We conclude that iNOS-mediated biosynthesis in endothelial cells (6–8). There are two groups of induced enzyme in cholesterol biosynthesis. Statins improve endothelial conditions (1–5). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, leading to decreased cholesterol biosynthesis. Emerging data indicate that statins stimulate apoptotic cell death in several types of proliferating tumor cells, including breast cancer cells, which is independent of its cholesterol-lowering property. The objective here was to elucidate the molecular mechanism(s) by which statins induce breast cancer cell death. Fluvastatin and simvastatin (5–10 μmol/L) treatment enhanced the caspase-3-like activity and DNA fragmentation in MCF-7 cells, and significantly inhibited the proliferation of MCF-7 cells but not MCF-10 cells (noncancerous epithelial cells). Statin-induced cytotoxic effects were reversed by mevalonate, an immediate metabolic product of the acetyl CoA/3-hydroxy-3-methylglutaryl CoA reductase reaction. Both simvastatin and fluvastatin enhanced nitric oxide (‘NO) levels which were inhibited by mevalonate. Statin-induced ‘NO and tumor cell cytotoxicity were inhibited by 1400W, a more specific inhibitor of inducible nitric oxide synthase (iNOS or NOS II). Both fluvastatin and simvastatin increased iNOS mRNA and protein expression. Stimulation of iNOS by statins via inhibition of geranylgeranylation by GGTI-298, but not via inhibition of farnesylation by FTI-277, enhanced the proapoptotic effects of statins in MCF-7 cells. Statin-mediated antiproliferative and proapoptotic effects were exacerbated by sepiapterin, a precursor of tetrahydrobiopterin, an essential cofactor of ‘NO biosynthesis by NOS. We conclude that iNOS-mediated ‘NO is responsible in part for the proapoptotic, tumoricidal, and antiproliferative effects of statins in MCF-7 cells. [Cancer Res 2007;67(15):7386–94]

Introduction

Statins, a class of hyperlipidemic blockbuster drugs, are routinely used for lowering serum cholesterol in the treatment and prevention of hypercholesterolemia and other cardiovascular conditions (1–5). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Statins improve endothelial and cardiovascular function by enhancing nitric oxide (‘NO) biosynthesis in endothelial cells (6–8). There are two groups of statins: (a) natural or fermentation-derived: lovastatin (Mevacor), simvastatin (Zocor), and pravastatin (Pravachol); (b) synthetic statins: fluvastatin (Lescol), atorvastatin (Lipitor), cerivastatin (Lipobay), and rosuvastatin (Crestor). Some statins such as simvastatin and lovastatin are more hydrophobic (i.e., lipid-soluble) than others (e.g., cerivastatin). A major biochemical function of statins is the ability to lower lipid levels, in particular, plasma cholesterol, low-density lipoprotein, and triglycerides. However, reports suggest that statins elicit vasoprotective and cardioprotective effects that are independent of their lipid-lowering effects (9–11). The pleiotropic effects (i.e., actions independent of the cholesterol-lowering property) of statins are dependent on their structural property (i.e., hydrophobicity; ref. 12).

Emerging research suggests that statins may prevent various types of cancers, including breast carcinoma, although results have been mixed (13–15). Statins inhibit cancer cell proliferation by arresting the cell cycle at the G1-S phase and inducing apoptosis (16, 17). Recent studies have shown that fluvastatin markedly attenuates epidermal growth factor–induced invasion of pancreatic cancer cells by inhibiting the translocation of RhoA from the cytosol to the membrane (18). Statins have been shown to inhibit in vivo tumor growth at clinically relevant doses and to diminish metastasis in animal models (19, 20). The objective of this study was to determine the molecular mechanisms by which statins exert cytotoxicity in human breast cancer cells. Results from this study revealed that statin-induced NO synthase (iNOS)/NO activity and inhibition of geranylgeranylation play a key role in enhancing MCF-7 cell apoptosis, and that supplementation with sepiapterin exacerbates statin-mediated breast cancer cell death via increased generation of ‘NO.

