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Membrane cholesterol interferes with neuronal apoptosis induced by soluble oligomers but not fibrils of the amyloid-β peptide

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ABSTRACT

Neuronal cell death in Alzheimer's disease (AD) is partly induced by the interaction of the amyloid-β peptide (Aβ) with the plasma membrane of target cells. Accordingly, recent studies have suggested that cholesterol, an important component of membranes that controls their physical properties and functions, plays a critical role in neurodegenerative diseases. We report here that the enrichment of the neuronal plasma membrane with cholesterol protects cortical neurons from apoptosis induced by soluble oligomers of the Aβ(1-40) peptide. Conversely, cholesterol depletion using cyclodextrin renders cells more vulnerable to the cytotoxic effects of the Aβ-soluble oligomers. Increasing the cholesterol content of small unilamellar liposomes also decreases Aβ-dependent liposome fusion. We clearly demonstrate that cholesterol protection is specific to the soluble conformation of Aβ, because we observed no protective effects on cortical neurons treated by amyloid fibrils of the Aβ(1-40) peptide. This may provide a new opportunity for the development of an effective AD therapy as well as elucidate the impact of the cholesterol level during AD development.

Key words: Alzheimer's disease • membrane fluidity • oxidative stress • cytoskeleton

A common feature of Alzheimer's disease (AD), the most common form of dementia, is the accumulation and the aggregation of the β-amyloid peptide (Aβ), a 39- to 43-amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein (APP) (1, 2). Although Aβ represents a key factor in AD (3), whether the fibrillar or the nonfibrillar peptides are more deleterious in early stages of AD remains a controversial issue (4). The amyloid cascade hypothesis causally links AD clinico-pathological process and neuronal cell death to the aggregation and deposition of Aβ (5–7). However, this hypothesis has been challenged by recent studies indicating that the nonfibrillar Aβ may be crucial in AD pathology

(8–13). Several studies with transgenic mice have demonstrated that neurodegeneration and specific spatial learning deficits might occur without amyloid plaque formation (14–17). According to these observations, our studies and others support the hypothesis of a close relationship between neuronal loss and a proapoptotic effect of soluble forms of Aβ (18–21). Indeed, it has been established that the amphiphilic nonaggregated Aβ may intercalate into the plasma membrane of neurons, directly altering membrane activities and inducing neuronal cell death (22–24). It is thus essential to identify and characterize the biological factors that are able to modulate both the interaction of Aβ with the plasma membrane of neurons and its destabilizing properties that depend on the conformation of Aβ peptide.

In the past few years, considerable attention has been focused on the possible association between cholesterol metabolism in the central nervous system (CNS) and the development of AD (25, 26). A relationship between cholesterol and AD has been clearly established (27, 28). Indeed, cholesterol has been shown to modulate the processing of APP, α- and β-secretase activities, and Aβ peptide production (29–31). Several lines of evidence indicate that lowering cholesterol levels prevents AD development by reducing Aβ production and secretion (32–34). These findings seem to contradict previous studies demonstrating that cholesterol protects PC12 cells from fibrillar Aβ peptide (35, 36) and that cholesterol depletion induces AD-type injuries in cultured hippocampal slices (37). Thus, we are still far from being able to understand how an alteration in the level and/or the distribution of intraneuronal cholesterol pathologically affects neurons, leading to the acceleration of AD-specific pathological processes, such as Aβ-induced cell death. In particular, it has not yet been described whether the cholesterol content of the plasma membrane of primary cortical neurons might affect the neurotoxicity of Aβ according to its different conformations.

In the present paper, we demonstrate that the cholesterol content of the plasma membrane of cortical neurons differently modulates cell death induced by soluble oligomers or fibrils of Aβ. Increasing the cholesterol level in the plasma membrane has no effect on the apoptosis induced by Aβ fibrils, whereas it specifically inhibits the molecular events leading to neuronal cell death triggered by this interaction of soluble oligomers of Aβ with the plasma membrane. It is likely that the inhibitory effect of cholesterol might be due to the modulation of the physical properties of the plasma membrane, because a depletion of cholesterol makes cells more susceptible to Aβ peptide-induced apoptosis.

MATERIALS AND METHODS

Materials

Aβ(1-40), Aβ(29-40), and caspase substrate peptides were purchased from Bachem (Voisins-le-Bretonneux, France) and DCFH-DA from Molecular Probes (Eugene, OR). Unless otherwise indicated, materials used for cell culture were obtained from Life Technologies (Grand Island, NY). Methyl-β-cyclodextrin (MβCD), water-soluble cholesterol, and all other chemicals were of high purity grade from Sigma (St. Louis, MO).

Peptide solubilization and aggregation

To overcome problems of amyloid peptide solubility at high concentrations, fresh peptide stock solutions were prepared at 5 mg/ml^{-1} in hexafluoro-2-propanol (Sigma), as described previously (18). For the incubation of the peptides with the neurons, aliquots of peptide stock solution were quickly dried under nitrogen and directly solubilized at the experimental concentrations into the culture medium. Peptide solutions were then applied to the cells. Under those conditions, all the amyloid peptides remained soluble for the determination of their neurotoxic properties (18). Fibrillar Aβ peptide was obtained by incubating 100 μM Aβ(1-40) in the culture medium for 96 h at room temperature, as described previously (18, 38).

Monitoring of liposome fusion

The fusion of small unilamellar liposomes (SULs) induced by the nonfibrillar Aβ(1-40) was monitored using a fluorescent probe dilution assay, as described previously (38, 39).

Neuronal cell culture

Cortical neurons from embryonic day 16–17 Wistar rat fetuses were prepared, as described previously (18). In brief, dissociated cells were plated at 4.5–5.0 $10^4 cells/cm^2$ in plastic dishes precoated with poly-L-ornithine (1.5 $\mu g/ml^{-1}$; Sigma). The cells were cultured in a chemically defined DMEM-F12 medium free of serum (Life Technologies, Grand Island, NY) and supplemented with insulin (5.10^{-7} M), putrescine (60 μM), sodium selenite (30 nM), transferrin (100 μM), progesterone (1.10^{-7} M), and 0.1 % (w/v) ovalbumin. Cultures were kept at 35°C in a humidified 6% CO_2 atmosphere. After 6–7 days in vitro (DIV), the cortical population was determined to be at least 95% neuronal by immunostaining as described previously (18).

Neuronal viability

Experiments were performed on 6–7 DIV neurons. Cell viability was first determined by morphological observation and cell counting after 5 min trypan blue staining (0.4%; Sigma) to evaluate membrane integrity and the metabolic activity assessed by the MTT reduction assay. Moreover, the release of LDH into the culture medium was assessed using a cytotoxicity detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations.

Monitoring of apoptosis

Cell nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI, Sigma). The cells, grown on glass coverslips, were washed in PBS, incubated at room temperature for 10 min with DAPI (0.1 $\mu g/ml^{-1}$), washed with PBS, and examined under a microscope equipped for epifluorescence. To evaluate the percentage of apoptotic cells, five independent fields of microscope were counted (~100 cells) in three separate experiments, with two determinations each. Under control conditions, neuronal cells exhibited 10–12% of apoptotic cells at 9 DIV. Alternatively, DNA fragmentation was monitored by ELISA for the detection of oligonucleosomes using a kit purchased from Boehringer Mannheim. In brief, cortical neurons were plated in 24-well dishes (~200,000 cells per well) and treated at 7 DIV for 24 h with Aβ.

After they were washed, cells were lyzed directly in wells, and oligonucleosomes were determined according to the manufacturer's recommendations.

