Simvastatin Potently Induces Calcium-dependent Apoptosis of Human Leiomyoma Cells*

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Statins are drugs commonly used for the treatment of high plasma cholesterol levels. Beyond these well known lipid-lowering properties, they possess broad-reaching effects in vivo, including antitumor effects. Statins inhibit the growth of multiple tumors. However, the mechanisms remain incompletely understood. Here we show that simvastatin inhibits the proliferation of human leiomyoma cells. This was associated with decreased mitogen-activated protein kinase signaling and multiple changes in cell cycle progression. Simvastatin potently stimulated leiomyoma cell apoptosis in a manner mechanistically dependent upon apoptotic calcium release from voltage-gated calcium channels. Therefore, simvastatin possesses antitumor effects that are dependent upon the apoptotic calcium release machinery.

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Significance: Statins may have antitumor properties significant for the treatment of human uterine leiomyomas.

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Statins are HMG-CoA reductase inhibitors that block an early step of the mevalonate pathway of cholesterol synthesis. They have been used for many years for treatment of hypercholesterolemia. In addition, statins have been demonstrated to have profound and broad-reaching effects on certain types of tissues beyond their well known properties as lipid-lowering drugs (1, 2). These include effects on endothelial cell function, oxidative stress, angiogenesis, anti-inflammatory and antiallergic actions, and effects on cellular proliferation (3–5). The effects of statins on proliferation are cell type-specific. Although they induce proliferation in certain cell types, e.g. endothelial progenitor cells (6), they inhibit proliferation of vascular smooth muscle cells, hepatocytes, and other cell types (7–10). Tumors whose growth have been shown to be inhibited by statins include breast cancer (9, 11), ovarian cancer (12, 13), prostate cancer (14), colon cancer (15) leukemia (16), and, more recently, certain variants of lung cancer (17).

The mechanisms by which statins inhibit tumor growth are incompletely understood. Multiple mechanisms have been proposed, including blocking protein geranylgeranylation (18), activation of the mitochondrial pathway of apoptosis (16, 19), arrest of cell cycle progression, and inhibition of invasion through modulating RhoA-dependent signaling pathways (20).

Although the effects of statins on certain tumors have been demonstrated to depend on HMG-CoA reductase expression (13), they have been found to be independent of HMG-CoA reductase in other cell types (21).

Uterine leiomyomas (fibroids and myomas) are the most common tumors of the female reproductive tract (22). Uterine fibroids are characterized by smooth muscle proliferation and excessive extracellular matrix deposition. Although several complex cellular and molecular signaling network abnormalities have been described as initiators and promoters in the development and growth of leiomyomas (23), their exact etiology is not well understood. In fact, multiple genetic, familial, sex steroid, and growth factor abnormalities have been associated with the development of uterine leiomyomas (24–26).

Here we report, for the first time, that simvastatin (a semi-synthetic lipophilic HMG-CoA reductase inhibitor) inhibits the proliferation of human leiomyoma cells. In addition, we demonstrate that this antiproliferative effect is associated with modulation of ERK1/2 signaling and alterations in cell cycle progression. Moreover, we demonstrate that simvastatin induces apoptosis in human leiomyoma cells. Intracellular calcium chelation completely inhibited apoptosis induced by simvastatin. Mechanistically, activation of L-type voltage-gated calcium channels likely mediates calcium-dependent apoptosis induced by simvastatin. Therefore, we identified a novel
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calcium-dependent pathway by which simvastatin induces apoptosis in tumor cells.

EXPERIMENTAL PROCEDURES

Materials—Simvastatin was purchased from Cayman Chemicals (Ann Arbor, MI). It was dissolved in dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (10 mM) were prepared and kept at −20 °C until use. The final concentration of DMSO in culture medium was 0.1% v/v. Complete protease inhibitor mixture without EGTA was purchased from Roche Applied Science. Z-DEVD-R110 used for the caspase-3 assay was purchased from American Peptide Co. (Sunnyvale, CA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was reagent was purchased from Calbiochem (Darmstadt, Germany). Collagenase and deoxyribonuclease I (used for primary cell isolation), propidium iodide, ribonuclease A (used for cell cycle analysis), and mibefradil and SKF96365 and the specific T-type voltage-gated calcium channel blocker NNC 55-0396 were purchased from Cayman Chemicals. Fura-2/AM and 1,2-Bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid tetrakis(acetoxy-methyl ester) (BAPTA-AM) were purchased from Molecular Probes and Invitrogen, respectively. U-73122, a cell-permeable, isotype non-selective phospholipase C inhibitor, was purchased from MP Biomedicals (Solon, OH). Xestospongin C, a cell-permeable, potent blocker of inositol 1,4,5-trisphosphate (IP3)-mediated calcium release, was purchased from EMD Millipore Corp. (Billerica, MA). Cells were treated with U-73122, xestospongin C, mibefradil, SKF96365, nimbipinone, and NNC 55-0396 for 30 min before simvastatin treatment.

Antibodies—Monoclonal anti-human smooth muscle actin (27) was purchased from Dako (Glostrup, Denmark). Alexa Fluor-labeled secondary antibody was purchased from Invitrogen. Monoclonal anti-ERK1/2, anti-phospho-ERK1/2, anti-proliferating cell nuclear antigen, and anti-Bim antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-α-tubulin antibody and anti-β-actin, used as a loading control, were purchased from Abcam (Cambridge, MA) and ThermoFisher Scientific (Rockford, IL), respectively. Rabbit polyclonal anti-pan α1 voltage-gated calcium channels (L-type) was purchased from Millipore Corp. Rabbit polyclonal anti-voltage-gated calcium channels (T-type) CACNA1G antibody was purchased from Novus Biologicals (Littleton, CO).

