Lovastatin Sensitizes Lung Cancer Cells to Ionizing Radiation

Modulation of Molecular Pathways of Radioresistance and Tumor Suppression

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Introduction: In this study, we investigated the effect of the 3-hydroxy-3-methylgutaryl-CoA reductase inhibitor lovastatin, as a sensitizer of lung cancer cells to ionizing radiation (IR).

Methods: A549 lung adenocarcinoma cells were treated with 0 to 50 μM lovastatin alone or in combination with 0 to 8 Gy IR and subjected to clonogenic survival and proliferation assays. To assess the mechanism of drug action, we examined the effects of lovastatin and IR on the epidermal growth factor (EGF) receptor and AMP-activated kinase (AMPK) pathways and on apoptotic markers and the cell cycle.

Results: Lovastatin inhibited basal clonogenic survival and proliferation of A549 cells and sensitized them to IR. This was reversed by mevalonate, the product of 3-hydroxy-3-methylgutaryl-CoA reductase. Lovastatin attenuated selectively EGF-induced phosphorylation of EGF receptor and Akt, and IR-induced Akt phosphorylation, in a mevalonate-sensitive fashion, without inhibition on extracellular signal-regulated kinase 1/2 phosphorylation by either stimulus. IR phosphorylated and activated the metabolic sensor and tumor suppressor AMPK, but lovastatin enhanced basal and IR-induced AMPK phosphorylation. The drug inhibited IR-induced expression of p53 and the cyclin-dependent kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$, but caused a redistribution of cells from G1-S phase (control and radiated cells) and G2-M phase (radiated cells) of cell cycle into apoptosis. The latter was also evident by induction of nuclear fragmentation and cleavage of caspase 3 by lovastatin in both control and radiated cells.

Conclusions: We suggest that lovastatin inhibits survival and induces radiosensitization of lung cancer cells through induction of apoptosis, which may be mediated by a simultaneous inhibition of the Akt and activation of the AMPK signaling pathways.

Key Words: Lovastatin, Lung cancer, A549 cells, Radiation sensitizer, Apoptosis, Akt, AMPK, Erk, EGFR, Cell cycle, Cleaved caspase 3.

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Radiotherapy is a widely used therapy in all stages of non-small cell lung cancer (NSCLC). However, NSCLC demonstrates intrinsic radioresistance that leads to failure of even high-dose thoracic radiation.[1] Therefore, there is an urgent need for rational development of effective radiation sensitizers for NSCLC, which are able to inhibit molecular pathways mediating radiation resistance.

Ionizing radiation (IR) elicits signal transduction leading to cell survival, apoptosis, and cell cycle regulation.[2] IR-induced DNA double-strand breaks are potentially lethal DNA damages leading to activation of phosphatidylinositol 3-kinase (PI3k)-like family protein kinases such as DNA-protein kinase and ataxia telangiectasia mutated (ATM).[3] ATM mediates phosphorylation of p53 leading to stabilization of this tumor suppressor and cell cycle arrest at the G1-S or the G2-M check points through induction of the *cip/kip* family cyclin-dependent kinase inhibitor (CDKI) $p21^{cip1}$.[4] $p27^{kip1}$, another *cip/kip* family CDKI, functions independently of the p53-$p21^{cip1}$ pathway and inhibits cyclin E-cyclin-dependent kinase 2 complex and cycle progression through the G1-S checkpoint.[4]

Recently, we reported that the energy sensor AMP-activated kinase (AMPK), an established effector of the tumor suppressor LKB1, is activated by IR in a variety of epithelial cancer cells.[5] IR activates AMPK in LKB1-independent but ATM-dependent manner leading to induction of p53 and $p21^{cip1}$, cell cycle arrest at the G2-M checkpoint, and modulation of the sensitivity of cells to IR. IR is also shown to regulate mediators of the signaling pathway of epidermal growth factor receptor (EGFR),[6,7] a well-established activator of cancer cell proliferation. IR activates the downstream

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effector pathways of EGFR such as the PI3k—Akt—mammalian target of rapamycin (mTOR) and the Raf—mitogen-activated protein kinase-kinase (Mek1)—mitogen-activated protein kinase p42/44 (also known as extracellular signal-regulated kinase [Erk1/2]) pathways. These are known to mediate cell survival and radiation resistance, gene expression, and protein synthesis.[8,9]

Small GTP-binding proteins of the Ras family such as Ras, Rac, and Rho (A/B) mediate signal transduction downstream of EGFR to activate the PI3k-Akt-mTOR and the Raf-Mek-Erk1/2 pathways. Ras mutations are frequent in lung cancer, and they occur in both the H- and K-Ras isoforms and were shown to induce radiation resistance in vitro.[10,11] For that, extensive work is focused on targeting Ras family members with inhibitors of prenylation, a posttranslational modification required for membrane targeting and function of Ras.[12]

Members of the statin family of 3-hydroxy-3-methylgutaryl-CoA (HMG-CoA) reductase inhibitors are widely used anticholesterol agents that inhibit the conversion of HMG-CoA to mevalonate, a rate-limiting step of the mevalonate—cholesterol biosynthesis pathway.[13,14] This pathway is also vital for the production of farnesyl and geranylgeranyl moieties required for the posttranslational modification and function of Ras and Rho, respectively.[13] For this reason, statins have been studied extensively as antitumor agents.

During the past 20 years, a large amount of studies have demonstrated the antiproliferative and proapoptotic effects of statins both in vitro and in animal models of cancer. Growth inhibition, cell cycle arrest, and induction of apoptosis in cancer cells have been demonstrated convincingly.[15] The interest in these drugs was enhanced by epidemiological studies indicating that patients on statins may have lower risk for development of colorectal carcinoma[16] and lung cancer.[17] The Veterans Affairs Health Care System study[17] showed that use of statins for more than 6 months could offer a 55% risk reduction on the incidence of lung cancer, indicating that these agents may have significant chemoprevention action. Further, in prostate cancer, statin use is suggested to decrease the risk for advanced and metastatic cancer in epidemiological studies,[18] to slow disease progression after radical prostatectomy,[19] and, importantly, to reduce disease recurrence in patients treated with curative radiotherapy.[20,21]

Lovastatin is probably the most widely studied statin in cancer. It has been shown to possess anticancer properties in vitro and in vivo.[14] The antiproliferative action of lovastatin has been demonstrated in lung cancer cells,[22] but its role as a potential IR sensitizer or adjunct to radiation has not been examined in lung cancer models. In this study, we examined the effects of lovastatin on clonogenic survival of lung cancer cells treated with or without IR and explored the effects of this drug on cell cycle, apoptosis, and signaling pathways involved in IR resistance.

