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

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We have shown previously that lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, induc...
constitutively active mutants of MEK1 prevented the apoptosis induced by nerve growth factor withdrawal in neuronally differentiated PC12 cells [27]. There is accumulating evidence that ERK1/2 may mediate the neuroprotective activity of several factors that protect 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, CREB (cAMP response element binding protein) 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 previously mentioned, we have shown that lovastatin induces apoptosis in rat brain neuroblasts [22]. The aim of the present study, 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 allow us to gain an insight into the growth inhibition and apoptosis induced by lovastatin and other statins in neuronal cells, and additionally will contribute to the elucidation of 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, U.S.A.). The inactive lactone of lovastatin was converted into the active form as described previously [29]. Mevalonate was purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Ham’s F-12 medium, FCS (foetal calf serum), L-glutamine, streptomycin, penicillin and trypsin/EDTA solution were from PAN Biotech (Aidenbach, Germany). Tissue culture flasks and dishes were from TPP (Trasadingen, Switzerland). MEK1/2 inhibitor PD98059 (2’-amino-3’-methoxyflavone) and PI3K (phosphoinositide 3-kinase) inhibitor LY294002 were from Calbiochem. MEK1/2 inhibitor PD184352 was provided by Dr Ana Cuenda (Departamento de Bioquímica y Biología Molecular, Universidad de Extremadura, Cáceres, Spain). Complete protease inhibitor cocktail tablets were from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Glutathione–Sepharose 4B was from Pharmacia (Freiburg, Germany). The caspase substrate Ac-DEVD-AMC (N-acetyl-DEVD-7-amino-4-methylcoumarin), Ras antibody, anti-(phospho-Ser472,473/474 PKB) (protein kinase B) and anti-PKB were from BD Biosciences Pharmigen (San Diego, CA, U.S.A.). Anti-ERK1/2, anti-(phospho- Thr183Tyr185 ERK) and anti-CREB antibodies were from Sigma–Aldrich. Anti-(phospho-Ser133 CREB) antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Goat anti-rabbit and goat anti-mouse antibodies conjugated to horseradish peroxidase were from Pierce (Rockford, IL, U.S.A.). Other reagents were obtained from different commercial sources and were of the highest purity available.

Cell culture and treatments

Spontaneously immortalized rat brain neuroblasts were used in the present study, which were obtained by spontaneous immortalization from cultures of 17-day-old foetal rat cerebral cortices [30] and were kindly provided by Dr Alberto Muñoz (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). The cells represented primitive neuroblasts that expressed NF68 and the primitive neuronal marker nestin, but lacked the astrocyte marker glial fibrillary acidic protein. After partial differentiation induction with dibutyryl-cAMP, the cells expressed further neuronal markers such as NF145, NF220 and neuron-specific enolase [30]. Cells were grown in Ham’s F-12 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin. Cells were seeded at 5 × 10^5 in a 75-cm² tissue culture flask and incubated at 37°C in a 5% CO₂/95% air atmosphere.

Confluent cells were trypsinized and seeded in tissue culture dishes at a density of 2 × 10^6 cells/cm². After 24 h, the medium was aspirated and replenished 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 1 h before lovastatin addition. For the time course experiments, cells were incubated with 10 µM 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 analyse the amount of active Ras as described previously [31]. Neuroblasts were seeded in 100-mm-diameter tissue culture dishes and, after the indicated treatments, the cells were harvested in PBS and lysed for 15 min in 100 µl of ice-cold lysis buffer A containing 20 mM Tris/HCl (pH 7.4), 5 mM MgCl₂, 1 mM EDTA, 0.2 mM sodium orthovanadate, 5 mM NaF, 10% (v/v) glycerol, 100 mM KCl, 1% (v/v) Triton X-100, 0.05% 2-mercaptoethanol and 4 µg/ml Complete protease inhibitor cocktail. Cell lysates were centrifuged at 20000 g for 10 min at 4°C and the protein concentration in the supernatants was determined by the Bio-Rad Laboratories (Hercules, CA, U.S.A.) protein assay, according to the instructions of the manufacturer. Protein-equalized supernatants (250 µg) were incubated for 2 h at 4°C with glutathione-Sepharose 4B beads pre-coupled with GST (glutathione S-transferase)–RBD (1 h at 4°C). The beads were washed four times in lysis buffer. Bound proteins were solubilized by the addition of 30 µl of Laemmli loading buffer and resolved by SDS/PAGE (12% gels). The amount of Ras in the bound fraction was analysed by Western blotting, as described below.

