing, in mmol/L, HEPES (pH 7.5) 50, MgCl2 10, EGTA 2.5, DTT 1, β-glycerophosphate 10, NaF 1, and sodium orthovanadate 0.1, and 20 μmol/L ATP. Samples were resuspended in 30 μL of kinase buffer containing 5 μg of histone H1 (Upstate Biotechnology), Inh 10 μM of [γ\(^{32}\)P]ATP (Amersham Pharmacia Biotech); after incubation at 30°C for 30 minutes with occasional mixing, reactions were stopped by adding 30 μL of hot 2× Laemmli sample buffer and boiling for 5 minutes. Samples were resolved by SDS-PAGE in a 12% gel and phosphorylated proteins were detected by autoradiography.

#### Apoptosis

Cells were harvested by trypsin digestion as described above. After the final wash, cells were incubated for 15 minutes in annexin V and propidium iodide using theVybrant apoptosis assay kit (catalog No. 13242) from Molecular Probes. Cells were analyzed within 60 minutes in the University of Colorado Flow Cytometry core.

#### Statistical Methods

Data are expressed as mean±SEM. Thymidine incorporation, cell cycle, apoptosis, and cell growth were compared using ANOVA with Fisher post hoc test; a probability value of \(P<0.05\) was considered significant.

#### Results

Mevastatin Causes G1 Arrest and Decreases DNA Synthesis After Serum Stimulation

PASMCs were growth-arrested in 0.1% FBS for 72 hours and then stimulated with 10% FBS in the presence of increasing doses of mevastatin. DNA synthesis as measured by [\(^{3}H\)]thymidine incorporation was decreased in a dose-dependent manner.
Midine incorporation at 24 hours was reduced only at the highest dose of mevastatin (80 \( \mu \)mol/L). Similarly, 80 \( \mu \)mol/L of mevastatin caused G0/G1 arrest as indicated by a reduction in the percentage of PASMCs in S phase at 24 hours (Figures 1A and 1B). Mevalonate, the downstream product of HMG-CoA reductase, reversed the effect of mevastatin.

**Mevastatin Blunts the Serum-Induced Decrease in p27 \(^{Kip1}\) in PASMCs at 24 Hours**

Previous studies show that mitogenic stimulation of PASMCs decreased the cdk inhibitor p27 \(^{Kip1}\) expression at 24 hours, which was coincident with peak DNA synthesis and cell cycle progression.24 One important mechanism of statin-induced cell cycle arrest involves the upregulation of p27 \(^{Kip1}\).21–23,25 To determine whether mevastatin-induced G1 arrest was due to an increase in p27 \(^{Kip1}\) levels, growth-arrested PASMCs were stimulated with 10% FBS in the presence of increasing doses of mevastatin and protein harvested after 24 hours. As expected, stimulated PASMCs showed a marked reduction in p27 \(^{Kip1}\) levels to \(~30\%\) baseline; the addition of mevalonate increased p27 \(^{Kip1}\) expression 2-fold above control (DMSO + 10% FBS) at 80 \( \mu \)mol/L (Figure 2), although there was some variability among the cells from different animals. Mevastatin at 80 \( \mu \)mol/L slightly decreased cyclin E levels to \(~70\%\) of control (67±13%, \( n=5, P<0.05 \), Figure 2), but it markedly reduced cyclin A and PCNA protein expression consistent with its ability to prevent G1/S transition (Figure 2).

**Mevastatin Inhibits Cyclin E Kinase Activity at 24 Hours**

Cell progression through G1 and the G1/S transition requires active cyclin E complexes to complete the hyperphosphorylation of Rb. Hyperphosphorylation leads to the release of the transcription factor E2F, which is important in the transactivation of genes required for DNA synthesis.26,27 We studied the effect of mevastatin on cyclin E activity in two ways—by determining the degree of Rb hyperphosphorylation in Western blots and by determining in vitro cyclin E kinase activity using histone H1 as a substrate. Figure 3A demonstrates that only the highest dose of mevastatin inhibited the hyperphosphorylation of Rb. The ability of cyclin E to phosphorylate histone H1 in vitro showed a similar dose-related effect (to 33±6% of control at 80 \( \mu \)mol/L, \( n=4, P<0.05 \), Figure 3B).

These results demonstrate that at high doses, mevastatin can prevent the accumulation of active cyclin E complexes consistent with its ability to prevent G1/S transition in PASMCs.