METHODS

Materials

Roswell Park Memorial Institute (RPMI) media, fetal bovine serum, trypsin, and antibiotic were purchased from Invitrogen (Burlington, ON). Antibodies against phospho-EGFR, phospho-Akt, phospho-Erk, p53, phospho-AMPK α-subunit, $p21^{cip1}$, $p27^{kip1}$, cleaved caspase 3, actin, and horseradish peroxidase-conjugated anti-rabbit secondary antibody were purchased from Cell Signal Technology (Mississauga, ON, Canada). Polyvinylidene difluoride membrane was purchased from Pall Corporation (Port Washington, NY). Lovastatin, mevalonate, and Hoechst 33258 were purchased from Sigma (Toronto, ON). A549 cells were from the American Type Culture Collection (Manassa, VA).

Cell Culture and Treatments

A549 cells were grown in Roswell Park Memorial Institute (RPMI) media containing 5 mM glucose, 10% (vol/vol) fetal bovine serum, and 1% (vol/vol) antibiotic-antimycotic at 37°C as described previously.[23] Cells were treated with the indicated concentrations of lovastatin 24 hours before radiation.

Clonogenic Assay

A549 cells were subjected to clonogenic assays as described earlier.[5] Briefly, 500 or 1000 cells were seeded into individual wells of a 6-well plate in triplicate and maintained at the indicated doses of lovastatin before radiation (2–8 Gy). After 7 days, cells were fixed and stained with methylene blue, and viable colonies (>50 cells) were counted. Results are expressed as cell survival fraction compared with untreated control. To evaluate radiation sensitization by lovastatin, data were fitted to the linear quadratic equation using Graphpad Prism version 5 software (La Jolla, CA) as described previously.[24]

Proliferation Assay

Approximately 2500 cells were seeded into a 96-well plate and treated with the indicated concentrations of lovastatin before being exposed to 0, 2, or 8 Gy IR. Ninety-six hours later, the cells were washed with phosphate-buffered saline, distilled H_2O was added to each well, and the plates were stored at −80°C until completely frozen. The plates were then thawed and stained with Hoechst working solution (20 μg/mL Hoechst 33258 in a Tris-Borate-EDTA buffer), and fluorescence was determined using the Cyto-Fluor Plate Reader (Applied Biosystems, Toronto, ON, Canada).

Immunoblotting

Twenty micrograms of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane as described earlier.[23] The primary antibody was detected with horseradish peroxidase-conjugated anti-rabbit secondary antibody and enhanced chemiluminescent detection reagent.

Cell Cycle Analysis

The propidium iodine method was used as described previously.[5] Cells were treated with lovastatin (10 μM) before treatment with 0 or 8 Gy of IR and incubated for the indicated times and were then subjected to flow cytometric cell cycle analysis using a FACScan flow cytometer (Beckton Dickinson, Mississauga, Canada).

Immunofluorescence Microscopy

Cells grown on glass coverslips for 24 hours were treated with lovastatin (10 μM) for the indicated times. Then the cells were stained with Hoechst 33258, and images were obtained as described previously.[5] Quantitation of apoptotic cells (showing nuclear fragmentation) was performed by counting the average proportion of apoptotic cells in four high-power fields on each slide (100 cells counted in each quadrant of each slide). Values were normalized to the untreated control.

Statistical Analysis

Statistical analyses was performed with unpaired T-test, using SPSS version 16.0 software (Somers, NY) and are presented as mean ± SEM of at least three separate experiments.

RESULTS

Lovastatin Inhibits NSCLC Cell Survival and Enhances the Cytotoxicity of IR

We initiated our studies with clonogenic survival assays. Lovastatin alone caused a dose-dependent inhibition of clonogenic survival in A549 cells (Figure 1*A*). The drug began inhibiting clonogenic survival at a dose of 5 μM (10% reduction in survival), inhibited the majority of clonogenic survival at 25 μM (95% reduction in survival), and completely abolished survival at 50 μM (Figure 1*A*).

Lovastatin sensitized A549 cells to IR (Figure 1*B*). Clonogenic assay values were fitted into a linear quadratic model. Both 5 and 10 μM of the drug showed significant radiosensitization of A549 cells to 2 to 8 Gy of IR. Almost

