

Lovastatin Protects Human Endothelial Cells from Killing by Ionizing Radiation without Impairing Induction and Repair of DNA Double-Strand Breaks

Tobias Nübel, Julia Damrot, Wynand P. Roos, Bernd Kaina, and Gerhard Fritz

Abstract Purpose: 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) are frequently used lipid-lowering drugs. Moreover, they are reported to exert pleiotropic effects on cellular stress responses, proliferation, and apoptosis. Whether statins affect the sensitivity of primary human cells to ionizing radiation (IR) is still unknown. The present study aims at answering this question. **Experimental Design:** The effect of lovastatin on IR-provoked cytotoxicity was analyzed in primary human umbilical vein endothelial cells (HUVEC). To this end, cell viability, proliferation, and apoptosis as well as DNA damage-related stress responses were investigated. **Results:** The data show that lovastatin protects HUVEC from IR-induced cell death. Lovastatin did not confer radioresistance to human fibroblasts. The radioprotective, antiapoptotic effect of lovastatin was observed at low, physiologically relevant dose level (1 $\mu\text{mol/L}$). Lovastatin affected various IR-induced stress responses in HUVEC: It attenuated the increase in p53/p21 protein level and impaired the activation of nuclear factor- κB , Chk-1, and Akt kinase but did not inhibit extracellular signal-regulated kinase activation. Exposure of HUVEC to IR did not change the level of Bax and Bcl-2 and did not cause activation of caspase-3, indicating that radioprotection by lovastatin does not depend on the modulation of the mitochondrial death pathway. Also, IR-induced DNA double-strand break formation and repair were not influenced by lovastatin. **Conclusions:** The data show that lovastatin has multiple inhibitory effects on IR-stimulated DNA damage-dependent stress responses in HUVEC. Because lovastatin causes radioresistance, it might be useful in the clinic for attenuating side effects of radiation therapy that are related to endothelial cell damage.

Cellular sensitivity to ionizing radiation (IR) is determined by numerous factors. Most important are DNA repair (1) and radiation-induced signaling mechanisms that cause changes in gene expression, cell cycle progression, and apoptosis (2). DNA damage induced by IR (e.g., DNA strand breaks) causes activation of the DNA damage-specific kinases ATM/ATR and DNA-PK cs (2–4). Subsequently, downstream functions, such as p53 and checkpoint kinases, become activated, resulting in changes in repair and cell cycle progression and, possibly, induction of cell death (5). Apart from DNA damage-triggered functions, IR also causes activation of cell surface receptors that eventually lead to the activation of mitogen-activated protein kinases and transcription factors, e.g., activator protein-1 (AP-1)

and nuclear factor- κB (NF- κB ; refs. 6–8). Similar to DNA damage-triggered stress responses, signal mechanisms originating from activated cell receptors also affect the cellular susceptibility to radiation (9, 10).

A pharmacologic approach for intervening with radiation-induced stress responses is based on the fact that Ras and Rho GTPases, which are required for genotoxic stress-stimulated activation of mitogen-activated protein kinases (8) and NF- κB (11), are subject to COOH-terminal prenylation (12). Attachment of a C15 or C20 lipid moiety to the cysteine of the COOH-terminal-located CAAX box is essential for the physiologic activity of Ras/Rho, because it is required for their correct localization at the cell membrane (12). The clinically highly relevant group of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins), which are widely used for lipid-lowering reason, cause depletion of the cellular pool of isopren precursor molecules. Thereby, statins eventually lead to a down-modulation of Ras/Rho-regulated signal mechanisms (13, 14). For example, it has been shown that lovastatin blocks UV-induced activation of c-Jun N-terminal kinase 1 and NF- κB to an extent that is similar to Rho-inactivating clostridial toxins (11, 15). Also, the IR-triggered activation of NF- κB gets attenuated by Rho inhibiting clostridial toxins as well as by overexpression of dominant-negative Rho mutants and by lovastatin (16). The Ras-related GTPase RhoB affects the susceptibility of cells to killing by γ -rays (17) and Ras-dependent mechanisms interfere with γ -ray-triggered cellular

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stress responses and cell survival as well (18–20). Furthermore, inhibitors of farnesylation, which affect Ras- and RhoB-regulated signaling (21), modulate cellular resistance to tumor-therapeutic drugs and radiation (22, 23). The same holds true for statins (23, 24). Various preclinical *in vitro* studies showed that statins impair G₁-S transition (25) and trigger apoptosis in tumor cells (26). This proapoptotic effect argues for a clinical usefulness of statins as anticancer drugs (23). Unfortunately, yet, their cytotoxic effect is not very tumor specific because statin-induced apoptosis has recently also been observed in primary rat pulmonary vein endothelial cells (27) as well as HUVECs (28). This opens the possibility that statins may exert undesired effects on specific nontumor compartments in the body if applied at higher dose level in tumor therapy. Thus far, no data are available regarding the effect of statins on the sensitivity of primary human cells under situations of coadministration of conventional anticancer drugs or radiation therapy. This issue, however, is of outmost importance in view of the question of whether intake of statins, e.g., for cardiovascular reasons, affects a patient's prognosis in case of tumor therapy. Therefore, in the present study, we addressed the question of whether the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor lovastatin influences the sensitivity of primary human umbilical vein endothelial cells (HUVEC) toward the cytotoxic and genotoxic effects of IR. Here, for the first time, we provide evidence that this is indeed the case.

