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**Lovastatin inhibits the extracellular signal-regulated kinases pathway in immortalized rat brain neuroblasts**

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Short title: ERK inhibition in lovastatin-induced neuronal apoptosis

## Abstract

We have previously shown that lovastatin, an HMG-CoA reductase inhibitor, induces apoptosis in spontaneously immortalized rat brain neuroblasts. In the present study, we analyzed the intracellular signal transduction pathways by which lovastatin induces neuroblast apoptosis. We showed that lovastatin efficiently inhibited Ras activation which was associated with a significant decrease in ERK1/2 phosphorylation. Lovastatin also decreased CREB phosphorylation and CREB-mediated gene expression. The effects of lovastatin on the Ras/ERK1/2/CREB pathway were time- and concentration dependent and fully prevented by mevalonate. In addition, we showed that two MEK inhibitors, PD98059 and PD184352, were poor inducers of apoptosis in serum-treated neuroblasts. However, these inhibitors significantly increased apoptosis induced by lovastatin treatment. Furthermore, we showed that pharmacological inhibition of both MEK and PI3K activities was able to induce neuroblast apoptosis with similar efficacy as lovastatin. Our results suggest that lovastatin triggers neuroblast apoptosis by regulating several signalling pathways, including the Ras/ERK1/2 pathway. These findings might also contribute to elucidate the intracellular mechanisms involved in the central nervous system side effects associated with statin therapy.

Key words: statins, apoptosis, neuroblasts, ERK1/2, PD98059, LY294002

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CNS, central nervous system; MEK, ERK kinase; ERK, extracellular signal-regulated kinase; CREB, cAMP response element binding protein; RBD, Ras-binding domain of Raf-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C.

## Introduction

3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG-CoA reductase inhibitors or “statins”) are widely used in the treatment of lipid disorders, especially hypercholesterolemia. They decrease the risk for vascular disease including vascular death, myocardial infarction and stroke. The statin family is composed of six members: lovastatin, simvastatin, atorvastatin, cerivastatin, pravastatin and fluvastatin. The last two are hydrophilic, whereas the others are lipid soluble, and each member differs in its specificity for HMG-CoA reductase [1-3].

HMG-CoA reductase is a rate-limiting enzyme that catalyses the conversion of HMG-CoA into mevalonate [3, 4]. It is well established that the mevalonate pathway plays an important role in cell growth and survival [4]. Several studies show that statins not only block the biosynthesis of mevalonate but, in addition, inhibit the proliferation and induce apoptosis of both normal and tumour cells [3, 5-12]. These pleiotropic effects of statins are thought to be mediated by their ability to block the biosynthesis of isoprenoid intermediates of the cholesterol pathway, such as farnesyl pyrophosphate and/or geranylgeranyl pyrophosphate which serve as lipid attachments for the small GTP-binding proteins Ras, Rho and Rac [3, 4]. This post-translational prenylation step (either farnesylation or geranylgeranylation) is required both for membrane association and cell signalling, leading to cell survival and proliferation [3, 13].

HMG-CoA reductase activity [14] and cholesterol biosynthesis [15, 16] are very high in the early phase of the ontogenetic development of the brain. These data indicate that the mevalonate pathway is essential to ensure normal growth, differentiation and maintenance of neuronal tissues. In agreement with this, recent reports show that the inhibition of this biosynthetic pathway during gestation by statin exposure is associated with severe central nervous system (CNS) defects [17, 18]. On the other hand, studies *in vitro* show that statins at micro- and millimolar concentrations have neurotoxic effects on primary neuronal cultures of cerebral cortex [19-21] and we have shown that lovastatin induces apoptosis of immortalized rat brain neuroblasts, and that its effect is associated with a decreased prenylation of Ras [22].

The Ras family of small GTPases are key regulators of signal transduction pathways that control cell proliferation, differentiation, survival and apoptosis. Ras and its relatives are activated in response to an extracellular or intracellular signal that

generates the GTP-bound form and energizes the signal transducing ability. Hydrolysis of the bound GTP by an intrinsic GTPase activity relaxes the conformation and terminates the signal [23-25]. Active Ras interacts with and modulates the activity of several downstream effectors. The best-characterized Ras effector pathway is the interaction with, and activation of, the three Raf serine/threonine kinases (A-Raf, B-Raf and Raf-1) leading to stimulation of the MEK1/MEK2 and ERK1/ERK2 (ERK1/2) mitogen-activated protein kinase (MAPK) cascade [23, 24]. In the nervous system, ERK1/2 cascade is critical for neuronal differentiation, plasticity and neuronal survival [26]. A role for ERK1/2 in neuronal survival was first indicated by experiments showing that overexpression of constitutively active mutants of MEK1 prevented the apoptosis induced by NGF withdrawal in neuronally differentiated PC12 cells [27]. Thereafter, although many studies supported a prosurvival activity of ERK1/2 in different neuronal cell types, several other studies suggested that ERK1/2 were not involved in the antiapoptotic signalling in neurons. Apart from those contradictory results, there is accumulating evidence that ERK1/2 may mediate the neuroprotective activity of several factors that neuroprotect against damaging insults and neuronal injury [26]. The mechanism by which the Ras/ERK1/2 signalling pathway promotes neuronal survival is under study, although it has been suggested that activation of a transcription factor, cAMP response element binding protein (CREB) and/or a direct inhibition of Bad, a pro-apoptotic member of the Bcl-2 family, may mediate the prosurvival activity of ERK1/2 in trophic-deprived cerebellar granule neurons [28].

As has been previously mentioned, we have shown that lovastatin induces apoptosis in rat brain neuroblasts [22]. The aim of the present work was to investigate the role of the Ras/ERK1/2 signalling pathway in regulating lovastatin-induced apoptosis in rat brain neuroblasts. The elucidation of the intracellular mechanisms affected by lovastatin will contribute to gain insight in the growth inhibition and apoptosis induced by lovastatin and other statins in neuronal cells, and additionally will contribute in establishing the important role that the mevalonate pathway plays in the development of the nervous system.

## Materials and Methods

### Reagents and antibodies

Lovastatin (Mevinolin, MK-803) was from Calbiochem (San Diego, CA, USA). The inactive lactone of lovastatin was converted to the active form as previously described [29]. Mevalonic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ham's F-12 medium, fetal calf serum, L-glutamine, streptomycin, penicillin and trypsin/EDTA solution were from PAN Biotech (Aidenbach, Germany, Europe). Tissue culture flasks and dishes were from TPP (Trasadingen, Switzerland, Europe). MEK1/2 inhibitor PD98059 (2'-amino-3'-methoxyflavone) and PI3K inhibitor LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] were from Calbiochem (San Diego, CA, USA). MEK1/2 inhibitor (PD184352) was kindly provided by Dr. Ana Cuenda (Departamento de Bioquímica y Biología Molecular, Universidad de Extremadura, Caceres, Spain). Complete protease inhibitor cocktail tablets were from Roche Molecular Biochemicals (Indianapolis, IN, USA). Glutathione-Sepharose 4B was from Pharmacia (Freiburg, Germany, Europe). The caspase substrate (Ac-DEVD-AMC), Ras antibody (cat. 610002), anti-phospho-Ser472/473/474 PKB (cat. 559029) and anti-PKB (cat. 559028) were from BD Biosciences Pharmingen (San Diego, CA, USA). ERK1/2 (cat. M3807) and phospho-ERK1/2 (Thr183 and Tyr185, cat. M8159) antibodies were from Sigma (St. Louis, MO, USA). CREB antibody (cat. 8977) was from Sigma, phospho-CREB (Ser 133, cat. 06-519) antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Goat anti-rabbit (cat. 31460) and goat anti-mouse (cat. 31430) conjugated to horseradish peroxidase were from Pierce (Rockford, IL, USA). Other reagents were obtained from different commercial sources and were of the highest purity available.