Western blot analysis

Treated and control cells were harvested in PBS and lysed for 15 min in ice-cold lysis buffer B containing 50 mM Hapes (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 5 mM MgCl₂, 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, the cell debris was removed by centrifugation at 20000g for 10 min at 4°C and protein concentrations determined in the supernatants as indicated above. Equal amounts of protein (20 µg) were separated by SDS/PAGE (10 or 12% gels) and blotted on to PVDF membranes (PolyScreen, NEN Life Science Products, Boston, MA, U.S.A.). The membranes were then blocked with antibody buffer [5% (w/v) non-fat dry milk in TBST (Tris-buffered saline, pH 7.5, containing 0.5% Tween 20)] and incubated with appropriate primary and horseradish peroxidase-conjugated secondary antibodies in antibody buffer. After required washes with TBST, proteins were analysed using an enhanced chemiluminescence detection system (SuperSignal® West Pico chemiluminescent substrate; Pierce, Rockford, IL, U.S.A.) and exposed to hyperfilm-ECL® (Amersham Pharmacia Biotech, Freiburg, Germany). Films were photographed and the intensity of each band was analysed using a ChemiDoc System (Documentation and Analysis

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System, Bio-Rad Laboratories). 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 (cAMP-response element) luciferase reporter, the SV-40 (simian virus 40) promoter in the vector pGL3-promoter (Promega) was excised with BglII and HindIII and replaced by a minimal HSV TK (Herpes simplex virus thymidine kinase) promoter (obtained by PCR amplification) containing nucleotides −109 to +17 flanked by BglII and HindIII restriction sites. A double-stranded oligonucleotide containing the CRE from the somatostatin promoter (5′ AGCCTGACGTCAAGA 3′) flanked by Asp718 and BglII restriction sites was then cloned into the vector to give 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, U.S.A.), following the manufacturer’s instructions. After transfection, cells were incubated for 24 h, the medium was replaced and the cells were incubated for an additional 24 h with 10 μM lovastatin in the absence or presence of 100 μM mevanolate. 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 β-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-diameter tissue culture dishes were used. At the end of each treatment, the medium was discarded, and the remaining viable adherent cells were stained with 0.03 % Crystal Violet in 2 % (v/v) ethanol for 5 min. Dishes were rinsed with tap water and 1 ml of 1 % SDS 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 non-specific adhesion of Crystal Violet to the plastic. Viable cells were calculated as a percentage of absorbance with respect to untreated cells.

**DNA fragmentation assay**

Neuroblasts were plated on 100-mm-diameter 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 C [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 0.1 mg/ml RNase A at 37°C for 45 min and then with 0.2 mg/ml proteinase K with 0.5 % SDS at 37°C for 45 min. Soluble DNA was then isolated by phenol/chloroform/isoamylalcohol (25:24:1; by vol.) extraction and ethanol precipitation. DNA was collected by centrifugation at 20000 g for 20 min, dissolved in autoclaved water and resolved on a 2 % (w/v) agarose gel in TBE buffer (1 x TBE = 45 mM Tris/borate/1 mM EDTA) containing 0.1 μg/ml ethidium bromide. DNA fragments were visualized under UV light and photographed using a ChemiDoc System (Documentation and Analysis System, Bio-Rad Laboratories).