Immortalized and Primary Cells—The immortalized human leiomyoma cell line (HuLM) was a gift from Dr. Salama A. Salama (Baylor College of Medicine, Houston, TX). In this cell line, human leiomyoma cells obtained from a patient after surgery were immortalized using a retroviral vector carrying human telomerase reverse transcriptase (27). Cells were con-firmed to maintain expression of molecular markers of human leiomyoma cells, including estrogen and progesterone receptors and smooth muscle actin (27). HuLM cells were cultured and maintained in smooth muscle basal medium containing 5% FBS, 0.1% insulin, 0.2% basic human fibroblast growth factor, 0.1% gentamicin/amphotericin B, and 0.1% human epidermal growth factor, all purchased from Lonza (Walkersville, MD). Cells were incubated in a 5% CO2 atmosphere at 37 °C and split when 70–80% confluent. Primary non-pregnant human leiomyoma and myometrial cells were obtained from patients undergoing a hysterectomy for symptomatic uterine fibroids at the University of Texas Medical Branch John Sealy Hospital (Galveston, TX). The study protocol was reviewed and approved by the institutional review board at the University of Texas Medical Branch. Primary leiomyoma cells and primary myometrial cells were isolated using a modification of a protocol described previously (28). In brief, tissues were obtained immediately after surgical excision. After washing in cold sterile DPBS solution without calcium or magnesium (Sigma-Aldrich), tissues were mechanically minced into approximately 1-mm pieces. Next they were agitated at 37 °C for 4 h in sterile Hank’s buffered salt solution without phenol, calcium, or magnesium (Sigma-Aldrich) to which collagenase and deoxyribonuclease I were added. The medium was subsequently filtered through a 70-μm filter and cultured in DMEM/F12 Ham 1:1 mixture (Sigma-Aldrich) supplemented with HEPES, pyridoxine, l-glutamine, 10% FBS, and 1% penicillin/streptomycin (v/v). The medium was changed on a daily basis. Cells were split when 70–80% confluent and used without further passaging to maintain the differentiated phenotype.

Immunocytochemistry—Immunocytochemistry was performed using a modification of a method described previously (29). In brief, cells were seeded on poly-l-lysine-coated coverslips. After reaching 70–90% confluence, the medium was aspirated, and cells were washed with 1× PBS solution. Slides were then fixed using a 4% paraformaldehyde solution, followed by quenching with 30 mm glycine and permeabilization with 0.25% Triton X-100 in 1% BSA solution. The slides were blocked with 2% BSA and stained with monoclonal anti-human smooth muscle actin followed by Alexa Fluor 594-labeled secondary antibody. Finally, the slides were exposed to DAPI nuclear stain and mounted. Images were acquired on a Nikon Eclipse TE2000-U epifluorescent inverted light microscope using a x40 oil immersion objective (SuperFluor, Nikon). QED Capture software, part of QED Imaging Solutions (Media Cybernetics, Inc., Rockville, MD) was used for image acquisition.

MTT Assay—An MTT assay was used to monitor cellular proliferation. HuLM cells were seeded into 96-well plates. Cells were treated with medium containing 0, 0.1, 0.5, 1, 5, and 10 μM simvastatin, and the MTT assay was performed as described previously (30). We chose to investigate doses of simvastatin ranging from 0.1–10 μM. This decision was made on the basis of dosages currently used to treat hypercholesterolemia (up to 1 mg/kg body weight/day), which is associated with peak serum levels of ~0.3 μM (31, 32). The maximum tolerated dose of simvastatin in humans has been found to be 15 mg/kg body weight/day (33), therefore providing the rationale of simvastatin concentrations of 0.1–10 μM.

3 The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BAPTA-AM, 1,2-Bis(2-aminoethoxy)ethane-NN,N',N'-tetracetic acid tetrakis(acetoxy-methyl ester); IP3, inositol 1,4,5-trisphosphate; IP,R, inositol 1,4,5-trisphosphate receptor; SIMV, simvastatin; DMSO, dimethyl sulfoxide.
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**Cell Cycle Analysis**—Cells were plated in medium containing serum and growth factors. The next day, the medium was changed, and the cells were kept overnight in serum-free, growth factor-free medium for cell cycle synchronization. Thereafter, the cells were treated for 3 h with simvastatin in serum-free, growth factor-free medium before replacing the medium with various simvastatin concentrations in serum- and growth factor-replete medium. After 48 h, cells were collected, and cell cycle analysis was performed as described previously (34), with some modifications. In brief, the cells were fixed and permeabilized by adding cold absolute acetone-free methanol while vortexing. Cells were saved in cold methanol until flow cytometric analysis (less than 7 days). Prior to flow cytometry, the cells were centrifuged, resuspended in a solution containing propidium iodide and ribonuclease A, and incubated in the dark for 30 min. Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences) using the FL1 parameter with an excitation wavelength at 488 nm and emission at 670 nm. Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Gating was used to exclude cell aggregates. Thereafter, cell cycle analysis was performed, and the percentage of cells in each cell cycle phase was calculated. In addition, the pre-G$_0$/G$_1$ (apoptotic) cell population was calculated.

**Cell Death Analysis**—Propidium iodide staining for the quantification of cell death was performed as described previously (35). The percentage of propidium iodide-positive cells was counted in a blinded manner. Caspase-3 activity was measured using a quantitative fluorometric assay as described previously (36).

