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, induces apoptosis in spontaneously immortalized rat brain neuroblasts. In the present study, we analysed 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 (extracellular-signal-regulated kinase 1/2) 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 [MAPK (mitogen-activated protein kinase)/ERK kinase] inhibitors, PD98059 and PD184352, were poor inducers of apoptosis in serum-treated neur-

oblasts. However, these inhibitors significantly increased apoptosis induced by lovastatin treatment. Furthermore, we showed that pharmacological inhibition of both MEK and phosphoinositide 3-kinase 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: apoptosis, extracellular-signal-regulated kinase 1/2 (ERK1/2), LY294002, neuroblast, PD98059, statin.

INTRODUCTION

HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, or statins, are widely used in the treatment of lipid disorders, especially hypercholesterolaemia. 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 develop-

ment 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 [17,18] show that the inhibition of this biosynthetic pathway during gestation by statin exposure is associated with severe CNS (central nervous system) defects. On the other hand, studies *in vitro* show that statins at micro- and milli-molar concentrations have neurotoxic effects on primary neuronal cultures of cerebral cortex [19–21], and we have shown [22] that lovastatin induces apoptosis of immortalized rat brain neuroblasts, and that its effect is associated with a decreased prenylation of Ras.

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 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/MEK2 and ERK1/2 cascade [23,24]. In the nervous system, the ERK1/2 cascade is critical for neuronal differentiation, plasticity and survival [26]. A role for ERK1/2 in neuronal survival was first indicated by experiments showing that overexpression of

Abbreviations used: Ac-DEVD-AMC, N-acetyl-DEVD-7-amino-4-methylcoumarin; CRE, cAMP-response element; CREB, CRE-binding protein; CNS, central nervous system; ERK, extracellular-signal-regulated kinase; FCS, foetal calf serum; GST, glutathione S-transferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RBD, Ras-binding domain of Raf-1; TBST, Tris-buffered saline, pH 7.5, containing 0.5% Tween 20.

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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 mentioned previously, 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 [2-(4-morpholinyl)-8-phenyl-4H-L-benzopyran-4-one] were from Calbiochem. MEK1/2 inhibitor PD184352 was provided by Dr Ana Cuenda (Departamento de Bioquímica y Biología Molecular, Universidad de Extremadura, Cáceres, Spain). CompleteTM 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-Ser^{472/473/474} PKB) (protein kinase B) and anti-PKB were from BD Biosciences Pharmingen (San Diego, CA, U.S.A.). Anti-ERK1/2, anti-(phospho-Thr¹⁸³Tyr¹⁸⁵ ERK1/2) 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 2×10^4 cells/cm². 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 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 CompleteTM 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 Hepes (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 CompleteTM protease inhibitor cocktail. After lysis, the 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 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

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 SV40 (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' AGCCTGACGTCAG-AGA 3') flanked by Asp718 and BglII restriction sites was then cloned into the vector to give pGL3-CRE. Neuroblasts were co-transfected with 1 μ g of pGL3-CRE and 1 μ g of β -galactosidase reporter vector using GenePORTERTM 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 μ M lovastatin in the absence or presence of 100 μ M 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 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 detached 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 $\times$ TBE = 45 mM Tris/borate/1 mM EDTA) containing 0.1 μ g/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 con-

ditions, 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 Ca^{2+} and Mg^{2+} . 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 μ g/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 G_0/G_1 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 NaH_2PO_4 , 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 μ l 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 μ M 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 spectrofluorometer 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 $\pm$ 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 μ M 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,

Figure 1 Time- and concentration-dependent effects of lovastatin on Ras activation

Neuroblasts cultured initially for 24 h in Ham's F12 medium with 10% (v/v) FCS were incubated in culture medium alone or with 10 μ M lovastatin for different periods of time (A), or for an additional 24 h in the medium alone (0) or various concentrations of lovastatin (B). (C) The effect of mevalonate treatment was measured using cells cultured initially for 24 h in Ham's F12 medium with 10% (v/v) FCS and then with lovastatin (Lov; 10 μ M) in the absence or presence of mevalonate (Mev; 100 μ M) for an additional 24 h. Control cells were incubated in the presence of medium or mevalonate alone. At the end of each experiment, Ras activation was measured by GST-RBD pull-down followed by Western blotting with anti-(pan)-Ras antibody. An aliquot of each lysate was also loaded on to another gel to analyse total Ras protein levels. A representative blot of each experiment is shown with the densitometric analysis corresponding to the means $\pm$ 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.

