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Inhibition of Cholesterol Production but Not of Nonsterol Isoprenoid Products Induces Neuronal Cell Death

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Abstract: Deficiency of nonsterol isoprenoids, intermediate metabolites of the cholesterol biosynthetic pathway, has been known to cause an inhibition of DNA synthesis and cell growth, and to induce apoptosis in nonneuronal cells. To investigate whether this is also the case in neurons, we examined the effect of a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor on the viability of neuronal cultures prepared from fetal rat brains. Treatment with compactin, a competitive inhibitor of HMG-CoA reductase, induced neuronal death in a dose-dependent manner. Concurrent treatment with cholesterol, β-migrating very low density lipoprotein, mevalonate, or squalene substantially inhibited the induction of neuronal death by compactin. Cell death was also induced by treatment with squalestatin, which specifically inhibits cholesterol biosynthesis at a site downstream from the generation of nonsterol metabolites. Furthermore, squalestatin-induced neuronal death was inhibited by concurrent incubation with squalene but not mevalonate. In contrast, cell growth of proliferating cells such as NIH 3T3 and PC12 cells was exclusively dependent on the level of nonsterol isoprenoid products and not that of cholesterol. The results of this study clearly indicate that the viability of neurons, different from that of nonneuronal cells, depends on the intracellular cholesterol content and not on the intermediate nonsterol isoprenoid products. **Key Words:** 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor—Compactin—Neuronal cell death—Isoprenoid products—Cholesterol—β-Migrating very low density lipoprotein.
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Unesterified cholesterol is essential for cellular viability, and homeostasis of cellular cholesterol is crucial to various cellular functions. This must be especially true for neurons because neurons have vast cellular membranes, and plastic membrane synthesis involving axonal growth and synaptogenesis requires the constant supply of lipid molecules including cholesterol. However, surprisingly little is known about the metabolism and physiological roles of cholesterol in neurons.

Compactin and its related compounds such as lovastatin, pravastatin, vastatin, and mevinolin are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in mevalonate metabolism, which yields cholesterol and nonsterol isoprenoid products. Previous in vitro work has demonstrated that these compounds inhibit the growth and proliferation of various kinds of cells, including human fibroblasts and lymphocytes, baby hamster kidney cells, vascular smooth muscle cells, rat hepatocytes, astrocytes, Swiss 3T3 cells, and mesangial cells, by inhibiting DNA synthesis (Kaneko et al., 1978; Quesney-Huneeus et al., 1979, 1980, 1983; Habenicht et al., 1980; Brown and Goldstein, 1980; Perkins et al., 1982; Fairbanks et al., 1984; Siperstein, 1984; Maltese and Sheridan, 1985; Cuts et al., 1989; Goldstein and Brown, 1990; Pavlov et al., 1995). In addition, it has been reported that the inhibition of DNA synthesis and cell growth induced by high concentrations of HMG-CoA reductase inhibitors could be reversed by mevalonate but not by cholesterol or other sterols in these cells (Quesney-Huneeus et al., 1979, 1983; Habenicht et al., 1980; Perkins et al., 1982; Fairbanks et al., 1984; Siperstein, 1984; Padayatty et al., 1997). Thus, inhibition of DNA synthesis and cell growth by inhibitors of HMG-CoA reductase in these cells is not a direct effect of reduced cholesterol availability but results from inhibition of the production of one or more nonsterol isoprenoid products that are synthesized from farnesyl pyrophosphate in the mevalonate pathway (cholesterol biosynthetic pathway). The importance of farnesyl pyrophosphate was also highlighted by the evidence of growth-regulating $p21^{ras}$ proteins (Casey et al., 1986; Hancock et al., 1989; Schafer et al., 1989; Maltese et al., 1990; Sinensky et al., 1990) and nuclear envelope proteins (Beck et al., 1988; Wolda and Glomset, 1988; Farnsworth et al., 1989) being covalently attached to farnesyl residues, which anchor them to cell membranes. Inhibition of HMG-CoA reductase is also known to induce cell death in mitotic cells by suppres-

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; β-VLDL, β-migrating very low density lipoprotein.

sion of the synthesis of nonsterol isoprenoid products (Sinensky et al., 1990; Jones et al., 1994; Dimitroulakos and Yeger, 1996; Satoh et al., 1996; Padayatty et al., 1997).

The critical role of nonsterol isoprenoid products in DNA synthesis, cell proliferation, and prevention of apoptosis in mitotic cells has been discussed; however, the biological effects of HMG-CoA reductase inhibitors on non- or postmitotic cells such as neurons remain unknown. Neurons are generated primarily prenatally (Frederiksen and McKay, 1988), and DNA synthesis does not occur in differentiated neurons, which do not divide.

