Lovastatin Induces Apoptotic Cell Death by Activation of Intracellular Ca\textsuperscript{2+} Signal in HepG2 Human Hepatoma Cells

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Abstract — Although lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, has been shown to have anti-cancer actions, the effect on human hepatoma cells was not investigated. Moreover, the exact mechanism of this action is not fully understood. In this study we investigated the mechanism by which lovastatin induces apoptosis using HepG2 human hepatoblastoma cells. Lovastatin induced apoptotic cell death in a dose-dependent manner in the cells, assessed by the flow cytometric analysis. Treatment with mevalonic acid, a precursor of cholesterol, did not significantly suppress the lovastatin-induced apoptosis. Lovastatin induced a rapid and sustained increase in intracellular Ca\textsuperscript{2+} concentration. Treatment with EGTA, an extracellular Ca\textsuperscript{2+} chelator did not significantly alter the lovastatin-induced intracellular Ca\textsuperscript{2+} increase and apoptosis, whereas intracellular Ca\textsuperscript{2+} reduction with BAPTA/AM and intracellular Ca\textsuperscript{2+} release blockers (dantrolene and TMB-8) completely blocked these actions of lovastatin. In addition, the lovastatin-induced apoptosis was significantly reduced by a calpain inhibitor, a broad spectrum caspase inhibitor z-VAD-fmk and inhibitors specific for caspase-9 and caspase-3 (z-LEHD-fmk and z-DEVD-fmk, respectively), but not by an inhibitor specific for caspase-8 (z-IETD-fmk). Collectively, these results suggest that lovastatin induced apoptosis of HepG2 hepatoma cells through intracellular Ca\textsuperscript{2+} release and calpain activation, leading to triggering mitochondrial apoptotic pathway. These results further suggest that lovastatin may be valuable for the therapeutic management of human hepatoma.

Keywords □ Lovastatin, apoptosis, Ca\textsuperscript{2+} signal, calpain, mitochondrial pathway, HepG2 cells

INTRODUCTION

Lovastatin is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, and is generally regarded as a safe and effective compound used extensively for the treatment of hypercholesterolemia (Goldstein and Brown, 1990). In addition, lovastatin has been shown to inhibit tumor growth and metastasis in vivo in several different implantable rodent tumorigenesis models, e.g. in mammary carcinoma (Alonso et al., 1998; Farina et al., 2002; Inano et al., 1997; Shibata et al., 2003), fibrosarcoma (Matur et al., 1998), colon adenocarcinoma (Broitman et al., 1996), melanoma (Jani et al., 1993) and pancreatic neoplasias (Kusama et al., 2002).

In breast cancer cell cultures lovastatin induces two cell cycle inhibitors, p21Waf1 and p27Kip1, and leads to cell cycle arrest in the G1 phase (Gray-Bablin et al., 1997; Keyomarsi et al., 1991; Shibata et al., 2003; Rao et al., 1998). In other in vitro studies apoptosis has been induced by lovastatin in cancer cells derived from various organs, including the breast (Alonso et al., 1998; Borner et al., 1995; Farina et al., 2002; Shibata et al., 2003). Apoptosis in response to stimulation by lovastatin appears to be caspase-3-dependent (Shellman et al., 2005) and it induces cytochrome c release (Shibata et al., 2004) and PARP cleavage (Marcelli et al., 1998). However, the molecular mechanism activated by lovastatin in enhancing apoptosis remains unclear.

Apoptosis is a highly organized cell death process characterized by ultrastructural modification (cytoskeletal disruption, cell shrinkage, and membrane blebbing), nuclear alteration (chromatin condensation and internucleosomal DNA cleavage), and biochemical changes (activation of proteases) (Kidd, 1998). Genetic changes resulting in loss of apoptosis are likely to be critical components of carcinogenesis (Schulte-Hermann et al., 1997). Many studies have demonstrated that carcinogenic processes are significantly linked to demeasurem of apo-

Thus, the main purposes of this study were to investigate whether lovastatin induces apoptotic cell death in HepG2 human hepatoma cells and to elucidate the mechanism of the lovastatin-induced apoptosis specifically focused on intracellular Ca$^{2+}$ signal.

