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The Inhibitory Effect of Apolipoprotein E4 on Neurite Outgrowth Is Associated with Microtubule Depolymerization*

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Evidence is presented for the differential effects of two isoforms of apolipoprotein (apo) E, apoE3 and apoE4, on neurite outgrowth and on the cytoskeleton of neuronal cells (Neuro-2a) in culture. In the presence of a lipid source, apoE3 enhances and apoE4 inhibits neurite outgrowth. Immunocytochemical studies demonstrate that there is a higher concentration of apoE3 than apoE4 in both the cell bodies and neurites. Cells treated with apoE4 showed fewer microtubules and a greatly reduced ratio of polymerized to monomeric tubulin than did cells treated with apoE3. The effect of apoE4 on depolymerization of microtubules was shown by biochemical, immunocytochemical, and ultrastructural studies. The depolymerization of microtubules and the inhibition of neurite outgrowth associated with apoE4 suggest a mechanism whereby apoE4, which has been linked to the pathogenesis of Alzheimer's disease, may prevent normal neuronal remodeling from occurring later in life, when this neurodegenerative disorder develops.

Apolipoprotein (apo)[1] E is a 34-kDa protein component of lipoproteins that mediates their binding to the low density lipoprotein (LDL) receptor and to the LDL receptor-related protein (LRP) (1–4). Apolipoprotein E is a major apolipoprotein in the nervous system, where it is thought to redistribute lipoprotein cholesterol among the neurons and their supporting cells and to maintain cholesterol homeostasis (5–7). Apart from this function, apoE in the peripheral nervous system functions in the redistribution of lipids during regeneration (8–10).

There are three common isoforms of apoE (apoE2, apoE3, and apoE4) that are the products of three alleles (ϵ2, ϵ3, and ϵ4) at a single gene locus on chromosome 19 (11). Apolipoprotein E3, the most common isoform, has cysteine and arginine at positions 112 and 158, respectively, whereas apoE2 has cysteine at both of these positions and apoE4 has arginine at both (1, 12).

Accumulating evidence demonstrates that the apoE4 allele (ϵ4) is specifically associated with sporadic and familial late-onset Alzheimer's disease and is a major risk factor for the disease (13–16). In accord with these findings, apoE immunoreactivity is associated with both the amyloid plaques and the intracellular neurofibrillary tangles seen in postmortem examinations of brains from Alzheimer's disease patients (17, 18). The mechanism by which apoE4 might contribute to Alzheimer's disease is unknown. However, our recent data demonstrating that apoE4 stunts the outgrowth of neurites from dorsal root ganglion (DRG) neurons suggest that apoE may have a direct effect on neuronal development or remodeling (19, 20). In an extension of these previous studies, we have now examined the effects of the apoE isoforms on neurite outgrowth and on the cytoskeleton of Neuro-2a cells, a murine neuroblastoma cell line. Apolipoprotein E4 inhibits neurite outgrowth from these cells, and this isoform-specific effect is associated with depolymerization of microtubules.

EXPERIMENTAL PROCEDURES

Cell Lines—Murine neuroblastoma (Neuro-2a) cells and murine fibroblasts (BALB/c) were obtained from American Type Culture Collection (Rockville, MD). Neuro-2a cells were maintained at 37 °C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium-nutrient mixture (DMEM/F12; 50%:50%) containing 10% fetal bovine serum, penicillin, and streptomycin. For experiments, Neuro-2a cells were plated in this medium in 6-well plates at 25,000 cells/well. After 3–6 h of incubation, the medium was replaced with N2 medium (DMEM/F12 containing growth supplements) alone (21), N2 medium containing rabbit β-migrating very low density lipoproteins (β-VLDL) alone (40 μg cholesterol/ml), or N2 medium containing β-VLDL and either purified human apoE3 (30 μg/ml) or purified human apoE4 (30 μg/ml), and the cells were incubated for an additional 48 h. BALB/c fibroblasts were maintained in DMEM containing 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in a humidified 7% CO_2 incubator. Experiments with BALB/c cells were performed in DMEM. Rabbit β-VLDL from cholesterol-fed animals were isolated as described (22), and human apoE was purified from the plasma of apoE3 and apoE4 homozygotes (23); biological activity was assessed by LDL receptor-binding assay (24).