**Mevastatin-Induced G1 Arrest Is Associated With Increased Binding of p27 \(^{Kip1}\) to Cyclin E**

Although the previous experiments clearly established that higher doses of mevastatin could inhibit cyclin E/CDK2 kinase activity, they did not explain the mechanism through which this occurs. We demonstrated in Figure 2 that mevastatin consistently increased total cellular p27 \(^{Kip1}\) in rat PASMCs. Given that the binding of p27 \(^{Kip1}\) to cyclin E/CDK2 complexes can reduce kinase activity, we immunoprecipitated cyclin E to determine whether mevastatin increased the amount of bound p27 \(^{Kip1}\). As Figure 3C shows, there was a 2.5-fold increase (\( n=3, P<0.05 \)) in p27 \(^{Kip1}\) binding to cyclin E at the highest dose of mevastatin. This suggests that the ability of mevastatin to cause G1/G1 arrest in PASMCs is due to an increase in p27 \(^{Kip1}\) binding to cyclin E.

![Figure 1. Mevastatin decreases DNA synthesis and arrests cell cycle progression in serum-stimulated PASMCs. Growth-arrested rat PASMCs were exposed to 10% FBS in the presence of increasing doses of mevastatin. A and B, [\(^{3}\)H]Thymidine incorporation (A) and percentage of cells in S phase (B) 24 hours after serum stimulation. Addition of mevalonate (L-mevalonic acid lactone) at 200 \( \mu \)mol/L reversed the inhibitory effect of mevastatin. Data are mean±SEM; \( n=5 \) separate experiments using PASMCs from 5 different animals; *\( P<0.05 \) vs DMSO control.](http://circres.ahajournals.org/content/3/3/119)
Figure 2. Mevastatin blunts the serum-induced decrease in p27^Kip1 levels. Western blot analysis of G0/G1 cell cycle proteins from cell lysates 24 hours after serum stimulation in the presence of increasing doses of mevastatin. Meva indicates coadministration of mevalonate at 200 μmol/L. Shown are representative blots from 4 separate experiments from 4 different animals.

Figure 3. Mevastatin inhibits cyclin E kinase activity and increases cyclin E–p27^Kip1 binding. Growth-arrested PASMCs were stimulated with 10% FBS in the presence of increasing doses of mevastatin and protein harvested 24 hours later. A, Western blot of cell lysates demonstrate that mevastatin blocks hyperphosphorylation of Rb (Rb-P). B, Autoradiograph demonstrates complete reduction of 32P incorporation into histone H1 from cyclin E immunoprecipitates in vitro. C, Cyclin E immunoprecipitates demonstrate an increase in p27^Kip1–cyclin E binding with increasing doses of mevastatin. All effects could be reversed by addition of the downstream product of HMG-CoA reductase, mevalonate. Results are representative of 4 different experiments from 4 different animals. IP indicates immunoprecipitation.
Mevastatin Reduces DNA Synthesis and Inhibits Cyclin E Kinase Activity in p27 Kip1-Deficient PASMCs

Our previous experiments suggested an important role for p27 Kip1 in mevastatin-induced G1 arrest through its binding and inhibition of cyclin E complexes. On the basis of these observations, we expected that mevastatin would be ineffective in causing G1 arrest in PASMCs lacking functional p27 Kip1. To test this hypothesis, growth-arrested PASMCs harvested from the main pulmonary artery of p27 Kip1-deficient mice were stimulated with 10% FBS in the presence of increasing doses of mevastatin. As Figure 4 indicates, mevastatin inhibited DNA synthesis and progression through the cell cycle despite the absence of p27 Kip1. Consistent with its ability to enforce G1 arrest, mevastatin also reduced cyclin E kinase activity as demonstrated by its inhibition of Rb hyperphosphorylation and the reduction of histone H1 phosphorylation in vitro (to 24 ± 6% of control, n = 3, P < 0.05, Figures 5A and 5B). In an attempt to explain this finding, we looked at other possible mechanisms through which mevastatin could block cyclin E activity in the absence of p27 Kip1. There was no increase in p21 Cip1 expression in mevastatin-treated p27 Kip1-deficient PASMCs (data not shown). Similarly, we did not detect an increase in the protein expression of p130, a member of the Rb family that has been shown to bind and inhibit cyclin E kinase activity in p27 Kip1-deficient mouse embryonic fibroblasts (data not shown).28 However, we did show that cyclin E protein levels were markedly reduced in p27 Kip1-null PASMCs treated with mevastatin (to 35 ± 9% of control, n = 6, P < 0.05) in contrast to the much smaller reduction in cyclin E levels seen in wild-type cells (Figure 5C). Reintroduction of p27 Kip1 using a replication-deficient adenovirus (provided by the University of Michigan Vector Core Laboratory) not only restored but increased cyclin E levels 2-fold above the vector control (2.2 ± 0.28 fold, n = 3, P < 0.05, Figure 5C). The increase in cyclin E occurred through a posttranscriptional mechanism, given that no difference in message could be detected by quantitative reverse transcriptase (RT)-PCR between the vector and p27 Kip1-infected cells (n = 3 experiments; data not shown). (The increase in cyclin E protein expression in control cells treated with vector + mevastatin relative to cells treated with mevastatin alone was due to a 7-fold increase in cyclin E transcription as determined by RT-PCR. This increase in cyclin E transcription was likely due to the inflammatory effect of the adenovirus on the cells and was the same in vector- and p27 Kip1-infected cells). Finally, we also showed that mevastatin blunted the phosphorylation of cdk2 on threonine 160—an event required for full kinase activity (Figure 5D).29