Materials and Methods

Materials. The 3-hydroxy-3-methylglutaryl CoA reductase inhibitor lovastatin and pan-caspase inhibitor Z-VAD-FMK were purchased from Calbiochem (Bad Soden, Germany). Bcl-2, Bax, extracellular signal-regulated kinase (ERK), p21, and p53 antibodies used in this study were obtained from Santa Cruz (San Diego, CA). Antibodies detecting the cleavage product of activated caspases and phosphospecific antibodies p-I κ B α , phosphorylated extracellular signal-regulated kinase (p-ERK), p-Chk-1, and p-Akt originate from New England Biolabs GmbH (Frankfurt, Germany). γ -H2AX and activating anti-Fas antibody were purchased from Upstate (Hamburg, Germany). Pifithrin is from Alexis Biochemicals (Grünberg, Germany).

Cell culture conditions and determination of cell viability. Primary human umbilical vein endothelial cells (HUVEC) and normal human dermal fibroblasts as well as the corresponding growth medium (EGM medium and fibroblast growth medium supplemented with 2% FCS) were obtained from Cambrex BioScience (Verviers, Belgium). Peripheral blood lymphocytes were isolated using Lymphoprep according to the protocol of the manufacturer. Cell viability was quantitated by use of the WST assay according to the protocol of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

Analysis of apoptotic cell death. The frequency of radiation-induced apoptotic cell death was determined by Annexin V/propidium iodide double staining and subsequent fluorescence-activated cell sorting (FACS) analysis. Alternatively, sub-G₁ population was determined by FACS. Activation of caspases was assayed by Western blot analysis using antibodies specifically detecting the cleaved (activated) form of caspase-3, caspase-8, or caspase-9.

Analysis of DNA replication. To assay the effect of lovastatin on IR-induced effects on DNA replication, cells were irradiated and pulse-labeled 12 to 24 hours later for 2 hours by the addition of BrdUrd. BrdUrd incorporation was determined by an ELISA-based method (Roche Diagnostics GmbH). DNA replication in IR-treated cells was related to that of the corresponding nonirradiated controls that were set to 100%.

Western blot analysis. Ten to 30 μ g of protein from total or nuclear extract were separated in 10% to 12.5% SDS polyacrylamide gels. After wet blotting to nitrocellulose and blocking of unspecific binding (5% dry milk in PBS/0.1% Tween 20; overnight at 4°C), filters were incubated for 2 hours at room temperature (or alternatively overnight at 4°C) with the corresponding primary antibody diluted 1:100 to 1:1,000 in 5% bovine serum albumin in PBS/0.1% Tween 20. After washing and incubation with secondary peroxidase-coupled antirabbit or antimouse antibody (1:5,000), proteins were visualized by autoradiography using the Renaissance enhanced luminol reagent (Du Pont NEN, Bad Homburg, Germany, Belgium).

Reverse transcription-PCR analysis. To determine the expression of Fas receptor and FAS ligand on mRNA level, reverse transcription-PCR analysis was done. Upon isolation of total RNA using Qiagen total RNA isolation kit, first-strand cDNA synthesis was done (Qiagen cDNA synthesis kit). For standard PCR reaction (35 cycles, annealing temperature 58°C), the following primers were used: FAS-R, 5'-AAGGGATTGGAATTGAGGAAGACTG-3' and 5'-GTGGAATTGG-CAAAAGAAGAAGAC-3'. FasL: 5'-CCCCTCCAGGCACAGTCTTCC-3' and 5'-CTTGTGGCTCAGGGGCAGGTTGT-3'. Glyceraldehyde-3-phosphate dehydrogenase: 5'-GAAGGTGAAGGTCCGAGTC-3' and 5'-GAA-GATGGTGATGGGATTTC-3'. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Analysis of DNA strand break induction. To quantitate the level of DNA strand break induction upon genotoxic treatment, the alkaline or the neutral comet assay was done as described (29). Under alkaline conditions, DNA single-strand breaks and apurinic sites become detectable, whereas the neutral comet predominantly detects DNA double-strand breaks (DSB). Briefly, at different time points after exposure, $\sim 10^4$ cells were embedded in low melting point agarose and transferred onto agarose-covered microscope slides. Cells were lysed for 1 hour in lysis buffer [2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% Na-laurylsarcosinate, 1% Triton X-100, and 10% DMSO (pH 10 alkaline or pH 7.5 neutral)]. In case of alkaline denaturation of DNA, cells were incubated in alkaline electrophoresis buffer [300 mmol/L NaOH and 1 mmol/L EDTA (pH 13)] for 25 minutes before electrophoresis (25 V, 300 mA for 15 minutes). Afterward, cells were washed with 0.4 mol/L Tris (pH 7.5), distilled H₂O, and finally with ethanol. In case of the neutral comet assay, electrophoresis was done with neutral running buffer [90 mmol/L Tris, 90 mmol/L boric acid, and 2 mmol/L EDTA (pH 7.5)]. DNA was stained with ethidium bromide. Comets were visualized by microscopy and quantitated by determination of the "Olive tail moment" using computer-based software (Komet 4.02, Kinetics Imaging, Liverpool, United Kingdom). Fifty cells were analyzed per measurement for calculation of the mean value. Induction of ATM or DNA-PK α -catalyzed phosphorylation of histone H2AX (γ -H2AX phosphorylation), which is indicative for DSBs (30, 31), was analyzed by Western blot analysis.