Materials and Methods

Reagents, cell lines, and culture conditions. Simvastatin, fluvastatin, N-[(2R)-amino-3-mercaptopropyl]aminopropan-2-aminobenzoyl(3-leucine methyl ester (GGTI-298), methyl [N-2-[phenyl-4-4-[2(R)-amino-3-mercaptopropylamino] benzoyl)]-methionine (FTI-277), and 4,5-diaminofluorescein diacetate (DAF-2-DA) were purchased from Calbiochem. Mevalonate, N-(3-aminomethyl)benzylacetamide (1400W), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), squalene, and sepiapterin were purchased from Sigma, Inc. ‘NO-fluvastatin (NCX 6553) was purchased from Cayman Chemicals. The culture medium (MEM) and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. All other chemicals were of reagent grade. All cell lines were purchased from the American Type Culture Collection. MCF-7 and MDA-MB-231 cells were grown in 10% MEM containing 10% FBS, 1-glutamine (4 mmol/L), penicillin (100 units/mL), and streptomycin (100 μg/mL), and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

MTT reduction cytotoxicity assay. MTT was taken up by cells and was reduced to a colored formazan product that could be detected by spectrophotometry (λmax = 562 nm). Reduction of MTT was dependent on the mitochondrial respiratory function, and thus, measures the relative number of viable cells in the culture. After the treatment was completed,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Current address for S. Kotamraju: Discovery Research Division, Institute of Life Sciences, University of Hyderabad Campus Gachibowli, Hyderabad, 500 046 India.

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Research Article

Statin-Induced Breast Cancer Cell Death: Role of Inducible Nitric Oxide and Arginase-Dependent Pathways

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Abstract

Statins are widely used cholesterol-lowering drugs that selectively inhibit the enzyme 3-hydroxy-3-methylglutaryl CoA reductase, leading to decreased cholesterol biosynthesis. Emerging data indicate that statins stimulate apoptotic cell death in several types of proliferating tumor cells, including breast cancer cells, which is independent of its cholesterol-lowering property. The objective here was to elucidate the molecular mechanism(s) by which statins induce breast cancer cell death. Fluvastatin and simvastatin (5–10 μmol/L) treatment enhanced the caspase-3-like activity and DNA fragmentation in MCF-7 cells, and significantly inhibited the proliferation of MCF-7 cells but not MCF-10 cells (noncancerous epithelial cells). Statin-induced cytotoxic effects were reversed by mevalonate, an immediate metabolic product of the acetyl CoA/3-hydroxy-3-methylglutaryl CoA reductase reaction. Both simvastatin and fluvastatin enhanced nitric oxide (‘NO) levels which were inhibited by mevalonate. Statin-induced ‘NO and tumor cell cytotoxicity were inhibited by 1400W, a more specific inhibitor of inducible nitric oxide synthase (iNOS or NOS II). Both fluvastatin and simvastatin increased iNOS mRNA and protein expression. Stimulation of iNOS by statins via inhibition of geranylgeranylation by GGTI-298, but not via inhibition of farnesylation by FTI-277, enhanced the proapoptotic effects of statins in MCF-7 cells. Statin-mediated antiproliferative and proapoptotic effects were exacerbated by sepiapterin, a precursor of tetrahydrobiopterin, an essential cofactor of ‘NO biosynthesis by NOS. We conclude that iNOS-mediated ‘NO is responsible in part for the proapoptotic, tumoricidal, and antiproliferative effects of statins in MCF-7 cells. [Cancer Res 2007;67(15):7386–94]

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**Figure 1.** Effects of statin and mevalonate on cell death and cell proliferation in MCF-7 and MCF-10A cells. A, MCF-7 cells were treated with simvastatin or fluvastatin (5–10 μM/L) for 24 to 48 h and cell survival was measured by the MTT assay. B, MCF-7 and MCF-10A cells were treated with simvastatin or fluvastatin (5–10 μM/L) for a period of 48 h and cell survival was measured by the MTT assay. C, the effect of mevalonate (20 μM/L) on cell death induced by simvastatin or fluvastatin was determined using the MTT assay. D, the effects of varying concentrations of simvastatin and fluvastatin in the presence or absence of mevalonate (20 μM/L) on cell proliferation were determined by measuring 3H-thymidine uptake into cells after a 48-h treatment. Columns, mean of three different experiments; bars, ± SD. * indicates significantly different compared with untreated conditions (P < 0.05); # indicates significantly different compared with simvastatin or fluvastatin alone (P < 0.05).

MCF-7 cells were washed twice with DPBS, resuspended in 1 mL of MEM without FBS, and incubated with 5 mg/mL of MTT solution for 1 h at 37°C. The medium was removed and the cells were solubilized in DMSO. The absorption was measured at 562 nm with reference at 630 nm.