In this work, we have examined the effect of an HMG-CoA reductase inhibitor on neuronal viability. In contrast to the previous studies on proliferating cells, here we report that death of neurons, which do not divide, induced by compactin, was inhibited by concurrent treatment of the cells with mevalonate, squalene, cholesterol, or β-migrating very low density lipoprotein (β-VLDL), indicating that compactin induces neuronal death by the reduced availability not of nonsterol isoprenoid products but of cholesterol. The critical role played by cholesterol in neuronal viability was also shown by the induction of cell death by squalestatin, an inhibitor of squalene synthase, and prevention of such cell death by concurrent treatment with squalene.

EXPERIMENTAL PROCEDURES

Materials

Compactin and N2 supplement (bovine insulin, human transferrin, putrescine, progesterone, and sodium selenite) were purchased from Sigma (St. Louis, MO, U.S.A.), and squalestatin was kindly provided by Dr. Simon Lister (Glaxo, U.K.). [^{14}C]Acetate (55 mCi/mmol) and [^{14}C]cholesterol (55 mCi/mmol) were purchased from NEN Life Science (Boston, MA, U.S.A.) and Amersham (Buckinghamshire, U.K.), respectively. Fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from GibcoBRL (Gaithersburg, MD, U.S.A.). DNase I was obtained from Boehringer-Mannheim (Mannheim, Germany).

Cell culture

Cerebral cortical neuron cultures were prepared from embryonic day 17 Sprague–Dawley rats. In brief, cortices were dissected, freed of meninges, and diced into small pieces; cortical fragments were incubated with 0.25% trypsin and 20 μg/ml DNase I in phosphate-buffered saline (PBS; 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, and 2.7 m*M* KCl, pH 7.4) at 37°C for 20 min. Cortical fragments were then dissociated into single cells by pipetting, suspended in the feeding medium, and plated onto poly-D-lysine-precoated 48-well plates for assay of cell death and 24-well plates for determination of cholesterol biosynthesis at a cell density of $1 \times 10^5/cm^2$. The feeding medium consisted of Dulbecco's modified Eagle's medium nutrient mixture (DMEM/F-12; 50%: 50%) containing N2 supplement. Six hours after plating, the cultures were incubated with the reagents under study until assayed.

For the culture of NIH 3T3 cells, a fibroblast cell line established from NIH Swiss mouse embryo cultures, the cells were plated onto 24-well plates at a cell density of $1 \times 10^5/cm^2$ and maintained in DMEM containing 10% FBS until use. The culture medium was switched to DMEM containing 5% lipid-deficient FBS and 5 μ*M* compactin when the reagents used were added.

For the culture of PC12 cells, a cell line established from a rat adrenal pheochromocytoma, the cells were plated onto 24-well plates at a cell density of $1 \times 10^5/cm^2$ and maintained in DMEM containing 8% FBS and 7% horse serum until use. The culture medium was switched to DMEM containing 10% lipid-deficient FBS and 5 μ*M* compactin when the reagents used were added.

To prepare stock solutions, compactin (10 m*M*), squalene (25 m*M*), and cholesterol (10 mg/ml) were solubilized in absolute ethanol, and squalestatin (1 m*M*) was solubilized in distilled water. Ethanol in the stock solutions did not cause any cytotoxic effects at the concentrations in the media used for the experiments. Mevalonic acid lactone was solubilized in PBS and then diluted in DMEM to produce 100 m*M* mevalonate salt. Aliquots of these solutions were stored at −20°C until use.

Effect of compactin and squalestatin on cholesterol biosynthesis

Neurons isolated from fetal rat cerebral cortices were cultured at a density of 1×10^5 cells/cm^2 on poly-D-lysine-precoated 24-well plates. The cultured cells maintained in serum-free N2 medium for 24 h were incubated with various concentrations of compactin or squalestatin, both inhibitors of cholesterol biosynthesis. After 5 h of incubation, the cultures were pulsed with 2 μCi/ml [^{14}C]acetate (1.64×10^5 photostimulated luminescence/nmol) at 37°C for 2 h. To determine the level of cholesterol biosynthesis from radioactive acetate, the cultures were washed in cold PBS, and the samples were extracted by incubation with 500 μl of hexane/isopropanol (3:2 vol/vol) for 30 min, evaporated under a nitrogen stream, and analyzed by thin-layer chromatography using the mobile phase of hexane/diethyl ether/acetic acid (70:30:1 by volume). The radioactive lipids that co-migrated with a purified standard of ^{14}C-labeled cholesterol were quantified using a Bio-Imaging Analyzer System-2500 Mac (Fuji Photo Film Co. Ltd., Japan). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as the standard. The amount of [^{14}C]acetate incorporated into cellular cholesterol per milligram of cell protein was calculated.