Results

To examine the influence of statins on the susceptibility of HUVEC to IR, cells were pretreated overnight with different concentrations of lovastatin before IR treatment. After a postincubation period of 48 hours, cell viability was analyzed using the WST assay. As shown in Fig. 1A, a low dose of lovastatin (1 μ mol/L) largely reduced the cytotoxicity induced by IR. At a higher concentration (i.e., 20 μ mol/L), radioprotection was even more enhanced. The radioprotective effect of lovastatin is cell type specific as it was not observed in primary human fibroblasts (Fig. 1B). To determine whether the statin-mediated increase in cell viability of irradiated HUVEC is related to cell proliferation, we analyzed DNA replication by measuring BrdUrd incorporation. In the absence of lovastatin, exposure of HUVEC to 20 Gy reduced BrdUrd incorporation

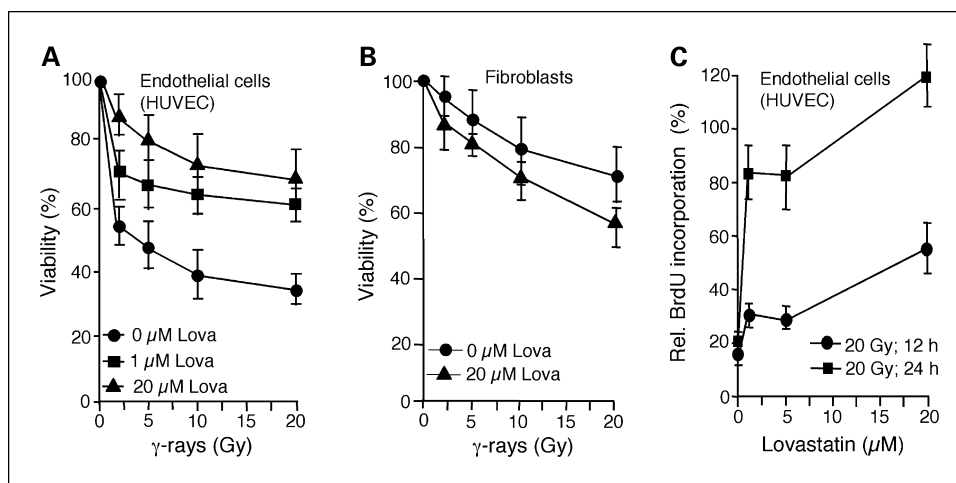


Fig. 1. Effect of lovastatin on cell viability and DNA replication after γ -irradiation of primary HUVEC and fibroblasts. *A* and *B*, primary HUVEC (*A*) and primary human fibroblasts (*B*) were pretreated overnight with the indicated concentration of lovastatin (*Lova*). Afterward, cells were irradiated with 2 to 20 Gy. After an incubation period of 48 hours, cell viability was assayed using the WST assay as described in Materials and Methods. Points, mean from three independent experiments each done in triplicate; bars, SD. *C*, primary HUVEC were pretreated with different concentration of lovastatin overnight. Afterward, cells were irradiated with 20 Gy. After an incubation period of 12 or 24 hours, cells were pulse-labeled with BrdUrd for 2 hours. Incorporation of BrdUrd was assayed as described in Materials and Methods. BrdUrd labeling of irradiated cells was related to that of the corresponding nonirradiated control, which was set 100%. Points, mean from one representative experiment done in quadruplicate; bars, SD.

by ~80% at 12 and 24 hours after radiation (Fig. 1C). Presumably, this effect is mainly due to radiation-induced cell cycle arrest (32). Pretreatment with increasing doses of lovastatin attenuated the IR-induced reduction in BrdUrd incorporation in a time- and dose-dependent manner (Fig. 1C). Twenty-four hours after irradiation, statin-pretreated cells revealed a similar proliferation rate as the corresponding nonirradiated controls (Fig. 1C). Apparently, lovastatin attenuates IR-induced cell cycle arrest and restores DNA synthesis in irradiated endothelial cells.