### Cell cultures and treatments

Spontaneously immortalized rat brain neuroblasts were used in this study. This cell line was obtained by spontaneous immortalization from cultures of 17-day fetal rat cerebral cortices [30] and was kindly provided by Dr. Alberto Muñoz (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). The cells represented primitive neuroblasts that expressed NF 68 and the primitive neuronal marker nestin, but lacked the astrocyte marker, glial fibrillary acidic protein (GFAP). After partial differentiation

induction with dibutyryl  $\alpha$ -cAMP, the cells expressed further neuronal markers such as NF 145, NF 220, and neuron-specific enolase [30]. Cells were grown in Ham's F-12 supplemented with 10% fetal calf serum, L-glutamine (2 mM), streptomycin (100  $\mu$ g/ml) and penicillin (100 U/ml). Cells were seeded at  $5 \times 10^5$  in a 75-cm<sup>2</sup> tissue culture flask and incubated at 37°C under a 5% CO<sub>2</sub>/95% air atmosphere.

Confluent cells were trypsinized and seeded in tissue culture dishes at a density of  $2 \times 10^4$  cells/cm<sup>2</sup>. After 24 h, the medium was aspirated and replaced with fresh medium alone or containing the indicated concentrations of lovastatin, mevalonate, PD98059, PD184352 or LY294002 and the incubation was continued for a further 24 h. When indicated, cells were incubated with PD98059 or PD184352 for 1h before lovastatin addition. For the time course experiments, cells were incubated with 10  $\mu$ M lovastatin for different periods of time.

### Measurement of Ras activation

The capacity of Ras-GTP to bind to RBD (Ras-binding domain of Raf-1) was used to analyze the amount of active Ras as previously described [31]. Neuroblasts were seeded in 100 mm tissue culture dishes and after indicated treatments, cells were harvested in phosphate-buffered saline (PBS) and lysed for 15 min in 100  $\mu$ l ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM sodium orthovanadate, 5 mM NaF, 10 % glycerol, 100 mM KCl, 1 % Triton X-100, 0.05 % 2-mercaptoethanol and 4  $\mu$ g/ml complete protease inhibitor cocktail. Cell lysates were centrifuged at 20000 g for 10 min at 4°C and protein concentrations in the supernatants were determined by using the Bio-Rad protein assay, according to the instructions of the manufacturer. Protein-equalized supernatants (250  $\mu$ g) were incubated for 2 h at 4°C with glutathione-Sepharose 4B beads pre-coupled with GST-RBD (1h at 4°C). The beads were washed four times in the lysis buffer. Bound proteins were solubilized by the addition of 30  $\mu$ l of Laemmli loading buffer and analyzed in 12 % SDS-PAGE gels. The amount of Ras in the bound fraction was analyzed by Western blot as described below.

### Western blot analysis

Treated and control cells were harvested in PBS and lysed for 15 min in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10%

glycerol, 5 mM MgCl<sub>2</sub>, 25 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 0,5 mM sodium orthovanadate and 4 µg/ml complete protease inhibitor cocktail. After lysis, cell debris was removed by centrifugation at 20000 g for 10 min at 4 °C and protein concentrations determined in the supernatants as indicated above. Equal amounts of protein (20 µg) were separated on 10 or 12% SDS-PAGE and blotted onto PVDF membranes (PolyScreen, NEN life Science Products, Inc. Boston, MA). The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.5) containing 0.5% tween 20 and incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies in antibody buffer [5% non-fat dry milk in Tris-buffered saline (pH 7.5) containing 0.05% tween 20 (TBS-T)]. After required washes with TBS-T, proteins were analyzed using an enhanced chemiluminescence detection system (Signal® West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) and exposed to hyperfilm-ECL (Amersham, Pharmacia Biotech, Freiburg, Germany). Films were photographed and the intensity of each band was analyzed using a ChemiDoc System (Documentation and Analysis System, Bio-Rad, Hercules, CA, USA). Results were expressed as percentage of control.

### Luciferase reporter construct and assay

The control β-galactosidase construct was pSV- β-Galactosidase (Promega). To construct the CRE response element luciferase reporter, the SV40 promoter in the vector pGL3-promoter (Promega) was excised with BglII and Hind III and replaced by a minimal HSV TK promoter (obtained by PCR amplification) containing nucleotides – 109 to + 17 flanked by BglII and Hind III sites. A double stranded oligonucleotide containing the CRE response element from the somatostatin promoter (AGCCTGACGTCAGAGA) flanked by Asp718 and BglII sites was then cloned into this vector (pGL3-CRE). Neuroblasts were co-transfected with 1 µg of pGL3-CRE and 1 µg of β-Galactosidase reporter vector using GenePORTER transfection reagent (Genlantis, San Diego, CA, USA), as recommended by the manufacturer. Following transfection the cells were incubated for 24 h, the medium was changed, and the cells were incubated for an additional 24 h with 10 µM lovastatin in the presence or absence of 100 µM mevalonate. The cells were then lysed and luciferase and β-galactosidase assays performed using Promega reagents as recommended by the manufacturer.



Luciferase activity was normalized to  $\beta$ -galactosidase activity. Results were expressed as percentage of control.

### Cell viability assay

Cell viability was evaluated by the crystal violet method [32]. For these experiments 35 mm tissue culture dishes were used. At the end of each treatment, the medium was discarded, and the remaining viable adherent cells were stained with crystal violet (0.03% in 2% ethanol) for 5 min. Dishes were rinsed with tap water and 1 ml of 1% sodium dodecyl sulfate was added to each plate to solubilize the stained cells. The absorbance of each plate was read at 560 nm. Dishes without cells were processed in parallel to correct the nonspecific adhesion of crystal violet to the plastic. Viable cells were calculated as percentage of absorbance with respect to untreated cells.

### DNA Fragmentation Assay

Neuroblasts were plated on 100 mm culture dishes. After indicated treatments, floating cells were harvested by centrifugation at 20000 g for 1 min and adherent cells were detached mechanically with a rubber policeman. Adherent and floating cells were mixed, washed twice in ice-cold PBS and lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA and 0.5 % Triton X-100) for 60 min at 4°C with agitation. After centrifugation at 20000 g for 30 min at 4°C, supernatants were incubated with RNase A (0.1 mg/ml) at 37°C for 45 min and then with Proteinase K (0.2 mg/ml) with 0.5% SDS solution at 37°C for 45 min. Soluble DNA was then isolated by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. DNA was collected by centrifugation at 20000 g for 20 min, dissolved in autoclaved water and resolved on 2% agarose-0.1  $\mu$ g/ml ethidium bromide gels in TBE buffer [80 mM Tris-borate (pH 8.0), 2 mM EDTA]. DNA fragments were visualized under ultraviolet light and photographed using a ChemiDoc System (Documentation and Analysis System, Bio-Rad, USA).