**Cytosolic Calcium Imaging**—HuLM cells were seeded onto coverslips in 6-well plates. Thereafter, cells were treated with 0.1 μM simvastatin as well as DMSO (control). After 24 h, resting cytosolic calcium levels were quantified using Fura-2/AM. In brief, the medium was replaced with imaging solution (107 mM NaCl, 7.25 mM KCl, 20 mM HEPES, 2.5 mM MgCl$_2$, and 11.5 mM glucose (pH 7.2)) containing 2.5 μM Fura-2/AM for 20 min at room temperature while protected from light. Next, the solution was aspirated, replaced with imaging solution without Fura-2/AM, and incubated for another 20 min at room temperature protected from light. Coverslips were then examined under ×40 oil immersion using a Nikon Eclipse TE2000 epifluorescence inverted microscope. Images were captured (340 and 380 nm excitation, 510 nm emission) and transferred to a personal computer using MetaFluor® imaging software (Molecular Devices). Five fields on each coverslip were chosen randomly, and cytoplasmic regions of interest in all cells in the field were quantified. This was repeated two more times for a total of three separate experiments comprising hundreds of cells. The ratiometric data were converted to calcium concentrations using a calibration curve generated from a Calcium Calibration buffer kit (Molecular Probes, Eugene OR). The data in Fig. A1 are represented as a histogram to better appreciate the heterogeneity in the cytoplasmic calcium. To measure calcium release kinetics continuously during the first 5 h of simvastatin exposure, we used the genetically encoded calcium indicator protein GCaMP6s (37). The expression plasmid driving the expression of GCaMP6s off a CMV promoter was provided by Dr. Douglas Kim (HHMI Janelia Farm) and obtained through Addgene (plasmid 40753). The HuLM cells were transfected with Lipopectamine 3000 and imaged after 48–72 h. Fluorescence was monitored by excitation at 480 nm and emission at 510 nm. Cells were imaged at 37 °C in growth medium. Images were taken every 30 s for 5 h. For each experiment, ~10–20 cells could be imaged simultaneously. After acquiring baseline calcium measurements, cells were treated with vehicle, 0.1 μM, 1 μM or 10 μM simvastatin. Each experiment was repeated 3 times with essentially identical results. The data in Fig. 4, D–G, are a representative experiment showing the calcium responses in all cells on a single coverslip. The number of cells responding to simvastatin treatment with a calcium transient during recording was quantified and expressed as percent responders in Fig. 4I. A response was defined as a transient calcium release event of amplitude at least 0.1 normalized fluorescence units.

**Mitochondrial Calcium Imaging**—Mitochondrial calcium was measured by loading the cells with 1 μM Rhod-2/AM for 30 min at 37 °C in imaging solution. Cells were imaged by excitation at 565 nm and monitoring emission at 610 nm. At least five fields on each coverslip were chosen randomly, and mitochondrial regions of interest in all cells in the field were quantified. This was repeated two more times for a total of three separate experiments from which the data were pooled.

**Mitochondrial Membrane Potential**—Mitochondrial membrane potential was measured using the cationic dye JC-1 (Invitrogen/Molecular Probes). This dye is red in polarized mitochondria and green in depolarized mitochondria. Cells were loaded with 10 μg/ml JC-1 for 10 min at 37 °C. Red and green emissions were monitored simultaneously by excitation at 480 nm and emission at 620 and 525 nm. The ratio of red: green was used as a measure of mitochondrial membrane potential, with a drop in this ratio indicative of depolarization. As in Rhod-2 imaging, five fields on each coverslip were chosen randomly, and mitochondrial regions of interest in all cells in the field were quantified. This was repeated two more times for a total of three separate experiments from which the data were pooled.

**Intracellular Calcium Chelation**—Cells were seeded into 6-well plates. Thereafter, three wells were loaded with BAPTA-AM at 1, 10, and 20 μM concentrations. After 30 min, the medium was changed to BAPTA-AM-free medium and treated with simvastatin (10 μM). In addition, three more wells were treated with DMSO (control). BAPTA-AM (10 μM) without simvastatin, and simvastatin (10 μM) without BAPTA-AM. Forty-eight hours later, the morphologic appearance of cells was observed and recorded. Thereafter, cells were harvested, cell lysates were obtained, and a caspase-3 activity assay was performed as described previously (36).

**Statistical Analysis**—Experiments were performed in triplicates and repeated independently at least three times. Whenever applicable, data are presented as mean ± S.E., and Student’s t test was used for statistical analysis. Results were considered statistically significant when $p < 0.05$. We used SigmaPlot software (Systat Software Inc., San Jose, CA) for statistical analysis.
RESULTS

Simvastatin Inhibits the Proliferation of Leiomyoma Cells—
Treatment of a human leiomyoma cell line (HuLM) (27) with simvastatin induced dose-dependent morphological changes consistent with the inhibition of cellular proliferation and induction of cell death (Fig. 1A). Almost identical results were found with primary leiomyoma cells isolated from a patient (Fig. 1B). We confirmed that isolated primary cells are smooth muscle-derived by performing immunocytochemistry for smooth muscle actin (38) (data not shown). To quantify the effects of simvastatin on cellular proliferation, we performed an MTT assay. Simvastatin induced dose-dependent and time-dependent inhibition of cellular proliferation (Fig. 1, C and D). These findings are evident at concentrations as low as 100 nM, whereas doses of 1 μM or more were associated with cell death. These effects were evident at 48 h and were more pronounced at 72 h of treatment (Fig. 1D). To confirm that simvastatin inhibited cellular proliferation, we investigated protein levels of proliferating cell nuclear antigen (PCNA, E) and phosphorylated and total ERK expression (F) along with quantification. α-Tubulin was used as a loading control. *, p < 0.05; **, p < 0.01 versus 0 μM.