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 μ M reaching the maximum effect at 10 μ M. 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 μ M lovastatin in the absence or presence of 100 μ M 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 μ M) 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 μ M, but the decrease in the phosphorylation of both kinases was only significant at higher concentrations (10 μ M and 20 μ M). Mevalonate (100 μ M) 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 μ M 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 p90^{sk}, 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 μ M 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 μ M lovastatin. Co-treatment with 100 μ M mevalonate prevented the decrease in CREB phosphorylation induced by lovastatin. Treatment of cells with mevalonate alone did not

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 $\pm$ S.E.M. of at least three independent experiments. ns, not significant; ** P < 0.01 and *** P < 0.001 compared with untreated cells.

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

To determine 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

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 $\pm$ 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.

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

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 μ M) in the absence or presence of mevalonate (Mev; 100 μ M) 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 $\pm$ 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 with lovastatin 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 Raf/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 μ M lovastatin alone or in combination with PD98059 (20 or 50 μ M) or PD184352 (1 or 2 μ M) 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

Figure 5 Effect of MEK inhibitors alone or in combination with lovastatin on (A) cell viability, (B) internucleosomal DNA fragmentation and (C) percentage of apoptotic cells

Cells cultured previously for 24 h in growth medium were incubated with different concentrations of PD98059 (20 or 50 μ M) or PD184352 (1 or 2 μ M) in the absence or presence of 10 μ 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 analysed by using electrophoresis on 2% (w/v) agarose gel (B), and the percentage of cells with hypodiploid DNA content was evaluated by flow cytometry (C). Results are means $\pm$ S.E.M. of at least three independent experiments performed in triplicate. ns, not significant; ** P < 0.01 and *** P < 0.001 compared with untreated cells. ^a P < 0.05, ^b P < 0.01 and ^c P < 0.001 compared with lovastatin-treated cells. A representative photograph of three independent experiments is shown in (B).

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 $\pm$ S.E.M. of two independent experiments performed in duplicate. ns, not significant; ** $P < 0.01$ and *** $P < 0.001$ compared with untreated cells. ^c $P < 0.001$ compared with lovastatin-treated cells. A representative blot of three independent experiments is shown in (B).

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 μ M), PD184352 (1 or 2 μ M) or LY294002 (20 μ M) 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,

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 μ M) or PD184352 (1 or 2 μ M) in the absence or presence of 20 μ M LY294002 (LY) and with 20 μ M LY or 10 μ M lovastatin (Lov) 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 the Materials and methods section. Results are means $\pm$ S.E.M. of at least three independent experiments performed in triplicate. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with their respective MEK-inhibitor-treated cells. ns, not significant; ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$ compared with LY-treated cells.

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

Figure 8 Effect of MEK inhibitors and PI3K inhibitor on both ERK1/2 and PKB phosphorylation

Cells were treated as described in the legend to Figure 7. At the end of each experiment, the cells were lysed and total proteins (20 μ g/lane) were separated by SDS/PAGE and analysed 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 analysed. A representative blot is shown.

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 results 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 their 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 ERK1/2 was only partially inhibited in lovastatin-treated cells compared with control cells. The sustained phosphorylation of ERK1/2 in lovastatin-treated neuroblasts may be due to the activation of other upstream intracellular signalling molecules, such as protein kinase C or Rac [40,41], which in turn induce the activation of the MEK/ERK pathway.