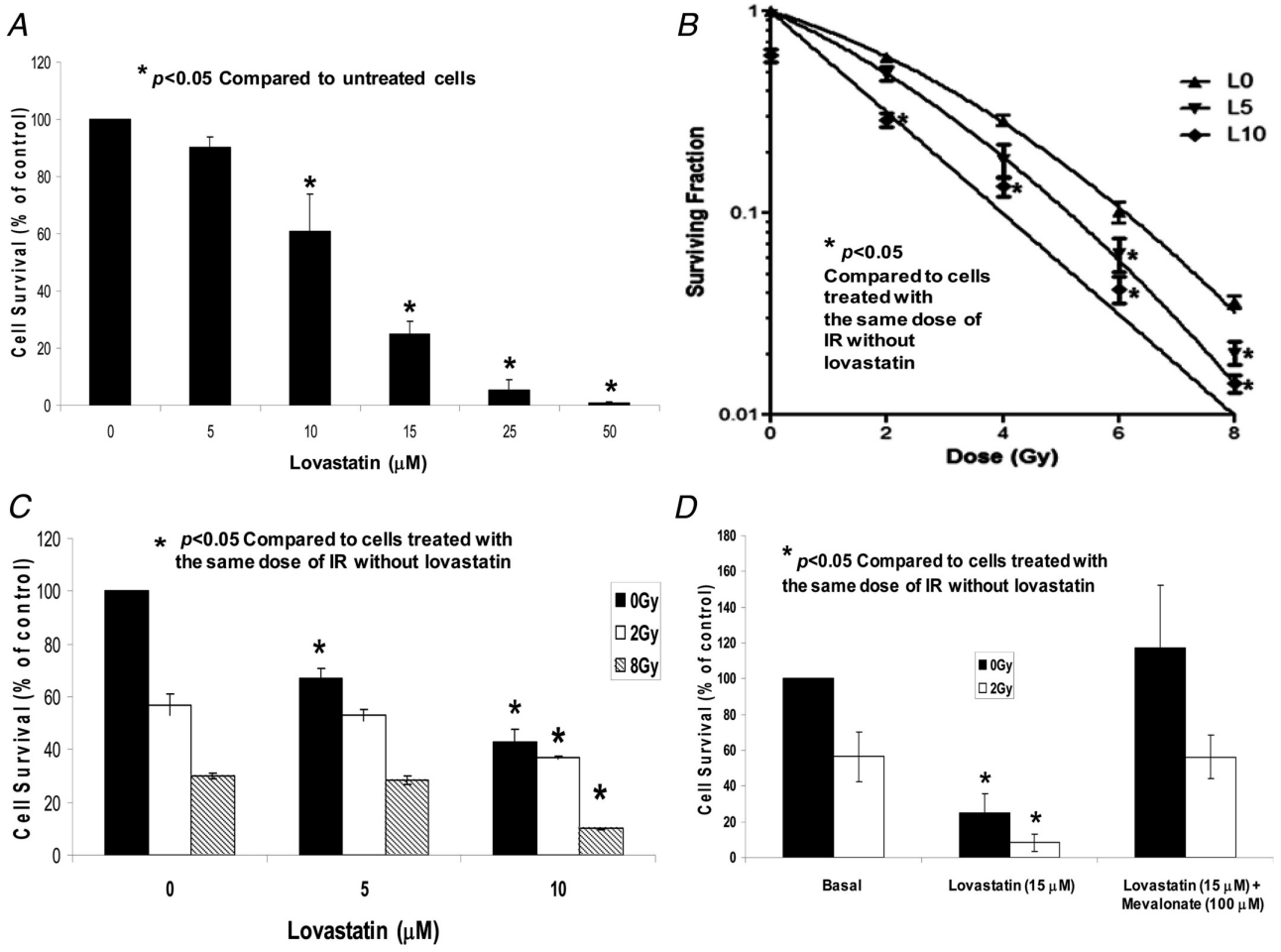

FIGURE 1. Lovastatin inhibits non-small cell lung cancer cell clonogenic survival and sensitizes cells to ionizing radiation. *A,* A549 cells were subjected to clonogenic assays as described in the Methods section. Results from five to eight independent experiments were normalized to the controls and are shown as the mean ± standard error (SE). *B,* Cells were subjected to increasing doses of ionizing radiation (IR) after incubation without (L0) or with 5 μM (L5) or 10 μM lovastatin (L10) lovastatin and subjected to clonogenic assays. Results from five to six independent experiments were normalized to the controls and are shown as the mean ± SE. *C,* Cell proliferation was evaluated after treatment with indicated concentrations of lovastatin for 24 hours before treatment with indicated doses of IR. Proliferation was evaluated 96 hours later as described in Methods section. Results from three independent experiments were normalized to the controls and are shown as the mean ± SE. *D,* Cells were subjected to 2 Gy of IR without or with preincubation with either lovastatin alone (15 μM) or mevalonate (100 μM) and lovastatin before treatment with 2Gy IR followed by clonogenic assays. Results are the mean ± SEM of three independent experiments.

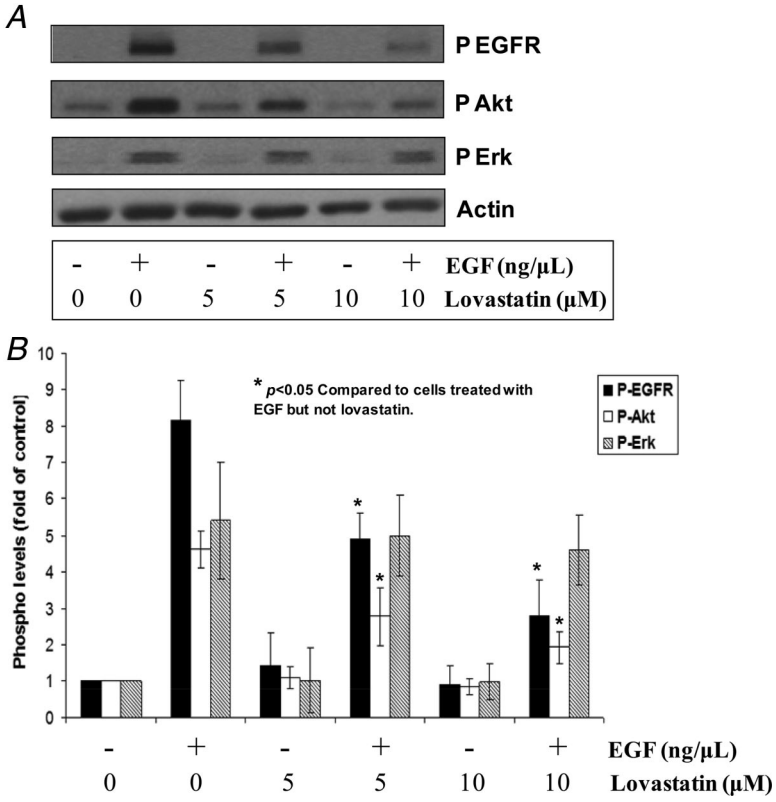

FIGURE 2. Lovastatin modulates epidermal growth factor (EGF)-stimulated Akt phosphorylation. *A,* Cells were preincubated with or without the indicated concentrations of lovastatin for 24 hours before exposure to EGF (1 ng/μL). Cells were then lysed 30 minutes after exposure to EGF, followed by immunoblotting with antibodies against phoshorylated EGFR, Akt, Erk, or total actin. Representative immunoblots are shown. *B,* Densitometry values (mean ± SEM) from three to four independent immunoblotting experiments are displayed in the graph.

complete inhibition of clonogenic survival was achieved with 10 μM lovastatin in combination with 8 Gy IR. In addition, we evaluated proliferation through DNA synthesis analysis using the Hoescht DNA staining method. Five micromolar of lovastatin inhibited basal cell proliferation (by 33%; compared with 0 Gy control) without affecting significantly the proliferation levels after IR (Figure 1*C*). However, at 10 μM, the drug inhibited dramatically cell proliferation in both control cells and those radiated with 2 or 8 Gy (by 63% and 90%, respectively).

Mevalonate Prevents Lovastatin-Induced Inhibition of Clonogenic Survival

We used mevalonate to examine the specificity of lovastatin for the mevalonate-cholesterol synthesis pathway (Figure 1*D*). Two gray of IR decreased clonogenic survival by 44% compared with control. Lovastatin alone (15 μM) inhibited survival by 75% and by 92% when combined with 2 Gy of IR. In these experiments, the higher concentration of 15 μM lovastatin was used to examine whether mevalonate is capable of reversing the effects of even high lovastatin doses. Mevalonate (100 μM) inhibited the lovastatin-induced decrease in cell survival in both nonradiated and radiated cells, suggesting that lovastatin mediates its cytotoxic action solely through inhibition of the mevalonate synthesis pathway.

Lovastatin Inhibits EGF-Stimulated Activation of EGFR and Akt

To analyze the mechanism of action of lovastatin, we examined first its effects on EGF-induced EGFR and downstream effector phosphorylation. EGF induced phosphorylation of EGFR and the Akt and Erk1/2 kinases (Figure 2). However, lovastatin inhibited EGFR and Akt phosphorylation, in a dose-dependent fashion, without affecting phosphorylation of Erk1/2 (Figure 2).

Modulation of IR-Stimulated Activation of Akt and AMPK

Control and lovastatin-treated cells were subjected to increasing doses of IR and were analyzed by immunoblotting. IR induced a consistent Akt phosphorylation, even with the lower dose of 2 Gy, but Erk phosphorylation was seen only after 4 to 6 Gy (Figure 3*A–C*). Interestingly, lovastatin abolished the IR activation of Akt but did not affect significantly Erk1/2 phosphorylation by IR (Figure 3*A–C*). The inhibition of IR-induced Akt phosphorylation by lovastatin was completely reversed by addition of mevalonate (Figure 3*D–E*), consistent with clonogenic survival results (Figure 1*D*). IR also caused a dose-dependent phosphorylation of AMPK that was accompanied by activation of this kinase shown by the enhanced phosphorylation of its established substrate acetyl CoA carboxylase, as observed earlier[5] (Figure 4*A–C*). Interestingly, lovastatin enhanced significantly both basal and radiation-induced AMPK phosphorylation and activity.