Mevastatin Induces Apoptosis in PASMCs

The previous experiments demonstrated that mevastatin could induce G0/G1 arrest and block DNA synthesis at high doses. We next wanted to determine whether inhibition of HMG-CoA reductase with mevastatin could decrease cell growth without inducing G1 arrest (ie, at lower doses of mevastatin that did not cause G1 arrest). We stimulated growth-arrested PASMCs with 10% FBS in the presence of increasing doses of mevastatin and determined cell growth (as determined by cell number) over 5 days. Figure 6 shows that cell number was reduced at days 3 and 5 at a dose of mevastatin (8 μmol/L) that did not induce G0/G1 arrest or block DNA synthesis at 24 hours (see Figures 1A and 1B). To determine whether the decrease in cell number at days 3 and 5 was due to the inhibition of cell proliferation by mevastatin or to its initiation of programmed cell death, we determined the amount of apoptosis at 72 hours. Figure 7 shows that both 8 and 80 μmol/L mevastatin caused apoptosis in PASMCs as...
determined by annexin V staining. The exclusion of propidium iodide confirmed that mevastatin did not cause cell necrosis. The addition of mevalonate, the direct downstream product of HMG-CoA reductase, completely reversed the mevastatin-induced apoptosis confirming a specific effect of HMG-CoA reductase inhibition. Apoptosis was confirmed by DNA laddering (not shown). These results indicate that G0/G1 arrest is not required for mevastatin-induced apoptosis.

Figure 5. Mevastatin can inhibit cyclin E kinase activity in the absence of functional p27Kip1. Growth-arrested PASMCs obtained from p27Kip1-deficient mice were stimulated with 10% FBS in the presence of increasing doses of mevastatin. A, Western blot of cell lysates demonstrates that mevastatin blocks hyperphosphorylation of Rb (Rb-P). B, Autoradiograph demonstrates decrease of P incorporation into histone H1 from cyclin E immunoprecipitates (IP) in vitro. C and D, Potential mechanisms to explain the p27Kip1-independent effect of mevastatin on cyclin E activity. C, Mevastatin markedly decreased cyclin E expression (to 35% control) in p27Kip1-deficient PASMCs—an effect that was reversed by reintroduction of functional p27Kip1 by adenoviral infection. D, Mevastatin partially inhibited the phosphorylation of cdk2 kinase on threonine 160 (cdk2-P) that is critical for full kinase activity. Shown are representative blots; n=3 different experiments from 2 different animals.

Figure 6. Mevastatin (Mev) decreases cell number over 5 days. Growth-arrested rat PASMCs were stimulated with 10% FBS and followed up over 5 days. Data are mean±SEM, n=5 separate experiments each done in quadruplicate using PASMCs from 5 different animals; *P<0.05 vs DMSO control.
Mevastatin Induces Apoptosis in p27 Kip1-Deficient PASMCs

To determine whether p27 Kip1 is required for mevastatin-induced apoptosis, we stimulated growth-arrested p27 Kip1–deficient PASMCs and determined cell number at 5 days and the amount of apoptosis at 3 days. As Figure 8A shows, mevastatin reduced cell number at 5 days, which is similar to results seen in wild-type PASMCs. Mevastatin also induced apoptosis at 3 days despite the absence of functional p27Kip1 (Figure 8B). The reintroduction of p27 Kip1 (by adenoviral infection) had no effect on mevastatin-induced apoptosis in p27Kip1-deficient PASMCs (data not shown). The addition of mevalonate prevented apoptosis.