It is accepted that ERK1/2 activation promotes neuronal survival by its capacity to activate p90^{rk}, which in turn phosphorylates and activates CREB and results in increased CREB-mediated gene expression [28,42]. In the present study, we have shown that the decrease in ERK1/2 phosphorylation/activity caused by lovastatin is associated with a reduction in both CREB phosphorylation and CREB-mediated gene expression. The down-regulation of the 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 shown previously [22] that lovastatin decreased the expression of Bcl-2 in neuroblasts undergoing apoptosis. As this pro-survival gene is a target of CREB in neurons [28], the results of the present study 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 studies that indicate that CREB is essential for neuronal survival [45].

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 PI3K 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.

We thank Ricardo Argent for his excellent technical assistance, Dr Ana Cuenda for kindly providing the MEK1/2 inhibitor (PD184352), and Mrs Mary Harper for help with the preparation of the manuscript (all from Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain). This work has been supported by grants SAF 2001-0154 from the Ministerio de Ciencia y Tecnología, and 2PR01B007 from the Junta de Extremadura. M. I. C.-G. is supported by a PhD fellowship from Junta de Extremadura, Spain.

REFERENCES

- 1 Blumenthal, R. S. (2000) Statins: effective antiatherosclerotic therapy. *Am. Heart J.* **139**, 577–583.
- 2 Chong, P. H., Seeger, J. D. and Franklin, C. (2001) Clinically relevant differences between the statins: implications for therapeutic selection. *Am. J. Med.* **111**, 390–400.