### Analysis of Nuclear DNA Content by Flow Cytometry

The ploidy determination of neuroblasts was estimated by flow cytometry DNA analysis. After culture under the indicated conditions, adherent cells were detached from dishes by the addition of a trypsin-EDTA solution. They were mixed with non-adherent cells and washed twice in ice-cold PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Cells were resuspended

in ice-cold 70% ethanol and fixed for 5 min. After centrifugation at 500 g for 5 min, cells were resuspended in PBS containing 1 mg/ml of RNase A and incubated at 37°C for 30 min. Cells were stained with 50 µg/ml propidium iodide for 30 min at room temperature and then flow cytometry analysis was done. DNA content per nucleus was evaluated in a CyAn MLE flow cytometer (DAKO, Gostrup, Denmark). This analysis was performed using a doublet discriminator system, which distinguishes between the signals coming from a single nucleus and the ones produced by two or more aggregated nuclei. For the computer analysis, only signals from single cells were considered (10000 cells/sample). This method of analysis of DNA content permits the identification and quantification of apoptotic cells, as revealed by the peak localized below the G0/G1 peak [33].

### Analysis of caspase activity

Caspase activity was measured using the synthetic substrate Ac-DEVD-AMC. Neuroblasts treated with different conditions were washed with cold PBS and lysed for 15 min in ice-cold lysis buffer containing 10 mM Tris (pH 7.5), 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 130 mM NaCl, 1% Triton X-100 and 10 mM sodium pyrophosphate. Cell extracts were obtained after centrifugation at 20000 g for 15 min at 4°C. For the assay, 20 µl of cell lysates were diluted in reaction buffer (20 mM HEPES pH 7.5, 10% glycerol, 2 mM dithiothreitol) and mixed with 20 µM of caspase substrate. The reactions were incubated at 37°C for different period of times and the release of AMC from the substrate was monitored in a spectrofluorometer using excitation and emission wavelengths of 380 nm and 440nm, respectively. The results were corrected for protein content of the lysates and expressed as the increase of fluorescence for 1 h per mg protein.

### Statistical analysis

Each experiment was repeated at least three times, with good agreement among the results of individual experiments. All data are expressed as the mean ± standard error of the mean. Results were analyzed by one-way analysis of variance (ANOVA) followed by Student's *t* test. *P* values of less than 0.05 were considered significant.

## Results

### Effect of lovastatin on Ras activation

We have previously shown that lovastatin induced apoptosis of neuroblasts in a dose- and time-dependent manner and also that the lovastatin effect was associated with an inhibition of Ras prenylation evaluated by the decrease of the level of Ras in the membrane compartment [22]. This post-translational prenylation step is required both for membrane association and Ras activation. Therefore, in the present work we first studied whether the inhibition of Ras prenylation by lovastatin was also associated with a decrease in its biological activity. To clarify this issue, time course and concentration-dependent experiments were done and Ras activation was analyzed by the GST-RBD-Raf-1 pull-down method. Time course experiments showed that in comparison to the control cells grown in 10% FCS media, lovastatin (10  $\mu$ M) inhibited Ras activation in a time dependent manner (Fig. 1A). The lovastatin effect started to be significant after 3 hours of treatment and was markedly inhibited 24 hours after lovastatin treatment. Inhibition of Ras activation occurred before induction of apoptosis that started to be significant after 12 hours of lovastatin treatment [22]. Additionally, neuroblasts were treated with increasing concentrations of lovastatin for 24 hours and as shown in Fig. 1B, Ras activation decreased in a concentration-dependent manner after lovastatin treatment. The effect of lovastatin was evident at a concentration as low as 1  $\mu$ M reaching the maximum effect at a concentration of 10  $\mu$ M. In order to know whether the lovastatin effect on Ras activation was specific of HMG-CoA reductase inhibition, cells were incubated with lovastatin (10  $\mu$ M) in presence or absence of mevalonate (100  $\mu$ M) for 24 h. Co-treatment with mevalonate prevented the decrease in Ras activation induced by lovastatin (Fig. 1C). Treatment of cells with mevalonate alone did not modify Ras activation. In all the experiments, lovastatin decreased Ras prenylation evaluated by the presence of the unprocessed form of Ras (unprocessed Ras migrates more slowly than its processed form in SDS-PAGE). Taken together, these data suggest that the decrease of Ras activation induced by lovastatin may be due to a specific failure of Ras prenylation.

### Effect of lovastatin on ERK1/2 phosphorylation

The best characterized Ras effector is the serine/threonine kinase Raf, which leads to the activation of the ERK1/2 pathway [23, 24]. Therefore, we next studied the

phosphorylation state of these kinases after lovastatin treatment by immunoblot analysis using phosphospecific antibodies. As shown in Fig. 2, lovastatin decreased ERK1/2 phosphorylation in a time- and concentration-dependent manner (Fig. 2A and Fig. 2B, respectively). Lovastatin (10  $\mu$ M) produced a decrease in ERK1/2 phosphorylation that was significant at 3 hours and was maximum at 12 hours of treatment (Fig. 2A). On the other hand, the lovastatin effect on ERK1/2 phosphorylation was evident at a concentration of 1  $\mu$ M, but the decrease in the phosphorylation of both kinases was only significant at higher concentrations (10  $\mu$ M and 20  $\mu$ M). Mevalonate (100  $\mu$ M) prevented the decrease in ERK1/2 phosphorylation induced by lovastatin, indicating that the lovastatin effect was specific (Fig. 2C). Treatment of neuroblasts with mevalonate alone did not affect ERK1/2 phosphorylation. Expression levels of ERK1/2 remained unchanged throughout the experiment (Fig. 2), suggesting that ERK1/2 phosphorylation was specifically affected by exposure to lovastatin. It is noteworthy that the lovastatin effect on ERK1/2 phosphorylation was less potent than the effect on Ras activation. In fact, neuroblast incubation with lovastatin 10  $\mu$ M for 24 hours reduced ERK1/2 phosphorylation by 45 % vs. control cells while the same treatment inhibited Ras activation by 90% vs. control.

### **Effect of lovastatin on CREB phosphorylation and CREB-mediated gene expression**

It has been shown that one of the mechanisms by which the Ras/ERK1/2 signalling pathway promotes neuronal survival is by activating p90<sup>rsk</sup>, which in turn phosphorylates the transcription factor CREB at serine 133. Activated CREB promotes cell survival, and inhibition of CREB phosphorylation triggers apoptosis. Therefore, we next evaluated the effect of lovastatin on the phosphorylation level of CREB. Treatment of neuroblasts with 10  $\mu$ M lovastatin during different periods of time led to a reduction in the phosphorylation at Ser133 of CREB that was significant at 6 hours. The maximum effect was observed after 12 hours of treatment (Fig. 3A). As shown in Fig. 3B, lovastatin treatment induced a decrease in the phosphorylation of CREB in a concentration-dependent manner with respect to untreated control cells, but in these experiments the maximum decrease in CREB phosphorylation was detected at 20  $\mu$ M lovastatin. Co-treatment with mevalonate (100  $\mu$ M) prevented the decrease in CREB phosphorylation induced by lovastatin. Treatment of cells with mevalonate alone did not

modify CREB phosphorylation (Fig. 3C). Lovastatin treatment did not modify CREB expression in any of the experimental conditions (Fig. 3).

To determinate whether the decrease in CREB phosphorylation induced by lovastatin was associated with an inhibition on CREB-mediated gene expression we next performed the luciferase reporter gene assay. As shown in Fig. 4, the incubation of the CRE-plasmid containing cells with 10  $\mu$ M lovastatin for 24 hour resulted in an inhibition in luciferase expression. This effect was not observed in the cells transfected with the plasmid control lacking CREB binding sites (data not shown). Again, co-treatment with mevalonate prevented the effect of lovastatin and completely restored CREB-mediated gene expression to control levels. Taken together, these data suggest that lovastatin not only decreases CREB phosphorylation in neuroblasts but this action also correlates with decreased CREB capacity to activate transcription.