Lipoproteins

β-VLDL ($d < 1.006$ g/ml) was prepared from the plasma of male New Zealand white rabbits as described previously (Michikawa and Yanagisawa, 1998). In brief, the plasma isolated from cholesterol-fed rabbits was centrifuged at $d = 1.006$ g/ml for 30 min at 27,000 *g* in a Ti-70.1 rotor (Beckman), and the floating fraction was discarded. The lower fraction was recentrifuged at $d = 1.006$ g/ml for 16 h at 110,000 *g*. The floating fraction was collected, passed through a Millipore filter (0.45 μm), and stored at 4°C until use within 10 days. The quality of the β-VLDL was determined using the Paragon reagent kit for lipoprotein electrophoresis (Beckman).

Viability assay

The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into culture medium was determined for the quantification of cell death. Fifty microliters of culture medium was transferred to a fresh 96-well flat-bottomed plate; a colorimetric LDH release assay was performed according to the instructions

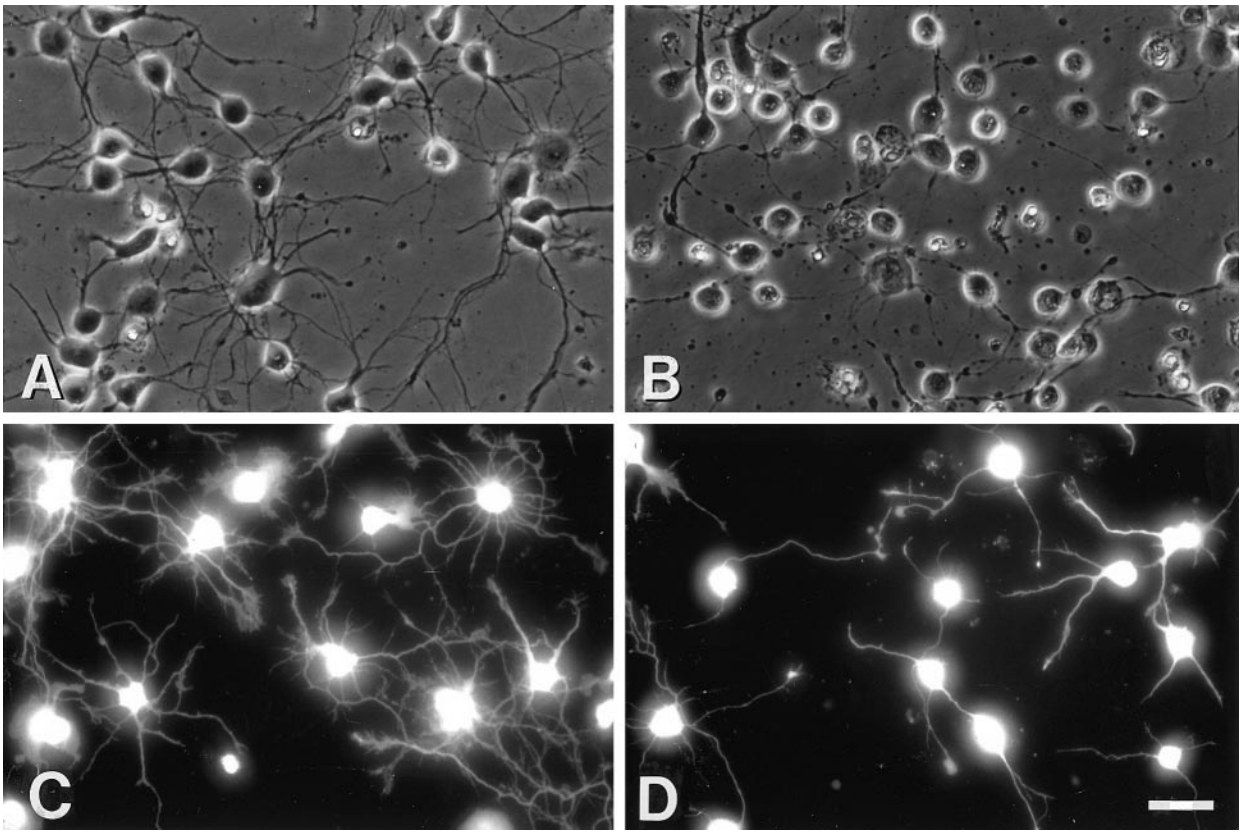

FIG. 1. Effect of compactin on neuronal cell morphology. Neuronal cells incubated with (**B**) or without (**A**) 2 μM compactin for 48 h were photographed under phase-contrast microscopy. Neuronal cells incubated with (**D**) or without (**C**) 2 μM compactin for 36 h were stained with calcein AM for 30 min and photographed under fluorescence microscopy. Bar = 20 μm.