**Thymidine uptake studies.** DNA synthesis was measured by monitoring the uptake of tritiated thymidine, [3H]TdR (Perkin-Elmer). Cells (5 x 10^4/mL) were cultured with different concentrations of simvastatin or fluvastatin (0–10 μM/L) in the presence or absence of mevalonate (20 μM/L), 1400W, or sepiapterin. Cells were pulse-chased with [3H]TdR [0.5 μCi (1.85 MBq)/well] during the last 3 h of a 24-h culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology), and radioactivity on the filters was measured by β-scintillation counting.

All experiments were done in triplicate and repeated thrice.

**Measurement of intracellular NO.** Intracellular NO levels were monitored using a DAF-2-DA fluorescence probe (21). After the treatments, cells were washed with DPBS and incubated in 2 mL of fresh culture medium without FBS. DAF-2-DA was added at a final concentration of 710 μM/L, and cells were incubated for 20 min. Cells were washed twice with DPBS and maintained in 1 mL of the culture medium for monitoring the fluorescence using a Nikon fluorescence microscope (excitation, 488 nm; emission, 500–550 nm) equipped with an FITC filter. Fluorescence intensity was calculated using the Metamorph software.

**Nitrate and nitrite measurements.** Nitrate and nitrite, the oxidative metabolites of NO, were measured by chemiluminescence, using a Sievers apparatus, following reduction with vanadium (III) chloride (22). Briefly, following treatments, the cells were washed with DPBS and then incubated for 30 min at 37°C in 1 mL of Hanks’ balanced salt mixture containing 25 μM/L of i-arginine. The medium was collected and centrifuged for 5 min at 5,000 rpm, and 50 μL of the clear supernatant was used for nitrate and nitrite analysis. Each sample was analyzed in triplicate.

**Western blot analysis.** After treatment with statins, cells were washed with ice-cold DPBS and resuspended in 150 μL of radioimmune precipitation assay buffer [20 mM/L Tris-HCl (pH 7.4), 2.5 mM/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM/L NaCl, and 100 mM/L sodium fluoride] containing 1 mM/L of sodium vanadate, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, and 10 μg/mL of pepstatin inhibitors. Cells were homogenized by passing the suspension through a 23-gauge needle (20 strokes). The lysate was centrifuged at 750 × g for 10 min at 4°C to pellet out the nuclei. The remaining supernatant was centrifuged for 30 min at 12,000 × g. Protein was determined using the Lowry method and 50 μg of the lysate was used for the Western blot analysis. Proteins were resolved using SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were washed with TBS (140 mM/L NaCl, 50 mM/L Tris-HCl; pH 7.2) containing 0.1% Tween 20 (TBST) and 5% skimmed milk to block nonspecific protein binding. Membranes were incubated with 1 μg/mL of rabbit anti-iNOS polyclonal antibody (Abcam), mouse anti-β-actin D1 antibody, mouse anti-cyclin D1 antibody, mouse anti-arginase II (BD Biosciences) or rabbit anti-p27 antibody (Chemicon International) in TBST for overnight at 4°C, washed five times with TBST, and then incubated with goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase–conjugated secondary antibody (1:5,000) for 1.5 h at room temperature. Immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham Biosciences).

**RT-PCR analysis.** Following the treatments, the medium was aspirated and 1 mL of TRizol reagent (Invitrogen) was added and total RNA was extracted using the manufacturer’s protocol. Five micrograms of RNA was used for the first-strand cDNA synthesis using a first-strand cDNA synthesis kit (Amersham Biosciences). Four microliters of the cDNA mixture was used for the first-strand cDNA synthesis using a first-strand cDNA synthesis apparatus, following reduction with vanadium (III) chloride (22). Briefly, following treatments, the cells were washed with DPBS and then incubated for 30 min at 37°C in 1 mL of Hanks’ balanced salt mixture containing 25 μM/L of i-arginine. The medium was collected and centrifuged for 5 min at 5,000 rpm, and 50 μL of the clear supernatant was used for nitrate and nitrite analysis. Each sample was analyzed in triplicate.

**Cell cycle analysis.** For DNA content analysis, harvested cells were centrifuged at 1,000 × g for 5 min, fixed by the gradual addition of ice-cold 70% ethanol, and washed with PBS. Cells were then treated with RNase (10 μg/mL) for 30 min at 37°C, washed once with PBS, and resuspended and stained in 1 mL of 69 μM/L of propidium iodide in 38 mM/L of sodium citrate for 30 min at room temperature. The cell cycle phase...
distribution was determined by analytic DNA flow cytometry as described previously (23). The percentage of cells in each phase of the cell cycle was analyzed using Modfit software (Verity Software House).