### **Effect of MEK inhibitors on neuroblast survival in absence or presence of lovastatin**

Our results suggested that lovastatin might induce neuroblast apoptosis by its capacity to inhibit the Ras/ERK1/2 signalling pathway. Therefore, we next studied whether the pharmacological inhibition of this pathway was able to induce neuroblast apoptosis. Neuroblasts were incubated for 24 hours with different concentrations of PD98059 and PD184352 that inhibit MEK1/2, the MAP kinase kinase responsible for ERK1/2 phosphorylation and then the ability of these inhibitors to induce apoptosis was assessed by different assays, neuroblast viability, internucleosomal DNA fragmentation, quantification of neuroblasts undergoing apoptosis by flow cytometry and caspase activation. As shown in Fig 5, the treatment of neuroblasts with PD98059 alone was associated with a concentration-dependent decrease in cell viability (Fig. 5A), the appearance of internucleosomal DNA fragmentation (Fig. 5B) and an increase in the percentage of apoptotic neuroblasts (Fig. 5C). Moreover, PD98059 also enhanced caspase activity (Fig. 6A). Although PD98059 inhibited ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 6B), the effects of this inhibitor were minimal when compared with control samples and never reached the parameters seen with lovastatin treatment (Fig. 5 and 6). Similar results were obtained when PD184352, other MEK1/2 inhibitor, was used (Fig. 5 and 6). These data suggest that the inactivation of the Ras/ ERK1/2 pathway may be necessary but not sufficient to evoke the lovastatin effect on neuroblast apoptosis.

It has been recently shown that the inhibition of the Raf/ERK pathway sensitizes cells to statins-induced apoptosis [10, 11]. To evaluate whether direct inhibition of this pathway can increase the efficacy of lovastatin to trigger neuroblast apoptosis, cells were treated with lovastatin (10  $\mu$ M) alone or in combination with PD 98059 (20  $\mu$ M and 50  $\mu$ M) or PD184352 (1  $\mu$ M and 2  $\mu$ M) for 24 hours. In these experiments, MEK inhibitors were added to the cells 1 hour before lovastatin treatment. As shown in Fig. 5 and 6, lovastatin effects were enhanced by pre-treatment with these inhibitors in a concentration-dependent manner. As expected, the down-regulation of ERK1/2 phosphorylation was also enhanced when lovastatin and PD98059 or PD184352 were used together (Fig. 6B).

#### **Effect of the inhibition of Ras/ERK1/2 and phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathways on neuroblast survival.**

We have shown that Ras/ERK1/2 and PI3K/PKB signalling pathways are down-regulated by lovastatin in neuroblasts and that the pharmacological inhibition of each of these pathways was not sufficient to induce the apoptosis degree elicited by lovastatin [34] and the present work. Therefore, we next studied the effect of co-incubation of PD98059 and PD184352 (MEK1/2 inhibitors) and LY294002 (PI3K inhibitor) on neuroblast survival and apoptosis. The treatment of neuroblasts with PD98059 (20 and 50  $\mu$ M), PD184352 (1 and 2  $\mu$ M) or LY294002 (20  $\mu$ M) alone during 24 h was associated with a decrease in cell viability (Fig. 7A), a slight increase in the percentage of cells in the apoptotic sub-G1 population (Fig. 7B), and an increase in caspase activity (Fig. 7C) when compared with control. However, the effects of both inhibitors were additive on all the parameters studied (Fig. 7) and only the concomitant use of both inhibitors resembled the lovastatin efficacy in inducing neuroblasts apoptosis (Fig. 7A,B,C). As shown in Fig. 8, MEK inhibitors decreased ERK1/2 phosphorylation but did not modify PKB phosphorylation and LY294002 inhibited the phosphorylation of PKB without affecting ERK1/2 phosphorylation.

## Discussion

It is known that the mevalonate pathway is essential to ensure normal growth, differentiation and maintenance of neuronal tissues [14-16]. We have recently shown that mevalonate pathway inhibition by lovastatin induces apoptosis of spontaneously immortalized rat brain neuroblasts [22]. In the present study, we investigated the molecular mechanisms underlying lovastatin-induced neuroblast apoptosis, focusing our work on the involvement of the Ras/ERK1/2/CREB signalling pathway.

In the present work, we have shown for the first time that lovastatin significantly decreased the activation of Ras stimulated by serum in spontaneously immortalized rat brain neuroblasts. The lovastatin effect on Ras activation was time- and concentration dependent and preceded neuroblast apoptosis [22]. Our results are in agreement with previous studies that show that statins inhibit Ras prenylation and activation in different non-neuronal cell types [7, 35-37] and strongly suggest that lovastatin may induce neuroblast apoptosis by its capacity to disrupt Ras-mediated signal transduction in the cells.

Activation of Ras has been demonstrated to initiate activation of Raf and MEK1/2, resulting in ERK activation [23, 24]. Since the Ras-MEK-ERK signalling pathway plays a key role in the transmission of cell survival signals, the phosphorylation of ERK 1/2 after lovastatin treatment was investigated. Our data show that lovastatin treatment led to a time- and concentration dependent decrease in the phosphorylation of ERK1/2 in neuroblasts that pointed to an inhibition of its kinase activity. These results suggest that the decrease in the phosphorylation of ERK1/2 may play a role in the apoptotic induction by lovastatin. Consistent with our findings, several reports show that statin-induced apoptosis and/or growth inhibition of various cell types correlates with down-regulation of ERK activity [7, 11, 12, 36-38]. In contrast, another study reports that statin-induced growth inhibition of rat RBL-2H3 cells did not involve down-regulation of ERK activity [39]. These controversial findings may be attributed to different biological actions of statins depending on the cell type. We have also shown that although the activation of Ras was almost completely inhibited by lovastatin, the serum-induced phosphorylation of the ERK1/2 was only partially inhibited in lovastatin-treated cells compared with control. The sustained phosphorylation of ERK1/2 in lovastatin-treated neuroblasts may be due to the activation of other upstream

intracellular signalling molecules, such as PKC or Rac [40, 41] which in turn produce the activation of the MEK/ERK pathway.

It is accepted that ERK1/2 activation promotes neuronal survival by its capacity to activate p90<sup>rsk</sup>, which in turn phosphorylates and activates CREB as well as increased CREB-mediated gene expression [28, 42]. In the present work, we have shown that the decrease in ERK1/2 phosphorylation/activity caused by lovastatin is associated with a reduction of both CREB phosphorylation and CREB-mediated gene expression. The turn off Ras/ERK/CREB signalling pathway has been related to apoptosis induced by other stimuli [28, 43, 44] but, to our knowledge, this is the first report that demonstrates that lovastatin inhibits the activation of this survival signalling pathway in neuronal cells. We have previously shown that lovastatin decreased the expression of Bcl-2 in neuroblasts undergoing apoptosis [22]. As this pro-survival gene is a target of CREB in neurons [28], our present data suggest that lovastatin may induce neuroblast apoptosis by decreasing the expression of Bcl-2 through the inhibition of the Ras/ERK/CREB signalling pathway. Our findings are in agreement with several works that indicate that CREB is essential for neuronal survival [45].

We have also shown that lovastatin effects were completely prevented by simultaneous exposure of cells to exogenous mevalonate, demonstrating that the inhibition of the Ras/ERK/CREB pathway induced by lovastatin was specifically due to a blockade of HMG-CoA reductase activity.