Measurement of caspase-like proteolytic activities

The caspase activities were measured by means of the cleavage of the substrates, DEVD-pNa, and IEPD-AMC (Bachem). In brief, at the indicated time points following peptide treatments, the cells were rinsed three times with ice-cold PBS and incubated for 20 min on ice in a buffer of 25 mM HEPES, pH 7.5, 1% (v/v) Triton X-100, 5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM PMSF, 10 μg/ml each of pepstatin and leupeptine, and 5 μg/ml aprotinin. The lysate was centrifuged for 15 min at 12,000 rpm and assayed for protein by Bradford (Bio-Rad, Hercules, CA). Protein (50 μg) was incubated for 2 h with 100 μM substrates initially dissolved in DMSO. The cleavage of the caspase substrate was monitored by absorbance measurements at 405 nm for DEVD-pNa or by fluorescence emission at 460 nm after exciting IEPD-AMC at 360 nm, using a Fluostar reader plate (BMG-Labtechnologies, Champigny-sur-Marne, France).

DCFH-DA assay

The measurement of cell oxidation is based on the oxidation of the nonfluorescent compounds, 2',7'-dichlorofluorescein diacetate (DCFH-DA), to a fluorescent derivative, DCF, in a peroxidase-mediated reaction. Increases in fluorescence emission reflect enhanced cellular oxidative stress. In brief, treated cortical neurons were loaded with 100 μM DCFH-DA for 45 min. Before analysis, cells were washed three times in PBS and DCF fluorescence was directly recorded on culture dishes by a Fluostar reader plate (BMG-Labtechnologies), using 488 nm excitation and 510 nm emission filters.

Immunofluorescence

For immunofluorescence studies, the neurons were cultured on glass coverslips that had been coated overnight with 15 μg/ml poly-L-ornithine. Following the treatments, the neurons were fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 made up in PBS containing 3% BSA for 30 min, and then incubated with a monoclonal anti-β-tubulin antibody (1:500) (Euromedex, Souffelweyersheim, France) for 1 h under constant agitation. After several washes in PBS, the cells were incubated for 1 h with a FITC-conjugated donkey anti-mouse IgG (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA), washed with PBS, labeled with DAPI as described above, and mounted in Fluoprep (BioMérieux, Marcy l'Etoile, France). The microtubules were visualized with a Nikon microscope using a PlanFluor X40/1.3 objective. For semiquantitative analysis of microtubule organization, at least five microscope fields/condition were imaged using a Nikon DXM1200 digital camera, and microtubule organization in 100–120 cells/field was classified as either normal or mildly or severely disrupted.

Cholesterol quantification

Cortical neurons (5.0×10^4cells/cm^2) were incubated at 37°C with or without increasing amount of soluble cholesterol for 3 h, or with MβCD for 30 min. After washing with PBS, neurons were

harvested in double distilled water and lyophilized. Lipids were extracted, and cholesterol was determined using cholesterol oxidase (40). In addition, filipin staining of cortical neurons was used as a visual indicator of cholesterol levels. After treatments, cells were fixed with 4% (w/v) paraformaldehyde and stained with 125 μg/ml filipin (Sigma) in PBS. Cells were post-fixed with 4% (w/v) paraformaldehyde for an additional 20 min before fluorescence visualization using FITC filters.

Statistical analysis

STAT VIEW computer software was used for the statistical analysis. Most of the data were from three separate experiments with three to four determinations each. Values were expressed as means ± SE. Differences between control and treated groups were analyzed using Student's *t* test. Multiple pair-wise comparisons among the groups of data were performed using ANOVA followed by a Scheffe's post hoc test. Statistical differences were determined at $P < 0.05$.

RESULTS

Cholesterol enrichment protects cortical neurons from soluble Aβ peptide-induced toxicity

We first investigated the effect of the enrichment of the plasma membrane in cholesterol on the neurotoxicity induced by soluble oligomers of the Aβ(1-40) peptide. Under our experimental conditions, the treatment of cortical neurons with 10 μg/ml cholesterol led to an increase of 30% of its content in membranes (Table 1). The cholesterol level within the plasma membrane of cortical neurons was monitored using filipin staining. Filipin is a fluorescent polyene antibiotic that forms complexes with free cholesterol that can be visualized by fluorescence microscopy (30). After treating the cells with exogenous cholesterol for 3 h (Fig. 1), the intensity of filipin staining increased relative to control cells, although the overall distribution remained unchanged. Moreover, as measured using the MTT or LDH assays, cholesterol enrichment did not affect cell viability, which remained >90% of control after 48 h of incubation with a concentration of cholesterol up to 25 μg/ml (data not shown).

As already reported (18, 41), the exposure of rat cortical neurons to 5 μM nonfibrillar Aβ(1-40) resulted in a time-dependent decrease of cell viability monitored by the MTT assay (Fig. 2*A*) or by the release of LDH into the culture medium (Fig. 2*B*). We observed a dose-dependent inhibitory effect of exogenous cholesterol when cells were preincubated with increasing amounts of cholesterol, as monitored using the MTT assay (Fig. 2*A*). The neuroprotective effects of cholesterol enrichment were statistically significant at all incubation times for a cholesterol supply of 10 μg/ml and after a 48 h treatment with Aβ for a cholesterol supply of 5.0 μg/ml (Fig. 2*A*). These effects were confirmed by the measurement of the release of LDH upon Aβ-peptide exposure (Fig. 2*B*). The neuroprotective effects of cholesterol were increased when cells were incubated with lower concentrations of Aβ peptide (data not shown). Furthermore, the addition of exogenous cholesterol to cortical neurons at the same time as Aβ(1-40) did not prevent neuronal cell death (data not shown), suggesting that the neuroprotective effect of cholesterol may not involve a direct interaction between cholesterol and Aβ. Interestingly, neither serum withdrawal- nor staurosporine-induced cell death was affected by cholesterol enrichment.

Indeed, preincubation of cells with 10 μg/ml cholesterol had no effect on the reduction of cell viability induced by 50 nM staurosporine, as measured with the MTT assay (Fig. 2*D*).

As described previously (41), the perturbation of the cytoskeleton network is an early hallmark of soluble Aβ peptide-induced neurotoxicity (Fig. 3). The preincubation of the cells with 10 μg/ml cholesterol resulted in the inhibition of the cytoskeleton perturbation induced by Aβ (Fig. 3*A*). This was confirmed by the quantitation of the number of cells exhibiting a perturbed cytoskeleton using immunofluorescence with a monoclonal anti-β-tubulin antibody (Fig. 3*B*). After a 6 h incubation, the microtubule network was mildly or severely disrupted in 30.3 ± 4.5% ($P<0.01$) of the cells treated with 5 μM soluble Aβ(1-40), and 68.9 ± 3.8% ($P<0.001$) neurons displayed disturbed microtubules after a 48 h incubation (Fig. 3*B*). Interestingly, the presence of cholesterol significantly abolished the microtubule perturbation induced by the nonfibrillar Aβ peptide even after 48 h of incubation (Fig. 3*B*).

Cholesterol enrichment inhibits apoptotic events induced by the soluble Aβ peptide

We next investigated whether the enrichment of the plasma membrane in cholesterol might modulate the apoptotic cell death induced by the soluble Aβ peptide. Preincubation of cortical neurons with exogenous cholesterol significantly reduced nuclear DNA condensation and fragmentation induced by the exposure of cells to 5 μM soluble Aβ(1-40) for 24 h (Fig. 4). Whereas the number of condensed and/or fragmented nuclei rose up to in 38.5 ± 2.6% ($P<0.01$) in the presence of Aβ, as monitored using DAPI staining, cholesterol enrichment significantly reduced the number of apoptotic nuclei, bringing it close to the control value (Fig. 4*A*). Similarly, the Aβ-induced increase in the amount of oligonucleosomes was reversed by preincubation of cortical neurons with cholesterol (Fig. 4*B*). Finally, cholesterol enrichment inhibited the activation of caspase-3 and the production of reactive oxygen species induced by the soluble Aβ(1-40) peptide (Fig. 4*C* and 4*D*, respectively).