Modulation of Cell Cycle Regulators and the Cell Cycle by Lovastatin

The Akt and AMPK pathways regulate cell cycle through modulation of p53 and CDKIs $p27^{kip1}$ and $p21^{cip1}$.[25]

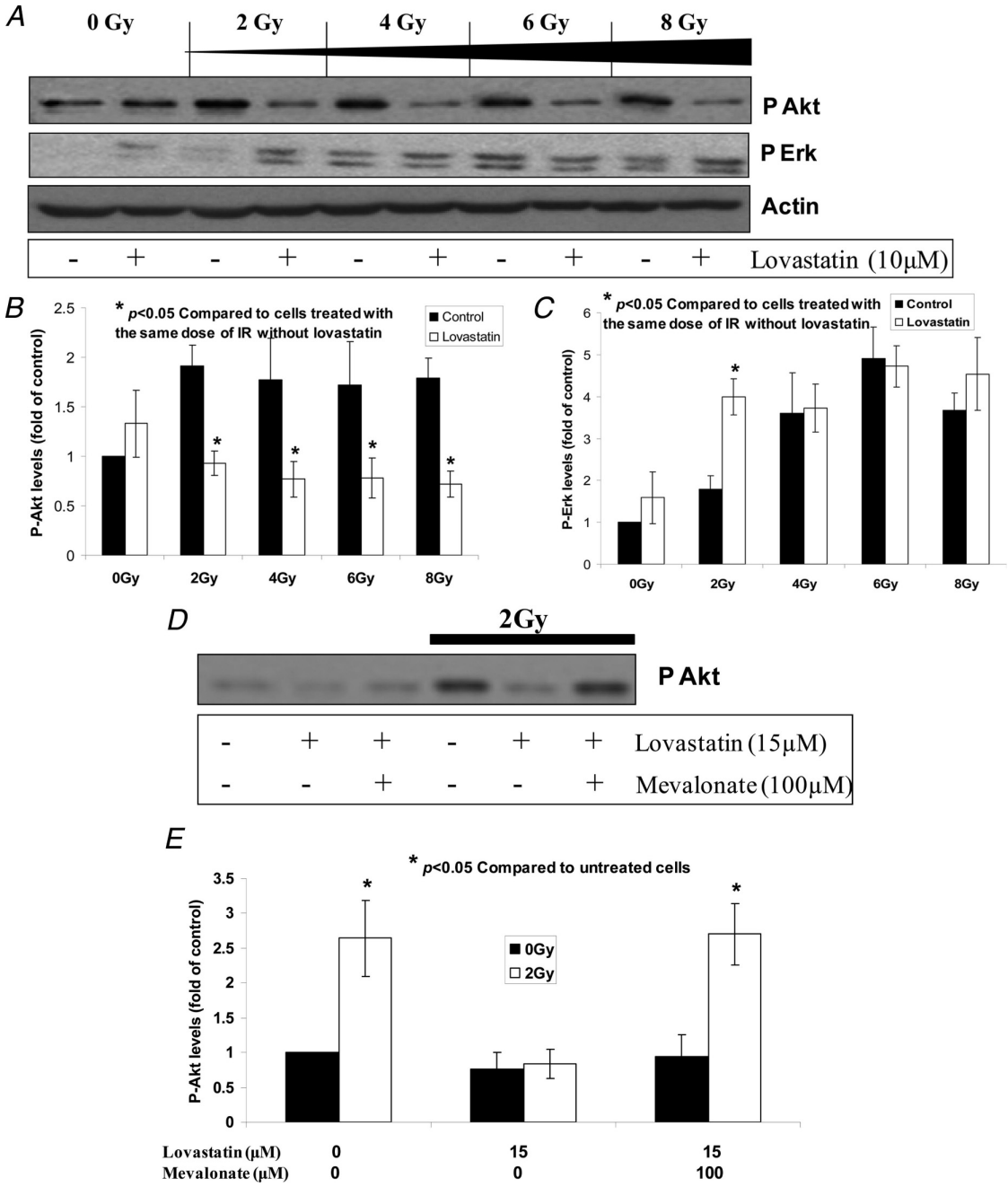

FIGURE 3. Lovastatin modulates ionizing radiation (IR)-induced Akt but not Erk phosphorylation. Cells were preincubated with or without lovastatin (10 μM) for 24 hours before exposure to the indicated dose of IR. *A,* Cells were then lysed 1 hour after the indicated doses of IR, followed by immunoblotting with indicated antibodies. *B* and *C,* Densitometry values of immunoblots (mean ± SEM) from three independent immunoblotting experiments are shown. *D,* Cells were treated with or without 15μM lovastatin or mevalonate (100 μM) for 24 hours before exposure to 2 Gy IR. Cell lysates were subjected to immunoblotting. A representative immunoblot is shown. *E,* Densitometry values (mean ± SEM) from three independent immunoblotting experiments are displayed.

Therefore, we examined whether lovastatin modulates the levels of these cell cycle inhibitors in control and IR-treated cells. We observed a significant increase in the expression of p53, $p27^{kip1}$, and $p21^{cip1}$ in response to IR (Figure 5*A*). However, lovastatin caused an early inhibition of the IR-induced expression of p53, $p27^{kip1}$, and $p21^{cip1}$, within 24 hours, and for that, we examined the levels of these three cell cycle regulators up to 96 hours after initiation of treatments. IR maintained enhanced p53, $p27^{kip1}$, and $p21^{cip1}$ levels up to 96 hours later (Figure 5*A*), but lovastatin inhibited this IR-