As said above, our results could suggest that lovastatin may induce neuroblast apoptosis by its capacity to inhibit the Ras/ERK/CREB pathway. Therefore, we investigated whether the pharmacological inhibition of this pathway was able to induce apoptosis in neuroblasts. Our results show that both PD98059 and PD184352, MEK inhibitors, were poor inducers of apoptosis in serum-treated neuroblasts. However, these inhibitors significantly increased apoptosis induced by lovastatin treatment. Taken together, these results suggest 1) that lovastatin does not trigger neuroblast apoptosis by down-regulating the Ras/ERK/CREB signalling cascade alone, and 2) that the sustained phosphorylation of ERK1/2 in lovastatin-treated neuroblasts may play a protective role [26]. Our findings are in agreement with other reports where MEK inhibitors enhance the apoptotic actions of statins and other drugs in different non-neuronal cell types [11, 43, 46].

Finally, in the present work we have shown that only the pharmacological inhibition of both MEK and PI3K activities was able to induce neuroblast apoptosis



with similar efficacy as lovastatin. These data suggest that lovastatin may induce neuroblast apoptosis by inducing the simultaneous inactivation of both Ras/ERK1/2 and PI3K/PKB signalling pathways. Whether neuroblast death induced by lovastatin involves other intracellular pathways is under study.

In conclusion, we have shown for the first time that HMG-CoA reductase inhibition by lovastatin leads to an inhibition of the Ras/ERK/CREB signalling pathway in spontaneously immortalized rat brain neuroblasts which may contribute to explaining its apoptotic effect. The lovastatin effects were time- and concentration-dependent and prevented by adding mevalonate to the medium, which indicates that they were due to an inhibition of mevalonate synthesis. Our results also suggest that Ras/ERK pathway inactivation appears insufficient to induce the apoptosis evoked by lovastatin and that apoptosis of neuroblasts induced by this statin could require the simultaneous inhibition of both the Ras/ERK and PI3K-PKB signalling pathways.

These findings could contribute to elucidate the molecular mechanisms by which statins induce growth suppression and/or apoptosis in neuronal cells and may also help to explain the CNS side effects associated with statin therapy.

## **Acknowledgments**

The authors thank Ricardo Argent for his excellent technical assistance and Dr. Ana Cuenda for kindly providing MEK1/2 inhibitor (PD184352). We also thank Mary Harper for the preparation of the manuscript. This work has been supported by Grants, SAF 2001-0154 from the Ministerio de Ciencia y Tecnologia, and 2PR01B007 from the Junta de Extremadura. M. I. Cerezo-Guisado is supported by a Ph D fellowship from Junta de Extremadura, Spain.

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## Figure legends

**Figure 1. Effect of lovastatin on Ras activation.** A: Time-dependent effect: Neuroblasts initially cultured for 24 hours in Ham's F12/10% FCS were incubated in culture media alone or with 10  $\mu$ M lovastatin for different periods of time. B: Concentration-dependent effect: cells initially cultured for 24 hours in Ham's F12/10% FCS were incubated for an additional 24 hours in the presence of media alone (0) or various concentrations of lovastatin. C: Effect of mevalonate treatment: Cells initially cultured for 24 hours in Ham's F12/10% FCS were incubated with lovastatin (Lov 10  $\mu$ M) in absence or presence of mevalonate (Mev 100  $\mu$ M) for an additional 24 hours. Control cells were incubated in presence of media or mevalonate alone. At the end of each experiment, Ras activation was measured by GST-RBD pull-down followed by Western blot with anti-(pan)-Ras antibody. An aliquot of each lysate was also loaded in another gel to analyze total Ras protein levels. A representative blot of each experiment is shown with the densitometric analysis corresponding to the mean  $\pm$  SE of at least three independent experiments. ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to untreated cells.

**Figure 2. Effect of lovastatin on ERK1/2 phosphorylation.** A: Time-dependent effect. B: Concentration-dependent effect. C: Effect of mevalonate treatment. Neuroblasts were treated as described in Fig. 1. At the end of each experiment, cells were lysed and total proteins (20  $\mu$ g/lane) were separated by SDS-PAGE and analyzed by Western blotting using an anti-phospho-ERK1/2 specific antibody. As a control of the amount of protein, total ERK1/2 were also analyzed in the same samples. A representative blot of each experiment is shown with the densitometric analysis corresponding to the mean  $\pm$  SE of at least three independent experiments. ns, not significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to untreated cells.

**Figure 3. Effect of lovastatin on CREB phosphorylation.** A: Time-dependent effect. B: Concentration-dependent effect. C: Effect of mevalonate treatment. Neuroblasts were treated as described in Fig. 1. At the end of each experiment, cells were lysed and total proteins (20  $\mu$ g/lane) were separated by SDS-PAGE and analyzed by Western blotting using an anti-phospho-CREB specific antibody. As a control of the amount of protein,

total CREB was also analyzed in the same samples. A representative blot of each experiment is shown with the densitometric analysis corresponding to the mean  $\pm$  SE of at least three independent experiments. ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to untreated cells.

**Figure 4. Effect of lovastatin on transcription from the CRE-response element.**

Neuroblasts initially cultured for 24 hours in Ham's F12/10% FCS were co-transfected with pGL3-CRE-luciferase and pSV- $\beta$ -Galactosidase reporter constructs. Following transfection, cells were incubated with lovastatin (Lov 10  $\mu$ M) in absence or presence of mevalonate (Mev 100  $\mu$ M) for 24 hours. Control cells were incubated in presence of media or mevalonate alone. Luciferase and  $\beta$ -Galactosidase activity were measured in the same extracts and the luciferase activity standardised for transfection efficiency by dividing the luciferase activity by  $\beta$ -Galactosidase activity. Results are expressed as the percentage relative to untreated cells. Each value represents the mean  $\pm$  SE of four independent experiments performed in triplicate. ns, not significant; \*\*,  $p < 0.01$ ; compared to untreated cells.

**Figure 5. Effect of MEK inhibitors alone or in combination with lovastatin on cell viability (A), internucleosomal DNA fragmentation (B) and percentage of apoptotic cells (C).** Cells previously cultured for 24 hours in growth media were incubated with different concentrations of PD98059 (20 and 50  $\mu$ M) or PD184352 (1 and 2  $\mu$ M) in absence or presence of 10  $\mu$ M lovastatin (Lov) for an additional 24 h. At the end of the experiment, cell viability was determined by the crystal violet method (A), internucleosomal DNA degradation was analyzed by using electrophoresis on 2% agarose gel (B) and the percentage of cells with hypodiploid DNA content was evaluated by flow cytometry (C). Each value represents the mean  $\pm$  standard error of at least three independent experiments made in triplicate. ns, not significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to untreated cells. a,  $p < 0.05$ ; b,  $p < 0.01$ ; c,  $p < 0.001$ ; compared to lovastatin-treated cells (A and C). A representative photograph of 3 independent experiments is shown in B.

**Figure 6. Effect of MEK inhibitors alone or in combination with lovastatin on caspase activation (A) and ERK1/2 phosphorylation (B).** Neuroblasts were treated as

described in Fig. 5. At the end of each experiment, cell extracts were obtained and used to analyze caspase activity (A) or ERK phosphorylation as described under Materials and Methods. Each value represents the mean  $\pm$  standard error of two independent experiments made in duplicate. ns, not significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to untreated cells. c,  $p < 0.001$ ; compared to lovastatin-treated cells. A representative blot of three independent experiments is shown in B.