Discussion

Reducing cholesterol synthesis by inhibiting the enzyme HMG-CoA reductase has been a cornerstone of treatment for coronary artery disease. Blocking the isoprenylation of small G proteins such as Ras, Rho, and Rac through the upstream inhibition of HMG-CoA reductase prevents their appropriate subcellular localization and has a profound inhibitory effect on cell proliferation. The specific mechanism through which blocking isoprenylation limits cell proliferation is not clear, however, and appears to vary depending on cell type. Given the important therapeutic implications of the antiproliferative effects of the statins on non–cholesterol-mediated vascular disease such as pulmonary arterial hypertension, we undertook these studies to determine the effect of HMG-CoA reductase inhibition on cell cycle progression, mitogenesis, and cell proliferation in PASMCs after serum stimulation. We demonstrate that mevastatin caused G1 arrest in PASMCs, but this effect was not dependent on p27Kip1. In addition, we found that mevastatin induced apoptosis at a dose that did not cause G1 arrest and that this apoptotic effect did not require functional p27Kip1. p27Kip1 is an important modulator of cell cycle progression that is upregulated in response to serum deprivation, cell-cell
contact, and transforming growth factor-β. In addition, the antiproliferative effects of rapamycin are mediated at least partly through increases in p27Kip1.32 p27Kip1 can bind cyclin D/cdk4, cyclin E/cdk2, and cyclin A/cdk2; inhibit their kinase activity; and cause cell cycle arrest. A number of studies have demonstrated that HMG-CoA reductase inhibitors increase cellular p27Kip1 levels, albeit through different mechanisms. In human vascular smooth muscle cells stimulated with platelet-derived growth factor, simvastatin increased p27Kip1 levels by preventing the isoprenoid modification and subsequent membrane activation of Rho and RhoA kinase.23 This prevented the Rho-mediated degradation of p27Kip1, decreased cdk2 and cdk4 kinase activity, and reduced DNA synthesis. Rao et al21 demonstrated that lovastatin increased p27Kip1 levels, but did so by blocking proteasome-mediated degradation of p27Kip1. Vidal et al20 demonstrated that inhibition of Rho activity by lovastatin can increase p27Kip1 translational efficiency. In normal and tumor breast cells, lovastatin did not increase p27Kip1 levels but did increase its binding to cdk2 through redistribution from cdk4.25 These studies, along with our observations in PASMCs that a decrease in p27Kip1 protein levels correlated with maximal DNA synthesis and cell cycle progression after serum stimulation,24 suggested that the ability of statins to increase or maintain p27Kip1 levels in response to mitogens was central to their antiproliferative effects. Our results demonstrate, however, that alternative mechanisms of inducing G1 arrest are available to the HMG-CoA reductase inhibitors.

In both wild-type and p27Kip1-deficient PASMCs, we demonstrated a reduction in cyclin E kinase activity in response to mevastatin. In cell lysates, Western blots showed a reduction in Rb hyperphosphorylation, and immunoprecipitates of cyclin E demonstrated reduced ability to phosphorylate histone H1 in vitro. An increase in both total and cyclin E–bound p27Kip1 was evident in wild-type cells treated with mevastatin, which is consistent with other reports suggesting that this may be an important pathway for enforcing G1 arrest in normal cells. Our observations that mevastatin could cause cell cycle arrest in p27Kip1-deficient PASMCs required us to look for alternative mechanisms through which mevastatin could reduce cyclin E kinase activity, however. We detected no increase in either total or cyclin E–bound p21Cip1 in p27Kip1-deficient PASMCs treated with mevastatin. We also studied whether mevastatin increased protein levels of p130, a member of the Rb family. Previously, Coats et al28 demonstrated that in mouse embryonic fibroblasts lacking both p21Cip1 and p27Kip1, p130 could bind to cyclin E and inhibit its kinase activity in serum-starved conditions. No increase in p130 levels could be detected even at the highest dose of mevastatin.