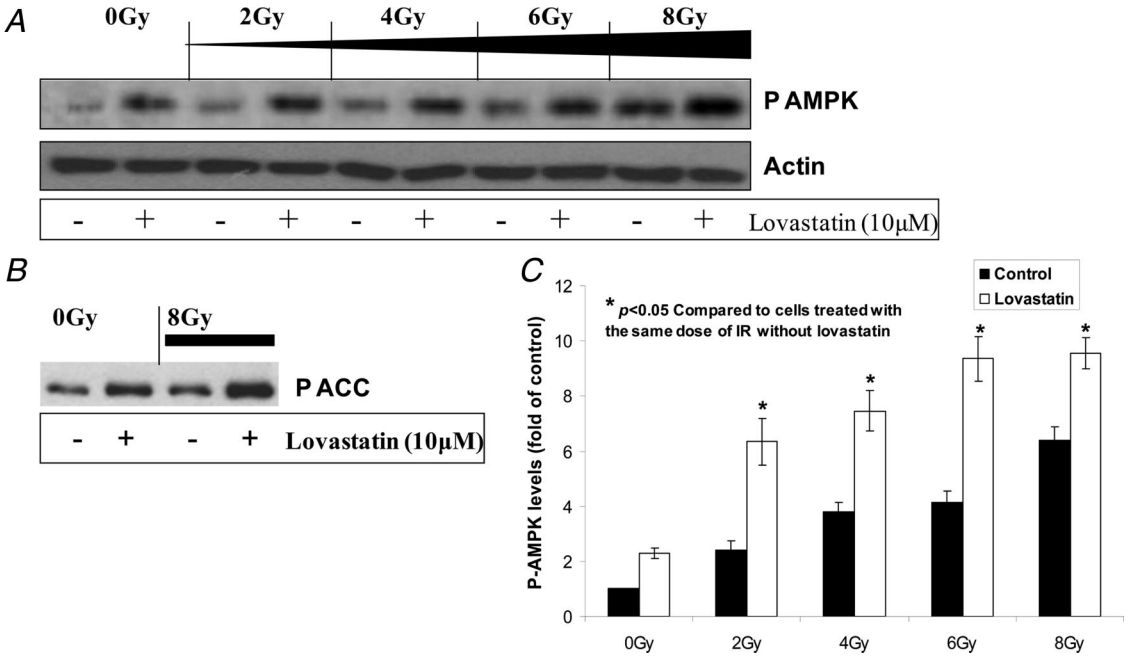

FIGURE 4. Lovastatin modulates ionizing radiation (IR)-induced AMP-activated kinase phosphorylation. *A,* Cells were preincubated with 10 μM lovastatin 24 hours before treatment with the indicated dose of IR, lysed, and subjected to immunoblotting with the indicated antibodies. A representative immunoblot from at least three independent experiments is shown. *B,* Cells were preincubated with 10 μM lovastatin 24 hours before treatment with 0 or 8 Gy IR. Immunoblotting was preformed with an antibody against phosphorylated acetyl CoA carboxylase (P-ACC). *C,* Mean ± SEM of densitometry values from three to four experiments performed in experiment A are shown.

induced expression, which was almost completely eliminated at 96 hours.

Effects on Cell Cycle Phase Distribution

Lovastatin alone did not affect significantly the distribution of cells in the phases of the cell cycle in the first 24 hours (Figure 5*B*). However, lovastatin caused a progressive significant shift of cells into G0/G1 phase, after 24 hours, compared with control, and eventually a marked induction of apoptosis by 72 to 96 hours (24 hours: 2%; 96 hours: 89%). IR alone caused a significant arrest of cells in G2/M phase at 48 hours (control: 0%; IR: 31%). However, lovastatin attenuated IR-induced G2/M arrest and shifted cells into G0/G1 phase and apoptosis (IR G2-M: 31%; Lovastatin + IR G2-M: 12%; Figure 5*C*).

Apoptosis Events Induced by Lovastatin

Finally, we examined the effects of lovastatin and IR on molecular and morphological markers of apoptosis. Cleaved caspase 3 levels, an established marker of apoptosis,[26] was analyzed by immunoblotting. Lovastatin alone caused a significant increase in cleaved caspase 3 levels and further potentiated IR-induced expression of this protein at 12 hours (Figure 7*A*). IR induction of cleaved caspase 3 dissipated after 12 hours, but lovastatin enhanced cleaved caspase 3 levels for up to 48 hours later and decreased thereafter. We analyzed apoptotic events also with morphological analysis of cells treated with lovastatin for 0 to 72 hours. Consistent with induction of cleaved caspase 3 (Figure 6*A*) and the cell cycle results (Figure 5), lovastatin caused a time-dependent nuclear fragmentation and induction of apoptotic bodies (Figure 6*B*, *C*).

DISCUSSION

Lovastatin was shown to sensitize human cervix cancer cells to IR.[27] Recently, another statin, simvastatin, was shown to inhibit small cell lung cancer growth in vitro and in vivo,[28] and Bellini et al.[29] showed that simvastatin inhibits the proliferation of A549 lung cancer cells. However, the potential benefit of combining statins with therapeutic doses of IR has not been examined in lung cancer models. To our knowledge, this is the first study to demonstrate that lovastatin acts as a radiation sensitizer in NSCLC cells.

Lovastatin Regulation of Clonogenic Survival in Control and Radiated Cells

We observed that lovastatin sensitized A549 lung cancer cells to therapeutic doses of IR of 2 to 8 Gy (Figure 1). This was mediated specifically through inhibition of the mevalonate pathway, as exogenous mevalonate completely reversed the decrease in lung cancer cell survival observed by lovastatin (Figure 1*D*). Fritz et al.[27] examined the sensitivity of a number of cancer cell lines to lovastatin, but only a few of them showed sensitivity to lovastatin at high doses. HeLa cells required 20 to 50 μM of lovastatin to demonstrate radiosensitization.[27] In this study, lung adenocarcinoma A549 cells showed higher sensitivity to the drug (at 5 and 10 μM), indicating that survival pathways in those cells may be more dependent on protein prenylation events.