**Figure 7. Effect of the coincubation with MEK inhibitors and PI3K inhibitor (LY294002) on cell viability (A), percentage of apoptotic cells (B) and caspase activation (C).** Cells previously cultured for 24 hours in growth media were incubated with different concentrations of PD98059 (20 and 50  $\mu$ M) or PD184352 (1 and 2  $\mu$ M) in absence or presence of LY294002 (LY 20  $\mu$ M) and with LY 20  $\mu$ M or lovastatin (Lov 10  $\mu$ M) alone for an additional 24 h. At the end of the experiment, cell viability (A), the percentage of cells with hypodiploid DNA content (B) and caspase activity (C) were determined as described in Materials and Methods. Each value represents the mean  $\pm$  standard error of at least three independent experiments made in triplicate. ns, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared to their respective PD-treated cells. Ns, not significant; a,  $p < 0.05$ ; b,  $p < 0.01$ ; c,  $p < 0.001$ ; compared to LY-treated cells.

**Figure 8. Effect of MEK inhibitors and PI3K inhibitor on both ERK1/2 and PKB phosphorylation.** Cells were treated as described in Fig. 7. At the end of each experiment, cells were lysed and total proteins (20  $\mu$ g/lane) were separated by SDS-PAGE and analyzed by Western blotting using anti-phospho-ERK1/2 and anti-phospho-PKB specific antibodies. As a control of the amount of protein, total ERK1/2 and PKB were also analyzed. A representative blot is shown.

Figure 1

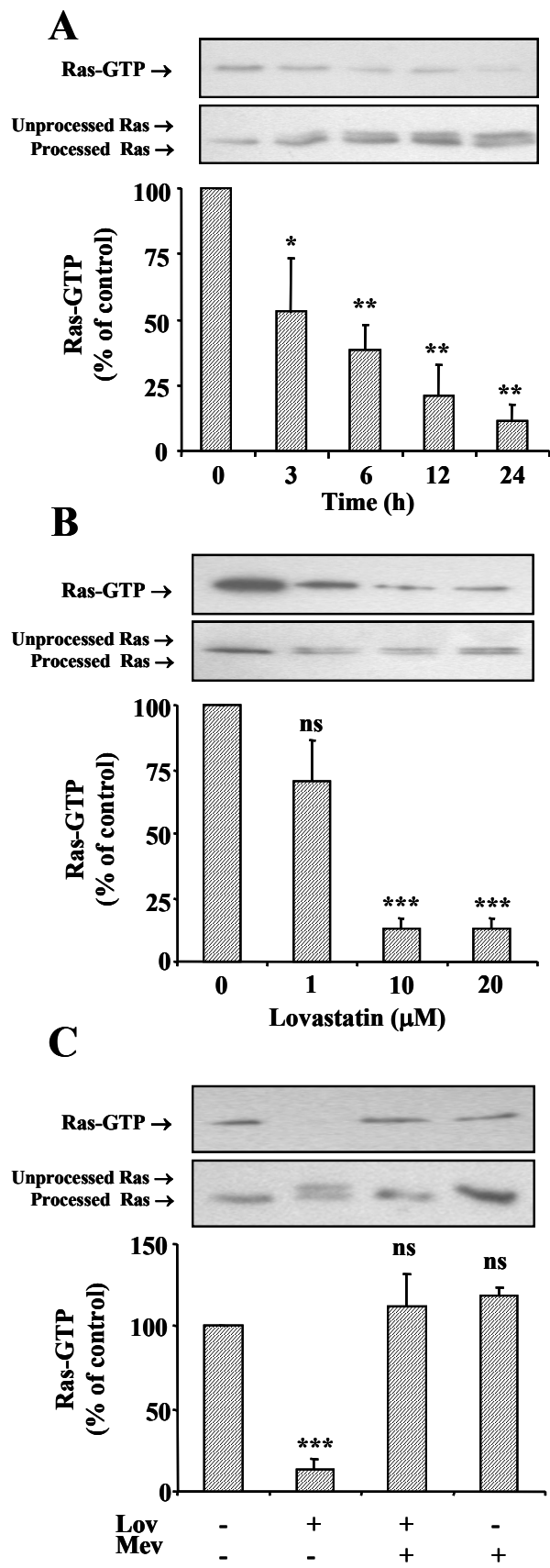
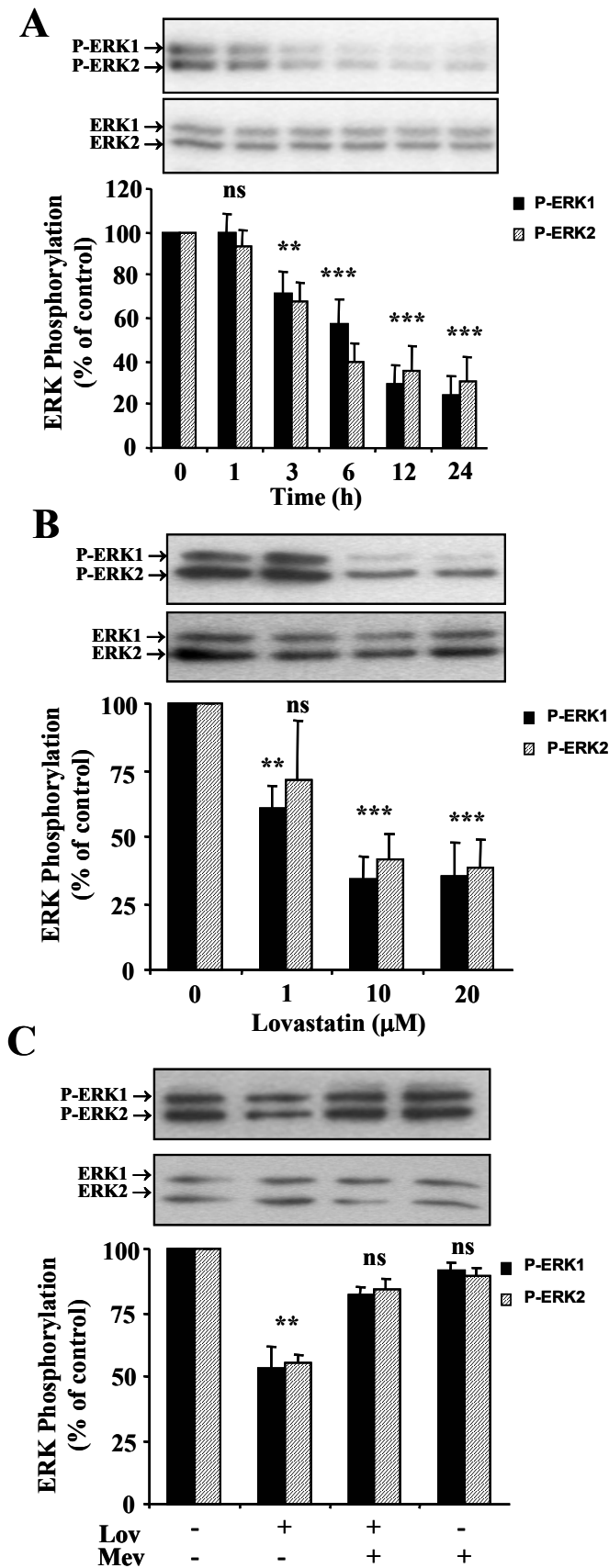
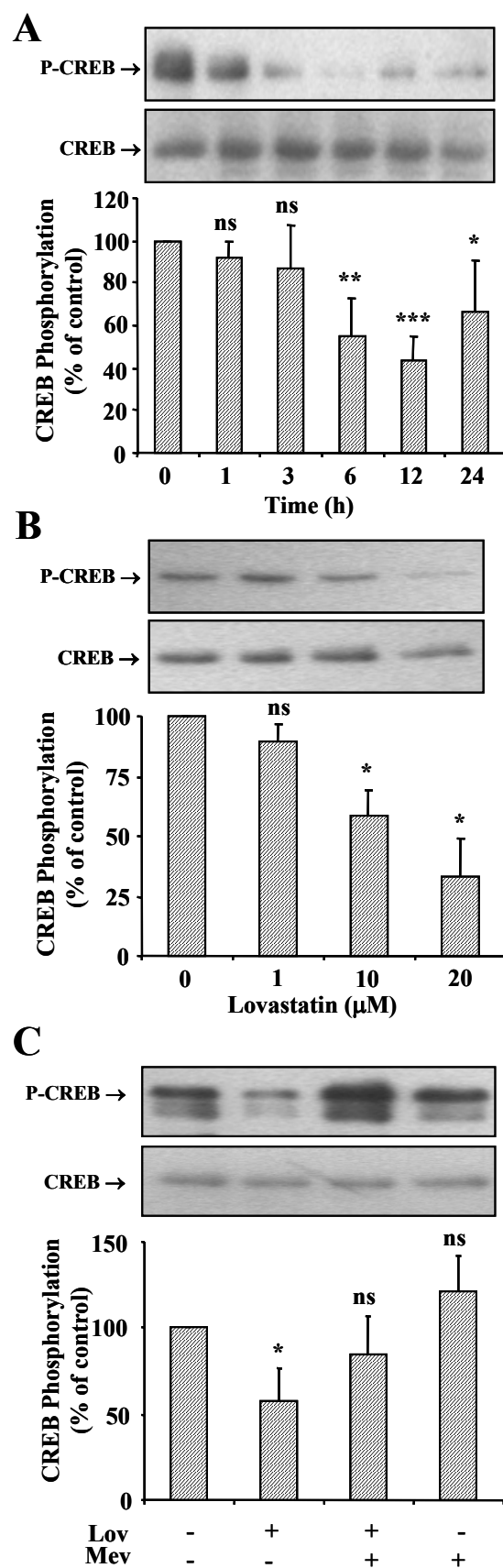