**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 Ca2+ and Mg2+. Cells were resuspended in ice-cold 70 % (v/v) ethanol and fixed for 5 min. After centrifugation at 500 g for 5 min, cells were resuspended in PBS containing 1 mg/ml 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 (25°C). 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 those 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 ice-cold PBS and lysed for 15 min in ice-cold lysis buffer containing 10 mM Tris (pH 7.5), 10 mM NaH2PO4, 130 mM NaCl, 1 % (v/v) 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 % (v/v) glycerol and 2 mM dithiothreitol] and mixed with 20 μM of caspase substrate. The reactions were incubated at 37°C for different time periods and the release of AMC from the substrate was monitored in a spectrophotometer using excitation and emission wavelengths of 380 nm and 440 nm respectively. The results were corrected for protein content of the lysates and are expressed as the increase of fluorescence for 1 h per mg of protein.

**Statistical analysis**

Each experiment was repeated at least three times, with good agreement among the results of individual experiments. All results are expressed as means ± S.E.M. Results were analysed by one-way ANOVA followed by Student’s t test. P < 0.05 was considered significant.

**RESULTS**

**Effect of lovastatin on Ras activation**

We have shown previously 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 in the level of Ras in the membrane compartment [22]. This post-translational prenylation step is required for both membrane association and Ras activation. Therefore in the present study we first investigated 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 analysed by the GST–RBD–Raf-1 pull-down method. Time course experiments showed that in comparison with the control cells grown in medium with 10 % (v/v) FCS, 10 μM lovastatin inhibited Ras activation in a time-dependent manner (Figure 1A). The lovastatin effect was significant 3 h after treatment and was markedly inhibited after 24 h. Inhibition of Ras activation occurred before induction of apoptosis, which was significant 12 h after lovastatin treatment [22]. Additionally,
neuroblasts were treated with increasing concentrations of lovastatin for 24 h and, as shown in Figure 1(B), Ras activation decreased in a concentration-dependent manner after lovastatin treatment. The effect of lovastatin was evident at a concentration as low as 1 µM reaching the maximum effect at 10 µM. In order to determine whether the lovastatin effect on Ras activation was a specific cause of HMG-CoA reductase inhibition, cells were incubated with 10 µM lovastatin in the absence or presence of 100 µM mevalonate for 24 h. Co-treatment with mevalonate prevented the decrease in Ras activation induced by lovastatin (Figure 1C), whereas treatment of the 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 compared with its processed form in SDS/PAGE). Taken together, these data suggest that the decrease in 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 phospho-specific antibodies. As shown in Figure 2, lovastatin decreased ERK1/2 phosphorylation in a time- and concentration-dependent manner (Figures 2A and 2B). Lovastatin (10 µM) produced a decrease in ERK1/2 phosphorylation that was significant after 3 h and was maximum after 12 h of treatment (Figure 2A). On the other hand, the lovastatin effect on ERK1/2 phosphorylation was evident at a concentration of 1 µM, but the decrease in the phosphorylation of both kinases was only significant at higher concentrations (10 µM and 20 µM). Mevalonate (100 µM) prevented the decrease in ERK1/2 phosphorylation induced by lovastatin, indicating that the lovastatin effect was specific (Figure 2C). Treatment of neuroblasts with mevalonate alone did not affect ERK1/2 phosphorylation. Expression levels of ERK1/2 remained unchanged throughout the experiment (Figure 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 10 µM lovastatin for 24 h reduced ERK1/2 phosphorylation by 45% compared with control cells, whereas the same treatment inhibited Ras activation by 90% compared with the 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 p90rsk, which in turn phosphorylates the transcription factor CREB on Ser133. 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 µM lovastatin for different times led to a reduction in the phosphorylation of CREB on Ser133 that was significant after 6 h, with the maximum effect observed after 12 h of treatment (Figure 3A). As shown in Figure 3(B), 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 µM lovastatin. Co-treatment with 100 µM mevalonate prevented the decrease in CREB phosphorylation induced by lovastatin. Treatment of cells with mevalonate alone did not
modify CREB phosphorylation (Figure 3C). Lovastatin treatment did not modify CREB expression in any of the experimental conditions (Figure 3).