- 3 Graaf, M. R., Richel, D. J., van Noorden, C. J. F. and Guchelarr, H. J. (2004) Effect of statins and farnesyltransferase inhibitors on the development and progression of cancer. *Cancer Treat. Rev.* **30**, 609–641
- 4 Goldstein, J. L. and Brown, M. S. (1990) Regulation of the mevalonate pathway. *Nature* **343**, 425–429
- 5 Padayatty, S. J., Marcelli, M., Shao, T. C. and Cunningham, G. R. (1997) Lovastatin-induced apoptosis in prostate stromal cells. *J. Clin. Endocr. Metab.* **82**, 1434–1439
- 6 Blanco-Colio, L. M., Villa, A., Ortego, M., Hernández-Presa, M. A., Pascual, A., Plaza, J. J. and Egido, J. (2002) 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. *Atherosclerosis* **161**, 17–26
- 7 Hillyard, D. Z., Cameron, A. J., McIntyre, A. H., Hadden, N. H., Marshall, C. E., Johnston, N. and Jardine, A. G. (2002) Inhibition of proliferation and signalling mechanisms in human lymphocytes by fluvastatin. *Clin. Exp. Pharmacol. P.* **29**, 673–678
- 8 Jaster, R., Brock, P., Sparmann, G., Emmrich, J. and Liebe, S. (2003) Inhibition of pancreatic stellate cell activation by the hydroxymethylglutaryl coenzyme A reductase inhibitor lovastatin. *Biochem. Pharmacol.* **65**, 1295–1303
- 9 Koyuturk, M., Ersoz, M. and Altioğlu, N. (2004) Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-Jun N-terminal kinase. *Neurosci. Lett.* **370**, 212–217
- 10 Takata, R., Fukasawa, S., Hara, T., Nakajima, H., Yamashina, A., Yanase, N. and Nizuguchi, J. (2004) Cerivastatin-induced apoptosis of human aortic smooth muscle cells through partial inhibition of basal activation of extracellular signal-regulated kinases. *Cardiovasc. Pathol.* **13**, 41–48
- 11 Wu, J., Wong, W. W., Khosravi, F., Minden, M. D. and Penn, L. Z. (2004) Blocking the Raf/MEK/ERK pathway sensitizes acute myelogenous leukaemia cells to lovastatin-induced apoptosis. *Cancer Res.* **64**, 6461–6468
- 12 Nishida, S., Matsuo, H., Tsubaki, M., Tanimori, Y., Yanae, M., Fujii, Y. and Iwaki, M. (2005) Mevastatin induces apoptosis in HL60 cells dependently on decrease in phosphorylated ERK. *Mol. Cell. Biochem.* **269**, 109–114
- 13 Zhang, F. L. and Casey, P. J. (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241–269
- 14 Maltese, W. A. and Volpe, J. J. (1979) Developmental changes in the distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase among subcellular fractions of rat brain. *J. Neurochem.* **33**, 107–115
- 15 Spady, D. K. and Dietschy, J. M. (1983) Sterol synthesis *in vivo* in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster and rat. *J. Lipids Res.* **24**, 303–315
- 16 Cavender, C. P., Turley, S. D. and Dietschy, J. M. (1995) Sterol metabolism in fetal, newborn and suckled lambs and their response to cholesterol after weaning. *Am. J. Physiol.* **269**, E331–E340
- 17 Edison, R. J. and Muenke, M. (2004) Central nervous system and limb anomalies in case reports of first-trimester statin exposure. *N. Engl. J. Med.* **350**, 1579–1582
- 18 Edison, R. J. and Muenke, M. (2004) Mechanistic and epidemiologic considerations in the evaluation of adverse birth outcomes following gestational exposure to statins. *Am. J. Med. Genet.* **131**, 287–298
- 19 Michikawa, M. and Yanagisawa, K. (1999) Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. *J. Neurochem.* **72**, 2278–2285
- 20 Tanaka, T., Tatsuno, I., Uchida, D., Moroo, I., Morio, H., Nakamura, S., Noguchi, Y., Yasuda, T., Kitagawa, M., Saito, Y. and Hirai, A. (2000) Geranylgeranyl-pyrophosphate, an isoprenoid of mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. *J. Neurosci.* **20**, 2852–2859
- 21 Schulz, J. G., Bosel, J., Stoeckel, M., Megow, D., Dirnagl, U. and Endres, M. (2004) HMG-CoA reductase inhibition causes neurite loss by interfering with geranylgeranylpyrophosphate synthesis. *J. Neurochem.* **89**, 24–32
- 22 García-Román, N., Álvarez, A. M., Toro, M. J., Montes, A. and Lorenzo, M. J. (2001) Lovastatin induces apoptosis of spontaneously immortalized rat brain neuroblasts: involvement of nonsterol isoprenoid biosynthesis inhibition. *Mol. Cell. Neurosci.* **17**, 329–341
- 23 Denhardt, D. T. (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem. J.* **318**, 729–747
- 24 Repasky, G. A., Chenette, E. J. and Der, C. J. (2004) Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? *Trends Cell. Biol.* **14**, 639–647
- 25 Mitin, N., Rossman, K. L. and Der, C. J. (2005) Signaling interplay in ras superfamily function. *Curr. Biol.* **15**, R563–R574