Cholesterol depletion increases the neurotoxicity of the soluble Aβ peptide

To further evaluate whether the cholesterol level directly modulates neuronal survival, we investigated the effects of cholesterol depletion on Aβ peptide-induced neurotoxicity. As shown in Table 1, the treatment of cortical neurons for 30 min at 37°C with 1 mM cyclodextrin (MβCD) resulted in a 41% decrease in the cholesterol level, similar to the reduction of cholesterol levels described in a recent study (42). Interestingly, cholesterol depletion rendered cells more susceptible to Aβ peptide-induced neurotoxicity as monitored using the MTT assay and the release of LDH (Fig. 5). In addition, we observed that the enhanced neurotoxicity of the soluble Aβ peptide generated by lowering the cholesterol content of the plasma membrane is partially reversed by 100 μM ZVAD-fmk, an inhibitor of several classes of caspases (Fig. 5).

Taken together, our results support the idea that cholesterol, a key component of the plasma membrane of cells, regulating its physical properties and controlling the activity of numerous membrane proteins, specifically modulates the neurotoxicity of soluble Aβ. This raised the possibility that the cholesterol level and its subcellular localization may play a crucial role in the neurodegenerative processes observed in AD.

Cholesterol has no effect on fibrillar Aβ-induced neurotoxicity

We thus investigated whether exogenous cholesterol might modulate the neurotoxicity of Aβ(1-40) fibrils. The preincubation of cells with 10 μg/ml cholesterol had no effect on the fibrillar Aβ-induced decrease of the cell viability measured using the MTT assay or the release of LDH into the culture medium (Fig. 6*A*, 6*B*), and on the apoptosis induced by 5 μM fibrillar Aβ(1-40) (Fig. 6*C*). One of the hallmarks of the neuronal cell death induced by the fibrillar Aβ peptide is the activation of caspase 8 (this study, Fig. 6*D*, and ref 43). Interestingly, the increase in the cholesterol content in the plasma membrane of neurons did not modulate the activation of caspase 8 induced by 5 μM fibrillar Aβ(1-40) as measured by the cleavage of its fluorescent substrate, IEPD-AMC (Fig. 6*D*).

The cholesterol level modulates the fusogenic properties of the soluble Aβ peptide

We have recently demonstrated that the lipid phase of the plasma membrane of neurons might represent an early target for the soluble Aβ peptide (41). We thus tested the effects of the cholesterol level on the rate of association of the soluble Aβ(1-40) peptide with the membrane of living cells. As described previously (18), cells were incubated at 35°C in the presence of 5 μM [^{14}C]Aβ(29-40) and the radioactivity associated with cells was counted according to incubation time. Cholesterol enrichment of cortical neurons did not affect the kinetics of association of the radiolabeled peptide (Fig. 7), suggesting that the inhibitory effects of cholesterol on Aβ peptide-induced cell death was not due to an inhibition of the interaction between the peptide and the membranes.

Another hypothesis would be that cholesterol might interfere with the perturbation of the membrane plasticity induced by soluble oligomers of Aβ (38, 41). The fusogenic properties of Aβ are estimated using an in vitro assay measuring the extent of lipid mixing of unilamellar vesicles, an indicator of the vesicular fusion (38). Interestingly, Figure 8 shows that the level of lipid mixing was inhibited by the addition of cholesterol in a dose-dependent manner. Similar results were obtained using the fusogenic Aβ(29-40) peptide (data not shown). These data strongly suggest that cholesterol inhibits the perturbation of the membrane organization induced by soluble oligomers of Aβ most likely by decreasing membrane fluidity and by modifying the shape of the liposomes.

DISCUSSION

The amyloid cascade hypothesis has been challenged by recent studies indicating that the nonfibrillar Aβ also plays a critical role in AD (9, 18, 20, 41, 44; see also 45 for review). Therefore, the soluble Aβ hypothesis could explain many observations that are not well explained by the amyloid cascade hypothesis, including the poor correlation between senile plaques and clinical symptoms of AD (17, 46). However, the molecular mechanisms associated with the cell death induced by soluble oligomers of Aβ remain largely unknown. Thus, it appears crucial to better characterize the molecular mechanisms associated to soluble Aβ peptide-induced cell death together with the physiological factors able to modulate the neurotoxicity of Aβ. In the majority of sporadic AD cases, a loss of HMG-CoA reductase activity has been reported in the frontal and temporal cortex, associated with a reduction of the level of cholesterol

in areas of vulnerability (47). Moreover, it has been suggested that the modulation of the cholesterol synthesis induces lysosomal dysfunction leading to pathologies characteristic of AD (37). However, whether the cholesterol level of cellular membranes might influence the neurotoxicity of soluble oligomers of Aβ has not been fully studied yet.

In this study, we have provided evidence supporting the hypothesis that cholesterol might selectively affect the sensitivity of primary neurons exposed to different conformations of the Aβ peptide. We have demonstrated that the neurotoxicity induced by the nonfibrillar Aβ(1-40) peptide could be modulated by an increase in the cholesterol content of the plasma membrane, whereas a depletion of cholesterol within the membrane makes the neurons more susceptible to Aβ insults. Prevention of the apoptotic events induced by the nonfibrillar Aβ(1-40) by cholesterol enrichment may be mediated by modifications of the physical properties of the plasma membrane, and not through a direct interaction between the Aβ(1-40) peptide and cholesterol. Our interpretation of these data is that the mechanism of the cholesterol effect is due to well-characterized, cholesterol-dependent changes in the properties of membranes. Interestingly, our data from experiments using model membranes show that the fusion of liposomes induced by soluble Aβ(1-40) peptide is also suppressed by increasing the mole fraction of cholesterol in the liposomes. These data indicate that under unphysiological conditions and without the interaction with any other mechanisms, the level of cholesterol in a membrane can dramatically modify the interactions between soluble Aβ and lipids. It is likely that the ability of the soluble Aβ peptide to fuse liposomes depends on the ability of the peptide to penetrate and disturb the membranes, as demonstrated in our previous studies (38, 39).

We have recently demonstrated that the C-terminal domain of Aβ (e.g., amino acids 29-40) displayed fusogenic properties (38) and induced neuronal cell death (18). We have found that the enrichment of plasma membranes with exogenous cholesterol also inhibited the apoptotic cell death induced by the Aβ(29-40) peptide (unpublished results). This strongly supports the hypothesis that the direct alteration of the structure and the functions of the neuronal plasma membrane represents one of the earliest molecular events involved in the Aβ-mediated cell death. By contrast, we show that the increase in the cholesterol level of the plasma membrane does not protect the cortical neurons from the neurotoxic effects of Aβ(1-40) amyloid fibrils. This was particularly illustrated by the lack of effect of cholesterol on the activation of caspase 8 induced by a fibrillar form of Aβ(1-40). These latter data on primary cortical neurons are in contradiction with previous studies demonstrating that the increase of the cholesterol level in the plasma membrane of cells might modulate the cytotoxicity of fibrillar Aβ (35, 36, 48). However, in all of these studies, the experiments have been performed using cell lines. It is likely that cholesterol homeostasis and distribution within membranes are different in cell lines and in primary neurons and that cholesterol differently modulates the sensitivity of these models to toxicity.

We have recently demonstrated that one of the earliest cellular targets of the soluble Aβ peptide is the cytoskeleton network (18, 41). Interestingly, it has been recently demonstrated that the structural integrity of the cytoskeleton in cultured neurons is also dependent on the level of cholesterol in the plasma membrane of cells (49). Moreover, alterations in the metabolism of cholesterol induce *tau* phosphorylation and a subsequent disruption of the cytoskeleton network, including microtubule depolymerization (50, 51). Altogether, these observations raise the

hypothesis that the cholesterol enrichment of the plasma membrane might protect cortical neurons from Aβ peptide-induced perturbations of the membrane structure but also from cellular insults triggered by the insertion of Aβ oligomers in the neuronal membrane. According to our data, it is more likely that the cholesterol bulk, by modifying the structure and the fluidity of the neuronal membrane, plays a critical role in AD by acting on Aβ dynamics rather than by complexing soluble Aβ oligomers and therefore by modifying the rate of association of the Aβ peptide with the plasma membrane.