of the manufacturer (Promega, WI, U.S.A.), and absorbances were read at 490 nm immediately thereafter. Calcein AM (Molecular Probes, OR, U.S.A.) staining was used to determine the number of viable cells in each well and demonstrate the morphological features of viable neurons. Cultured neurons were incubated with calcein AM (2 μM) at 37°C for 30 min and rinsed with PBS, and the number of calcein AM-positive viable cells in each well was determined with Fluoroskan Ascent (Labosystems, Helsinki, Finland). The morphological features of viable neurons were examined under a fluorescence microscope (Olympus, Japan). Fluorescence images were photographed using a 35-mm film, and prints were obtained.

RESULTS

Compactin suppresses cholesterol biosynthesis and induces neuronal cell death

To investigate the effect of the inhibitor of HMG-CoA reductase on neuronal cell viability, we established a neuron-rich culture system, where >98% of cells were immunostained with anti-microtubule-associated protein 2 antibody until day 4 in culture (data not shown). Cells dissociated from fetal rat cortices were grown in serum-free N2 medium in the presence of various combinations of the reagents under study. In cultures treated with 2 μM compactin for 48 h, damaged neurons with fragmented neurites and floating debris were observed (Fig. 1B), whereas in control cultures, neurons looked healthy with well-developed neurites (Fig. 1A). There were a great number of viable neurons after a 36-h incubation with 2 μM compactin; however, treated cells had fewer neurites than control cells, as demonstrated by calcein AM staining (Fig. 1C and D). We further determined the neurotoxic activity of compactin in culture. To ascertain the dose of compactin required for the suppression of cholesterol biosynthesis, neuronal cultures maintained in serum-free N2 medium were incubated with compactin at various concentrations. The cells were incubated with compactin for 5 h at 37°C and then exposed to [^{14}C]acetate for another 2 h. The incorporation of [^{14}C]acetate into newly synthesized cholesterol was determined by quantitating the radioactivity on thin-layer chromatography plates. Compactin suppressed cholesterol biosynthesis in a dose-dependent manner. The level of cholesterol biosynthesis in cells cultured in the presence of compactin at concentrations higher than 1 μM was suppressed to <26% of the control level (Fig. 2). Viability assays for neurons were performed 48 h after the start of incubation with compactin at various concentrations. Incubation with compactin at concentrations higher than 1 μM induced marked neuronal cell death as demonstrated by LDH assay (Fig. 2). The time course of compactin

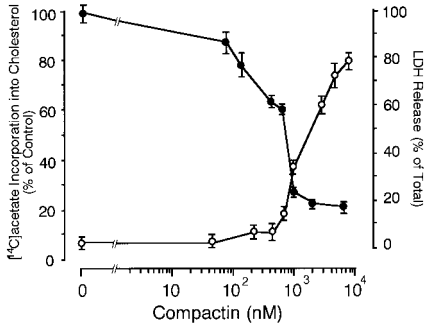

FIG. 2. Effect of compactin at various concentrations on the incorporation of [^{14}C]acetate into [^{14}C]cholesterol (●) and LDH release (○) in neuronal cultures. On day 2, each of the neuronal cultures was exposed to the indicated concentration of compactin. After incubation with compactin for 2 h at 37°C, the cultures were pulse-labeled with 2 μCi/ml [^{14}C]acetate for 5 h at 37°C, after which cells were harvested for determination of their [^{14}C]cholesterol content, as described in Experimental Procedures. For cell viability assay, the cultures were exposed to various concentrations of compactin at 6 h after plating and maintained for 48 h at 37°C, after which the medium was harvested for LDH assay.

(2 μM)-induced cell death, as demonstrated by LDH assay in neuronal cultures, is shown in Fig. 3. LDH release from cells cultured with 2 μM compactin was 0, 8.3, 10.4, 20.8, 75.0, and 91.7% compared with that in control cells at 3, 15, 24, 36, 48, and 72 h, respectively.