FIGURE 1. Antiproliferative effects of simvastatin on human leiomyoma cells. A and B, morphologic effects of simvastatin (SIMV) treatment for 48 h on HuLM cells (A) and primary human leiomyoma cells (B). C and D, MTT proliferation assay results for cells treated with 0–10 μM SIMV with treatment end points at 24, 48, and 72 h showing a dose-response curve (C) and a time-response curve (D), a.u., arbitrary units. In D, the significance signs in the lower part of the graph apply to both 48 and 72 h. E and F, Western blotting showing the effects of SIMV treatment for 48 h on proliferating cell nuclear antigen (PCNA, E) and phosphorylated and total ERK expression (F) along with quantification. α-Tubulin was used as a loading control. *, p < 0.05; **, p < 0.01 versus 0 μM.
cells with different simvastatin concentrations for 48 h and monitored ERK phosphorylation. Simvastatin treatment was associated with significantly decreased ERK1/2 phosphorylation at concentrations of 1 μM and higher (Fig. 1F). Interestingly, there was a moderate increase in ERK phosphorylation at the lowest dose tested (0.1 μM). However, the significance of this effect is unclear because this dose was associated with reduced proliferation (Fig. 1C). Simvastatin increased the total amount of ERK1/2 at all concentrations, suggesting compensatory up-regulation of the kinase by the tumor cells.

**Simvastatin Induces Changes in Cell Cycle Progression**—To determine the effects of simvastatin on cell cycle progression, we performed flow cytometry. Synchronized cells were treated with different concentrations of simvastatin in addition to vehicle. After 48 h, the cells were collected and cell cycle analysis was performed. Simvastatin induced a dose-dependent decrease in the number of cells in S phase and a concomitant increase in the sub-G₀/G₁ (apoptotic) population (Fig. 2, A–H).

**Simvastatin Induces Apoptosis of Leiomyoma Cells**—We next examined whether simvastatin induces apoptotic cell death. As
shown in Fig. 3A, simvastatin induces a dose-dependent increase in cell death, as demonstrated by the percentage of propidium iodide (PI)-positive cells. We next examined whether simvastatin-induced cell death was apoptotic in nature by measuring caspase-3 enzymatic activity. As shown in Fig. 3B, simvastatin treatment resulted in a dose-dependent and robust increase in caspase-3 activation, which was evident at concentrations as low as 0.1 μM. These results are consistent with the effects on morphology, proliferation, and cell cycle progression, suggesting that simvastatin potently induces leiomyoma cell death at therapeutically relevant concentrations.

Simvastatin Increases Expression of Bim—We next wanted to further elucidate the underlying mechanism of simvastatin-induced apoptosis and to examine whether it is linked to its proliferation-inhibitory effect. It is known that ERK1/2 promotes the degradation of the Bim spliceform BimEL (41). BimEL is a proapoptotic BH3-only member of the Bcl-2 family that induces apoptosis by binding to and antagonizing antiapoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL. BimEL expression and activity goes up following decreases in ERK/MAPK activity and, therefore, is a cytosolic proapoptotic sensor of growth factor deprivation. Therefore, we examined the effect of simvastatin treatment on BimEL protein levels. As shown in Fig. 3C, simvastatin treatment increases BimEL protein levels, consistent with decreased ERK activation (Fig. 1F) and the induction of apoptosis (Fig. 3, B and C). The doses at which we observed increased BimEL expression were somewhat lower than those observed for pERK (Fig. 1F). However, the effects of simvastatin on ERK activity and expression are complex, with even the lowest dose (0.1 μM) increasing total ERK protein levels, suggesting ERK inhibition at this dose and a concomitant compensatory increase in ERK protein levels. Regardless of the mechanism, simvastatin dose-dependently increases BimEL levels with direct implications for apoptotic signaling.

Simvastatin Induces Cytosolic Calcium Release—We next sought to further determine the mechanism by which simvastatin induces apoptosis of leiomyoma cells. Given reports that statins can affect cellular calcium homeostasis (42) and the central role of calcium in many apoptotic paradigms (36), we determined whether simvastatin-mediated apoptotic cell death is associated with engagement of the calcium signaling machinery. Treatment with 0.1 μM simvastatin for 24 h increased resting cytosolic calcium in HuLM cells to an extent consistent with the activation of calcium-dependent apoptosis in other paradigms (Fig. 4A) (43). Chronically elevated cytosolic calcium during apoptosis, as shown in Fig. 4A, results in mitochondrial accumulation of calcium, mitochondrial depolarization, and release of proapoptotic factors. Consistent with apoptotic calcium release, we found that treatment with 0.1 μM simvastatin for 24 h significantly increased mitochondrial calcium levels (Fig. 4B) and associated mitochondrial depolarization (Fig. 4C). Many apoptotic stimuli induce calcium release very early in the apoptotic program (minutes to hours) (44), and this is useful to discern whether the calcium elevations are causative or a consequence of the apoptotic program (45). To measure the effects of simvastatin early in the apoptotic program, we measured cytosolic calcium continuously for the first 5 h after simvastatin administration using the genetically encoded calcium indicator GCaMP6s (37). Simvastatin dose-dependently increased the spiking activity of HuLM cells, consistent with apoptotic calcium release (Fig. 4, D–F). Finally, to determine whether cytosolic calcium release is absolutely required for simvastatin-induced apoptosis, we chelated intracellular calcium by loading the cells with BAPTA-AM. Loading HuLM cells with BAPTA dramatically and dose-dependently protected HuLM cells against simvastatin-induced morphologic changes (Fig. 4I) and prevented simvastatin-induced induction of caspase-3 activity (Fig. 4J) and cell death (Fig. 4K). Therefore, simvastatin-in
duced cytosolic calcium elevations are required for leiomyoma cell apoptosis.