**Soft agar assay for colony formation.** After cells were treated under various conditions, they were seeded in six-well plates. The plates were first covered with phenol red–free MEM containing 0.6% agar and 10% FBS. The middle layer contained cells (5 × 10⁵) in phenol red–free MEM with 0.35% agar and 10% FBS. The top layer, consisting of the medium, was added to prevent drying of the agar in the plates. The plates were incubated for 21 days, after which the plates were stained in 0.5 mL of 0.005% crystal violet for 1 h and the cultures were inspected and photographed. The colony efficiency was determined by a count of the number of colonies >15 mm in diameter, which was calculated as the average number of colonies counted at 50× magnification in five individual fields manually (24).

**Caspase-3–like proteolytic activity.** Cells were washed twice in cold DPBS and lysed in buffer containing 10 mmol/L of Tris-HCl, 10 mmol/L of NaH₂PO₄/Na₂HPO₄ (pH 7.5), 130 mmol/L of NaCl, 1% Triton, and 10 mmol/L of sodium PPi. Cell lysates were incubated with a caspase-3 fluorogenic substrate N-acetyl-DEVD-7-amido-4-trifluoromethylcoumarin at 37°C for 1 h. 7-Amido-4-trifluoromethylcoumarin liberated from the substrate was measured using a fluorescence plate reader (Perkin-Elmer Life Sciences) with λ exc = 400 nm and λ em = 505 nm (25). The fluorescence intensity was normalized to the protein levels measured with the Bradford protein assay kit (Sigma).

**Measurement of apoptosis by terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.** The terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay was used for microscopic detection of apoptosis (26). This assay is based on labeling...
Results

Statin-induced MCF-7 cell cytotoxicity. We assessed the effectiveness of simvastatin and fluvastatin to induce cytotoxicity in MCF-7 cells. MCF-7 breast cancer cells were treated with fluvastatin or simvastatin at different concentrations (0.5–20 μmol/L) for 24 to 48 h. The changes in the number of viable cells were determined using the MTT assay, which monitors the intracellular conversion of MTT to formazan spectrophotometrically (λ_max = 562 nm). As shown in Fig. 1A, statins potently diminished the number of viable MCF-7 cells. Statins induced cytotoxicity in both MCF-7 breast cancer (malignant) cells (Fig. 1A and B) and MDA-MB-231 (metastatic breast cancer cell lines; data not shown). Fluvastatin and simvastatin did not affect noncancerous mammary epithelial cells, MCF-10A (Fig. 1B). To determine whether statin-induced MCF-7 cell cytotoxicity was due to the inhibition of HMG-CoA reductase activity, cells were pretreated with mevalonate prior to adding simvastatin and fluvastatin. Results show that mevalonate significantly reversed the cytotoxic effects of statins (Fig. 1C), suggesting that the HMG-CoA reductase activity (leading to cholesterol biosynthesis or protein isoprenylation) plays a pivotal role in statin-induced tumor cell cytotoxicity. However, pretreatment with squalene, an immediate precursor of cholesterol biosynthesis, did not prevent statin-induced cytotoxicity (data not shown). This suggests that modulation of isoprenylation of proteins may play a key role in statin-mediated effects in MCF-7 cells.

To further confirm the loss of cell proliferation (as detected by the MTT assay), we measured DNA synthesis in MCF-7 cells treated with simvastatin or fluvastatin for a period of 24 h and monitored the uptake of ³H-thymidine during the last 3 h of the incubation. As seen in Fig. 1D, both simvastatin and fluvastatin inhibited the uptake of ³H-thymidine, and this inhibition was partially reversed by mevalonate (Fig. 1D).