It has been reported that statins provoke apoptotic death of tumor cells (26, 33) and endothelial cells on their own (27, 28). We did not observe any apoptotic effect of lovastatin on HUVEC when applied overnight at low (physiologic relevant) concentration of 1 μ mol/L (Fig. 2), whereas, at this concentration, it largely blocked IR-induced apoptotic death (Fig. 2). Time course analysis showed that the radioprotective effect of 1 μ mol/L lovastatin is detectable at all time points measured (i.e., 48–96 hours after irradiation; Fig. 3A). Pretreatment of HUVEC with a 20-fold higher dose of lovastatin (20 μ mol/L) also protected HUVEC from IR-induced apoptotic death as measured up to 72 hours after irradiation (Fig. 3A). At later time points, however (i.e., 96 hours after radiation), 20 μ mol/L of lovastatin promoted radiation-induced apoptosis (Fig. 3A), presumably because it caused apoptosis on its own (Fig. 3A and B). It seems that the effect of lovastatin on apoptotic cell death triggered by radiation treatment is dose dependent, with antiapoptotic effects occurring at low and proapoptotic effects at a high concentration (detectable at very late time points). In a lipid-lowering therapeutic situation, the concentration of lovastatin that is achieved in man is in the low micromolar range (34). Therefore, we suppose that the antiapoptotic rather than the proapoptotic effects of lovastatin are relevant under therapeutic situation in men.

DSBs are considered to be the main cytotoxic lesion induced by IR and are known to trigger genomic instability and apoptosis (35). Yet, neither initial DSB formation nor the

amount of residual DSBs remaining after a postirradiation period of 4 hours were affected by lovastatin pretreatment (Fig. 4A). Essentially the same result was obtained by measuring IR-triggered γ -H2AX phosphorylation (Fig. 4B), which is an indicator of DSB formation and repair (30). The induction and repair of DNA single-strand breaks also remained unaffected by lovastatin (data not shown). Thus, it seems that lovastatin-mediated radioprotection of HUVEC is independent of the formation and repair of DNA strand breaks induced by IR.

Well-known players in the regulation of radiation-induced apoptosis are NF- κ B (10) and p53 (36). Although NF- κ B is believed to act in a radioprotective way (10), p53-related functions can contribute both to radioresistance and radiosensitivity (36). Irradiation of HUVEC results in a rapid activation of NF- κ B, as indicated by phosphorylation of I κ B α , which is abrogated by lovastatin (Fig. 5A; ref. 16). IR also provoked a clear increase in the levels of p53 and p21 protein in HUVEC (Fig. 5A). Radiation-induced stabilization of p53 and increased expression of p21 were clearly attenuated by lovastatin (Fig. 5A). p53 and NF- κ B are part of the DNA damage response, which is triggered by sensors of DNA damage, such as ATM/ATR and DNA-PK $_{cs}$ (4, 37). To check whether lovastatin interferes with elements of the IR-induced DNA damage response, the activation of checkpoint kinase Chk-1, which is regulated by ATM/ATR and DNA-PK $_{cs}$ (37), was investigated. As shown in Fig. 5B, IR leads to an increase in Chk-1 phosphorylation that was blocked by lovastatin (Fig. 5B). Lovastatin also inhibits Akt kinase (Fig. 5B), which can also be activated in a DNA damage and ATM/ATR-dependent manner (38). On the other hand, IR-induced activation of ERK, which is triggered in a DNA damage-independent manner via activation of epidermal growth factor receptor and Ras (39), was not blocked by lovastatin (Fig. 5B). To substantiate that the inhibition of p53/p21-regulated functions might be most relevant for lovastatin-mediated radioprotection, we examined whether inhibition of p53 by pifithrin is able to mimic the radioprotective effect of lovastatin. As shown in Fig. 5C, pifithrin slightly increased IR resistance in

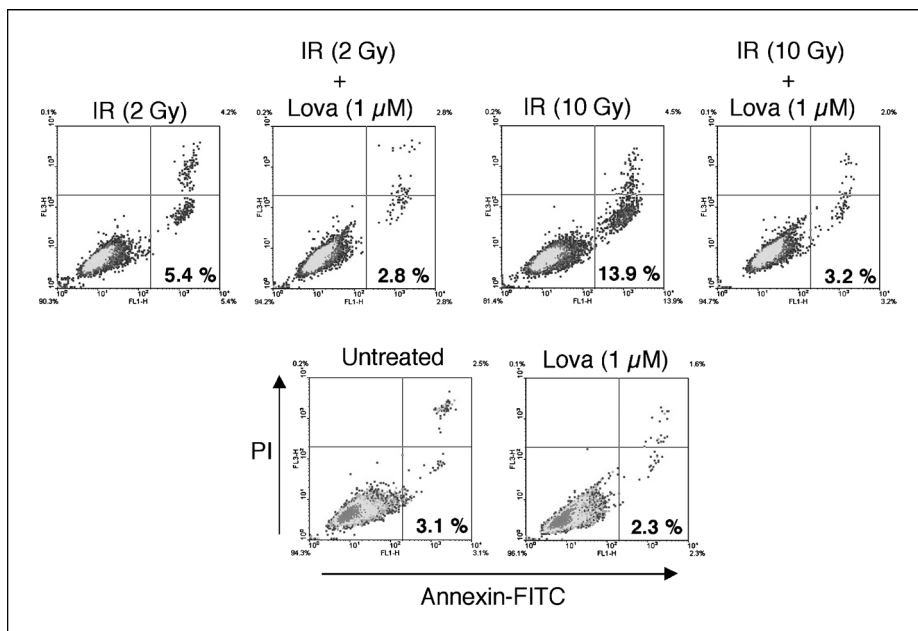


Fig. 2. Lovastatin protects HUVEC from γ -ray – induced apoptosis. After overnight pretreatment with lovastatin (1 μ mol/L), HUVEC were exposed to different doses of γ -rays (2 or 10 Gy). After an incubation period of 72 hours, cells were harvested and the frequency of apoptotic cell death was quantitated using FACS-based Annexin V method.