Quantitation of Neurite Outgrowth—To assess neurite outgrowth, Neuro-2a cells were grown in test reagents and then nonspecifically stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) and fixed with 4% paraformaldehyde, as described previously (20). Neurons were imaged in fluorescence mode with a confocal laser scanning system (MRC-600, Bio-Rad), and the images were digitized with an Image-1/AT image analysis system (Universal Images, West Chester, PA) (20). The neuronal images were coded, and the neurite extension (the distance from the cell body to the end of the longest neurite) was measured for each neuron. Approximately 50–60 treatment-responsive cells (cells with at least one neurite longer than the diameter of the cell body) were measured for each treatment condition, and the data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. The value for the N2 medium alone was set at 100%. Data are

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[1] The abbreviations used are: apo, apolipoprotein; LDL, low density lipoprotein; LRP, LDL receptor-related protein; DRG, dorsal root ganglion; Neuro-2a, murine neuroblastoma; BALB/c, murine fibroblasts; DMEM, Dulbecco's modified Eagle's medium; β-VLDL, β-migrating very low density lipoproteins; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid.

presented as the mean ± S.E. Statistics were done using Stat View II software.

Immunocytochemistry—Immunocytochemistry of apoE was performed on Neuro-2a cells and fibroblasts incubated for 48 h with β-VLDL and either apoE3 or apoE4. Cells were fixed for 3 days at 4 °C in PBS containing 3% paraformaldehyde and 0.1% glutaraldehyde and quenched with 150 mM sodium acetate in PBS containing 0.1% milk powder (quench buffer). The cells were further incubated for 15 min at room temperature in quench buffer with or without 0.5% Triton X-100. The cells were then washed with PBS containing 15 mM sodium acetate and 0.1% nonfat dry milk (wash solution) and incubated with a polyclonal antibody to apoE (GHE) at a concentration of 1:1000 in wash solution containing goat serum (1:50 dilution) for 1 h at room temperature followed by extensive washes with wash solution. The secondary antibody (anti-rabbit IgG conjugated to Texas Red, Vector Laboratories, Burlingame, CA) was incubated for 1 h before use at a 1:100 dilution in PBS containing 10% fetal bovine serum. The cells were washed with PBS and coverslipped, and serial optical sections (~1 μm in thickness) were made using a confocal laser scanning microscope.

Immunocytochemistry to detect tubulin was performed on Neuro-2a cells and fibroblasts grown for 48 h in medium alone, with β-VLDL alone, or with β-VLDL and either apoE3 or apoE4. Following incubation with test reagents, the medium was aspirated, and the cells were washed twice with PBS. The cells were fixed for 1 h at room temperature in 10 mM HEPES, pH 7.2, containing 100 mM KCl, 3 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 0.5% Triton X-100, 2% paraformaldehyde, and 0.1% glutaraldehyde, followed by several quick washes with PBS. The cells were quenched with 0.05 M ammonium chloride in PBS for 5 min at room temperature and blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) in PBS. Immunocytochemistry was performed for 45 min at room temperature, using a monoclonal antibody to β-tubulin (Boehringer Mannheim) at a concentration of 1 μg/ml in PBS containing 1% BSA. Following incubation, the cells were washed five times with PBS containing 0.1% BSA and then incubated for 30 min in the dark with goat anti-mouse IgG (Zymed Laboratories Inc., South San Francisco, CA) conjugated to fluorescein isothiocyanate (10 μl/ml) in PBS containing 1% BSA. Cells were coverslipped, and optical sections of 0.5 μm thickness were made using a Bio-Rad MRC-600 confocal laser scanning microscope; the sections were overlaid to obtain a composite image.

For localization of actin, the medium was aspirated, and the cells were washed twice with PBS. Cells were fixed with 3% paraformaldehyde in PBS, washed twice with PBS, and permeabilized for 5 min at room temperature with 0.25% Triton X-100 in PBS containing 1% BSA. The permeabilized cells were washed twice with PBS and incubated for 30 min at room temperature in PBS containing 5 units/ml of rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR). The cells were washed twice with PBS and coverslipped, and optical sections were made as described above.

In immunocytochemistry experiments, 15–20% of the cells did not respond to the treatments and were similar to control neurons. These cells may represent cells injured during plating.