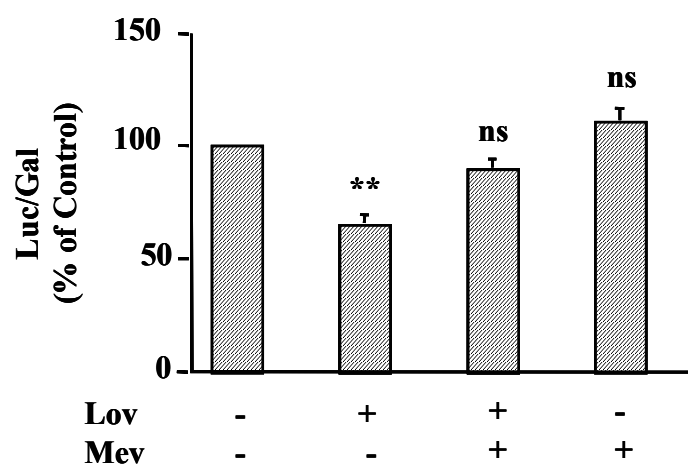
Figure 2



**Figure 3**



**Figure 4**



**Figure 5**

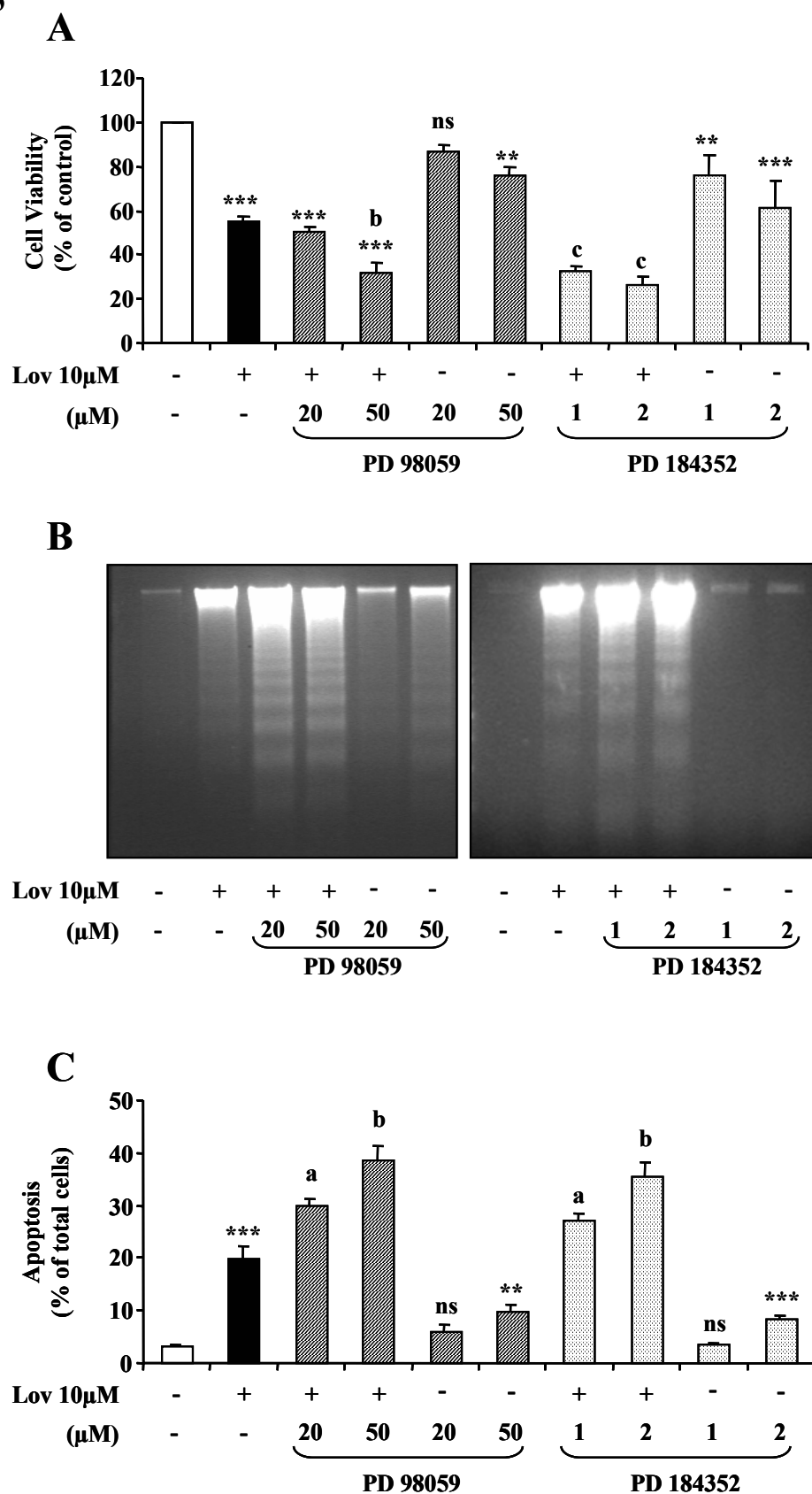
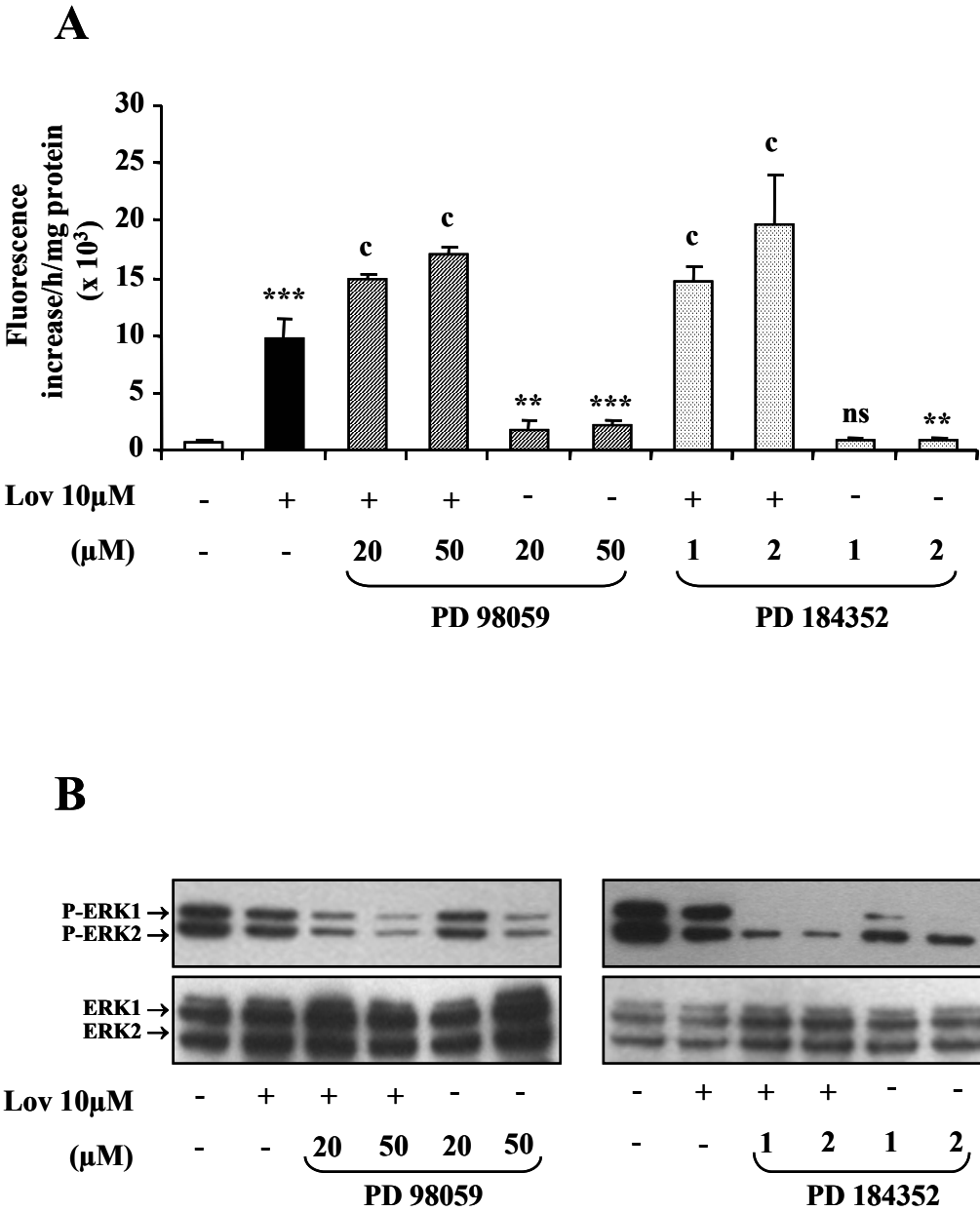