Simvastatin-induced Apoptosis Is Not Mediated by IP₃Rs—As calcium-mediated apoptosis is most commonly associated with inositol 1,4,5-trisphosphate receptor (IP₃R)-mediated calcium release from the endoplasmic reticulum (36, 44, 46, 47), we examined the role of IP₃R in simvastatin-induced apoptosis. We used xestospongin C, a cell membrane-permeable inhibitor of IP₃R and the endoplasmic reticulum Ca²⁺/H⁺ ATPase (48, 49). We found that xestospongin C did not prevent simvastatin-
induced apoptosis (Fig. 5A). To further confirm that IP$_3$R do not contribute to simvastatin-induced cell death, we used the cell membrane-permeable phosphoinositide-specific PLC inhibitor U73122 (50, 51). We found that U73122 has no effect on simvastatin-induced apoptosis, as measured by caspase-3 activity. Therefore, the phospholipase C/IP$_3$R pathway does not appear to be involved in simvastatin-induced apoptosis of HuLM cells.

Simvastatin-induced Apoptosis Requires Voltage-gated Calcium Channel Activity—We next examined whether activation of plasma membrane calcium channels contributes to simvas-

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**FIGURE 5.** Simvastatin-induced apoptosis in human leiomyoma cells is not dependent on ER calcium release. A and B, pretreating cells with the IP$_3$R inhibitor xestospongin C (XesC) (A) or the cell membrane-permeable phosphoinositide-specific PLC inhibitor U73122 (B) does not prevent simvastatin-induced apoptosis, as measured by caspase-3 activity.

**FIGURE 6.** Simvastatin-induced apoptosis is inhibited by non-selective voltage-gated calcium channel inhibitors. A and B, pretreating cells with the voltage-gated calcium channels blockers mibebradil (A) or SKF96365 (SKF) (B) prevents simvastatin-induced apoptosis, as measured by caspase-3 activity. C, pretreatment with the highly selective L-type voltage-gated calcium channel inhibitor nimodipine prevents simvastatin-induced apoptosis, as measured by caspase-3 activity. *, $p < 0.05$; **, $p < 0.01$ versus 10 $\mu$M SIMV. D, Western blotting for expression of L-type (L-Ca$_{v}$, top blot) and T-type (T-Ca$_{v}$, bottom blot) voltage-gated calcium channels in human leiomyoma cells. We observed no significant changes in expression levels after simvastatin treatment.
Simvastatin-induced Apoptosis Requires L-type Voltage-gated Calcium Channel Activity—We further sought to determine whether simvastatin-induced apoptosis requires L- or T-type channels. Treatment with the L-type selective voltage-gated calcium channel blocker nimodipine potently inhibited simvastatin-induced apoptosis in a dose-dependent manner (Fig. 6C). Treatment of cells with the T-type selective voltage-gated calcium channel blocker NNC 55-0396 by itself led to rapid detachment and death of cells, which precluded analysis.

Simvastatin Treatment Does Not Affect the Expression of L- or T-type Voltage-gated Calcium Channels—We next examined whether the effects of simvastatin are mediated through modulating the expression of L- or T-type voltage-gated calcium channels. Western blotting showed that treatment with simvastatin does not significantly affect the expression of either L- or T-type channels (Fig. 6D). This is consistent with simvastatin increasing the activity of existing L-type calcium channels (either directly or indirectly) to increase apoptotic calcium release.

**DISCUSSION**

The results of this study demonstrate that simvastatin inhibits the proliferation of human uterine leiomyoma cells. In addition, we have shown that inhibition of proliferation is associated with inhibition of ERK phosphorylation, a common pathway for growth factor signaling. Furthermore, our results show that simvastatin causes cell cycle progression arrest and induces calcium-dependent apoptosis in human leiomyoma cells. We were not able to determine whether the effects of simvastatin were specific for leiomyoma cells versus normal myometrium because there are no reliable models for the culture and propagation of human myometrial smooth muscle. However, several lines of evidence suggest that statins may have specific antitumor effects in vivo. First, unlike normal myometrium, leiomyoma growth is dependent upon the autocrine production of steroids (59–61), which would be expected to be dramatically reduced in response to statin treatment. Second, leiomyoma growth, also unlike normal myometrium, is dependent upon continual stimulation with a variety of growth factors, such as insulin-like growth factor I (25, 26), platelet-derived growth factor (40), and epidermal growth factor (28). This growth factor “addiction” would make the tumor more susceptible to statin-dependent inhibition of Ras/ERK/MAPK signaling. Future in vivo studies will examine these possibilities.