^{125}I-ApoE Binding Assay—The cells were grown in 12-well plates until they reached half of maximal confluence. Cells were washed with medium and incubated with β-VLDL (40 μg cholesterol/ml) along with 30 μg/ml of either ^{125}I-apoE3 or ^{125}I-apoE4 for 48 h. Following incubation, the medium was removed and the cells were washed four times with PBS containing 0.2% BSA at 4 °C. The cells were solubilized with 0.1 N NaOH and assayed for protein, and radioactivity was determined by gamma counting. Apolipoprotein E was iodinated using Bolton-Hunter reagent (Amersham Corp.) according to the manufacturer's instructions. The average specific activity was 50 counts/min/ng apoE.

Electron Microscopy of Neuro-2a Cells—Neuro-2a cells were incubated with test reagents as described above. Following incubation, the cells were lifted from the plates using 0.05% trypsin and 0.5 mM EDTA and pelleted by centrifugation. Cells were fixed for 1 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed for 1 h in 2% OsO_4. The cells were dehydrated, embedded in Epon 812, sectioned (80 nm) using a Reichert Ultracut E, and stained with uranyl acetate and lead citrate. The cells were photographed using a JEOL CX-100II electron microscope. Consistent results were obtained in three independent experiments performed with fresh preparations of apoE and β-VLDL.

Binding of ^{125}I-ApoE to Microtubules—Neuro-2a cells in a 100-mm plate were grown to confluence as described above, scraped from the plate in PBS, and lysed by sonication in PME buffer, pH 7.2 (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin), containing 1 mM GTP. The solution was centrifuged and pelleted cell debris discarded. The supernatant was incubated at 37 °C for 1 h to facilitate tubulin polymerization. The resultant pellet of microtubules was resuspended in PME buffer, aliquoted into microfuge tubes, and incubated at 37 °C with either ^{125}I-apoE3 (2 μg/ml) or ^{125}I-apoE4 (2 μg/ml). Following incubation, the samples were centrifuged for 1 h at 100,000 × *g* to separate the free ^{125}I-apoE from that bound to microtubules. The radioactivity associated with the supernatant and pellet was estimated by gamma counting.

Immunoblotting of Tubulin—Neuro-2a cells were incubated with test reagents, and cell extracts were prepared as described above. An aliquot of each extract containing an equal amount of protein was centrifuged through a sucrose cushion (PME + 20% sucrose) at 100,000 × *g* for 1 h at 37 °C to separate the microtubules (polymeric tubulin preparation) from the tubulin (monomeric tubulin preparation). The monomeric tubulin preparation was polymerized for 1 h at 37 °C by incubating the sample in PME buffer containing 20% sucrose and 1 mM GTP, followed by centrifugation as described above (25). The polymeric tubulin preparation was subjected to a temperature-dependent depolymerization-polymerization cycle to remove cellular debris, followed by centrifugation to obtain the microtubules. Aliquots of the cell extract (total tubulin) and the monomeric and polymeric tubulin preparations were subjected to 12% SDS-polyacrylamide gel electrophoresis under reducing conditions and immunoblotted using a monoclonal antibody to α-tubulin (ICN, Irvine, CA) at a dilution of 1:100, as described by the manufacturer. The tubulin bands in the immunoblots were quantitated by densitometry (Ambis Systems, San Diego, CA). The value obtained with N2 medium alone was set at 100%, and the data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. Data are presented as the mean ± S.E. Statistics were done using Stat View II software.

Other Assays and Methods—Protein assay was performed as described (26). The amount of cholesterol in cells incubated for 48 h with β-VLDL (40 μg cholesterol/ml) and human apoE3 or apoE4 (30 μg/ml) was assayed using a commercially available kit (Monotest, Boehringer Mannheim). The [^{3}H]thymidine incorporation assay was performed with Neuro-2a cells incubated with β-VLDL and apoE3 or apoE4 using a previously published procedure (27). The lactate dehydrogenase assay was performed as described (28). The DiI-labeled β-VLDL uptake was performed as described (19).