Role of L-arginine metabolizing enzymes in statin-induced cytotoxicity. As statins are known to protect against endothelial dysfunction by modulating the NOS and *NO levels in endothelial cells (6–8), we surmised that statins might also regulate NOS and *NO levels in MCF-7 cells. To this end, we initially measured the DAF–derived green fluorescence. Both simvastatin and fluvastatin significantly increased *NO-mediated DAF fluorescence (Fig. 2A). To identify the source of *NO, we initially monitored the eNOS protein levels by Western blotting in MCF-7 cells treated with and without statins, and found no detectable eNOS protein levels in control and treated MCF-7 cells (data not shown). However, quite unexpectedly, iNOS protein and iNOS mRNA levels were up-regulated in cells treated with statins (Fig. 2B). To further confirm that increased expression of iNOS protein corresponds to increased activity, we initially measured the levels of NO and citrulline, L-arginine can also be metabolized by arginases to  ornithine and urea within the urea cycle and is subsequently converted to polyamines (27). Polyamines are known to increase cell proliferation (28). Because iNOS is significantly induced by statin treatment, it was of interest to measure the levels of arginases (Arg I and Arg II) in statin-treated MCF-7 cells. Arg I transcript levels could not be detected in MCF-7 cells, but Arg II
levels were significantly down-regulated in statin-treated cells which was reversed by mevalonate (Fig. 2C). This result suggests a "cross-talk" between arginase and iNOS that plays a role in statin toxicity in MCF-7 cells. As statins increased *NO levels, we wondered whether N-nitroso-fluvastatin (*NO-fluvastatin) supplementation in MCF-7 would be more effective in causing MCF-7 cell death as compared with fluvastatin alone. *NO-fluvastatin is a hybrid molecule comprised of both statin and *NO activities (29). Results show that *NO-fluvastatin was more potent than fluvastatin alone in causing MCF-7 cell death (Fig. 2D). This clearly implicates a major role for *NO in statin-induced MCF-7 cell death.

Inhibition of geranylgeranylation by statins induces iNOS expression and cell death in MCF-7 cells. The present data showed that cholesterol-independent pathways were responsible for statin-induced effects. Statins have been reported to deplete the availability of prenylated substrates (30). Posttranslational prenylation of small GTPases by the addition of a geranylgeranyl or farnesyli moiety is critical for cellular localization and signaling activity (31). To further confirm the involvement of isoprenoids on statin-induced, iNOS-dependent cell death, we investigated the effects of isoprenylation inhibitors. Pretreatment of MCF-7 cells with geranylgeranyltransferase inhibitor (GGTI-298), but not farnesyltransferase inhibitor (FTI-277) decreased the number of viable cells, as indicated by the MTT assay (Fig. 3D), and decreased cell proliferation, as indicated by diminished ^3H-thymidine uptake (Fig. 3B). To investigate whether inhibition of geranylgeranylation or farnesylation was responsible for enhanced iNOS expression, iNOS protein levels were measured in the presence of either GGTI-298 or FTI-277. As shown, GGTI and not FTI-277 dose-dependently increased iNOS protein levels (Fig. 3C). Concomitantly, inhibition of geranylgeranylation, but not farnesylation, increased NO_2^-/NO_3^- levels (Fig. 3D). However, treatment of noncancerous mammary epithelial cells with fluvastatin and simvastatin did not induce NO_2^-/NO_3^- levels (Supplemental Fig. S1). Based on these results, we conclude that GGTI mimics the effects of statins, and therefore, it is likely that statin-mediated iNOS/*NO induction and cytostatic/cytotoxic effects in MCF-7 cells occur through reduced geranylgeranylation of downstream signaling targets, such as Rho or Rac GTGases.

Contrasting effects of iNOS inhibitor and iNOS activator on statin-induced MCF-7 cell apoptosis. Pretreatment with 1400W, a specific inhibitor of iNOS (32), partially reversed the statin-dependent antiproliferative effects (Fig. 4A) and diminished statin-dependent NO_2^-/NO_3^- generation (Fig. 4B). Conversely, treatment with sepiapterin (which is a precursor of the NOS cofactor BH_4) significantly enhanced the statin-dependent increase in NO_2^-/NO_3^- levels (Fig. 4B). Sepiapterin also enhanced the GGTI-298-dependent increase in NO_2^-/NO_3^- levels (compare Figs. 3D with 4C). Sepiapterin treatment alone or in the presence