FIGURE 7. Schematic cartoon showing the proposed mechanism of simvastatin inhibition of proliferation and induction of calcium-dependent apoptosis in human leiomyoma cells. Proximal effects include inhibition of growth factor signaling. Downstream effects include increased expression of the proapoptotic Bcl-2 family member protein Bim through decreased ERK-mediated degradation. This leads to increased Bim<sub>EL</sub> activity and mitochondrial leakage of apoptosis-initiating proteins, including cytochrome c. Permeabilization of mitochondria also requires activation of L- or T-type voltage-gated calcium channels and calcium influx into the mitochondria. RTK, receptor tyrosine kinase; CytC, cytochrome c; L-Ca<sub>V</sub>, L-type voltage-gated calcium channels.

The antitumor effects of statins have been described in a number of neoplasms, including those of the breast, ovaries, prostate, and colon, and leukemia (7–17). However, the exact mechanism of these antitumor properties is not completely understood. In this study, we found that calcium release is absolutely required for these effects and that it is mediated by voltage-gated calcium channels. Importantly, calcium chelation with BAPTA or incubation with calcium channel blockers completely suppressed the effects of simvastatin, suggesting that cytosolic calcium elevation is necessary for inducing leiomyoma cell death. T-type calcium channels, which are a class of low-voltage activated channels, are known to be up-regulated in tumors and are thought to be proproliferative (62). In contrast, the high voltage-activated L-type calcium channels have been shown to contribute to cell death in multiple models by promoting mitochondrial calcium uptake and cytochrome c release (Fig. 7) (63–65). Our results demonstrating that nimodipine potently suppresses simvastatin-induced toxicity (Fig. 6C) suggest that simvastatin increases the activity of L-type calcium channels (Fig. 7, dashed arrow). This leads to mitochondrial calcium influx and increased release of proapoptotic factors such as cytochrome c. Interestingly, it has been hypothesized by others that statins can directly increase the expression of L-type calcium channels in vascular smooth muscle cells (66). In our experimental paradigm, simvastatin did not alter total protein levels of L- or T-type channels. Future work will directly examine the contribution of L-type calcium channels in leiomyoma cell death and potential mechanisms of activation. Specifically, future experiments should study the effects of...
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L-type calcium channel blockers on calcium spikes, elevated basal calcium levels, and Rhod-2 fluorescence and better link these changes with simvastatin-induced apoptosis in leiomyoma cells.

We found that simvastatin decreases ERK activation, which may be related to the ability of statins to decrease isoprenylation of certain proteins such as Ras. Therefore, statin-induced leiomyoma cell death may represent an example of “trophic factor deprivation cell death,” as demonstrated previously (67) and supported by our findings of decreased ERK activation and increased Bim expression. As mentioned above, multiple growth factors affect the development and growth of uterine fibroids (39), including insulin-like growth factor I (25, 26), platelet-derived growth factor (40), and epidermal growth factor (28). Increased signaling through the Raf-MEK-ERK pathway can drive tumor growth (68). In addition, it is known that ERK phosphorylates the proapoptotic BH3-only protein BimEL, which leads to its degradation (41, 69, 70). BimEL is a central regulator of cell death induced by trophic factor deprivation (71) and is negatively regulated by ERK activity (41, 69, 70). Therefore, BimEL activation after simvastatin treatment is a plausible mechanism for increased mitochondrial calcium, membrane depolarization, cytochrome c release, and cell death (Fig. 7). Future studies will determine whether simvastatin inhibits leiomyoma growth in vivo.