RESULTS

Differential Effects of ApoE3 and ApoE4 on Neurite Outgrowth—Our previous studies examined the effects of apoE and lipoproteins on the outgrowth of neurites from primary rabbit DRG neurons *in vitro* (19, 20). The addition of purified human apoE3, together with rabbit β-VLDL (cholesterol-rich lipoproteins), increased neurite extension from these peripheral nervous system neurons, whereas apoE4 when added to the cells together with β-VLDL inhibited neurite outgrowth.

In this study, we found a similar differential effect of apoE3 and apoE4 on the outgrowth of neurites from Neuro-2a cells. In the presence of β-VLDL, apoE3 and apoE4 had dramatic isoform-specific effects on neurite outgrowth from Neuro-2a cells, as assessed by phase contrast microscopy (Fig. 1). Incubation of the cells with β-VLDL (Fig. 1*B*) stimulated neurite outgrowth slightly as compared with cells grown in N2 medium alone (Fig. 1*A*); however, a more dramatic effect was seen with the addition of apoE. Cells incubated with apoE3 and β-VLDL (Fig. 1*C*) had more neurite extension than cells incubated with β-VLDL, whereas cells incubated with apoE4 and β-VLDL had less neurite extension (Fig. 1*D*). These observations were confirmed when neurite outgrowth was quantitated using an image analysis system (Fig. 2). Incubation of the cells with β-VLDL enhanced neurite extension, as compared with cells maintained in N2 medium alone (Fig. 2). Addition of human apoE3 with β-VLDL further increased the extension ($p < 0.005$), whereas human apoE4 along with β-VLDL significantly reduced neurite extension as compared with the extension observed from cells incubated with β-VLDL ($p < 0.001$).

Three different studies were performed to rule out a general toxic effect of apoE4 on neurons. We assayed lactate dehydro-