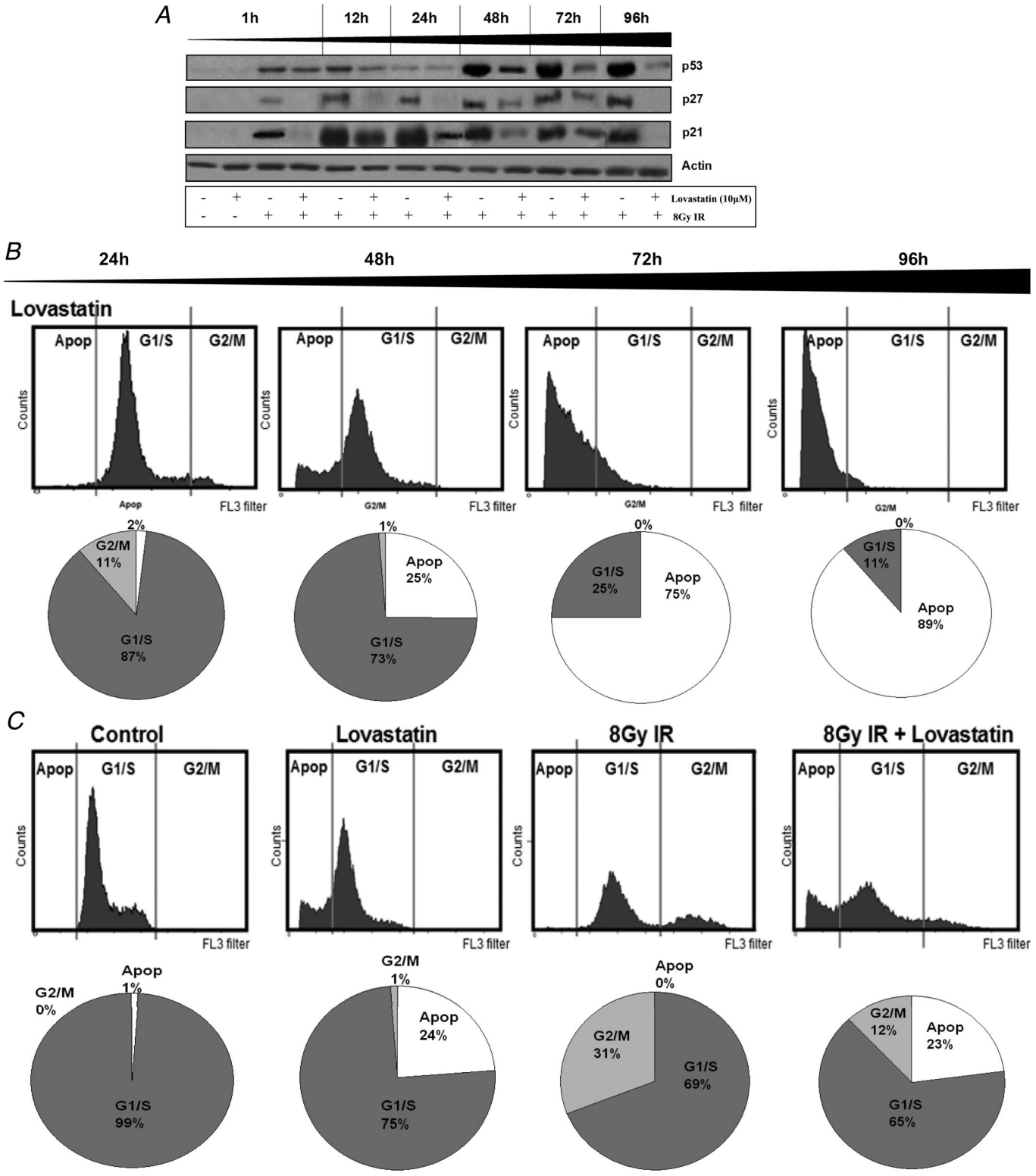

FIGURE 5. Lovastatin modulates ionizing radiation (IR)-induced expression cell cycle inhibitors and the cell cycle. *A,* Cells were treated with 10 μM lovastatin with or without a single dose of 8 Gy IR. Cells were then lysed at the indicated times (1–96 hours), followed by immunoblotting with antibodies that recognize p53, $p27^{kip1}$, and $p21^{Waf/cip}$. A representative immunoblot from at least three independent experiments is shown. *B,* Cells were treated with 10 μM lovastatin for the indicated times, followed by cell cycle analysis. The results were quantified as % distribution in apoptosis (Apop), G1/S, and G2/M phases. *C,* Cells were treated without or with lovastatin were exposed to 0 or 8 Gy IR. Cell cycle analysis was preformed 48 hours later.

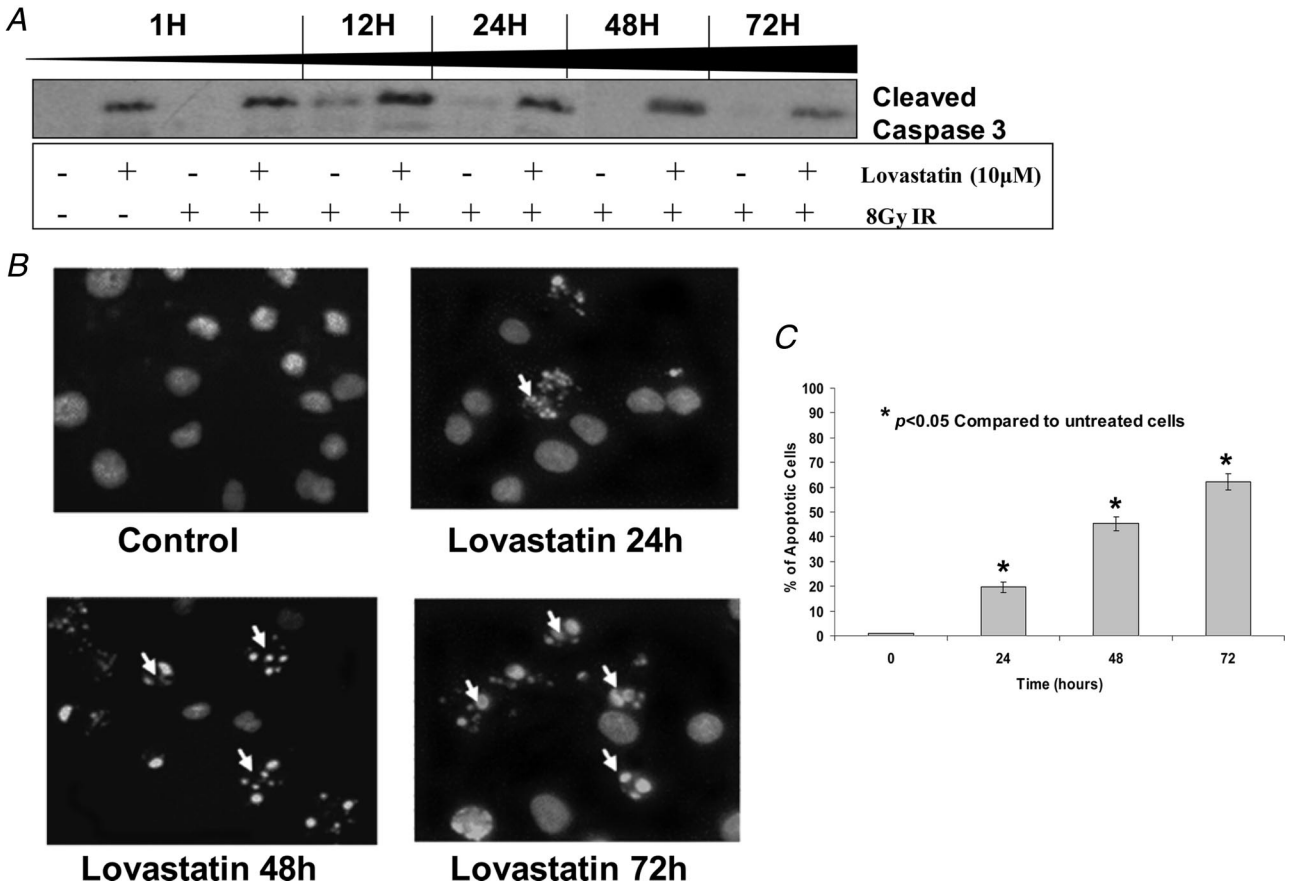

FIGURE 6. Lovastatin induces cancer cell apoptosis. *A,* Cells were pretreated with or without lovastatin before exposure to 8 Gy ionizing radiation (IR) and were lysed at the indicated times after radiation (1–72 hours). Cleaved caspase 3 induction was analyzed by immunoblotting. *B,* After treatment with lovastatin (10 μM) for the indicated times, cells were fixed and stained with Hoechst, and the nuclear morphology was analyzed with fluorescence microscopy. A representative from three independent experiments is shown. *C,* Nuclear fragmentation was quantitated in three independent experiments as described in the Methods section. Results are normalized to control untreated cells (0 time with lovastatin).