Cumulative evidence emphasizes a strong relationship between cholesterol and AD (27, 30). Several reports have suggested that high serum cholesterol levels are involved in the formation of AD pathology (28, 32) and that a 75% decreased prevalence of AD and dementia are associated with the use of statins (33). In these retrospective studies, the beneficial effect of simvastatin or lovastatin, both of which cross the blood-brain barrier, might not be entirely attributed to their cholesterol-lowering effect. Indeed, nonstatin lipid-lowering agents had no significant effect on the risk of dementia (33), but it is uncertain that nonstatin lipid-lowering drugs directly influence cholesterol metabolism in the CNS. Recently, statins have been shown to modulate leukocyte migration, MHC class II and costimulatory signal activities, expression of inflammatory mediators, and NO synthase activity (for review, see ref 52). Whether these pharmacological effects are directly related to statin's specific inhibitory effect on HMG-CoA reductase and cellular cholesterol synthesis or due to as yet undefined pharmacological mechanisms is unclear. Because of the possible pleiotropic effects of statins on neuron metabolism, we have chosen in our study to directly modulate cellular membrane cholesterol content without using these pharmacological agents. Studies of statin effects on neuronal death in conjunction or independently of variation of the cellular cholesterol content are currently underway.

At this stage, our direct experimental approach shows that cholesterol protects neurons from direct toxicity of soluble oligomeric Aβ, but not from that of fibrillar Aβ. Reducing cellular cholesterol by statins decreases β and γ secretase activities both in vitro and in vivo, thereby reducing the production of the Aβ peptide (30, 31, 34). These findings contradict our present results and other studies demonstrating that cholesterol is one of the factors preventing Aβ-induced membrane alterations and neurotoxicity (36, 53–55). Hence, the reduction of cellular cholesterol content might prevent the production of toxic Aβ while increasing cell sensitivity to the soluble forms of the peptide. These results strongly argue for caution in the use of cholesterol synthesis lowering drugs as a means of reducing AD dementia. It is indeed possible that some individuals suffering mostly from fibrillar Aβ toxic effects will benefit from such therapeutics while the progression of the disease would remain unchanged or even exacerbated in others in which soluble Aβ is the principle cause of cell death. The relative contribution of either soluble or fibrillar Aβ in the early and late stages of AD is currently under debate (4, 56, 57). Specific diagnostic probes allowing identification of the precise mechanism responsible for the progression of dementia in each individual are needed. At this stage, it is prudent to restrict the use of statins to those that do not cross the blood-brain barrier for people at risk for AD.

Therefore, the novelty and physiological relevance of our results are that cholesterol controls the sensitivity of primary cortical neurons to the soluble oligomeric Aβ. Importantly, the model membrane study and the cell study lead to the same general conclusion. Taking these

observations and our results together, one might propose that, by modulating the fluidity of the membranes, the cholesterol content of neuronal membranes may specifically influence the insertion of soluble Aβ in the plasma membrane and its properties to disturb the membrane structure and ultimately trigger cell death.

In conclusion, the data from all the experiments described in this paper clearly demonstrate that the neurotoxicity induced by the nonfibrillar Aβ peptide is specifically modulated by the amount of cholesterol in the surface membrane. By affecting the physical properties of the membrane, cholesterol modulates the membrane-destabilizing activity of soluble Aβ oligomers. However, our data showing no inhibitory effect of cholesterol toward the fibrillar Aβ(1-40) peptide-induced toxicity of primary cortical neurons highlight the idea that the modulation of cholesterol and/or other factors for the treatment of AD will depend on which Aβ conformations are mainly involved during the disease.

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Table 1

Cholesterol content after different treatments[a]

Treatment	Cholesterol (ng/well)	Cholesterol (% of control)
Control	459 ± 179	100
Cholesterol	598 ± 203	130.3 ± 7.9
MβCD	269 ± 102	58.6 ± 6.3

[a]Six-day-old cortical neurons were treated for 3 h with 10 μg/ml cholesterol or exposed to 1 mM MβCD for 30 min at 37°C, before determination of the cholesterol level as described in Materials and Methods. Results are means ± SD of three independent analyses.

Fig. 1

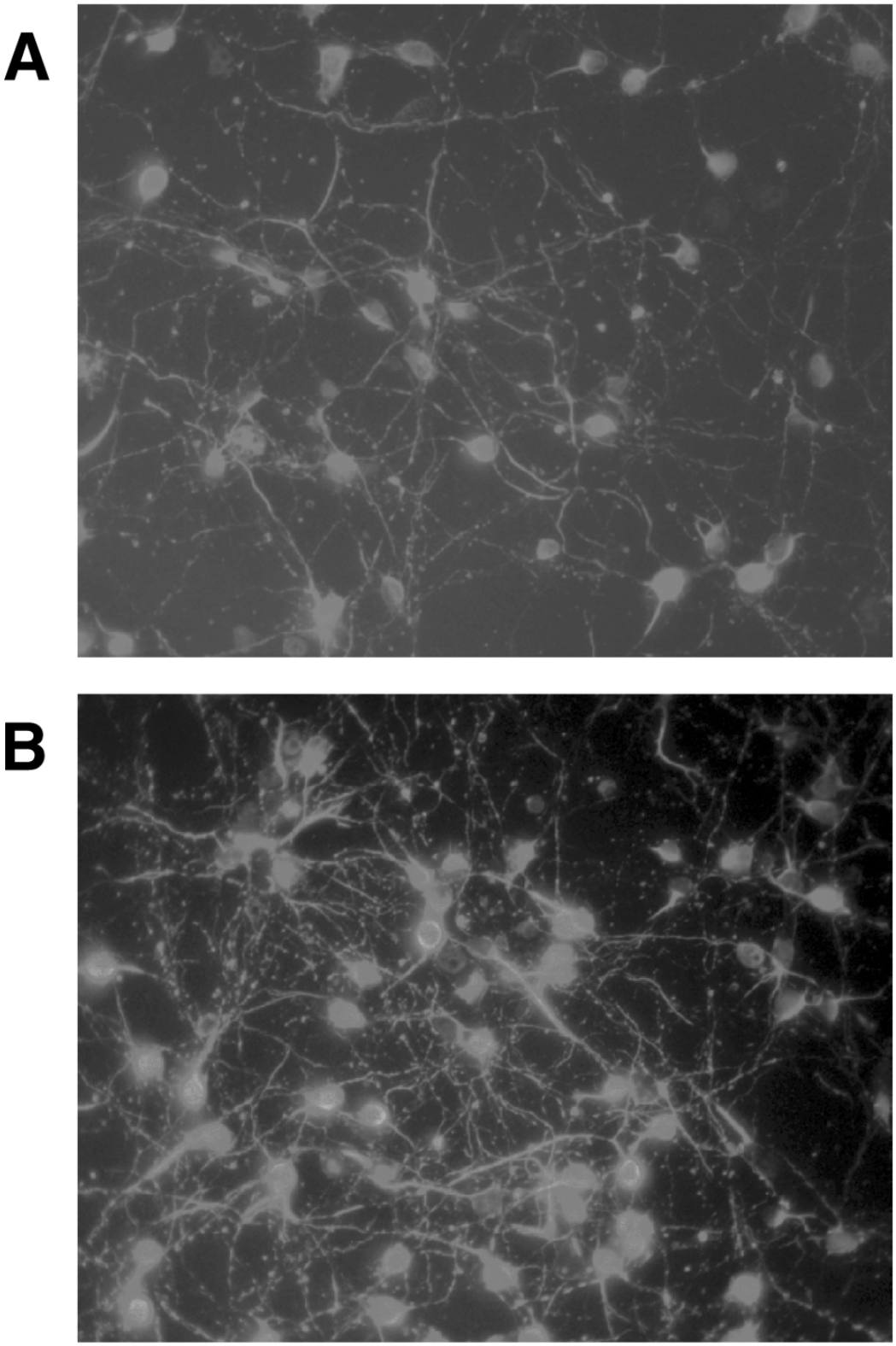

Figure 1. Cholesterol enrichment of the plasma membrane of cortical neurons. Cholesterol modulation of cortical neurons was monitored using filipin staining followed by fluorescence microscopy. ***A*)** Filipin binds to free cholesterol on both neurites and cell soma. ***B*)** The addition of exogenous cholesterol to cells results in a more intense filipin staining but does not affect the overall staining pattern.