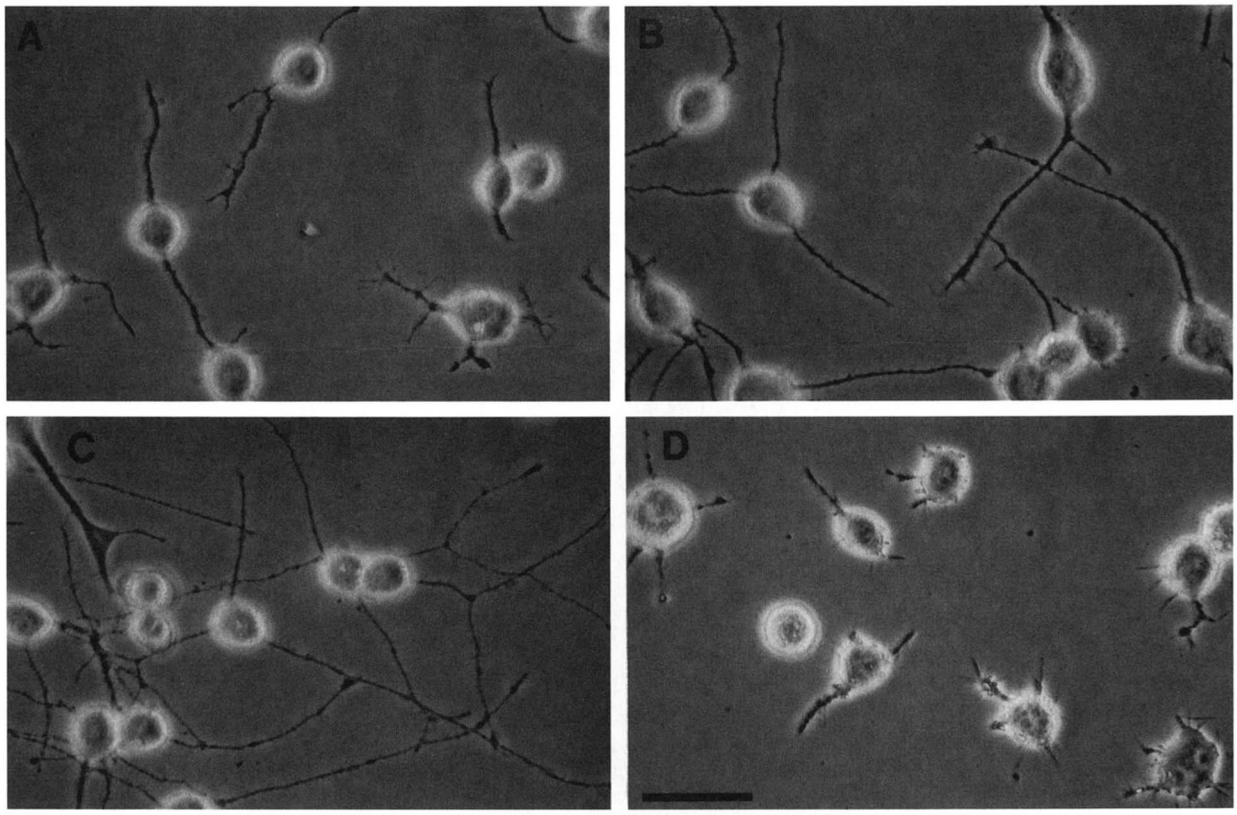

FIG. 1. **Effect of apoE on neurite outgrowth from Neuro-2a cells.** Neuro-2a cells were grown for 2 days in N2 medium alone (*A*), in N2 medium with β-VLDL (40 μg of cholesterol/ml) (*B*), or in N2 medium with β-VLDL (40 μg of cholesterol/ml) and 30 μg/ml of either human apoE3 (*C*) or human apoE4 (*D*). Cells were photographed using a phase contrast microscope. *Scale bar* = 25 μm.

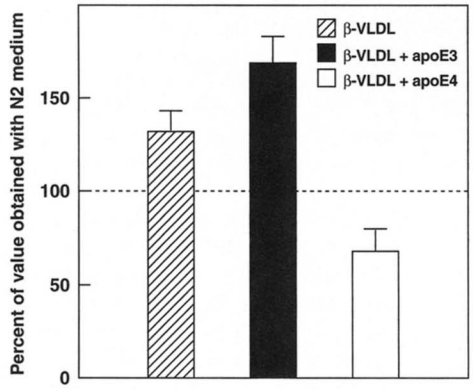

FIG. 2. **Quantitation of the effect of apoE on neurite extension from Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Neurite extension then was measured for 50–60 neurons from each group as described under "Experimental Procedures." Data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the various experiments then were averaged. The value for the N2 medium alone was set at 100% (*dashed line*). Data are presented as the mean ± S.E.

genase activity, a commonly used indicator of cell death, measured thymidine incorporation into DNA as an indication of cell replication, and examined the ability of the cells to develop neurites following incubation with apoE4 and β-VLDL. No significant differences in lactate dehydrogenase activity (apoE3 + β-VLDL, 212 ± 26.2 units/ml; apoE4 + β-VLDL, 205 ± 22 units/ml) or [^{3}H]thymidine incorporation into DNA (apoE3 + β-VLDL, 17.37 ± 1.66 counts/min × 10^6; apoE4 + β-VLDL, 18.13 ± 1.42 counts/min × 10^6) were seen with cells incubated with β-VLDL and either apoE3 or apoE4. The inhibitory effect of apoE4 was reversible, since removal of apoE4 and β-VLDL from the cells in culture and addition of medium alone or medium containing β-VLDL resulted in normal outgrowth (data not shown). These findings taken together demonstrate that the inhibitory effect of apoE4 on neurite outgrowth is not due to a cytotoxic effect of apoE4 on the cells.

To determine if lipoproteins were required for the differential effects of apoE3 and apoE4, the cells were incubated with free apoE for 48 h and the effects on neurite outgrowth examined. In the absence of lipoprotein, neither apoE3 nor apoE4 had an effect on neurite extension (data not shown). This result suggests that receptor-mediated endocytosis of apoE is necessary for the differential effects on neurite outgrowth, as previous studies have shown that apoE is a ligand for the LDL receptor and the LRP only when it is present on lipoproteins (1–3, 29).

Metabolism and Localization of ApoE3 and ApoE4 in Neuro-2a Cells—In several nonneuronal cell lines it has been demonstrated that apoE-containing lipoproteins follow a classic receptor-mediated endocytotic pathway by which the ligands are delivered to lysosomes where they are degraded (1–3, 12). Both apoE3- and apoE4-containing lipoproteins exhibit similar binding activity in nonneuronal cells (1, 22, 30). However, recent studies showing apoE immunoreactivity in the cytoplasm of neurons (31) suggest differences between neuronal and nonneuronal cells in the metabolism of apoE.