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**Figure 4.** Effects of 1400W, sepiapterin, and mevalonate on statin-induced cell death and *NO levels in MCF-7 cells. A, cells were treated with simvastatin or fluvastatin (10 µmol/L) in the presence or absence of a specific iNOS inhibitor, 1400W (10 µmol/L) for 48 h and cell death was measured by the MTT assay. B, the cells were treated as described in (A) or treated with statins in the presence or absence of sepiapterin (50 µmol/L) for 40 h, and NO_2^-/NO_3^- levels were measured using the *NO analyzer. C, the cells were treated with GGTI or FTI (20 µmol/L) for 48 h and NO_2^-/NO_3^- levels were measured at the end of the experiment using the *NO analyzer. D, the cells were treated with GGTI (5–10 µmol/L) in the presence or absence of 1400W (10 µmol/L) for a period of 48 h and cell proliferation was determined by measuring ^3H-thymidine uptake as described in Materials and Methods. Columns, means of at least three independent experiments; bars, SD. * significantly different compared with untreated conditions (P < 0.05); # significantly different compared with simvastatin or fluvastatin alone (P < 0.05; A and B) or GGTI alone (D).
of FTI-277 had no effect on NO\textsubscript{2}/NO\textsubscript{3} levels (Fig. 4B and C). These findings support the model that statin-mediated inhibition of geranylgeranylation causes an iNOS-dependent increase in NO\textsubscript{2}/NO\textsubscript{3} levels, which promotes cell death or loss of cell proliferation. Consistent with this model, we found that 1400W partially suppresses the antiproliferative effects of GGTI-298 (Fig. 4D), just as 1400W partially suppresses the antiproliferative effects of statins (Fig. 4A). Further support for this model is provided by the contrasting effects of 1400W and sepiapterin on statin-induced apoptosis (Fig. 5). Treatment with simvastatin or fluvastatin promotes apoptosis, as indicated by increased caspase-3–like activity in the cells (Fig. 5A) and increased TUNEL staining (Fig. 5B). These statin-induced indicators of apoptosis were diminished by treatment with the iNOS inhibitor 1400W and enhanced by treatment with sepiapterin (Fig. 5A and B). Taken together, these results indicate that *NO modulation plays a key role in the antiproliferative and proapoptotic effects of statins in tumor cells.

**Effect of statins on cell cycle distribution: role of *NO.** As *NO has previously been reported to exert tumor cell cycle alterations (33), we investigated the cytostatic effect of statins in MCF-7 cells. MCF-7 cells were treated with simvastatin and fluvastatin for 48 h in the presence and absence of 1400W (10 \textmu mol/L) and mevalonate (20 \textmu mol/L). Cell cycle progression was examined using FACScan flow cytometry analysis. As shown in Table 1, both simvastatin and fluvastatin (5–10 \textmu mol/L) arrested MCF-7 cells in G\textsubscript{0}/G\textsubscript{1} phase, and as a result, the number of cells in the S phase was decreased. Similar effects were observed with *NO-fluvastatin at a much lower concentration (1 \textmu mol/L) as compared with native fluvastatin (Table 1). Statin-induced cell cycle alterations were partially reversed by the iNOS inhibitor (1400W) and almost completely reversed by mevalonate (Table 1).

As cell cycle progression from G\textsubscript{0} through G\textsubscript{1} phase involves activation of the cell regulatory proteins, cyclins D and E, we investigated the effects of statins and iNOS inhibitor on the cell cycle proteins. As expected, the cell cycle regulatory proteins, cyclin D\textsubscript{1} and cyclin E (which are responsible for driving the cell cycle progression from G\textsubscript{0}/G\textsubscript{1} to S phase transition) were significantly decreased with statin treatments and restored in part by 1400W and mevalonate (Fig. 6A). The levels of the cyclin-dependent kinase inhibitor, p27, were also down-regulated by statin treatments (Fig. 6A). Therefore, under our experimental conditions, it seems that the decrease in cell cycle regulatory proteins was independent

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NOTE: The cell cycle distribution of MCF-7 cells treated with either simvastatin or fluvastatin (5–10 \textmu mol/L) for 48 h in the presence or absence of 1400W (10 \textmu mol/L) or mevalonate (20 \textmu mol/L) was measured by flow cytometry of propidium iodide–stained cells, as described in Materials and Methods.
of the levels of cyclin-dependent kinase (cdk) inhibitor(s) and possibly other cdk-independent regulatory mechanisms are involved.

Effects of statins on anchorage-independent growth of MCF-7 cells. The extent of malignancy of cells corresponds to the attainment of anchorage-independent growth (34). To test the effects of statins on anchorage-independent growth, the long-term effects of statins, GGTI, and FTI on the clonogenic growth of MCF-7 cells were determined. MCF-7 cells were treated with simvastatin or fluvastatin in the presence or absence of either 1400W or mevalonate or sepiapterin. In separate experiments, cells were treated with either GGTI-298 or FTI-277. At the end of the treatments, \(-5 \times 10^3\) cells were seeded onto soft agar to determine their clonogenic efficiency after 21 days. Simvastatin and fluvastatin (10 \(\mu\)mol/L) and GGTI-298 (10 \(\mu\)mol/L) but not FTI-277 (10 \(\mu\)mol/L) drastically lowered the visible colony formation in soft agar (Fig. 6B). In the presence of either 1400W or mevalonate, the colony formation was restored in statin-treated cells (Fig. 6B). Sepiapterin supplementation completely inhibited the colony growth at a lower concentration of simvastatin or fluvastatin (5 \(\mu\)mol/L; Fig. 6B). For quantitative estimation of the number of colonies, please refer to Supplemental Fig. S2. Taken together, these results indicate that statins are able to inhibit cell proliferation and anchorage-independent growth of MCF-7 cells by modulating \(\ast\)NO levels through a pathway involving the inhibition of geranylgeranylation.