Fig. 2

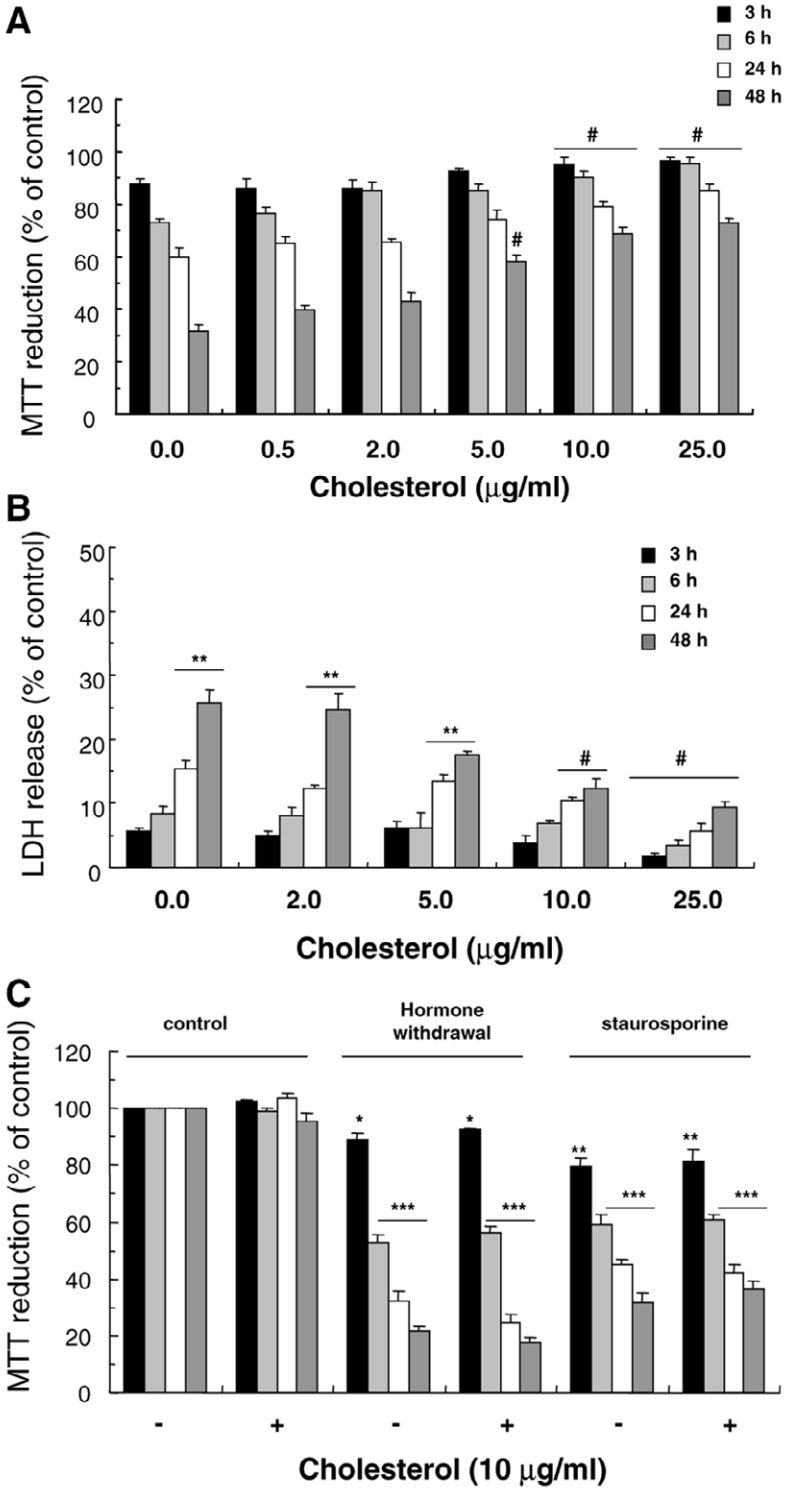

Figure 2. Cholesterol enrichment specifically modulates the neurotoxicity induced by soluble oligomers of Aβ peptide. Before Aβ peptide treatment, cortical neurons were incubated in the absence or in the presence of increasing concentrations of cholesterol for 3 h. After washing in order to remove the excess cholesterol, cells were treated for the indicated time with 5 μM soluble Aβ(1-40) peptide. The cell viability was monitored by the MTT assay (***A***) and the release of LDH (***B***). Alternatively, cell death was induced by hormone withdrawal or 50 nM staurosporine, and cell viability was followed by the MTT assay (***C***). Data are means ± SE of three different experiments with four determinations each and are normalized to the effect of vehicle, designated as 100% (**P*<0.05; ***P*<0.01; ****P*<0.001). Differences among the subgroups for each condition were performed by ANOVA followed by a Scheffe's post hoc test. $^{\#}P < 0.05$ between cells treated with Aβ alone and cells treated with Aβ in the presence of cholesterol.