Figure 6



**Figure 7**

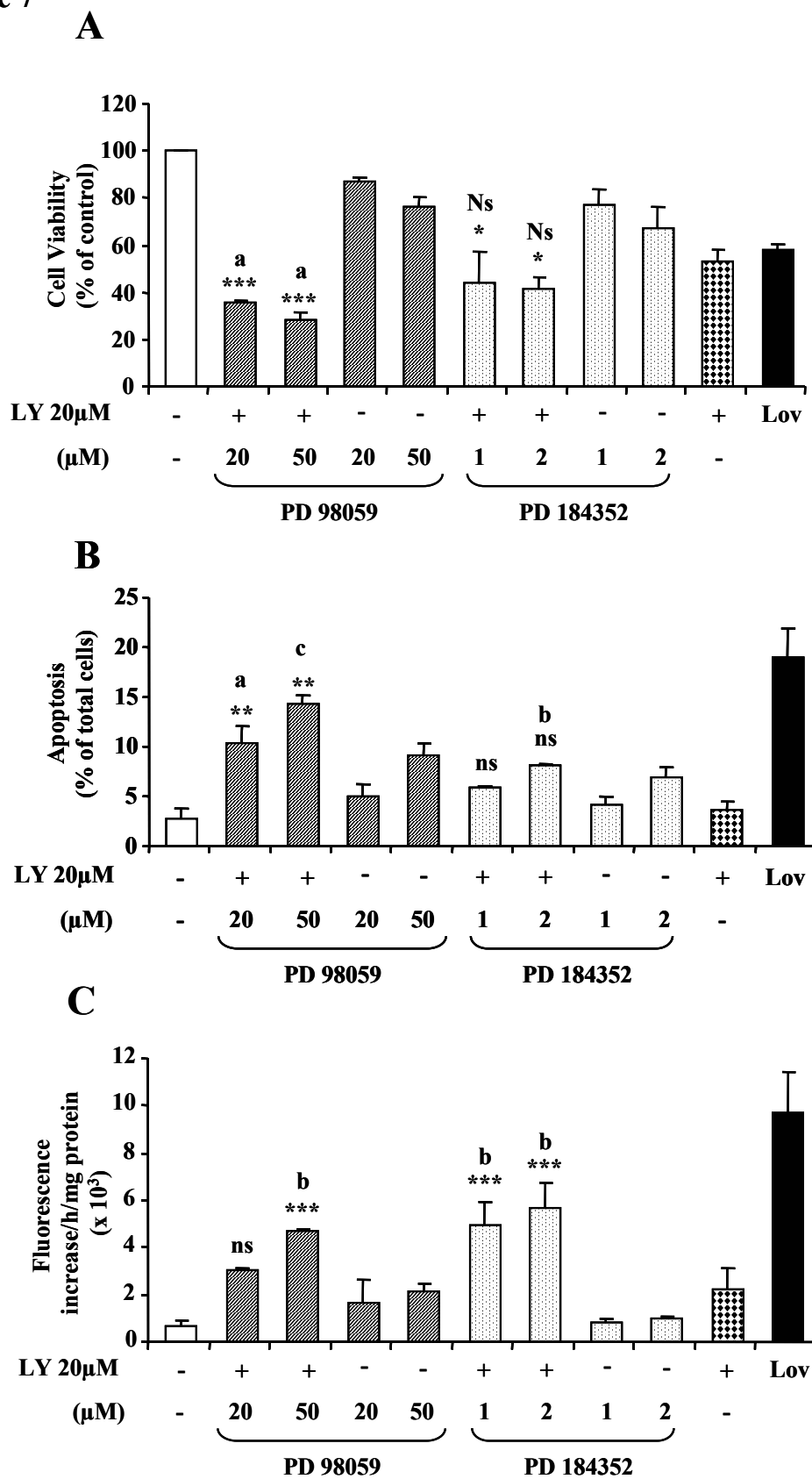


Figure 8