Discussion

In this study, we report the following: (a) statins diminish proliferation and promote apoptosis in MCF-7 breast cancer cells through elevation of inducible NOS expression and \(\ast\)NO formation from oxidation of l-arginine to l-citrulline using BH4 as a cofactor; (b) supplementation with sepiapterin, a precursor to BH4 biosynthesis, enhanced statin-mediated proapoptotic and antiproliferative effects in MCF-7 cells; (c) statin-mediated tumoricidal effects occur through inhibition of geranylgeranylation and not through inhibition of farnesylation.

Statins as cancer-therapeutic/cancer-preventive agents. Recently, statins have been studied as potential cancer therapeutics. As early as 1992, it was reported that lovastatin treatment diminished tumor cell proliferation in xenografts of pancreatic cancer in nude mice (35). Epidemiologic studies support the effectiveness of statins as anticancer therapeutics (36–39). Cancer cells usually exhibit constitutively elevated levels of HMG-CoA reductase and low-density lipid receptor levels to satisfy their increased need for isoprenoids and lipids, and potentially making them more sensitive than normal cells to the isoprenoid-depleting effects of statins (40). The clinical reports on the antitumor effects of statins in breast cancer have yielded mixed results, ranging from no association to both positive and negative associations (41, 42). However, results from a recent study conducted in a large population of postmenopausal women with breast cancer concluded that there exists a significant beneficial association between breast cancer and hydrophobic or lipophilic statin therapy (43). However, when statins were considered together as a class including both hydrophobic and hydrophilic statins, no statistically significant association with breast cancer regression was detected in the same group (43). For instance, the use of pravastatin (hydrophilic statin) did not show any positive effect in association with breast cancer (43). This lack of an in vivo effect of pravastatin...
was consistent with the results of in vitro cell culture studies in which only hydrophobic statins (e.g., lovastatin) but not hydrophilic statins (e.g., pravastatin) induced significant antiproliferative and antitumor effects in breast cancer cells. These findings have been confirmed by other investigators (17). By and large, the in vitro cell culture experiments are a reasonable predictor of the in vivo antitumor and/or chemopreventive effects of statins in mammary carcinogenesis models (17). Therefore, defining the biological mechanisms by which statins induce apoptosis and exert antiproliferative effects in breast tumor cells is important. The present study shows a novel mechanism by which statins (lipophilic) exert proapoptotic effects in MCF-7 cells.

*NO as a tumoricidal agent. The cytostatic and cytotoxic effects of *NO in tumor cells are well-established (33, 44, 45). Potential strategies for treating cancer through modulation of *NO biosynthesis and its downstream signaling pathway have previously been described (45). Inducible NOS activity has been implicated in macrophage-induced antitumor activity. Tumor cells transfected with iNOS gene (NOS II gene) exhibited a less aggressive phenotype in both in vitro and in vivo assays due to increased apoptosis (46). Inducible NOS gene transfection in human lung carcinoma cells was shown to down-regulate the expression of the antiapoptotic protein, survivin (47), leading to enhanced tumor cell death.

The geranylgeranylated members of the Rho family of small GTPases may participate in the statin-mediated increase of *NO levels in MCF-7 cells. This probability is supported by our finding that GGTI mimics the effects of statins by increasing iNOS expression, increasing *NO levels, and decreasing the proliferation of the cells. Previous studies indicate that inhibiting isoprenylation of RhoA with statins or GGTI, or inhibiting the RhoA activity with bacterial toxins, increases *NO levels in a variety of cell types. RhoA can modulate *NO levels by regulating iNOS expression (48, 49). Thus, the loss of RhoA activity in statin-treated cells may contribute to increased iNOS expression and elevated *NO levels.

*NO production is compromised by the interplay between NOS, arginases, and argininosuccinate synthetase in tumor cells (27). L-Arginine is a common substrate for both NOS and arginase enzymes. L-Arginine is oxidized by NOS enzymes to L-citrulline and *NO through the formation of an intermediate, N-hydroxy-L-arginine, which is also a potent competitive inhibitor of arginase. Arginase, both cytosolic and mitochondrial forms, metabolizes L-arginine to L-ornithine, the precursor of polyamines that are essential for cell proliferation (27). Although the \( K_m \) for arginine is 1,000-fold higher for arginase as compared with the NOS enzymes, the \( V_{max} \) of arginase is >1,000 times that of the NOS enzymes (27). This suggests similar rates of L-arginine utilization by arginase and NOS enzymes. Thus, *NO production can be regulated by controlling the availability of L-arginine, by inhibiting the arginase enzyme, or by stimulating the NOS activity. In MCF-7 cells, we were able to detect only Arg II transcript levels, but not Arg I levels, and its levels were significantly decreased by statin treatments.