HUVEC, indicating that at least part of the radioprotective effect of lovastatin is due to the inhibition of p53-regulated proapoptotic mechanisms.

Apoptosis in HUVEC triggered by IR could be executed by the death receptor or mitochondrial death pathway or by another caspase-independent mechanism. Both the expression of CD95 receptor (CD95-R) and its ligand (CD95-L) are stimulated by IR in HUVEC (Fig. 5D). IR-provoked stimulation of CD95-L (FAS-L) mRNA expression was attenuated by lovastatin (Fig. 5D), whereas the mRNA and protein expression

of CD95-R remained unaffected (Fig. 5D). FAS-activating antibody, which is able to induce cell death in primary human lymphocytes (Fig. 6B), did not cause cell death in HUVEC (Fig. 6A). Furthermore, activation of caspase-8, which is a downstream event of FAS activation, was not detected after irradiation of HUVEC (data not shown). Based on these data, we suggest that activation of the FAS system is not involved in IR-stimulated apoptosis in HUVEC. Bax and Bcl-2 are key players in the regulation of the mitochondrial death pathway. Radiation treatment of HUVEC did not change the expression level of Bax

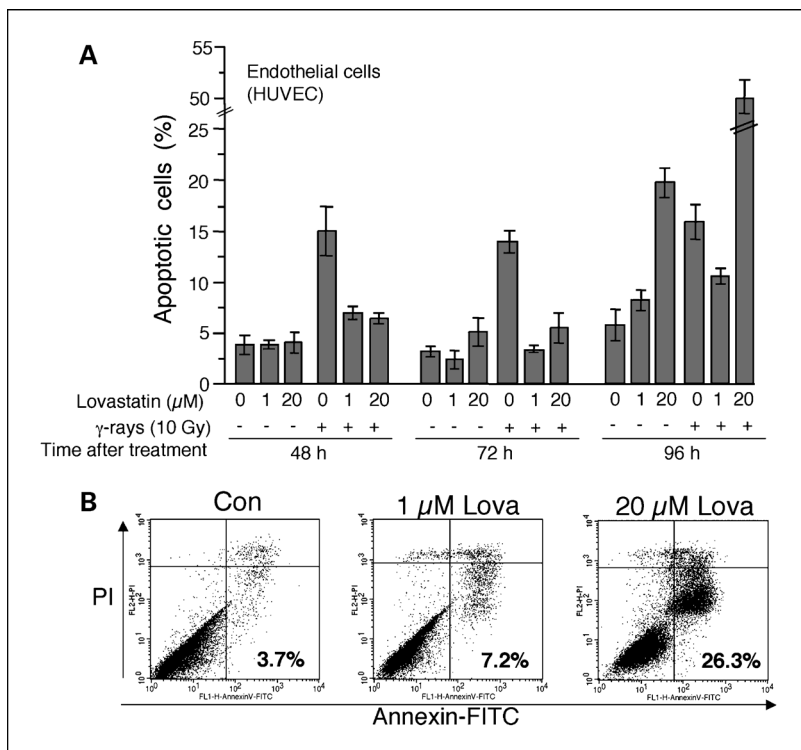


Fig. 3. Dose- and time-dependent pro- and antiapoptotic effects of lovastatin on basal and IR-induced apoptosis in HUVEC. **A.** after overnight treatment with either low (1 μ mol/L) or high dose (20 μ mol/L) of lovastatin, HUVEC were irradiated (10 Gy). After incubation periods of 48 to 96 hours, cells were harvested for determination of apoptotic death using the Annexin V methods as described in Materials and Methods. Columns, mean from at least two independent experiments each done in duplicate; bars, SD. Identical results were obtained upon quantitation of apoptotic death by measuring sub-G₁ population (data not shown). **B.** representative analysis of the effect of lovastatin provoked 96 hours after overnight pretreatment of HUVEC with low (1 μ mol/L) and high (20 μ mol/L) statin concentration (no irradiation).