Fig. 3

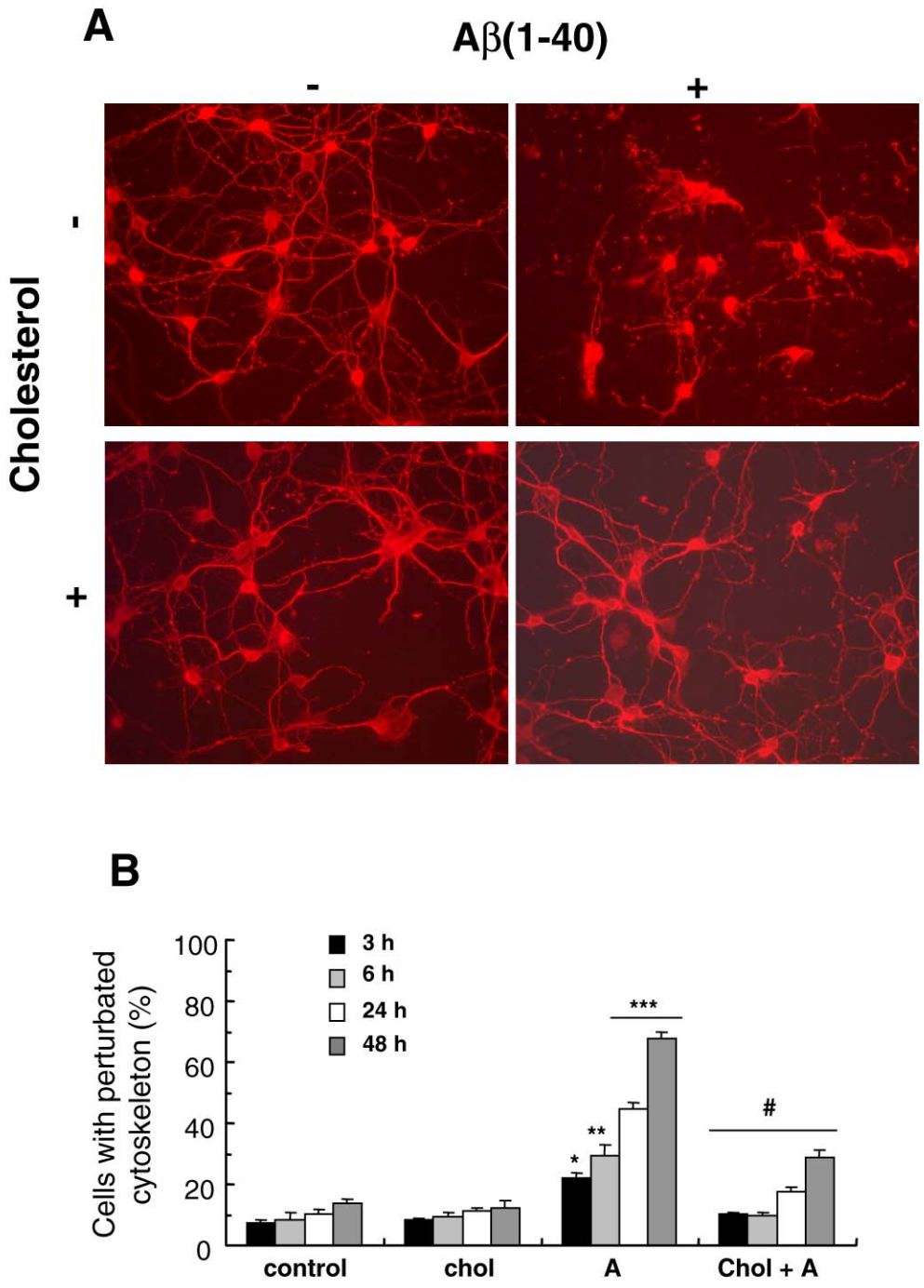

Figure 3. Cholesterol enrichment protects cortical neurons from soluble Aβ peptide-induced cytoskeleton disruption. Cells were preincubated for 3 h in the presence of 10 μg/ml cholesterol and were then exposed to 5 μM soluble Aβ(1-40) for 24 h. ***A*)** The microtubule network was visualized using immunofluorescence with an anti-β-tubulin monoclonal antibody. No differences were found between cholesterol-treated and control cells. ***B)*** The microtubule perturbations induced by the soluble Aβ(1-40) peptide were quantified at the indicated time using immunofluorescence with an anti-β-tubulin monoclonal antibody as previously described (see ref 41). Data are means ± SE of three different experiments with four determinations each and are normalized to the effect of vehicle, designated as 100% (**P*<0.05; ***P*<0.01; ****P*<0.001). Differences among the subgroups for each condition were performed by ANOVA followed by a Scheffe's post hoc test. $^{\#}P < 0.05$ between cells treated with Aβ alone and cells treated with Aβ in the presence of cholesterol. No significant differences were found between cholesterol-treated and control cells.

Fig. 4

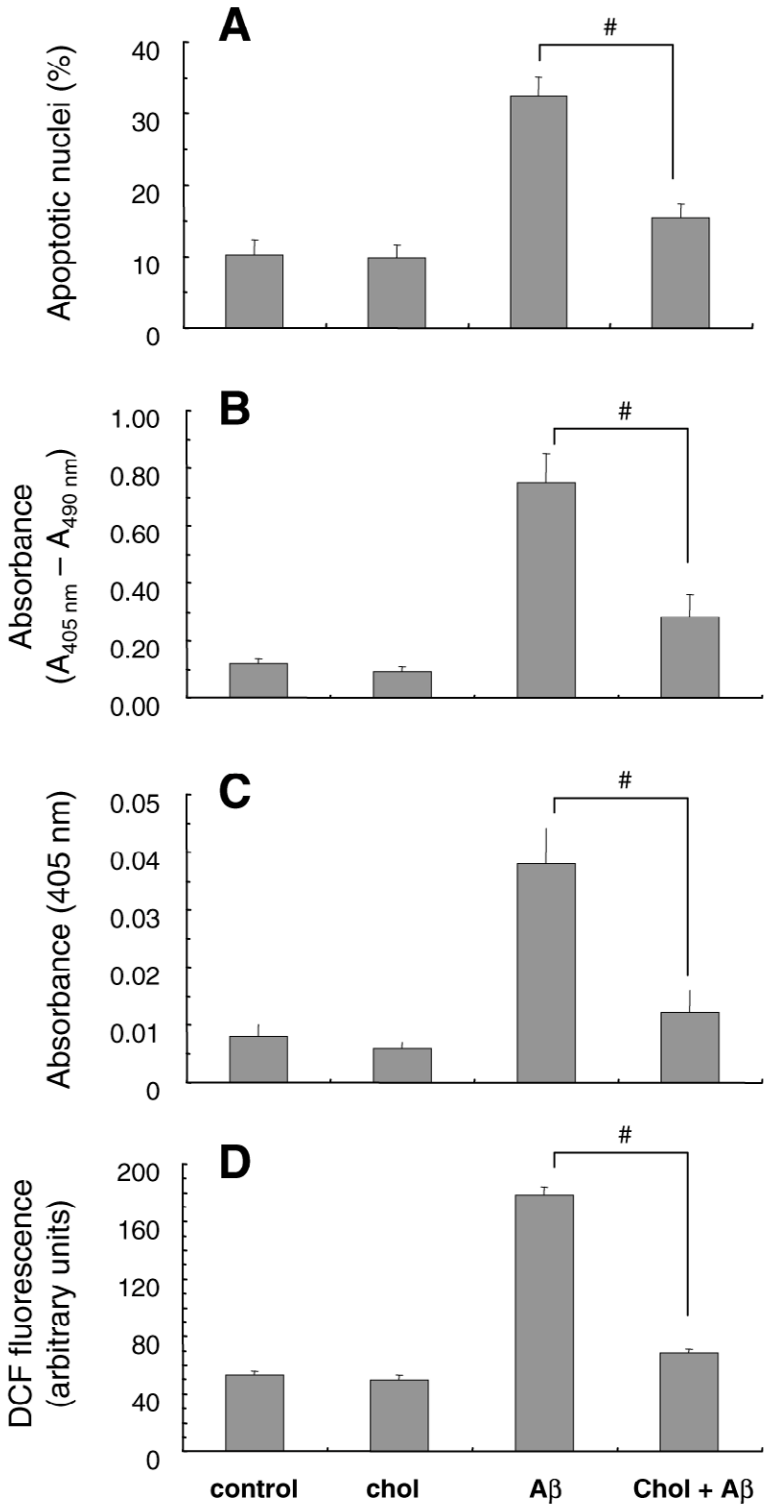

Figure 4. Cholesterol enrichment inhibits the apoptotic events induced by the soluble Aβ peptide. Cells were preincubated for 3 h in the presence of 10 μg/ml cholesterol and were then exposed to 5 μM soluble Aβ(1-40) for 24 h. Apoptotic nuclei were visualized and quantified after DAPI staining (***A***), and oligonucleosome production was monitored using an ELISA test as described in Materials and Methods (***B***). The activation of caspase-3 (***C***) and the production of reactive oxygen species (***D***) were monitored as described in Materials and Methods. Data are means ± SE of three different experiments with four determinations each and are normalized to the effect of vehicle, designated as 10%. No significant differences were found between caspase inhibitor-treated and control cells.