**MATERIALS AND METHODS**

**Materials**

The HepG2 human hepatoma cell line was purchased from American Type Culture Collection (Rockville, MA). Powdered Eagle’s minimum essential medium (MEM) and Earle’s basal salt solution (EBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dantrolene, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester (TMB-8), ethylene glycol-bis(aminomethyl) ether)$\cdot$N,N,N’N’-tetraacetic acid (EGTA), mevalonic acid (MA), trypsin solution, sodium pyruvate, probenecid, propidium iodide (PI), ribonuclease A and all salt powders were obtained from Sigma Chemical Co. (St. Louis, MO). Calpain inhibitor I (N-acetyl-Leu-Leu-Nle-CHO) was obtained from Calbiochem (San Diego, CA). 1(2,5-Carboxyoxazol-2-yl-6-amino)benzotriazol-1-carboxylate-8-(diethylamino)-octyl ester (BAPTA/AM) and bis-(o-aminophenoxy)-ethane-N,N,N’N’-tetraacetoxylmethyl ester (Fura-2/AM) were prepared as stock solutions in dimethyl sulfoxide (DMSO), then diluted with aqueous medium to the final desired concentrations. The stock solution of drugs was sterilized by filtration through 0.2 µm disc filters (Gelman Sciences: Ann Arbor, MI).

**Cell culture**

HepG2 cells were grown at 37°C in a humidified incubator under 5% CO$_2$/95% air in a MEM supplemented with 10% FBS, 200,000 IU/L penicillin, 200 µg/L of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

**Cell viability assay (MTT staining)**

Cell viability was assessed by the MTT staining method (van de Loosdrecht et al., 1991). Cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10$^4$ cells/well. The volume of the medium in the wells was 1 mL. In control experiments cells were grown in the same media containing drug-free vehicle. After incubation with drug for 48 hr, 100 µL of MTT (5 mg MTT/mL in H$_2$O) were added and cells incubated for a further 4 hr. Two hundred microliters of DMSO were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

**Flow cytometry assay**

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer, pH 7.4. After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 µg/mL PI and 50 µg/mL ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine percentage of nuclei with hypodiploid content indicative of apoptosis (Bombeli et al., 1997).

**Measurement of intracellular Ca$^{2+}$ concentrations**

Aliquots of the HepG2 cells were washed in EBSS. Then, 5 µM Fura-2/AM was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM was removed by centrifugation at 150g for 3 min. Cells were resuspended at a density of
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2×10\(^6\) cells/L in Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 5 mM NaHCO\(_3\), 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, 0.5 mL aliquot of Fura-2-loaded cells was equilibrated at 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. In the data intracellular free Ca\(^{2+}\) concentrations were presented by the 340:380 nm fluorescence ratios.

Data analysis
All experiments were performed four times. Data were expressed as mean± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Induction of apoptotic cell death by lovastatin
The effect of lovastatin on cell viability of the HepG2 cells were examined using the MTT staining method. Lovastatin decreased cell viability in a dose-dependent manner as depicted in Fig. 1. A significant cytotoxicity by lovastatin was started at the concentration of 5 µM. Lovastatin reduced cell viability to 50% at around 9 µM. To determine whether the reduced cell viability by lovastatin is due to apoptotic cell death, its effect on apoptosis of the HepG2 cells was studied. Flow cytometry assay was used for detection of apoptosis (Bombeli et al., 1997). As shown in Fig 2, lovastatin increased the number of cells with hypodiploid nuclei, indicative of apoptosis, in a dose-

![Graph](image)

Fig. 1. Effects of lovastatin on cell viability in HepG2 human hepatoma cells. Cells were incubated with or without each concentration of lovastatin (Lova) for 48 hr. Cell viability assay was done by the MTT staining method. Results are expressed as percent change of control condition in which cells were grown in medium without drugs. Data points represent the mean values of four replications with bars indicating SEM.

![Graph](image)

Fig. 2. Lovastatin induces apoptotic cell death of HepG2 human hepatoma cells. In figure A, after cells were treated with or without lovastatin (Lova, 20 µM) for 48 hr, the cells were stained with PI and analyzed by flow cytometry. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G\(_0\)/G\(_1\) peak, designated A\(_o\), was defined as cells undergoing apoptosis-associated DNA degradation. In figure B, the cells were incubated with Lova for 48 hr at each designated concentration. The data represent the mean values of four replicates with bars indicating SEM.
dependent manner. These results clearly showed that lovastatin induced apoptotic cell death in the HepG2 cells.