To determinate whether the decrease in CREB phosphorylation induced by lovastatin was associated with an inhibition of CREB-mediated gene expression, we performed luciferase reporter assays. As shown in Figure 4, incubation of CRE-plasmid-containing cells with 10 µM lovastatin for 24 h resulted in the inhibition of luciferase activity. This effect was not observed in the cells transfected with the plasmid control lacking CREB binding sites (results not shown). Again, co-treatment with mevalonate prevented the effect of lovastatin and completely restored CREB-mediated reporter activity to control levels. Taken together, these results suggest that lovastatin not only decreases CREB phosphorylation but also inhibits CREB-mediated gene expression.

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 the legend to Figure 1. At the end of each experiment, cells were lysed, and total proteins (20 µg/lane) were separated by SDS/PAGE and analysed by Western blotting using an anti-(phospho-ERK1/2) specific antibody. As a control of the amount of protein, total ERK1/2 was also analysed in the same samples. A representative blot of each experiment is shown with the densitometric analysis corresponding to the means ± S.E.M. of at least three independent experiments. ns, not significant; **P < 0.01 and ***P < 0.001 compared with 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 the legend to Figure 1. At the end of each experiment, cells were lysed, and total proteins (20 µg/lane) were separated by SDS/PAGE and analysed by Western blotting using an anti-(phospho-CREB) specific antibody. As a control of the amount of protein, total CREB was also analysed in the same samples. A representative blot of each experiment is shown with the densitometric analysis corresponding to the means ± S.E.M. of at least three independent experiments. ns, not significant; * P < 0.05, ** P < 0.01 and ***P < 0.001 compared with untreated cells.
Figure 4  Effect of lovastatin on reporter activity driven by CRE

Neuroblasts initially cultured for 24 h in Ham's F12 medium containing 10% (v/v) FCS were co-transfected with pGL3-CRE-luciferase and pSV-β-galactosidase reporter constructs. Following transfection, cells were incubated with lovastatin (Lov; 10 μM) in the absence or presence of mevalonate (Mev; 100 μM) for 24 h. Control cells were incubated with either medium alone or medium plus mevalonate. Luciferase (Luc) and β-galactosidase (Gal) activity were measured in the same extracts, and the luciferase activity was standardized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. Results are expressed as the percentage relative to untreated cells. Results are means ± S.E.M of four independent experiments performed in triplicate. ns, not significant; **P < 0.01 compared with untreated cells.

phosphorylation in neuroblasts, but this action also correlates with decreased CREB-mediated transcription activation.

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

Our results suggested that lovastatin might induce neuroblast apoptosis through 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 h with different concentrations of PD98059 and PD184352, which inhibit MEK1/2, the MAPK kinase responsible for ERK1/2 phosphorylation. 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 Figure 5, the treatment of neuroblasts with PD98059 alone was associated with a concentration-dependent decrease in cell viability (Figure 5A), the appearance of internucleosomal DNA fragmentation (Figure 5B) and an increase in the percentage of apoptotic neuroblasts (Figure 5C). Moreover, PD98059 also enhanced caspase activity (Figure 6A). Although PD98059 inhibited ERK1/2 phosphorylation in a concentration-dependent manner (Figure 6B), the effects of this inhibitor were minimal when compared with control samples and never reached the effects seen withLovastatin treatment (compare Figure 5 with Figure 6). Similar results were obtained when PD184352, an alternative MEK1/2 inhibitor, was used (compare Figure 5 with Figure 6). These results 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 shown recently [10,11] that inhibition of the Ras/ERK pathway sensitizes cells to statin-induced apoptosis. To evaluate whether direct inhibition of this pathway can increase the efficacy of Lovastatin to trigger neuroblast apoptosis, cells were treated with 10 μM Lovastatin alone or in combination with PD98059 (20 or 50 μM) or PD184352 (1 or 2 μM) for 24 h. In these experiments, MEK inhibitors were added to the cells 1 h before Lovastatin treatment. As shown in Figures 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 (Figure 6B).