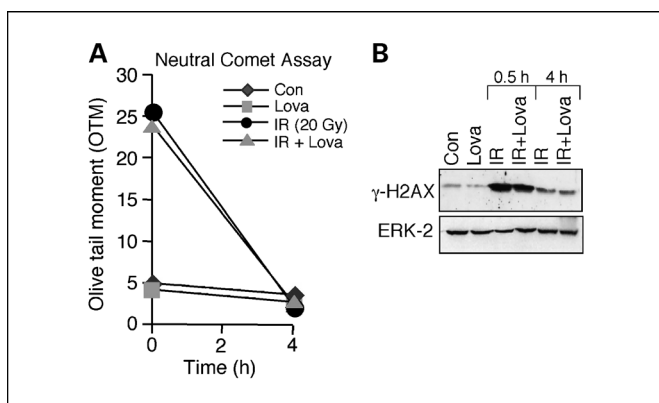


Fig. 4. IR-induced formation and repair of DNA DSBs are not affected by lovastatin. **A**, after overnight pretreatment with lovastatin (1 $\mu\text{mol/L}$), HUVEC were irradiated (20 Gy). Immediately after irradiation or after postincubation period of 4 hours, cells were harvested and the level of DSBs was quantitated by the neutral comet assay as described in Materials and Methods. Shown is the result of a representative experiment. **B**, HUVEC were pretreated with lovastatin as described in (A). Thirty minutes and 4 hours after irradiation (20 Gy), nuclear extracts were prepared and analyzed for the level of phosphorylated histone ($\gamma\text{-H2AX}$), which is a generally accepted marker for DSBs.

or Bcl-2 protein (Fig. 6C). Furthermore, IR-induced activation of caspase-9 (data not shown) and caspase-3 (Fig. 6D) was not detectable in HUVEC. Because cisplatin and a high dose of lovastatin caused caspase-3 activation in HUVEC (Fig. 6D), we conclude that HUVEC are not generally compromised in the activation of caspase-3. This is in line with a previous report (28). The pan-caspase inhibitor Z-VAD attenuated IR-induced increase in apoptotic death of HUVEC by $\sim 70\%$ (Fig. 6E), indicating that IR-induced cell death in HUVEC is caspase dependent. Therefore, IR-stimulated apoptosis of HUVEC seems to be regulated by executive caspases other than caspase-3.

Discussion

3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) have pleiotropic biological effects that are partially

independent from their cholesterol-lowering activity. It is believed that statins disturb the COOH-terminal prenylation and, therefore, the membrane localization and function of low molecular weight GTPases, in particular Ras and Rho (13). Ras/Rho GTPases are well-known regulators of genotoxin-inducible stress responses controlling gene expression, proliferation, DNA repair, and apoptosis (14). Previously, we have shown that IR-triggered activation of NF- κB and subsequent expression of the cell adhesion molecule E-selectin in primary HUVEC requires Rho proteins and is abrogated by lovastatin (16). Bearing in mind that (a) severe side effects of radiation therapy are related to the induction of endothelial dysfunction (40), (b) Rho proteins affect cellular susceptibility to $\gamma\text{-rays}$ (17, 41), and (c) NF- κB protects cells from radiation-induced cell death (10), the question arose as to whether lovastatin has an effect on the cytotoxicity of IR in primary HUVECs.

Measuring cell viability, DNA synthesis, and apoptosis, we observed that pretreatment of HUVEC with a low physiologically relevant dose of lovastatin (1 $\mu\text{mol/L}$) protects against the cytotoxic effects of a subsequent radiation exposure. Changes in the basal level of apoptosis were not observed under our experimental conditions. Only at a high dose level (20 $\mu\text{mol/L}$) did lovastatin potentiate IR-triggered late apoptosis (i.e., after 96 hours) and provoke apoptosis on its own. Apparently, depending on the dose, lovastatin can either protect or promote cell death: At low dose, it has a radioprotective function, whereas at high dose it can act as a radiosensitizer. It has been suggested that statins induce apoptosis specifically in tumor cells (26, 33), making statins highly attractive as anticancer drugs (23). Yet, recent reports showed that statins also cause apoptosis in primary endothelial cells of rodent and human origin (27, 28). In all of these studies, clear apoptosis-inducing effects have been observed only at relatively high dose level ($>10 \mu\text{mol/L}$). Because the serum concentration of statins, which is therapeutically relevant for lipid lowering, is in the low micromolar range (34), statins are not expected to cause cytotoxic side effects on endothelial cells *in vivo*. This is in line with the fact that statins are well tolerated (as known from their broad clinical application)