**Sepiapterin enhances statin-mediated proapoptotic effects.** BH4 is an indispensable cofactor for *NO generation by all isoforms of NOS (50). In the absence of BH4, NOS switches its activity from *NO synthesis to superoxide generation (Supplemental Fig. S2). Inducible NOS transfection of fibroblast cells deficient in BH4 synthesizing enzymatic machinery failed to produce *NO in the absence of exogenous supplementation with BH4 (50). This clearly shows the critical need for intracellular BH4 for iNOS-dependent generation of *NO. De novo biosynthesis of BH4 is catalyzed by guanosine-5'-triphosphate (GTP) cyclohydrolase 1. Mammalian cells can also generate BH4 by a "salvage pathway" which converts sepiapterin to BH4 by sepiapterin reductase and dihydrofolate reductase (50). Intracellular BH4 is also manipulated by inhibiting either GTP cyclohydrolase 1 with 2,4-diamino-6-hydroxyprymidine, or dihydrofolate reductase using methotrexate. Alternatively, treatment of cells with sepiapterin or 7,8-dihydrobipterin increases BH4. Thus, pharmacologic manipulation of BH4 levels is a good strategy to modulate *NO in cancer cells. Determining how statins modulate L-arginine metabolism through the arginase and iNOS pathways would likely have a significant effect on our understanding of the stimulation and inhibition of breast cancer growth. As shown in the present study, treatment of MCF-7 cells with sepiapterin enhances the potency of statins via enhanced synthesis of *NO. As shown in Supplemental Fig. S3, supplementation with drugs activating iNOS activity may synergistically enhance statin-induced antiproliferative and proapoptotic effects in breast cancer cells.

**Role of *NO as a cytostatic agent.** Recent research suggests that one of the potential areas of intervention to arrest breast cancer progression is to block the activity of HMG-CoA reductase and lower the levels of mevalonate and other products (39). This pathway controls critical cellular functions including cell signaling and cell cycle progression associated with tumor initiation and tumor metastasis. Cell cycle progression is mainly governed by the activity of a family of protein kinases (cdk). Cyclins were shown to promote cell cycle transitions by binding and activating specific cyclins. Cyclin D is involved in the early to mid-G1 phase, whereas cyclin E activation occurs in the late G2 to early S phase (Fig. 6C). The molecular mechanism(s) by which statins induce G1 arrest in breast cancer cells are not known. The present results show that statins inhibit cyclin D and cyclin E expression in MCF-7 cells, and that this inhibition was abrogated by pretreatment with mevalonate and 1400W, a selective inhibitor of iNOS (Fig. 5A). This finding implicates a role for statin-derived *NO in the modulation of the cell cycle regulatory proteins (Fig. 5A). Previous reports have shown that DETA-NONOate, a nitric oxide donor, induced cytosasis in human breast cancer cells MDA-MB-231, and that the arrest of cells in the G1 phase of the cell cycle was accompanied by cyclin D1 down-regulation (33). However, our results show that although cyclin D1 and cyclin E were significantly down-regulated by statins, it seems that these events were not controlled by cdk inhibitor p27, the levels of which were also decreased with statin treatments. These results were consistent with a recent report showing that the decrease in cell cycle regulatory proteins by statin treatments was not dependent on p21 and p27 protein levels in prostate cancer cells (16).

In conclusion, the present study shows, for the first time, that (a) *NO generated via the induction of iNOS by selective inhibition of geranylgeranylation plays an important role in statin-induced cell death in MCF-7 breast cancer cells and (b) supplementation with sepiapterin exacerbates the proapoptotic effects of statins in these cells through augmentation of *NO synthesis.

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References


Correction: iNOS Statins and Breast Cancer Cell Apoptosis

In the article on iNOS statins and breast cancer cell apoptosis in the August 1, 2007 issue of Cancer Research (1), the correct spelling of the second author’s name is Carol L. Williams.

Statin-Induced Breast Cancer Cell Death: Role of Inducible Nitric Oxide and Arginase-Dependent Pathways

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