We did identify two potential mechanisms through which mevastatin could limit cyclin E kinase activity in the absence of p27Kip1. First, we showed that mevastatin markedly decreased cyclin E levels in p27Kip1-deficient cells. Although it did slightly reduce cyclin E levels in wild-type cells (to ~70% of control), it had a much more profound effect in reducing cyclin E levels in p27Kip1-deficient PASMCs (to ~35% of control). It appeared that the absence of p27Kip1 made cyclin E less stable in response to mevastatin because reintroduction of p27Kip1 into these cells increased cyclin E levels 2-fold above cells treated with the vector control. This increase above vector control was due to posttranscriptional effects, given that no difference in message could be detected by RT-PCR. Despite this increase in cyclin E after p27Kip1 overexpression, cyclin E kinase activity remained inhibited with cells arrested in G1 as demonstrated by the presence of hypophosphorylated Rb.

We also demonstrated a second potential pathway through which mevastatin could reduce cyclin E kinase activity in the absence of p27Kip1—by inhibiting the phosphorylation of

Figure 8. Mevastatin (Mev) can induce apoptosis in p27Kip1-deficient PASMCs. Growth-arrested PASMCs lacking p27Kip1 were stimulated with 10% FBS in the presence of increasing doses of mevastatin and followed up for 5 days. A, Cell number over 5 days. B, Percentage of cells undergoing apoptosis at 72 hours as determined by flow cytometry analysis of propidium iodide and annexin V–positive cells. Data are mean ± SEM, n=3 separate experiments using PASMCs from 2 different animals; *P<0.05 vs DMSO control.
cdk2 on threonine 160. Phosphorylation on this site by the cdk activating kinase (CAK) is critical for complete cdk2 activation. Mevastatin was able to reduce, although not completely block, phosphorylation on threonine 160. Mevalonate, but not reintroduction of p27Kip1, reversed this effect on phosphorylation. Whether this degree of inhibition is sufficient to reduce cdk2, and ultimately cyclin E, kinase activity is not clear.

We also show that mevastatin caused apoptosis in PASMCs and did so at a dose much lower than that required to induce G1 arrest. Although there was a tendency toward G1 arrest at a lower dose of mevastatin (8 μmol), significant arrest occurred only at a higher dose (80 μmol). In contrast, 8 μmol caused marked apoptosis at 72 hours, demonstrating that G1 arrest is not required for mevastatin-induced apoptosis. The selective staining for annexin V with the exclusion of propidium iodide confirmed that mevastatin induced apoptosis rather than necrosis in these cells. Mevastatin was also able to induce apoptosis in PASMCs lacking p27Kip1, confirming that this cdk inhibitor is not required for this effect.

The cholesterol-independent antiproliferative effects of the HMG-CoA reductase inhibitors make them attractive therapeutic agents in a variety of diseases. A 43% reduction in newly diagnosed colorectal cancer cases during a 5-year follow-up in patients with coronary artery disease was documented in patients taking pravastatin. In prostate, breast, and colon cancer cell lines, HMG-CoA reductase inhibitors have been shown to induce G1 arrest. Therefore, understanding the mechanism through which these agents work in different cell types is of great interest. One important putative pathway through which these agents work is through inhibition of cell cycle progression via the upregulation of the cdk inhibitor p27Kip1. Our studies are consistent with these observations. We extend these findings, however, to demonstrate that p27Kip1 is not required for the ability of mevastatin to cause G1 arrest or apoptosis. This has important implications not only for diseases such as colon, breast, and prostate cancer, in which decreased p27Kip1 levels portend a poor prognosis, but also in primary pulmonary hypertension, in which p27Kip1 expression is reduced or absent.

In summary, we have shown that inhibiting the enzyme HMG-CoA reductase with mevastatin can cause cell cycle arrest and apoptosis in PASMCs. The dose needed to cause apoptosis is much lower than that required to induce cell cycle arrest and indicates that these two processes are not linked in PASMCs. We also established that, although the cdk inhibitor p27Kip1 may be important in the antiproliferative and apoptotic effects of mevastatin under normal conditions, it is not required for its function. The ability of the statins both to block cell proliferation and to induce apoptosis makes them important potential therapies for non–cholesterol-mediated vascular disease such as pulmonary arterial hypertension. These agents would be expected to work even in diseases in which the cdk inhibitor p27Kip1 has been downregulated or lost.

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