Effects of intermediate metabolites in cholesterol synthetic pathway on compactin-induced neuronal cell death

Previous studies have shown that treatment of cells with HMG-CoA reductase inhibitors resulted in cell death that could be prevented by the addition of mevalonate but not by exogenously supplied cholesterol or other sterols (Satoh et al., 1996; Padayatty et al., 1997). To determine whether neuronal cell death induced by compactin is a result of the decreased synthesis of nonsterol isoprenoid products, we examined the effects of intermediate metabolites in the cholesterol synthetic pathway on compactin-induced neurotoxicity. Concurrent treatment with mevalonate inhibited compactin-induced neuronal death, as demonstrated by LDH assay, in a dose-dependent manner (Fig. 4A). Concurrent treatment with squalene also inhibited neuronal death induced by compactin in a dose-dependent manner (Fig. 4B). Figure 4C shows the additive effect of mevalonate and squalene in inhibiting compactin-induced neuronal death, as demonstrated by LDH assay.

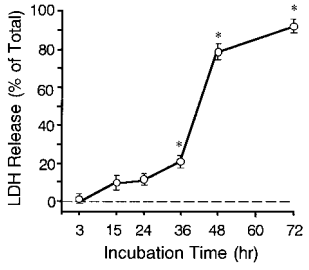

FIG. 3. Time course of LDH release in the culture medium following incubation with compactin. The cultures were exposed to 2 μM compactin at 6 h after plating and incubated for 3, 15, 24, 36, 48, and 72 h at 37°C, after which the medium was harvested for LDH assay. The data are means ± SE for four cultures. *p < 0.005 vs. cultures at time 3 h (ANOVA).

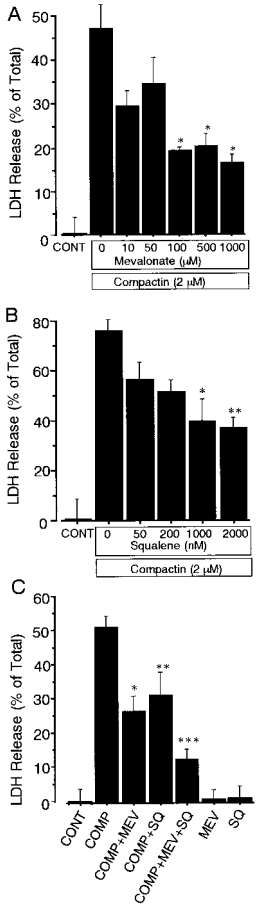

FIG. 4. Intermediate metabolites of the cholesterol biosynthetic pathway protect neurons from compactin-induced toxicity. Six hours after plating, neuronal cultures were exposed to the reagents indicated for 48 h in serum-free N2 medium. Cell viability was assayed using an LDH release kit. Results are expressed as percent LDH released relative to total LDH in the culture. The data are means ± SE for four cultures. **A:** Concurrent treatment with mevalonate attenuates toxicity induced by 2 μM compactin in a dose-dependent manner. *p < 0.05 vs. 2 μM compactin-treated cells (ANOVA). **B:** Concurrent treatment with squalene attenuates neurotoxicity induced by 2 μM compactin in a dose-dependent manner. *p < 0.05, **p < 0.01 vs. 2 μM compactin-treated cells (ANOVA). **C:** Additive effect of mevalonate (MEV) and squalene (SQ) in preventing neuronal death induced by compactin (COMP). *p < 0.05, **p < 0.01 vs. 2 μM compactin-treated cells (ANOVA). Concentrations of the reagents used are 2 μM compactin, 1 mM mevalonate, and 2 μM squalene. ***p < 0.05 vs. COMP + MEV- and COMP + SQ-treated cells (ANOVA).

Cholesterol and β-VLDL prevent compactin-induced cell death

Both mevalonate and squalene have been shown to rescue neurons from cell death induced by compactin, indicating that endogenously synthesized cholesterol is critical for neuronal cell survival. We further examined whether exogenous cholesterol is equivalent to endogenous in preventing compactin-induced cell death. Concurrent treatment with cholesterol exerted a dose-dependent inhibitory effect on compactin-induced neuronal death (Fig. 5A). Supply of cholesterol via rabbit β-VLDL also rescued neurons from cell death induced by compactin in a dose-dependent manner (Fig. 5B).