We first examined the ability of β-VLDL together with either apoE3 or apoE4 to deliver lipids to the cells. Incubation of the cells with β-VLDL and apoE3 or apoE4 for 48 h did in fact lead to similar levels of lipid accumulation (apoE3 + β-VLDL, 115 ± 3.9 μg cholesterol/mg of cell protein; apoE4 + β-VLDL, 121 ± 5.8 μg cholesterol/mg of cell protein), suggesting that the stimulation of β-VLDL uptake was similar with the two apoE isoforms. In comparison, Neuro-2a cells accumulated 20 ± 0.9 and 86 ± 2.6 μg of cholesterol/mg of cell protein when incubated

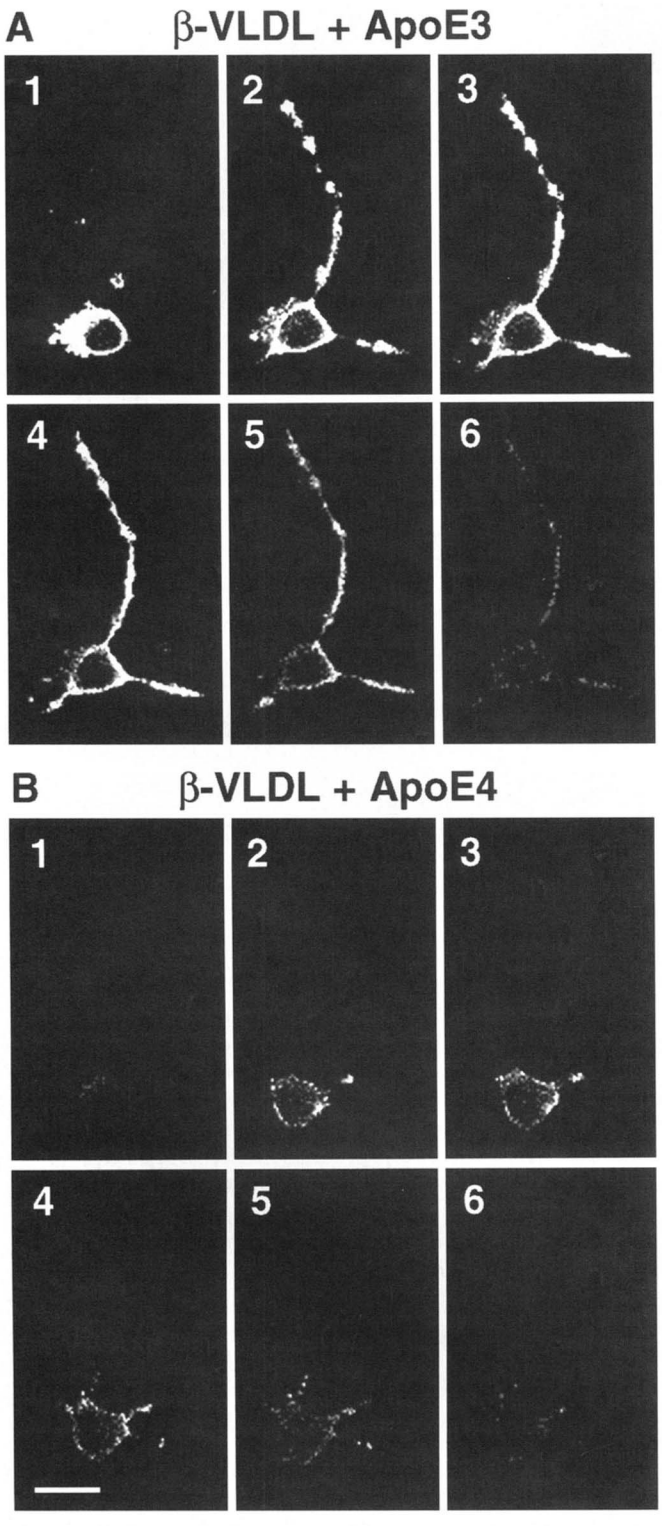

FIG. 3. **Immunocytochemical localization of apoE in Neuro-2a cells.** Neuro-2a cells were grown for 2 days in medium containing β-VLDL (40 μg of cholesterol/ml) together with 30 μg/ml of either human apoE3 (*A*) or human apoE4 (*B*). Immunocytochemistry was performed as described under "Experimental Procedures." Serial optical sections (~1 μm in thickness) were made from the top (*section 1*) to the bottom (*section 6*) of the cells using a confocal laser scanning microscope. *Scale bar* = 15 μm.

with N2 medium and β-VLDL alone, respectively. Examination of the uptake of fluorescently labeled β-VLDL in the presence of either apoE3 or apoE4 supported the conclusion that apoE3 and apoE4 stimulated β-VLDL uptake similarly.