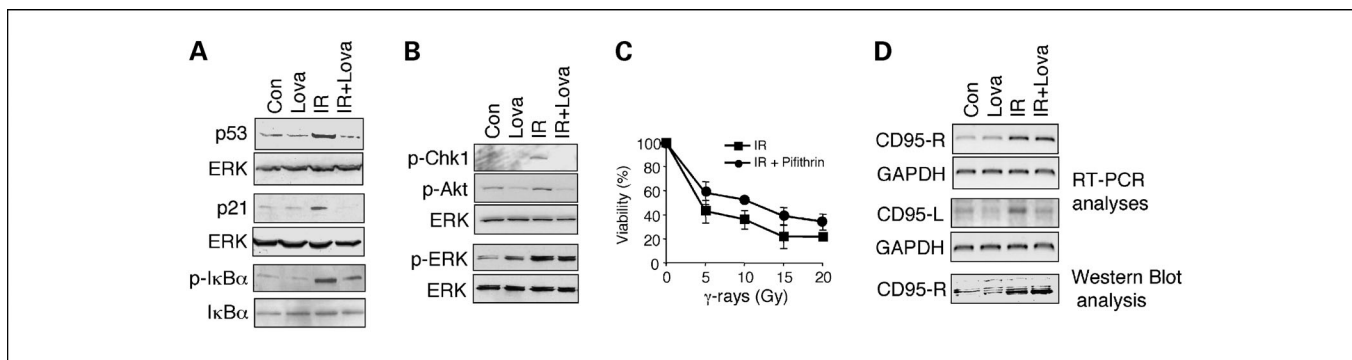


Fig. 5. Lovastatin exerts pleiotropic inhibitory effects on IR-inducible stress responses. **A**, after overnight pretreatment with lovastatin (1 $\mu\text{mol/L}$), HUVECs were exposed to $\gamma\text{-rays}$ (IR, 10 Gy). After incubation periods of 2 and 24 hours, expression of p53 (nuclear extract) and p21 (total extract) was analyzed by Western blot analysis. As a loading control, filters were reprobbed with anti-ERK2 (ERK) antibody. Phosphorylation of I $\kappa\text{B}\alpha$ (p-I $\kappa\text{B}\alpha$), which is indicative of activation of NF- κB , was determined 60 minutes after irradiation. I $\kappa\text{B}\alpha$, nonphosphorylated protein. **B**, lovastatin pretreated (1 $\mu\text{mol/L}$, overnight) or nonpretreated HUVECs were exposed to $\gamma\text{-rays}$ (10 Gy). Six hours after exposure, cells were harvested and phosphorylated (activated) forms of checkpoint kinase (p-Chk1) and Akt (p-Akt) kinase were determined by Western blot analysis. Activated form of ERK (p-ERK) was analyzed 2 hours after radiation exposure. **C**, HUVECs were pretreated or not with the p53 inhibitor pifithrin (30 $\mu\text{mol/L}$) for 1 hour. Afterward, cells were irradiated with different doses. Seventy-two hours after irradiation, viability of the cells was analyzed by the WST assay as described in Materials and Methods. **D**, lovastatin and radiation treatments were done as described in (A). Twelve hours after irradiation, mRNA expression of CD95-L and CD95-R was analyzed by reverse transcription-PCR (RT-PCR). mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Shown are ethidium bromide-stained gels (inverse presentation). CD95-R expression was additionally analyzed on the level of the protein 24 hours after radiation treatment (Western blot analysis).

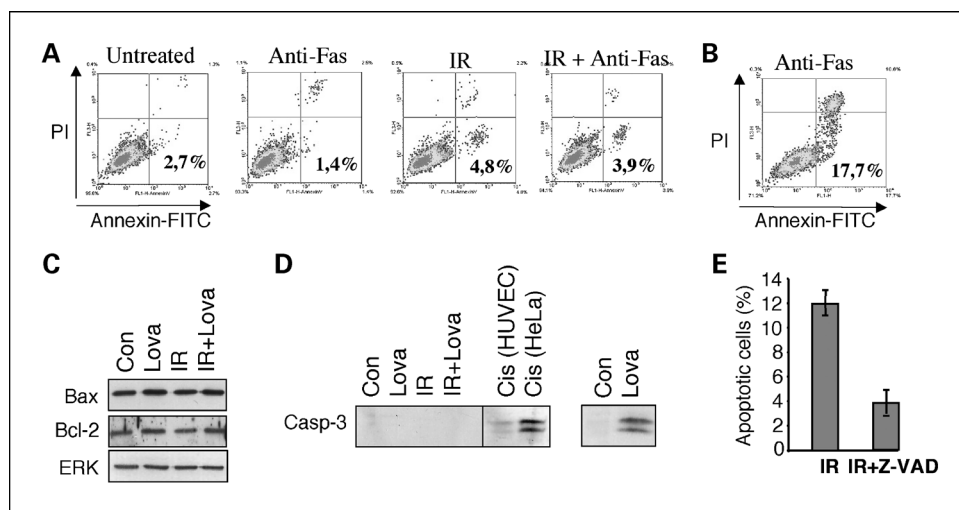


Fig. 6. IR-induced apoptosis of HUVEC is independent of activation of caspase-3. *A*, nonirradiated HUVEC or 8 hours after IR (2 Gy), HUVECs were treated with activating anti-FAS antibody (1 $\mu\text{g}/\text{mL}$). After further incubation period of 24 hours, cells were harvested for determination of cell death using the FACS-based Annexin V method. *B*, untreated human peripheral lymphocytes were exposed to activating anti-FAS antibody as described above. Frequency of apoptotic death was quantitated by FACS-based Annexin V analysis. *C*, analysis of the expression level of Bax and Bcl-2 protein 24 hours after IR exposure (10 Gy) of lovastatin pretreated or not pretreated HUVEC. To confirm equal protein loading, filter was re probed with ERK-specific antibody. *D*, after pretreatment with lovastatin (1 $\mu\text{mol}/\text{L}$, overnight) HUVECs were exposed to IR (10 Gy). Seventy-two hours later, activated caspase-3 was determined by Western blot analysis. As controls, the effect of cisplatin (50 $\mu\text{mol}/\text{L}$, 3-hour treatment) on caspase-3 activation in HUVEC and HeLa cells was analyzed. Furthermore, data showing the effect of treatment of HUVEC with high dose of lovastatin (20 $\mu\text{mol}/\text{L}$) on caspase-3 activation was included as a further positive control. *E*, effect of the pan-caspase inhibitor Z-VAD on IR-induced apoptosis in HUVEC. After irradiation (10 Gy), cells were postincubated in the presence or absence of the pan-caspase inhibitor Z-VAD for 48 hours before the frequency of apoptotic cells was determined by FACS (Annexin V measurement). Shown is the radiation-induced increase in the percentage of apoptotic cells in the presence or absence of caspase inhibitor. Columns, mean from two experiments; bars, SD. Basically identical result was obtained after postincubation period of 72 hours (data not shown).