**Role of intracellular Ca\(^{2+}\) signal in the lovastatin-induced apoptosis**

Since lovastatin is a cholesterol-lowering drug that exerts pleiotropic functions by preventing the synthesis of mevalonic acid (MA), the precursor of non-steroidal isoprenoid compounds that play a key role in a number of cellular processes, such as post-translational modification of proteins (McTaggart, 2006), cell differentiation (Buhaescu and Izzedine, 2007) and cell proliferation (Alegret and Silvestre, 2006), we investigated whether administration of MA can inhibit the lovastatin-induced apoptosis. As shown in Fig. 3B, treatment with MA did not abrogate the lovastatin-induced apoptosis. These results suggest that lovastatin may induce apoptotic cell death through a mevalonate-independent mechanism.

Intracellular Ca\(^{2+}\) signal appears to be commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1997). Thus we examined whether lovastatin alters intracellular Ca\(^{2+}\) concentration using Fura-2 fluorescence technique (Grynkiewicz et al., 1985). Lovastatin at the concentration inducing apoptosis (20 \(\mu\)M) rapidly increased intracellular Ca\(^{2+}\) concentration, as shown in Fig. 3A. Treatment with BAPTA/AM, an intracellular Ca\(^{2+}\) chelator, significantly suppressed the lovastatin-induced apoptosis as shown in Fig. 3B. These results suggest that lovastatin-induced apoptosis may be due to increased intracellular Ca\(^{2+}\).

To determine the source of the lovastatin-induced intracellular Ca\(^{2+}\) increase, we measured intracellular Ca\(^{2+}\) concentration using a nominal Ca\(^{2+}\)-free medium containing 1 mM EGTA. This experimental protocol can effectively reduce extracellular free Ca\(^{2+}\) concentration, and thus, blunt available Ca\(^{2+}\) influx. Under these conditions cellular Ca\(^{2+}\) response to lovastatin was not significantly altered as illustrated in Fig. 4A. Treatment with dantrolene and TMB-8, specific inhibitors for ryanodine receptor and IP\(_3\) receptor, respectively (Ehrlich et al., 1994), almost completely inhibited the Ca\(^{2+}\)-increasing effect of lovastatin. These results indicate that the increased intracellular Ca\(^{2+}\) concentration by lovastatin may be due to Ca\(^{2+}\) release from the internal Ca\(^{2+}\) stores. Moreover, treatment with these inhibitors (dantrolene, TMB-8) also significantly prevented the apoptosis induced by lovastatin, as depicted in Fig. 4B. These results strongly support that intracellular Ca\(^{2+}\) release results in the lovastatin-induced apoptosis.

**Involvement of mitochondrial pathway in the lovastatin-induced apoptosis**

There are two pathways currently proposed to play major roles in regulating apoptosis in mammalian cells: an extrinsic pathway mediated by one or more death receptors and an intrinsic pathway mediated by mitochondria (Hengartner, 2000). Thus in this study we examined which pathway is involved in the lovastatin-induced apoptosis using various caspase inhibitors. As shown in Fig. 5, the results show that the lovastatin-induced apoptosis was significantly reduced by both
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The broad spectrum caspase inhibitor z-VAD-fmk and inhibitors specific for caspase-9 and caspase-3 (z-LEHD-fmk and z-DEVD-fmk, respectively) but that apoptosis was not reduced by exposure to z-IETD-fmk, an inhibitor specific for caspase-8. Considering that caspase-8 appears to play a role in triggering only the extrinsic pathway of apoptosis (Gogvadze and Orrenius, 2006), these indirect evidence suggest that the intrinsic mitochondrial pathway may be, at least in part, engaged in the lovastatin-induced apoptosis.

Since the Ca\(^{2+}\)-dependent protease calpain seems to be involved in the mechanism of Ca\(^{2+}\)-associated apoptosis (Goll et al., 2003), we investigated whether a cell permeable calpain inhibitor suppresses the lovastatin-induced apoptosis. Indeed, the calpain inhibitor significantly abrogated the apoptosis, as depicted in Fig. 5. These results further indicate that intracellular Ca\(^{2+}\) increase by lovastatin may trigger the activation of calpain, in turn, resulting in apoptosis.