To determine whether apoE3 and apoE4 were processed

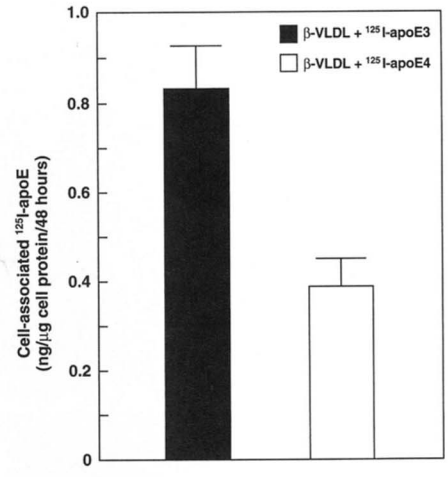

FIG. 4. **Cell association of ^{125}I-apoE with Neuro-2a cells.** Neuro-2a cells were grown for 2 days in medium containing β-VLDL (40 μg of cholesterol/ml) together with 30 μg/ml of either ^{125}I-apoE3 or ^{125}I-apoE4. Following incubation, the radioactivity associated with the cells (representing bound and internalized apoE) was determined as described under "Experimental Procedures." The experiment was repeated five times, each time with a fresh preparation of ^{125}I-apoE and β-VLDL. The data are presented as the mean ± S.E.

differently by neurons and by fibroblasts, we incubated Neuro-2a cells or murine fibroblasts with apoE3 or apoE4 in the presence of β-VLDL and examined the accumulation of apoE in the cells. Immunocytochemical detection of apoE in Neuro-2a cells incubated with β-VLDL and either apoE3 or apoE4 revealed that both isoforms were present within neurons (Fig. 3). There was, however, a substantial difference in the intensity of reactivity of apoE3 (Fig. 3*A*) and apoE4 (Fig. 3*B*). Apolipoprotein E3 was present both in the cell body and in the neurites at a substantially higher concentration than was apoE4 (Fig. 3, *A* and *B*). Both apoE3 and apoE4 were observed in nearly all serial optical sections made throughout the cell, suggesting that apoE was intracellular (Fig. 3, *A* and *B*).

The possibility that apoE was localized intracellularly was examined using two additional approaches. First, Neuro-2a cells were incubated with β-VLDL and either apoE3 or apoE4 and treated with suramin (a polyanion known to remove lipoproteins nonspecifically bound to the cell surface and specifically bound to their receptors), followed by immunocytochemistry for apoE. Treatment with suramin did not significantly reduce the apoE immunoreactivity associated with the cells. Second, immunocytochemical studies were performed in cells that were not permeabilized to permit access of antibody to the cytoplasm. No immunoreactivity of apoE was observed in nonpermeabilized cells incubated with β-VLDL together with either apoE3 or apoE4. These results demonstrated that the apoE was intracellular. The observed intracellular accumulation of apoE was unexpected, since lipoproteins and their apoproteins, when internalized by nonneuronal cell types, are rapidly degraded. To determine if the accumulation of apoE was specific to neurons, we performed similar immunocytochemistry experiments in murine fibroblasts. In these studies no apoE immunoreactivity was observed in the fibroblasts incubated with β-VLDL and either apoE3 or apoE4, suggesting either that apoE enters neurons and fibroblasts through different pathways or that in neurons apoE, especially apoE3, can escape lysosomal degradation. These results demonstrate that apoE3 is retained in Neuro-2a cells to a greater extent than apoE4 and that the metabolism of apoE in neuronal and nonneuronal cells is different.