when used at lipid-lowering dose. Thus, the proapoptotic effects of statins, as observed at high concentration *in vitro*, may not be relevant for the *in vivo* situation. Rather, anti-apoptotic effects of low concentration of statins might be more relevant in men, especially when genotoxic stress is applied. The fact that qualitatively opposite effects can be provoked by statins is an important aspect if discussing their therapeutic usefulness as anticancer drugs, in particular if a high-dose application of statins (aimed at killing tumor cells) is considered as a therapeutic option.

Apparently, gain of radioresistance by lovastatin is independent of mechanisms related to the induction of DNA damage or its repair. Therefore, we analyzed the effect of lovastatin on radiation-induced signal mechanisms relevant for cell survival and death. We found that lovastatin impairs radiation-induced activation of NF- κ B, blocks activation of Chk-1 and Akt kinase, and attenuates the increase in p53/p21 protein levels. Yet, IR-induced activation of ERK is not abrogated by lovastatin, which is line with data reported for UV exposure (15). Because ATM/ATR and DNA-PK $_{cs}$ -mediated sensing of DNA damage is involved in the regulation of NF- κ B (42), Akt (38), p53, and Chk-1 (3), we suggest that lovastatin interferes with the activity of DNA damage sensors. Noteworthy, this inhibitory effect of lovastatin is specific because ATM/ATR or DNA-PK $_{cs}$ -induced phosphorylation of H2AX is not affected by the statin. The inhibitory effect of lovastatin on the IR-stimulated increase in p53 might be due to the promotion of Mdm2-catalyzed degradation of p53 (43). Whereas NF- κ B and Akt kinase-regulated mechanisms are considered to be antiapoptotic, p53 is capable of either promoting or inhibiting apoptotic cell death (36). Therefore, we suggest that inhibition of radiation-induced p53/p21-related proapoptotic mechanisms by lovastatin contributes to radioresistance of HUVEC. This assumption is

supported by the fact that inhibition of p53 by pifithrin was also radioprotective in HUVEC although to a lesser extent than lovastatin. Based on the data, we furthermore suggest that the antiapoptotic potency of lovastatin is related to the inhibition of caspases other than caspase-3 and that FAS-R and Bax/Bcl are not involved in IR-triggered caspase-regulated cell death of HUVEC.

The clinically most relevant aspect of the data pertains to the role of lovastatin and other statins, which can be assumed to have similar physiologic activities as lovastatin (27, 33), in the radiation response of endothelial cells *in vivo*. An important question that needs to be answered is whether the beneficial therapeutic outcome and/or the severity of side effects of radiation therapy is altered in cancer patients who are already treated with statins (for reason of cardiovascular protection) during radiotherapy. Regarding the effect of statins on radioresistance of human tumor cells, *in vitro* studies showed that lovastatin is able to reverse radiation resistance caused by oncogenic Ras (18). The effect of lovastatin on radiosensitivity of human tumor cells that harbor wild-type Ras seems to be cell type specific, whereby HeLa cells show enhanced susceptibility to radiation-induced apoptosis (44). Also, primary human acute myelogenous leukemia cells have recently been shown to be sensitized toward radiochemotherapy if pretreated with lovastatin (45). Injury of endothelial cells is a main source of severe side effects following radiation therapy. Apart from protecting against the cell killing effects of IR, as shown in the present work, IR-induced inflammatory responses are also reduced by statins (46). Here, inhibition of NF- κ B-regulated expression of adhesion molecules (16, 46) and interleukin-6 and interleukin-8 (46) are considered as underlying mechanisms. By attenuating endothelial stress responses and by protecting them from radiation-induced cell killing, statins might be highly

useful for the reduction of side effects of radiotherapy that originate from radiation-induced dysfunction of the endothelial cell barrier. The hypothesis that statins are promising compounds for use in radio-oncology by reducing side effects is also supported by *in vitro* data, indicating that pravastatin is able to reduce late radiation enteritis (47, 48). Notably, statins might be able to interfere with the therapeutic ratio of anticancer drugs, too. Thus, it has been shown that lovastatin potentiates antitumor activity and attenuates cardiotoxicity of doxorubicin (49). In line with this report, we observed that doxorubicin-induced cell killing of HUVEC is reduced by lovastatin.¹

¹ Unpublished data.

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