**DISCUSSION**

HMG-CoA reductase inhibitors, statins, reduce the proliferation of a wide variety of cell types in vitro by inducing cell-cycle arrest at G1 phase (Kocar et al., 2004; Koyuturk et al., 2004; Mo and Elson, 2004; Muck et al., 2004). Evidence is accumulating that statins may inhibit carcinogenesis because of their central action on important cellular functions (Hindler et al., 2006). It has been suggested that the anticancer effect of statins can be potentially exploited for the cancer therapy. Retrospective studies concluded that the long-term use of statins reduces the risk of pancreatic, prostate, and colorectal cancers (Poynter et al., 2005). In addition, several cancer trials have shown evidence supporting the use of statins as therapeutic anticancer agents (Brower, 2003; Chan et al., 2003). The mecha-

**Fig. 4.** Activation of Ca\(^{2+}\) release mechanism mediates the lovastatin-induced intracellular Ca\(^{2+}\) increase and apoptosis in HepG2 human hepatoblastoma cells. Intracellular Ca\(^{2+}\) concentration and apoptosis were assessed by Fura-2 fluorescence technique and flow cytometry, respectively. In figure A, dantrolene (50 \(\mu\)M) and TMB-8 (20 \(\mu\)M) were added 5 min before lovastatin (Lova, 20 \(\mu\)M) application. In the EGTA group, a nominal Ca\(^{2+}\)-free medium containing 100 \(\mu\)M EGTA was used. The data are expressed as percent changes of intracellular Ca\(^{2+}\) levels compared to the control condition in which the cells were incubated with Lovastatin-free medium. In figure B, Lova (20 \(\mu\)M) was treated for 48 hr. Dantrolene (50 \(\mu\)M), TMB-8 (20 \(\mu\)M) and EGTA (1 mM) were added 30 min before Lova treatment. The data (A and B) represent the mean values of four replications with bars indicating SEM. *\(P<0.05\) compared to control.

**Fig. 5.** Effects of inhibitors of calpain and caspases on the lovastatin-induced apoptosis in HepG2 human hepatoma cells. After cells were incubated with Lova (20 \(\mu\)M) for 48 h, the cells were stained with PI and analyzed by flow cytometry. The number of apoptotic cells was measured by flow cytometry as described in text. In these experiments, z-VAD-fmk, z-IETD-fmk, z-LEHD-fmk and z-DEVD-fmk were used as inhibitors of the broad spectrum caspase, the caspase-8, the caspase-9 and the caspase-3, respectively. These caspase inhibitors (100 \(\mu\)M) and the calpain inhibitor (CI, 10 \(\mu\)M) were given 30 min before Lova application. The data represent the mean values of four replicates with bars indicating SEM. *\(P<0.05\) compared to control in which the cells were incubated with drug-free medium. *\(P<0.05\) compared to Lova alone.
anism responsible for action of statins is considered to be the inhibition of cholesterol biosynthesis through inhibition of HMG-CoA reductase. This results in depletion of mevalonate, which is a precursor of cholesterol (Goldstein and Brown, 1990). Interestingly, elevated mevalonate synthesis has been reported in malignant breast (Dalenec et al., 2005), leukemia and lymphoma (Fernández et al., 2004; van de Donk et al., 2006), and prostate carcinoma cells (Mo and Elson, 2004). In this study we clearly showed, for the first time, that lovastatin induces apoptotic cell death in human hepatoma cells (Fig. 2).

Treatment with mevalonic acid did not abrogate the lovastatin-induced apoptosis (Fig. 3B), suggesting that apoptosis of the hepatoma cells by lovastatin may be induced through a mevalonate-independent mechanism. Although this discrepancy between this study and others is not clearly understood, the mechanism of the lovastatin-induced apoptosis may be cell type specific.

Accumulating evidence implies that intracellular Ca\(^{2+}\) is commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1997). Consistently, in this study we also showed that lovastatin appeared to induce apoptosis through intracellular Ca\(^{2+}\) rise, since lovastatin induced a rapid and sustained increase in intracellular Ca\(^{2+}\) concentration (Fig 3A), and intracellular Ca\(^{2+}\) chelation with BAPTA/AM effectively prevented the lovastatin-induced apoptosis (Fig. 3B). In addition to apoptosis, cell proliferation and differentiation have been linked to the stimulation of intracellular Ca\(^{2+}\) signal (Munaron et al., 2004). Interestingly, Ca\(^{2+}\) channel antagonists have decreased cell proliferation in a variety of cancer cells in vitro (Yoshida et al., 2003) and in vivo (Taylor et al., 1992).