- 26 Hetman, M. and Gozdz, A. (2004) Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. *Eur. J. Biochem.* **271**, 2050–2055
- 27 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326–1331
- 28 Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A. and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. *Science* **286**, 1358–1362
- 29 Kita, T., Brown, M. S. and Goldstein, J. L. (1980) Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. *J. Clin. Invest.* **66**, 1094–1100
- 30 Muñoz, A., Wrighton, C., Seliger, B., Bernal, J. and Beug, H. (1993) Thyroid hormone receptor/c-erbA: control of commitment and differentiation in the neuronal/chromaffin progenitor line PC12. *J. Cell Biol.* **121**, 423–438
- 31 Villalonga, P., Lopez-Alcala, C., Chiloeches, A., Gil, J., Marais, R., Bachs, O. and Agell, N. (2002) Calmodulin prevents activation of Ras by PKC in 3T3 fibroblasts. *J. Biol. Chem.* **277**, 37929–37935
- 32 Drysdale, B. E., Zacharchuk, C. M. and Shin, H. S. (1983) Mechanism of macrophage-mediated cytotoxicity: production of a soluble cytotoxic factor. *J. Immunol.* **131**, 2362–2367
- 33 Darynkiewicz, Z., Bruno, S., Bino, G. D., Gorczyca, W., Hotz, M. A., Lassota, P. and Traganos, F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**, 795–808
- 34 Cerezo-Guisado, M. I., García-Marín, L. J., Lorenzo, M. J. and Bragado, M. J. (2005) Lovastatin inhibits the growth and survival pathway of phosphoinositide 3-kinase/protein kinase B in immortalized rat brain neuroblasts. *J. Neurochem.* **94**, 1277–1287
- 35 Nakagawa, H., Mutoh, T., Kumano, T. and Kuriyama, M. (1998) HMG-CoA reductase inhibitor-induced L6 myoblast cell death: involvement of the phosphatidylinositol 3-kinase pathway. *FEBS Lett.* **438**, 289–292
- 36 Bassa, B. V., Roh, D. D., Vaziri, N. D., Kirschenbaum, M. A. and Kamanna, V. S. (1999) Effect of inhibition of cholesterol synthetic pathway on the activation of Ras and MAP kinase in mesangial cells. *Biochim. Biophys. Acta* **1449**, 137–149
- 37 Miura, S., Matsuo, Y. and Saku, K. (2004) Simvastatin suppresses coronary artery endothelial tube formation by disrupting Ras/Raf/ERK signaling. *Atherosclerosis* **175**, 235–243
- 38 Johnson, M. D., Woodard, A., Okediji, E. J., Toms, S. A. and Allen, G. S. (2002) Lovastatin is a potent inhibitor of meningioma cell proliferation: evidence for inhibition of a mitogen associated protein kinase. *J. Neurooncol.* **56**, 133–142
- 39 Graham, T. E., Pfeiffer, J. R., Lee, R. J., Kusewitt, D. F., Martinez, A. M., Fautz, T., Wilson, B. S. and Oliver, J. M. (1998) MEK and ERK activation in Ras-disabled RBL-2H3 mast cells and novel roles for geranylgeranylated and farnesylated proteins in FcεRI-mediated signaling. *J. Immunol.* **161**, 6733–6744
- 40 Skalez-Rorowski, A., Müller, J. G., Kroke, A., Waltenberger, J., Pulawski, E., Pinkernell, K. and Breithardt, G. (2001) Lovastatin blocks basis fibroblast growth factor-induced mitogen-activated protein kinase signalling in coronary smooth muscle cells via phosphatase inhibition. *Eur. J. Cell. Biol.* **80**, 207–212
- 41 Negre-Aminou, P., van Leeuwen, R. E., van Thiel, G. C., van den IJssel, P., de Jong, W. W., Quinland, R. A. and Cohen, L. H. (2002) Differential effect of simvastatin on activation of Rac(1) vs. activation of the heat shock protein 27-mediated pathway upon oxidative stress, in human smooth muscle cells. *Biochem. Pharmacol.* **64**, 1483–1491
- 42 Riccio, A., Ahn, S., Davenport, C. M., Blendy, J. A. and Ginty, D. D. (1999) Mediation by CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* **286**, 2358–2361
- 43 Rabelo, F. L. A., Ropert, C., Ramos, M. G., Bonjardim, C. A., Gazzinelli, R. T. and Alvarez-Leite, J. I. (2003) Inhibition of ERK1/2 and CREB phosphorylation by caspase-dependent mechanism enhances apoptosis in fibrosarcoma cell line treated with butyrate. *Biochem. Biophys. Res. Commun.* **303**, 968–972
- 44 Hansen, H. H., Briem, T., Dzielko, M., Siffringer, M., Voss, A., Rzeski, W., Zdzisinska, B., Thor, F., Heumann, R., Stepulak, A. et al. (2004) Mechanisms leading to disseminated apoptosis following NMDA receptor blockade in the developing rat brain. *Neurobiol. Dis.* **16**, 440–453
- 45 Walton, M. R. and Dragunow, M. (2000) Is CREB a key to neuronal survival? *Trends Neurosci.* **23**, 48–53
- 46 Brantley-Finley, C., Lyle, C. S., Du, L., Goodwin, M. E., Hall, T., Swzedo, D., Kaushal, G. P. and Cambers, T. C. (2003) The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide. *Biochem. Pharmacol.* **66**, 459–469