Interestingly, in recent experiments investigating the effects of lovastatin in SK-MES lung cancer cells, a cell line of squamous cell carcinoma origin, we have observed an even greater sensitivity to the drug. These results are shown in Figure s1 (Supplemental Digital Content) and indicate a 20 to 50 times greater sensitivity of SK-MES cells to lovastatin compared with adenocarcinoma A549 cells. We are currently investigating in depth the molecular etiology of this higher sensitivity of SK-MES cells and its implications. However, overall, our results demonstrate that lung cancer cells show significant radiosensitization in response to lovastatin that should be explored further in preclinical in vivo and in clinical studies.

As other statins, beyond lovastatin, have shown antiproliferative effects in cancer cells,[15] one wonders whether lung cancer cell radiosensitization is a phenomenon unique to lovastatin. For that, we began to explore the effects of other statins in A549 cells. In preliminary studies, we observed that simvastatin is also able to inhibit proliferation of A549 cells (as shown earlier[29]) and to sensitize lung cancer cells to IR. This indicates that radiosensitization is likely a common effect for this class of agents (Figure s2, Supplemental Digital Content).

Effects on EGFR and Effector Kinases

Adenocarcinoma A549 cells have a genetic profile that offers a survival advantage including a *K-Ras* (Gly12-Ser) mutation.[30] K-Ras activates the PI3k-Akt pathway[31] and that is required for NSCLC tumorigenesis in K-Ras mutant mice.[32] Because it inhibits posttranslational modification of Ras GTP-binding proteins, lovastatin is expected to abrogate oncogenic K-Ras and EGFR signaling. In this study, we observed that lovastatin selectively abrogated EGF-stimulated phosphorylation of EGFR and Akt but not Erk1/2. This discrepancy was observed also by Mantha et al.[33] in SCC9 head and neck tumor cells and suggests that (1) persistent EGFR phosphorylation may not be required for Erk1/2 activation and (2) activation of Erk1/2 alone is not adequate to confer radiation resistance. Our observations suggest that in lung cancer cells, lovastatin is able to inhibit selectively the key prosurvival pathway of Akt. This alone could account for the antiproliferative and proapoptotic effects of the drug.

Lovastatin Regulation of IR-Activated Signals

Effects on Akt

Similar to EGF-induced signals, lovastatin attenuated IR-activation of Akt, in a mevalonate-dependent fashion but did not affect IR-activation of Erk1/2 (Figure 3*A–C*). Similarly, Mistafa and Stenius[34] found that statins primarily target the Akt pathway to sensitize pancreatic cancer cells to chemotherapeutic drugs, without effecting Erk. Studies in K-Ras mutant cells, including A549, have shown that in these cells, activation of the EGFR-PI3k-Akt pathway confers radioresistance[35] and that inhibition of this axis by EGFR inhibitors sensitizes cells to IR.[36] Further, Akt is an established mediator of radiation resistance in many cancer cells.[37] The effect of lovastatin to inhibit IR activation of Akt illustrates a key property of this drug that luckily mediates its radiosensitization action.

Effects on AMPK

A549 cells also carry a point mutation of the *LKB1* gene (codon 37 [Q-Ter]) that generates a truncated LKB1 product.[38] Therefore, these cells lack LKB1-regulated AMPK activation, an event that is shown to lead to aberrant activation of the Akt—mTOR pathway activating protein synthesis and survival.[39] In this study, in agreement with earlier studies with statins,[40] we observed that lovastatin alone activated AMPK. However, we observed that it also potentiated its activation by IR (Figure 4*C*). Recently, we observed that IR activates AMPK in LKB1 null A549 cells.[5] Our observations in the same cells here suggest that lovastatin also activates AMPK in an LKB1-independent fashion. These observations are significant because AMPK is (1) shown to dephosphorylate and inhibit Akt through increased protein phosphatase 2A activity[41] and (2) to inhibit the mTOR pathway by directly phosphorylating either its upstream regulator tuberous sclerosis 2 or its binding partner Raptor.[39]

Importantly, AMPK activation also mimics statin action because this kinase is known to inhibit HMG-CoA reductase.[42] Therefore, AMPK activation by stimuli such as IR can work synergistically with lovastatin to augment the effects of inhibition of the mevalonate pathway. Taking these notions together with the discussion earlier, lovastatin seems to be a highly attractive agent with dual potential to enhance the activity of AMPK and inhibit the Akt pathway through a number of potential molecular steps.

Modulation of Cell Cycle

Cell Cycle Regulators

IR regulates cell cycle through the induction of p53 and CDKIs, $p21^{cip1}$ and $p27^{kip1}$, expression to mediate mainly an arrest at the G2-M checkpoint.[43] We hypothesized that lovastatin's antiproliferative effects may involve arrest of the cell cycle through enhanced expression of p53 and CDKIs. Although we did observe a potent induction of p53, $p21^{cip1}$, and $p27^{kip1}$ expression by IR alone, lovastatin inhibited IR induction of p53 and CDKI expression (Figure 5*A*). This may be due to either (1) effects of the drug on global gene transcription and translation or (2) a dependence of p53 and CDKI expression on specific events inhibited by lovastatin. Statins were shown to inhibit mTOR-dependent phosphorylation or deactivation of the translational repressor eukaryotic initiation factor 4E-binding protein, leading to suppression of initiation of cap-dependent mRNA translation.[44] It should be stressed that we did not detect in our study any significant effects on the levels of any other proteins, including signaling molecules or actin, suggesting that a global effect on gene expression is unlikely. Conversely, Akt activity, which is inhibited by lovastatin, is required for the DNA damage–induced stabilization of p53,[45] and this mechanism may be active in lovastatin-treated cells. A decrease in $p21^{cip1}$ levels with statin treatment was observed by other investigators in A549 cells.[22] Consistent with observations in HeLa cells,[27] our work suggests that the mechanism of radiosensitization of A549 cells is independent of p53 and the CDKIs $p21^{cip1}$ and $p27^{kip1}$.

Cell Cycle

Lovastatin was shown to inhibit cell cycle progression at G0/G1 phase and promote apoptosis in thyroid cancer,[46] breast cancer,[47] glioblastoma,[48] cervical cancer cells,[27] and squamous cell carcinomas.[49] In this study, we observed that lovastatin treatment shifted cells into G0/G1 phase with a markedly increased proportion of cells moving into apoptosis after 48 hours of treatment (Figure 5*B*). Prolonged treatment with lovastatin (96 hours) induced marked induction of apoptosis in nonradiated cells and caused a reversal of the G2-M checkpoint arrest induced by IR and a G0/G1 and apoptotic distribution (Figure 5*B*, *C*). It is possible that inhibition of the IR-induced G2-M arrest by lovastatin induced radiosensitization through prevention of DNA repair and induction of genomic instability.