Lovastatin appeared to elevate intracellular Ca\(^{2+}\) level through internal Ca\(^{2+}\) release, since this action of lovastatin was not altered by reducing extracellular Ca\(^{2+}\) concentration with a nominal Ca\(^{2+}\)-free medium containing 1 mM EGTA, but completely inhibited by intracellular Ca\(^{2+}\) release blockers, dantrolene and TMB-8 (Fig 4A). Our results are in line with the previous report that simvastatin, another inhibitor of HMG-CoA reductase, induced intracellular Ca\(^{2+}\) release from IP\(_3\)-sensitive pools mediated by activation of phospholipase C-\(\gamma\) 1 (Mutoh et al., 1999). Significant inhibition of the lovastatin-induced apoptosis by these Ca\(^{2+}\) release blockers (Fig 4B) implies that Ca\(^{2+}\) release from internal stores may act as a major trigger for the lovastatin-induced apoptotic cell death in the HepG2 cells.

There are two pathways currently proposed to play major roles in regulating apoptosis in mammalian cells: an extrinsic pathway mediated by one or more death receptors and an intrinsic pathway mediated by mitochondria (Hengartner, 2000). In the extrinsic death receptor/ligands pathway, caspase activation occurs as a direct consequence of death receptor ligation, with upstream caspase-8 cleaving and activating downstream proteases such as caspase-9 and caspase-3. In the intrinsic mitochondrial pathway, Bax, a member of the Bcl-2 family, plays the leading role. Bax normally resides in the cytosol in a quiescent state. After an apoptotic stimulus, Bax is translocated into the mitochondria and promotes the release of cytochrome c (Jurgensmeier et al., 1998), possibly by forming a pore (Saito et al., 2000) or a voltage-dependent anion channel (Shimizu et al., 1999) in the outer mitochondrial membrane. Both the death receptor and the mitochondrial pathways are linked by cleavage of Bid by caspase-8. It is known that cleaved Bid also translocates to mitochondria and induces Bax-dependent release of cytochrome c (Luo et al., 1998). Once in the cytosol, cytochrome c activates Apaf-1, which then activates procaspase-9, which, in turn, activates caspase-3, triggering apoptosis. Lovastatin-induced apoptosis was significantly reduced by both the broad spectrum caspase inhibitor z-VAD-fmk and inhibitors specific for caspase-9 and caspase-3 (z-LEHD-fmk and z-DEV-D-fmk, respectively), but not by exposure to z-ETD-fmk, an inhibitor specific for caspase-8 (Fig. 5), which strongly suggests that the intrinsic mitochondrial pathway is engaged in the lovastatin-induced apoptosis.

Calpains, a family of Ca\(^{2+}\)-dependent cytoplasmic cysteine proteases are activated through an increase in intracellular Ca\(^{2+}\) (Molinar and Carnegioli, 1997). The two major calpain isofoms, m- and \(\mu\)-, differ in the amount of Ca\(^{2+}\) required for activation. Calpain is considered to be primarily responsible for both apoptosis (Nath et al., 1996) and necrosis (Wang et al., 1996), probably as a consequence of a loss of Ca\(^{2+}\) homeostasis (Squier et al., 1994). Calpain has been shown to act upstream (McCullum et al., 2002), or downstream of caspase activation and to contribute to the degradation phase of apoptotic cell death (Wood and Newcomb, 1999 and Wood et al., 1998). Lovastatin rapidly mobilized Ca\(^{2+}\) from intracellular stores (Fig. 2A) and that the lovastatin-induced apoptosis was significantly prevented by a calpain inhibitor (Fig. 5), suggesting that Ca\(^{2+}\)-dependent activation of calpain(s) may be involved in the lovastatin-induced apoptosis in the hepatoma cells.

In conclusion, lovastatin induced apoptosis of HepG2 human hepatoma cells through intracellular Ca\(^{2+}\) release and calpain activation, leading to triggering intrinsic mitochondrial pathway. These results suggest that lovastatin may be a good
candidate for the therapeutic management of human hepatoma.

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