Squalestatin suppresses cholesterol biosynthesis and induces neuronal cell death

Although compactin, when added to cultures, inhibits biosynthesis of both cholesterol and a number of non-

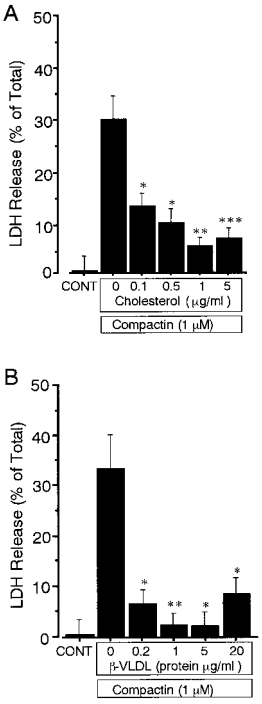

FIG. 5. Cholesterol and β-VLDL inhibit compactin-induced neuronal death. Six hours after plating, neuronal cultures were exposed to the reagents indicated for 48 h in serum-free N2 medium. Cell viability was assessed using an LDH detection kit. Results are expressed as percent LDH released relative to total LDH in the culture. The data are means ± SE for six cultures. **A:** Concurrent treatment with cholesterol attenuates neurotoxicity induced by 1 μ*M* compactin at concentrations higher than 0.1 μg/ml. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. 1 μ*M* compactin-treated cells (ANOVA). **B:** Concurrent treatment with β-VLDL, at concentrations higher than 0.2 μg of protein/ml, attenuates neurotoxicity induced by 1 μ*M* compactin. **p* < 0.05, ***p* < 0.01 vs. 1 μ*M* compactin-treated cells (ANOVA).

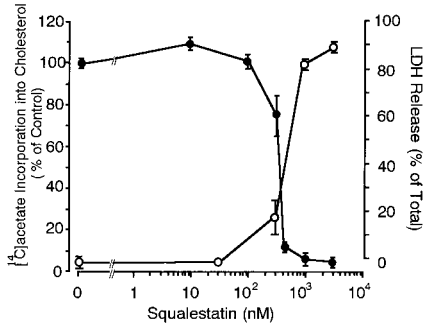

FIG. 7. Effect of squalestatin at various concentrations on the incorporation of [^{14}C]acetate into [^{14}C]cholesterol (●) and LDH release (○) in neuronal cultures. On day 2, each of the neuronal cultures was exposed to the indicated concentration of squalestatin. After incubation with compactin for 2 h at 37°C, the cultures were pulse-labeled with 2 μCi/ml [^{14}C]acetate for 5 h at 37°C, after which cells were harvested for determination of their [^{14}C]cholesterol content. For cell viability assay, the cultures were exposed to various concentrations of compactin 6 h after plating and incubated for 48 h at 37°C, after which the medium was harvested for LDH assay.

sterol isoprenoid products (Fig. 6), the present result of neuronal death induced by compactin being inhibited by squalene indicates that compactin-induced neuronal cell death is due to reduced cholesterol availability. To further confirm that cholesterol availability is critical for neuronal cell viability, we employed a more selective inhibitor of cholesterol biosynthesis, squalestatin, without interfering with other critical reactions involving farnesyl pyrophosphate. Squalestatin suppressed cholesterol biosynthesis in cultured neurons in a dose-dependent manner (Fig. 7). The level of cholesterol synthesized in neurons treated with squalestatin at concentrations higher than 500 n*M* was <13% of the control level. Viability assays for neuronal cells were performed 48 h after the start of incubation with squalestatin at various concentrations. Treatment with squalestatin at concentrations above 1 μ*M* induced marked neuronal death, as demonstrated by LDH assay (Fig. 7). The concentrations of squalestatin that caused neuronal death corresponded to those at which cholesterol biosynthesis was suppressed to 8% of the control level. Treatment with squalene also rescued neurons from squalestatin-induced cell death (Fig. 8B); however, treatment with mevalonate did not (Fig. 8A).

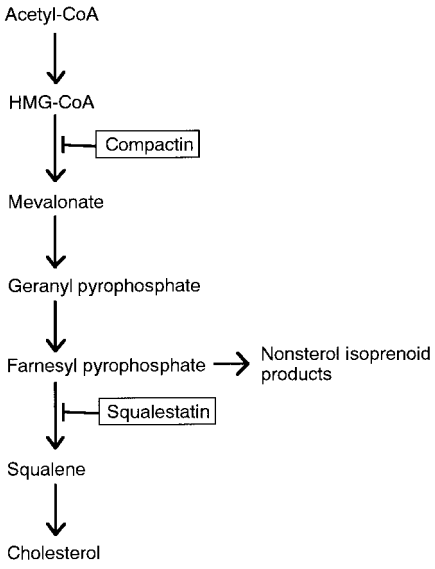

FIG. 6. Intermediate metabolites of the cholesterol biosynthetic pathway. HMG-CoA reductase is formed from three molecules of acetyl-CoA. The rate-limiting enzyme in the pathway, HMG-CoA reductase, is the target of the pharmacological agent compactin.