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REFERENCES

1. McFarlane, S. I., Muniyappa, R., Francisco, R., and Sowers, J. R. (2002) Clinical review 145: pleiotropic effects of statins: lipid reduction and beyond. J. Clin. Endocrinol. Metab. 87, 1451–1458
2. Liao, J. K., and Laufs, U. (2005) Pleiotropic effects of statins. Annu. Rev. Pharmacol. Toxicol. 45, 89–118
3. McKay, A., Leung, B. P., McInnes, I. B., Thomson, N. C., and Liew, F. Y. (2004) A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. J. Immunol. 172, 2903–2908
4. Bruner-Tran, K. L., Osteen, K. G., and Duleba, A. J. (2009) Simvastatin protects against the development of endometriosis in a nude mouse model. J. Clin. Endocrinol. Metab. 94, 2489–2494
5. Wassmann, S., Laufs, U., Bäumer, A. T., Müller, K., Ahlborg, K., Linz, W., Itter, G., Rösen, R., Böhm, M., and Nickeng, G. (2001) HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. Hyperension 37, 1450–1457
6. Assmus, B., Uribich, C., Aicher, A., Hofmann, W. K., Haendeler, J., Rössig, L., Spyridopoulos, I., Zeiher, A. M., and Dimmeler, S. (2003) HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. Circ. Res. 92, 1049–1055
7. Demierre, M. F., Higgins, P. D., Gruber, S. B., Hawk, E., and Lippman, S. M. (2005) Statins and cancer prevention. Nat. Rev. Cancer 5, 930–942
8. Rombouts, K., Kisanga, E., Hellemans, K., Wielant, A., Schuppan, D., and Geerts, A. (2008) Effect of HMG-CoA reductase inhibitors on proliferation and protein synthesis by rat hepatocyte stellate cells. J. Hepatol. 38, 564–572
9. Seeger, H., Wallwiener, D., and Mueck, A. O. (2003) Statins can inhibit proliferation of human breast cancer cells in vitro. Exp. Clin. Endocrinol. Diabetes 111, 47–48
10. Stamm, J. A., and Ornstein, D. L. (2005) The role of statins in cancer prevention and treatment. Oncology 19, 739–750; discussion 753–734
11. Mäck, A. O., Seeger, H., and Wallwiener, D. (2004) Inhibitory effect of statins on the proliferation of human breast cancer cells. Int. J. Clin. Pharmacol. Ther. 42, 695–700
12. Martirosyan, A., Clendening, J. W., Goard, C. A., and Penn, L. Z. (2010) Lovastatin induces apoptosis of ovarian cancer cells and synergizes with doxorubicin: potential therapeutic relevance. BMC Cancer 10, 103
13. Kato, S., Smalley, S., Saradangani, A., Chen-Lin, K., Oliva, B., Brañas, J., Carvajal, J., Gejman, R., Owen, G. I., and Cuello, M. (2010) Lipophilic but not hydrophilic statins selectively induce cell death in gynaecological cancers expressing high levels of HMGCoA reductase. J. Cell Mol. Med. 14, 1180–1193
14. Hoque, A., Chen, H., and Xu, X. C. (2008) Statins induce apoptosis and cell growth arrest in prostate cancer cells. Cancer Epidemiol. Biomarkers Prev. 17, 88–94
15. Cho, S. J., Kim, J. S., Kim, J. M., Lee, J. Y., Jung, H. C., and Song, I. S. (2008) Simvastatin induces apoptosis in human colon cancer cells and in tumor xenografts, and attenuates colitis-associated colon cancer in mice. Int. J. Cancer 123, 951–957
16. Chapman-Shimshoni, D., Yuleka, M., Radnay, J., Shapiro, H., and Lishner, M. (2003) Simvastatin induces apoptosis of B-CLL cells by activation of mitochondrial caspase 9. Exp. Hematol. 31, 779–783
17. Ramakrishna, S., Andrei, A. C., Varlotto, J., Colosimo, A., Shelkey, J., Sehgal, V., Medford-Davis, L., Meyerson, S., de Hoyos, A., and Decamp, M. (2012) Statin use is associated with decreased local recurrence and improved overall survival in resectable non-small cell lung cancer (NSCLC). Chest 142, 925A
18. Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. (2001) Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia 15, 1398–1407
19. Cafforio, P., Dammaco, F., Gernone, A., and Silvestris, F. (2005) Statins activate the mitochondrial pathway of apoptosis in human lymphoblasts and myeloma cells. Carcinogenesis 26, 883–891
20. Denoyelle, C., Vasse, M., Körner, M., Mishal, Z., Ganné, F., Vannier, J. P., Soria, J., and Soria, C. (2001) Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis 22, 1139–1148
21. Rao, S., Porter, D. C., Chen, X., Herleczek, T., Lowe, M., and Keyomarsi, K. (1999) Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. Proc. Natl. Acad. Sci. U.S.A. 96, 7797–7802
22. Okolo, S. (2008) Incidence, aetiology and epidemiology of uterine fibroids. Best Pract. Res. Clin. Obstet. Gynaecol. 22, 571–588
23. Islam, M. S., Prodic, O., Storton, P., Grechi, G., Lamanna, P., Petraglia, F., Castellucci, M., and Ciarmela, P. (2013) Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. Fertil. Steril. 100, 178–193
24. Walker, C. L., and Stewart, E. A. (2005) Uterine fibroids: the elephant in the room. Science 308, 1589–1592
25. Peng, L., Wen, Y., Han, Y., Wei, A., Shi, G., Mizuguchi, M., Lee, P., Hernando, E., Mittal, K., and Wei, J. J. (2009) Expression of insulin-like growth factors (IGFs) and IGF signaling: molecular complexity in uterine leiomyomas. Fertil. Steril. 91, 2664–2675
26. Burroughs, K. D., Howe, S. R., Okubo, Y., Fuchs-Young, R., LeRoith, D., and Walker, C. L. (2002) Dysregulation of IGF-I signaling in uterine leiomyoma. J. Endocrinol. 172, 83–93
27. Carney, S. A., Tahara, H., Swartz, C. D., Risinger, J. I., He, H., Moore, A. B., Haseman, J. K., Barrett, J. C., and Dixon, D. (2002) Immortalization of human uterine myometrial tissue and smooth muscle cells after induction of telomerases activity: molecular and phenotypic characteristics. Lab. Invest. 82, 719–728
28. Rossi, M. I., Chegini, N., and Masterson, B. J. (1992) Presence of epidermal growth factor, platelet-derived growth factor, and their receptors in human myometrial tissue and smooth muscle cells: their action in smooth muscle cells in vitro. Endocrinology 130, 1716–1727
29. Malik, M., and Catherino, W. H. (2012) Development and validation of a three-dimensional in vitro model for uterine leiomyoma and patient-matched myometrium. *Fertil. Steril.* **97**, 1287–1293

30. Salama, S. A., KameI, M., Awad, M., Nasser, A. H., Al-Hendy, A., Botting, S., and Arrastia, C. (2008) Catecholcholastogens induce oxidative stress and malignant transformation in human endometrial glandular cells: protective effect of catechol-O-methyltransferase. *Int. J. Cancer* **123**, 1246–1254

31. Schmidtmaier, R., Baumann, P., Bumeder, I., Meinhard, G., Straka, C., and Emerich, B. (2007) First clinical experience with simvastatin to overcome drug resistance in refractory multiple myeloma. *Eur. J. Haematol.* **79**, 240–243

32. Corsini, A., Bellosta, S., Baetta, R., Fumagalli, R., Paioletti, R., and Bernini, F. (1999) New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol. Ther.* **84**, 413–428

33. van der Spek, E., Bloem, A. C., van de Donk, N. W., Bogers, L. H., van der Giended, R., Kramer, M. H., de Weerdt, O., Wittebol, S., and Lokhorst, H. M. (2006) Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma or lymphoma. *Haematologica* **91**, 542–545