Effect of the inhibition of Ras/ERK1/2 and 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
Figure 6 Effect of MEK inhibitors alone or in combination with lovastatin on (A) caspase activation and (B) ERK1/2 phosphorylation

Neuroblasts were treated as described in the legend to Figure 5. At the end of each experiment, cell extracts were obtained and used to analyse caspase activity (A) or ERK phosphorylation (B) as described in the Materials and methods section. Results are means ± S.E.M. of two independent experiments performed in duplicate. ns, not significant. **P < 0.01 and ***P < 0.001 compared with untreated cells. ♠P = 0.001 compared with lovastatin-treated cells. A representative blot of three independent experiments is shown in (B).

Figure 7 Effect of the co-incubation with MEK inhibitors and PI3K inhibitor (LY294002) on (A) cell viability, (B) percentage of apoptotic cells, and (C) caspase activation

Cells cultured previously for 24 h in growth medium were incubated with different concentrations of PD98059 (20 or 50 µM), PD184352 (1 or 2 µM) or LY294002 (20 µM) alone for 24 h was associated with a decrease in cell viability (Figure 7A), a slight increase in the percentage of cells in the apoptotic sub-G₁ population (Figure 7B) and an increase in caspase activity (Figure 7C) compared with control cells. However, the effects of both types of inhibitor were additive in all the parameters studied (Figure 7) and only the concomitant use of both types of inhibitor resembled the lovastatin efficacy to induce neuroblast apoptosis (Figure 7). As shown in Figure 8, MEK inhibitors decreased ERK1/2 phosphorylation, but did not modify PKB phosphorylation, whereas 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 shown previously that mevalonate pathway inhibition by lovastatin induces apoptosis of spontaneously immortalized rat brain neuroblasts [22]. In the present study, pharmacological inhibition of each of these pathways was not sufficient to induce apoptosis to the level elicited by lovastatin ([34] and the present study). Therefore we next studied the effect of co-incubation of PD98059 and PD184352, and the PI3K inhibitor LY294002 on neuroblast survival and apoptosis. The treatment of neuroblasts with PD98059 (20 or 50 µM), PD184352 (1 or 2 µM) or LY294002 (20 µM) alone for 24 h was associated with a decrease in cell viability (Figure 7A), a slight increase in the percentage of cells in the apoptotic sub-G₁ population (Figure 7B) and an increase in caspase activity (Figure 7C) compared with control cells. However, the effects of both types of inhibitor were additive in all the parameters studied (Figure 7) and only the concomitant use of both types of inhibitor resembled the lovastatin efficacy to induce neuroblast apoptosis (Figure 7). As shown in Figure 8, MEK inhibitors decreased ERK1/2 phosphorylation, but did not modify PKB phosphorylation, whereas LY294002 inhibited the phosphorylation of PKB without affecting ERK1/2 phosphorylation.

we have investigated the molecular mechanisms underlying lovastatin-induced neuroblast apoptosis, focusing on the involvement of the Ras/ERK1/2/CREB signalling pathway.

In the present study, 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
We have also shown that the 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 the blockage of HMG-CoA reductase activity.

As described 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 the MEK inhibitors PD98059 and PD184352 were both poor inducers of apoptosis in serum-treated neuroblasts. However, these inhibitors significantly increased apoptosis induced by lovastatin treatment. Taken together, these results suggest that: first, lovastatin does not trigger neuroblast apoptosis by down-regulating the Ras/ERK/CREB signalling cascade alone; and secondly: 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 study, we have shown that only the pharmacological inhibition of both MEK and PKB activities was able to induce neuroblast apoptosis with similar efficacy as lovastatin. These results 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 currently being studied.

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 these effects were prevented by the addition of mevalonate to the medium, which indicates that they were due to an inhibition of mevalonate synthesis. Our results also suggest that inactivation of the Ras/ERK pathway appears to be insufficient to induce apoptosis evoked by lovastatin, and that apoptosis in 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.

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REFERENCES
ERK inhibition in lovastatin–induced neuronal apoptosis