Fig. 5

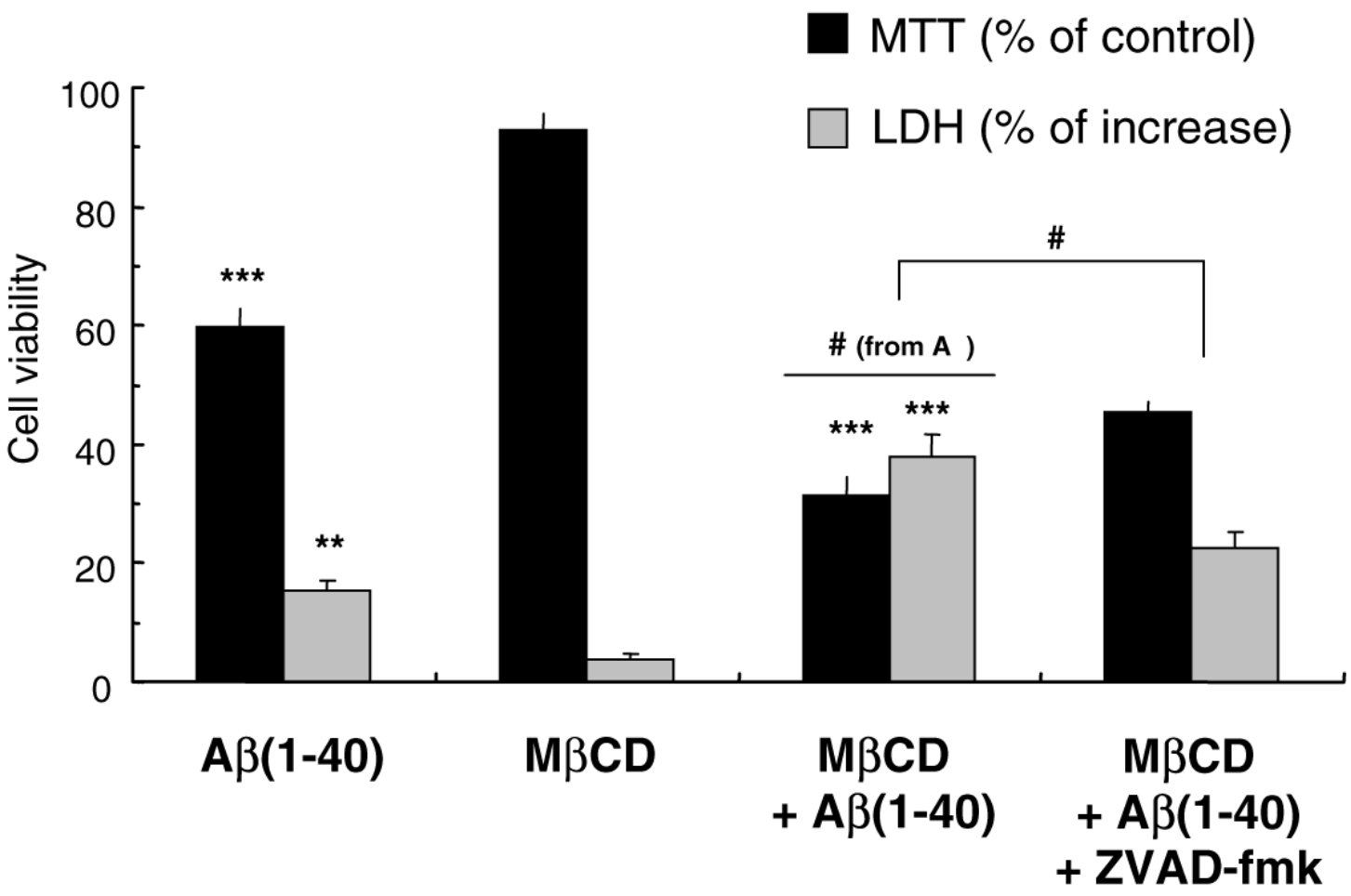

Figure 5. Cholesterol depletion makes cells more susceptible to Aβ insults. Cells were preincubated for 30 min at 37°C in the presence of 1 mM MβCD and were then exposed to 5 μM soluble Aβ(1-40) for 24 h in the absence or presence of 100 μM ZVAD. Cell viability was monitored by the MTT assay and the release of LDH. Data are means ± SE of three different experiments with four determinations each and are normalized to the effect of vehicle, designated as 100% (**P*<0.05; ***P*<0.01; ****P*<0.001). No significant differences were found between MβCD-treated and control cells.

Fig. 6

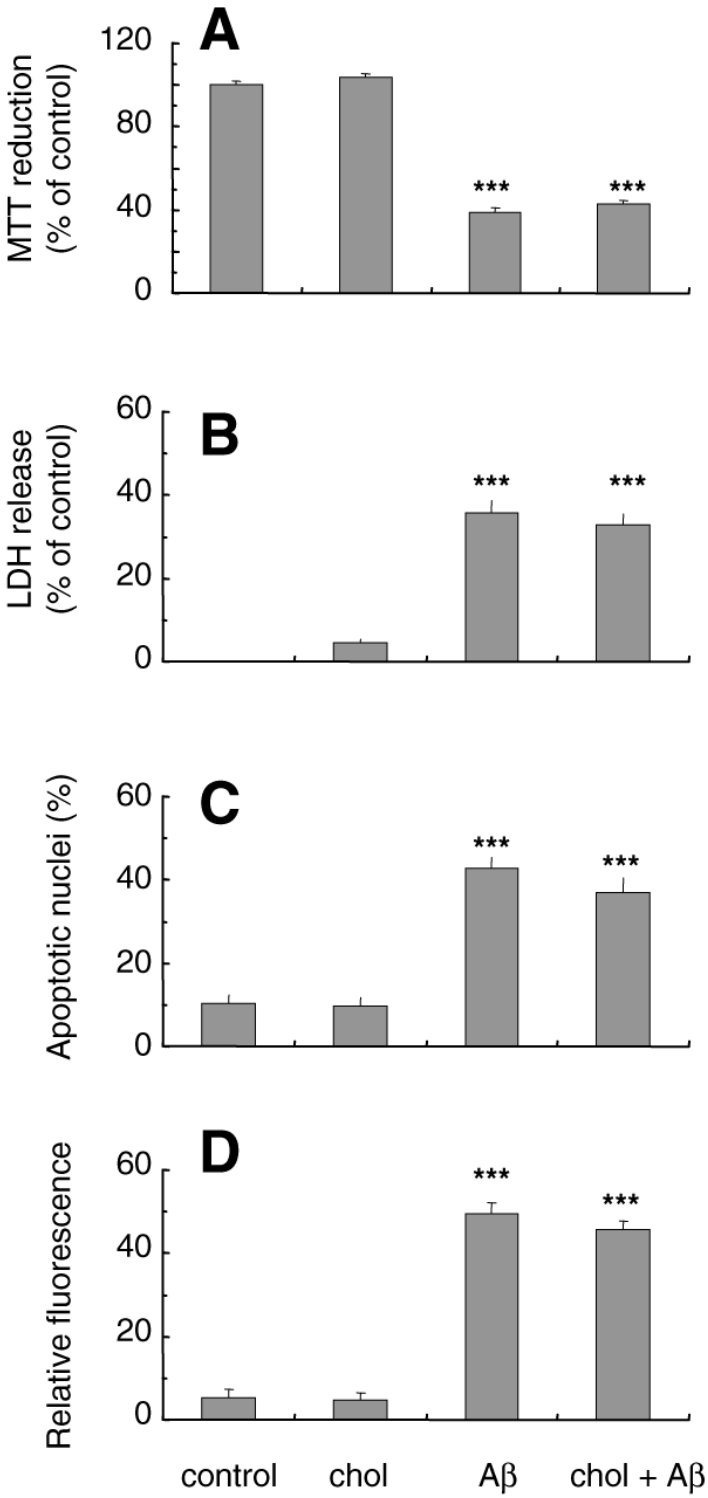

Figure 6. The neurotoxicity of the fibrillar Aβ peptide is not modulated by exogenous cholesterol. Before Aβ peptide treatment, cortical neurons were incubated in the absence or in the presence of increasing concentrations of cholesterol for 3 h. After they were washed in order to remove the excess cholesterol, cells were treated for the indicated time with 5 μM fibrillar Aβ(1-40) peptide. Cell viability was monitored by the MTT assay (***A***) and the release of LDH (***B***). Apoptotic nuclei were visualized and quantified after DAPI staining (***C***), and the activation of caspase-8 (***D***) was monitored as described in Materials and Methods. Data are means ± SE of three different experiments with four determinations each and are normalized to the effect of vehicle, designated as 100% (**P*<0.05; ***P*<0.01; ****P*<0.001). No significant differences were found between caspase inhibitor-treated and control cells.