34. Salama, S. A., KameI, M., Botting, S., Sahib, S. M., Borahay, M. A., Hamed, A. A., Kilic, S. G., Saeed, M., Williams, M. J., and Diaz-Arrastia, C. R. (2009) Catechol-O-methyltransferase expression and 2-methoxyestradiol affect microtubule dynamics and modify steroid receptor signaling in leiomyoma cells. *PloS ONE* **4**, e7356

35. Stieren, E. S., El Ayadi, A., Xiao, Y., Siller, E., Landsverk, M. L., Oberhauser, A. F., Barral, J. M., and Boehringer, D. (2011) Ubiquitin-1 is a molecular chaperone for the amyloid precursor protein. *J. Biol. Chem.* **286**, 35689–35698

36. Boehringer, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosaki, T., and Snyder, S. H. (2005) Simvastatin binds to inositol (1,4,5) triphosphate receptors, amplifying calcium-dependent apoptosis. *Nat. Cell Biol.* **8**, 1051–1061

37. Chen, T. W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., Schreiter, E. R., Kerr, R. A., Orger, M. B., Jayaraman, V., Looger, L. L., Svoboda, K., and Kim, D. S. (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**, 295–300

38. Mack, C. P., Somlyo, A. V., Hautmann, M., Somlyo, A. P., and Owens, G. K. (2001) Smooth muscle differentiation marker gene expression is regulated by inositol 1,4,5-trisphosphate receptors, amplifying calcium-dependent apoptosis. *PLoS ONE* **6**, e14357

39. Marsh, E. E., and Bulun, S. E. (2006) Steroid hormones and leiomyomas. *Obstet. Gynecol. Clin. North Am.* **33**, 59–67

40. Sozen, I., and Arici, A. (2002) Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyoma. *Fertil. Steril.* **78**, 1–12

41. Luciano, F., Jacquel, A., Colosetti, P., Herrant, M., Cagnol, S., Pages, G., and Auburger, P. (2003) Phosphorylation of Bim EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* **22**, 6785–6793

42. Sirvent, P., Mercier, J., Vassort, G., and Lacampagne, A. (2005) Simvastatin blocks intrinsic and extrinsic cell death pathways. *Fertil. Steril.* **83**, 33–41

43. Leung, Y. M., and Kwan, C. Y. (1999) Current perspectives in the pharmacological studies of store-operated Ca(2+)-entry blockers. *Jn. J. Pharmacol.* **81**, 253–258

44. Singh, A., Hildebrand, M. E., Garcia, E., and Snutch, T. P. (2010) The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. *Br. J. Pharmacol.* **160**, 1464–1475

45. Bulun, S. E., Simpson, E. R., and Word, R. A. (1994) Expression of the CYP19 gene and its product aromatase cytochrome P450 in human uterine leiomyoma tissues and cells in culture. *J. Clin. Endocrinol. Metab.* **78**, 736–743

46. Shozu, M., Murakami, K., Segawa, T., Kasai, T., and Inoue, M. (2003) Successful treatment of a symptomatic uterine leiomyoma in a perimenopausal woman with a nonsteroidal aromatase inhibitor. *Fertil. Steril.* **79**, 628–631

47. Shozu, M., Murakami, K., and Inoue, M. (2004) Aromatase and leiomyoma of the uterus. *Semin. Reprod. Med.* **22**, 51–60

48. Dziegielewski, B., Gray, L. S., and Dziegielewski, J. (2014) T-type calcium channels blockers as new tools in cancer therapies. *Pflugers Arch.* **466**, 801–810

49. Wildburger, N. C., Lin-Ye, A., Baird, M. A., Lei, D., and Bao, J. (2009) Neuroprotective effects of blockers for T-type calcium channels. *Mol. Neurodegener.* **4**, 44

50. Cano-Abad, M. F., Villarroya, M., García, A. G., Gablan, N. H., and López, M. G. (2001) Calcium entry blockers as new tools in cancer therapies. *Pflugers Arch.* **466**, 801–810

51. Dziegielewski, B., Gray, L. S., and Dziegielewski, J. (2014) T-type calcium channels blockers as new tools in cancer therapies. *Pflugers Arch.* **466**, 801–810

52. Tanaka, T., Nangaku, M., Miyata, T., Inagi, R., Ohse, T., Ingelfinger, J. F., and Fujita, T. (2004) Blockade of calcium influx through L-type calcium channels attenuates mitochondrial injury and apoptosis in hypoxic renal tubular cells. *J Am. Soc. Nephrol.* **15**, 2320–2333

53. Clunn, G. F., Sever, P. S., and Hughes, A. D. (2010) Calcium channel...
regulation in vascular smooth muscle cells: synergistic effects of statins and calcium channel blockers. *Int. J. Cardiol.* **139**, 2–6

67. Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, E. M., Jr., Snider, W. D., and Korsmeyer, S. J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* **17**, 401–411

68. Kolch, W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem. J.* **351**, 289–305

69. Ley, R., Ewings, K. E., Hadfield, K., Howes, E., Balmanno, K., and Cook, S. J. (2004) Extracellular signal-regulated kinases 1/2 are serum-stimulated “Bim(EL) kinases” that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover. *J. Biol. Chem.* **279**, 8837–8847

70. Harada, H., Quearry, B., Ruiz-Vela, A., and Korsmeyer, S. J. (2004) Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 15313–15317

71. Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. A., Strasser, A., and Johnson, E. M. (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* **29**, 615–628