Compactin inhibits growth of NIH 3T3 and PC12 cells due to deficiency of nonsterol isoprenoids but not of cholesterol

We further examined the effect of compactin on NIH 3T3 and PC12 cells, both of which are proliferating cell types. As compactin is supposed to cause cell death or growth arrest in these cells, we employed two assay systems: the LDH release assay to determine the extent of cell death and calcein AM staining to determine the cell number. Compactin at a concentration of 5 μ*M* did not induce cell death as determined by the LDH release assay (data not shown). However, it inhibited cell proliferation, as demonstrated by calcein AM staining, in both NIH 3T3 and PC12 cells, which could not be reversed by exogenously added cholesterol or lipoprotein (Fig. 9). In contrast, mevalonate could reverse the effect of compactin, suggesting that a nonsterol intermetabolite of the mevalonate pathway is important for this recovery of the cells from growth arrest.

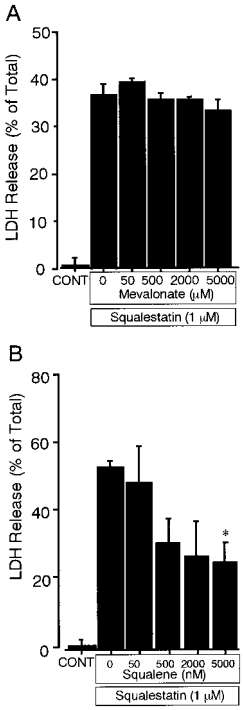

FIG. 8. Effect of intermediate metabolites of the cholesterol biosynthetic pathway on squalestatin-induced neurotoxicity. Neuronal cultures were exposed to the reagents indicated for 48 h. Cell viability was assessed using an LDH detection kit. Results are expressed as percent LDH released relative to total LDH in the culture. The data are means ± SE for seven cultures. **A:** Concurrent treatment with mevalonate does not attenuate neurotoxicity induced by 1 μM squalestatin. **B:** Concurrent treatment with squalene attenuates neurotoxicity induced by 1 μM compactin in a dose-dependent manner. *$p < 0.01$ vs. 1 μM squalestatin-treated cells (ANOVA).

DISCUSSION

Cholesterol is the predominant sterol in the plasma membrane and is therefore required for cell growth and maintenance of cell functions (Brown et al., 1978; Kaneko et al., 1978; Faust et al., 1979; Quesney-Huneeus et al., 1979; Habenicht et al., 1980). Changes in the cholesterol content of the membrane can modulate lipid fluidity, thereby affecting various cellular functions including antigen expression (Basu et al., 1980), enzyme activities (Papahadjopoulos et al., 1973; Farias et al., 1975; Sinha et al., 1977), endocytosis (Heiniger et al., 1976), and receptor functions (Heron et al., 1980; Papaphilis and Deliconstantinos, 1980). In contrast, it has generally been believed that inhibition of HMG-CoA reductase suppresses DNA synthesis (and/or cell growth) and induces apoptosis via deficiency of nonsterol isoprenoid products, but not of cholesterol, in nonneuronal cells (Quesney-Huneeus et al., 1979; Habenicht et al., 1980; Perkins et al., 1982; Fairbanks et al., 1984; Siperstein, 1984; Satoh et al., 1996; Padayatty et al., 1997). The importance of isoprenoid products as opposed to cholesterol in compactin-induced growth arrest was also confirmed using NIH 3T3 and PC12 cells in the present study (Fig. 9).

However, in the present study, we found, for the first time, that it is the inhibition of cholesterol biosynthesis, and not a deficiency of nonsterol isoprenoid products, that is crucial to the induction of neuronal death in culture, when the neuronal cultures are treated with compactin. One of the most likely explanations for these apparently conflicting results concerns the phenotypic differences between the cells studied, whether they are mitotic or postmitotic. Nonsterol isoprenoid products are not crucial for the survival of neurons, which are postmitotic, whereas they are for nonneuronal proliferating cells, which synthesize DNA. Furthermore, prolonged treatment, for at least 6 days, with an HMG-CoA reductase inhibitor at concentrations that inhibit protein prenylation is necessary for the induction of cytotoxic effects on proliferating cells (Sinensky et al., 1990) and astrocytes (data not shown), whereas cytotoxicity is induced by the inhibitor within 36 h in neuronal cells, suggesting that the mechanism in neurons is different from that in proliferating cells.