Fig. 7

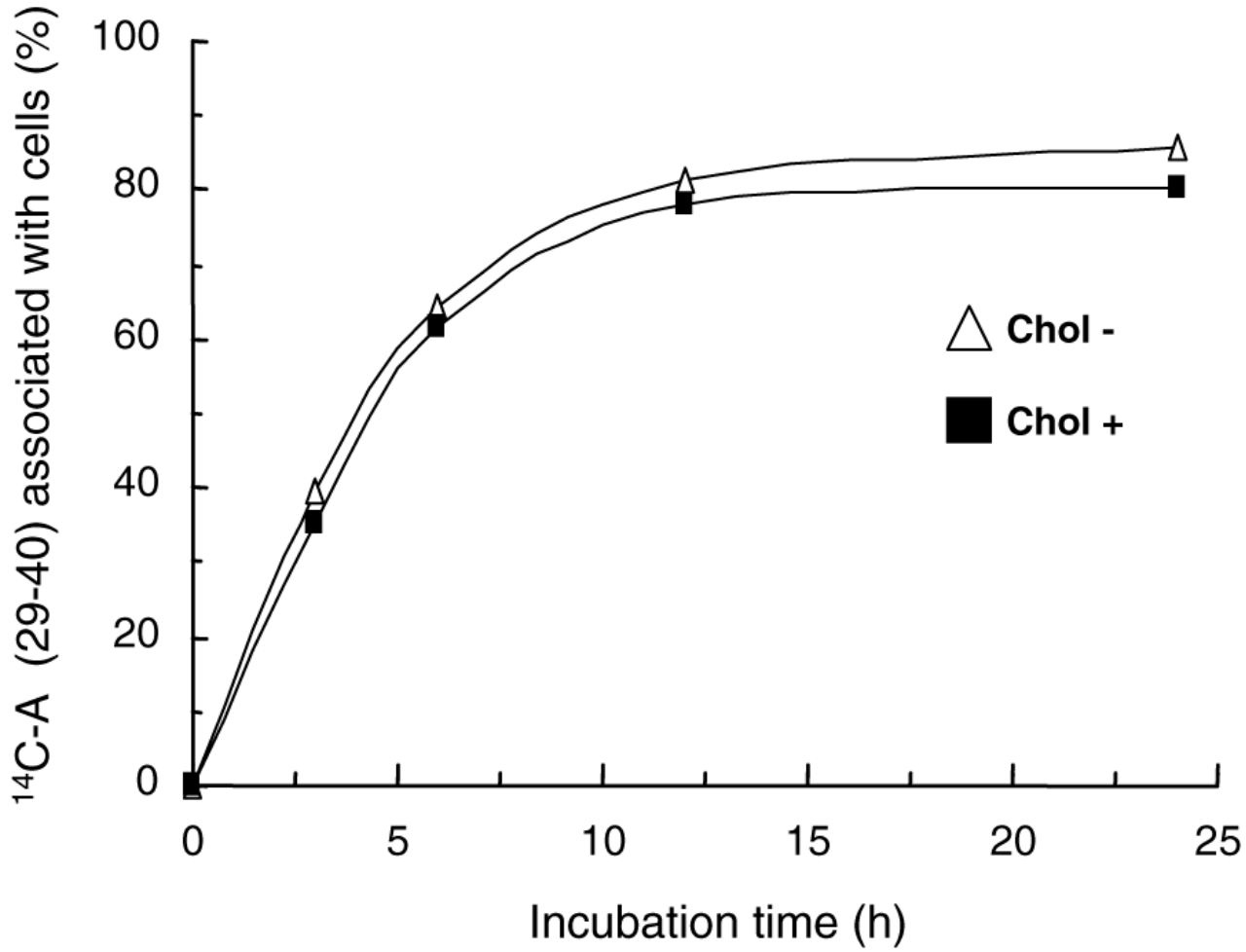

Figure 7. Cholesterol effects on the association of the soluble Aβ peptide with the plasma membrane of cortical neurons. Cells were preincubated for 3 h in the presence of 10 μg/ml cholesterol were then exposed to 5 μM soluble [^{14}C]Aβ(29-40), and the radioactivity associated with cells was monitored as a function of incubation time. Data are means of three independent experiments.

Fig. 8

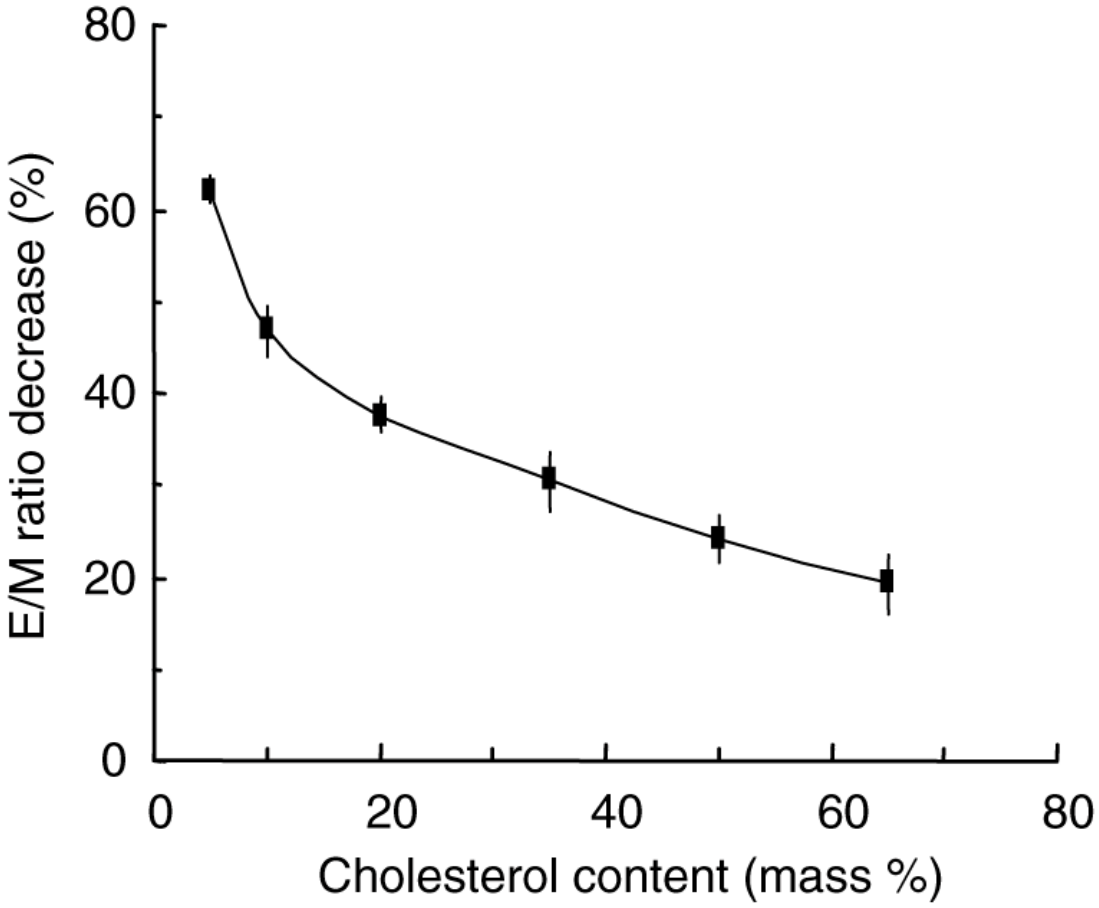

Figure 8. Cholesterol inhibits the liposome fusion induced by the soluble Aβ peptide. The extent of lipid mixing of PC/PE/PS/SM/cholesterol SULs induced by the soluble Aβ(1-40) peptide was monitored as described in Materials and Methods. Percentages of E/M ratio decrease, as an indication of liposome fusion, were plotted vs. the liposome cholesterol content, after 10-min incubation of SULs and peptides. Data are means of two independent experiments.