Induction of Apoptosis

Consistent with the cell cycle analysis results, we observed that lovastatin alone induced cleaved caspase 3, a significant contributor to protein degradation. Although IR caused a reversible induction of this marker that was not detectable after 24 hours, lovastatin enhanced and prolonged the IR-induced cleaved caspase 3 formation for up to 72 hours (Figure 6*A*). Furthermore, morphological analysis verified a progressive formation of apoptotic bodies with continued incubation with lovastatin (Figure 6*B*, *C*). Overall, our results are consistent with other studies,[22,50] suggesting apoptosis as major mechanism of the cytotoxic action of lovastatin and suggests that this is also a predominant mode of action of the drug when combined with radiation in lung cancer cells.

Potential for Clinical Development in Lung Cancer in Combination with Radiotherapy

A number of clinical studies explored the potential of lovastatin to achieve tumoricidal doses in human patients. Typical doses of lovastatin aiming to control cholesterol levels in humans are approximately 1 mg/kg/d and are shown to yield serum concentrations in the range of 0.15 to 0.3 μM.[51] Early phase dose-escalation studies have explored a number of regiments, and in a study of 7 consecutive days treatment, in 4-week cycles, doses up to 25 mg/kg/d were

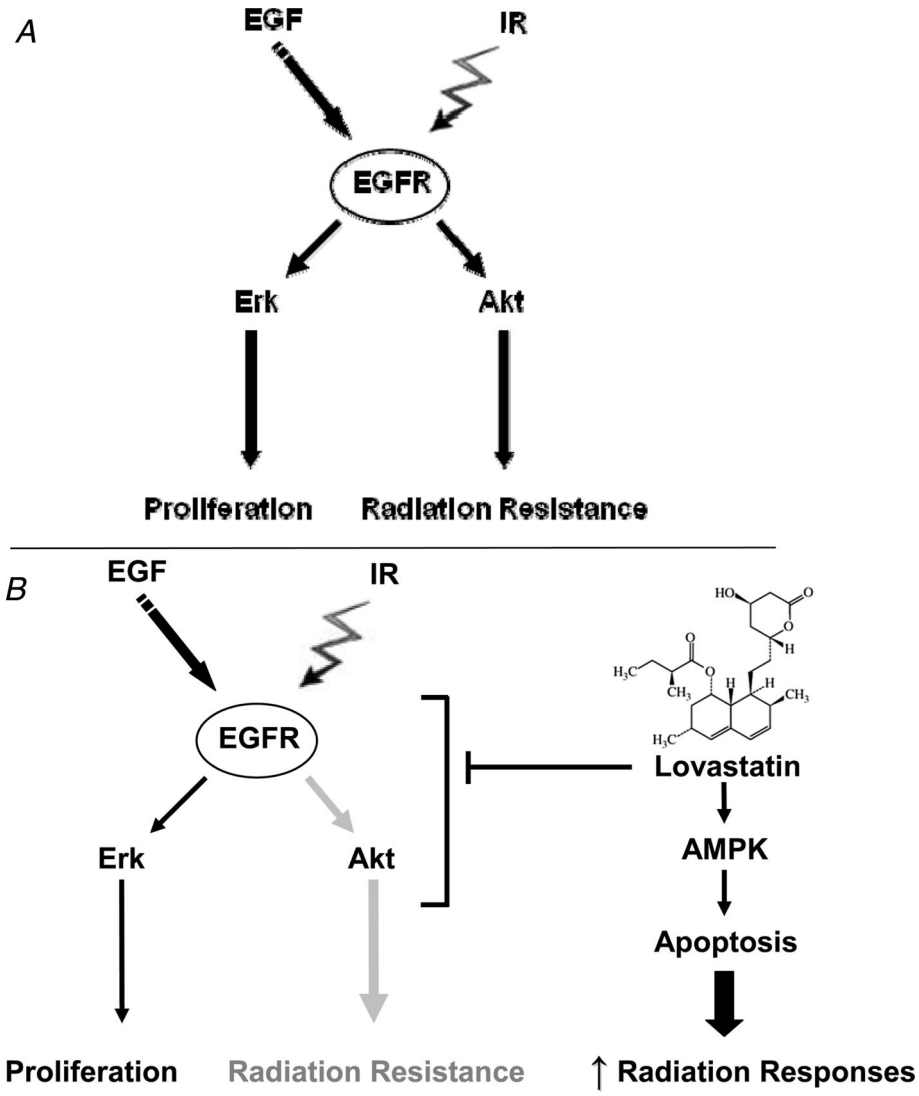

FIGURE 7. Model of lovastatin-induced sensitization of lung cancer cells to radiation. *A,* Stimulation of epidermal growth factor receptor (EGFR) signaling and activation of Erk and Akt stimulate proliferation and induce radiation resistance in lung cancer cells. *B,* Lovastatin inhibits Akt and stimulates activation of AMPK leading to apoptosis and radiation sensitization. IR, ionizing radiation.

tolerated without severe myopathy.[51] Ubiquinone is used to address myopathy. Under these conditions, maximum tolerated doses were not reached, and systemic drug concentrations reached 0.1 to 3.92 μM.[51] In a study with end-stage head and neck and cervix cancers patients,[52] a regiment of 7.5 mg/kg/d for 21 consecutive days in 4-week cycles was defined as maximum tolerated doses in patients with good renal function. Although no objective responses were seen in this study, where lovastatin was used as a single agent, the authors still reported a 23% rate of stable disease at 3 months,[52] which is indeed encouraging in patients with end-stage disease.

The aforementioned studies suggest that it is possible to achieve safely tumoricidal and radiosensitizing doses of lovastatin in cancer patients. Our work indicates that some lung cancer tumors may exhibit sensitivity to lovastatin even in the high nanomolar range (Figure s1, Supplemental Digital Content and discussion earlier) making it even more plausible that lovastatin will sensitize tumors to IR in human patients. Overall, these data indicate that this drug deserves further investigation with in vivo preclinical and clinical studies. Although, other statins may also be able to radiosensitize lung tumor cells (Figure s2, Supplemental Digital Content and discussion above), lovastatin remains the best studied agent in this class, in both the preclinical and the clinical setting and, therefore, is the most favorable candidate for further development.

CONLCUSIONS

Figure 7 illustrates our model of the action of lovastatin in lung cancer cells. Our work suggests that lovastatin is a promising agent with significant antitumor properties as a single agent and a radiation sensitizer. Lovastatin seems to function mainly through induction of apoptosis. This effect may be mediated by a unique simultaneous inhibition of the prosurvival Akt and activation of the tumor suppressor AMPK pathways. This work presents compelling evidence that support further investigation of lovastatin as a radiation sensitizer in vivo. Work in animal models of lung cancer will

expedite the development of this drug to the clinical setting in early phase studies in combination with radiotherapy.

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