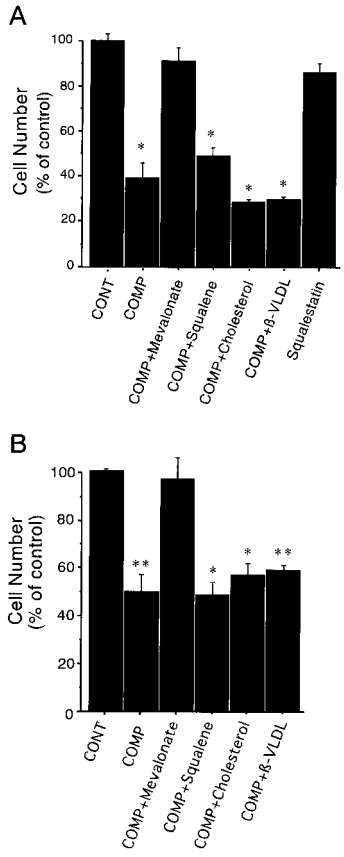

FIG. 9. The effect of compactin and squalestatin on nonneuronal cells in culture. Proliferating PC12 and NIH 3T3 cells were plated onto 12-well plates and maintained in DMEM containing 5% FBS when the reagents examined were added. For determination of the cell number, the cultures were exposed to 2 μM calcein AM and incubated for 30 min at 37°C. The number of calcein AM-positive cells in each well was determined with Fluoroskan Ascent (Labosystems, Helsinki, Finland). **A:** Compactin (COMP; 5 μM) inhibited proliferation of PC12 cells. Concurrent treatment with mevalonate (4 mM) could reverse the effect of compactin; however, squalene (10 μM), cholesterol (5 μM), or β-VLDL (5 μg of protein/ml) could not. In addition, squalestatin (1 μM) did not inhibit cell growth. **B:** Compactin (5 μM) inhibited proliferation of NIH 3T3 cells. Concurrent treatment with mevalonate (4 mM) could reverse the effect of compactin; however, squalene (10 μM), cholesterol (5 μM), or β-VLDL (5 μg of protein/ml) could not. n = 4 each. *$p < 0.0001$, **$p < 0.002$ vs. control cultures (ANOVA).

We also propose an alternative hypothesis as follows: Neurite outgrowth and formation of a neuronal network via synapse formation, both of which strongly depend on cholesterol availability (Vance et al., 1994), are required for neuronal viability. In contrast to other types of cells, neurons have an enormously extended shape, with a long axon and dendrites connecting it through synapses to other cells. The cholesterol availability in axons of neurons treated with compactin, when cultured in lipid-free medium, is very restrictive for their outgrowth. The limited supply of cholesterol into axons results in the inhibition of axonal outgrowth (Vance et al., 1994), followed by poor formation of a neuronal network with synapses. As compactin-treated cells, before cell death, possess fewer neurites with fewer branches than nontreated neurons (Fig. 1C and D), formation of a neuronal network and subsequent neuronal interactions, including anterograde and retrograde electrical or chemical signaling, are probably impaired, resulting in cell death (Pettmann and Henderson, 1998).

The effects of mevalonate and squalene were partial, and concurrent incubation with mevalonate and squalene resulted in additive inhibition of compactin-induced cell death. However, the present results of exogenously added cholesterol or cholesterol via β-VLDL rescuing neurons completely from compactin-induced cell death indicate that a deficiency of cholesterol, but not of nonsterol isoprenoid products, is crucial for the neurotoxicity induced by HMG-CoA reductase inhibition. The experiments using squalestatin support this hypothesis. The effect of squalene in inhibiting squalestatin-induced cell death was also partial. Thus, it is likely that insufficient supply of mevalonate or squalene to the cholesterol biosynthetic pathway is responsible for the partial effects of these compounds in inhibiting compactin-induced cell death.

Neuronal culture systems have been only infrequently used in studies on the metabolism and functions of cholesterol because of the difficulties in the preparation of neuronal cultures for biological assays. However, the different responses of neurons and nonneuronal cells, as revealed by this study, to an HMG-CoA reductase inhibitor and intermediate metabolites in the cholesterol biosynthetic pathway may highlight the importance of using neuronal culture systems for studying the novel aspects of cholesterol metabolism.

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