The differences observed in the accumulation of apoE3 and apoE4 were confirmed by incubating the neurons with ^{125}I-

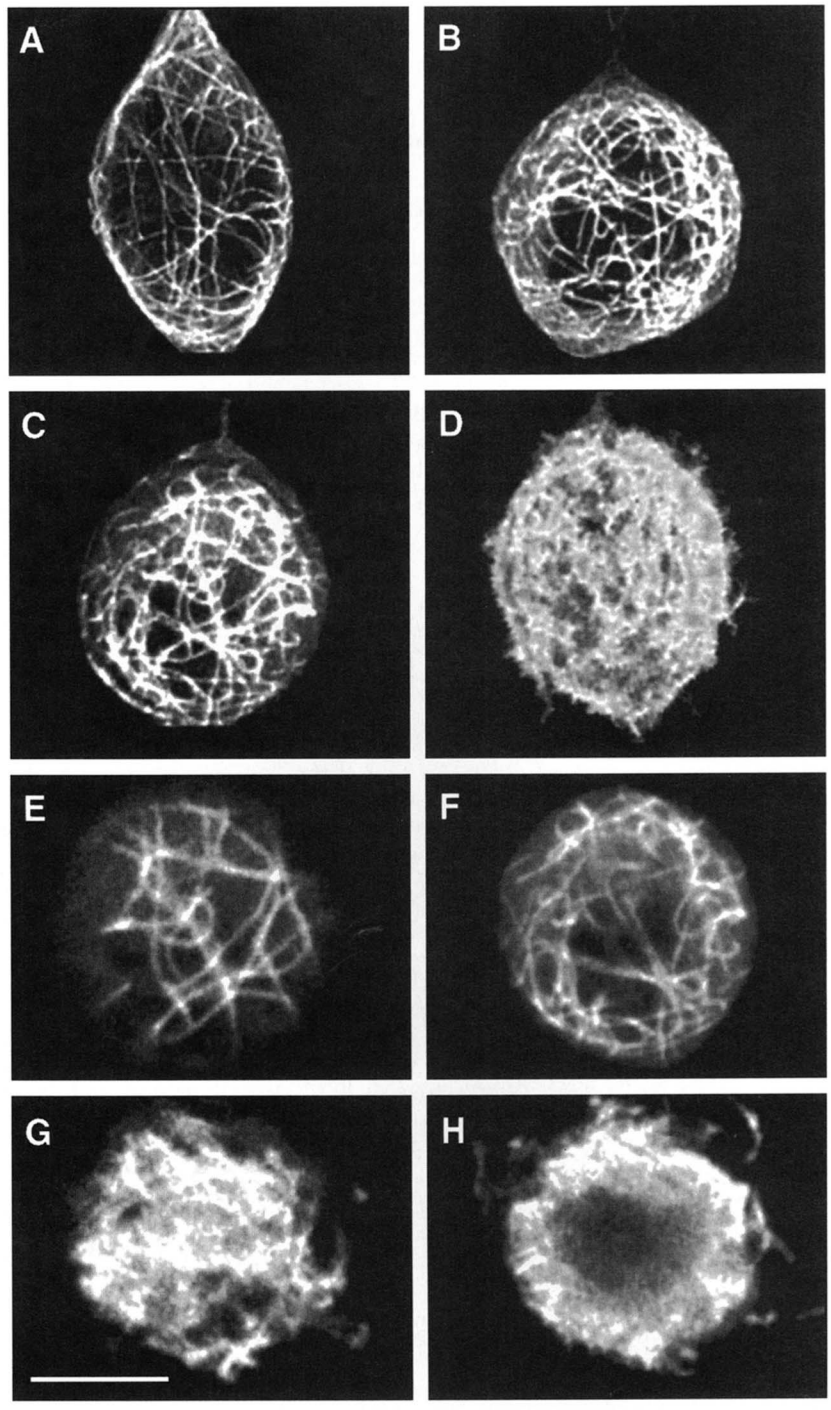

FIG. 5. **Immunocytochemical localization of tubulin in Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Immunocytochemistry was performed using a monoclonal antibody to β-tubulin and visualized using a fluorescein-labeled secondary antibody. Optical sections (0.5 μm in thickness) were made using a confocal microscope, and sections were overlaid to obtain a reconstructed image. Reconstructed images of cells incubated in medium alone (*A*), in medium containing β-VLDL (40 μg cholesterol/ml) (*B*), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 (*C*) or human apoE4 are shown (*D*). Representative individual optical sections near the top (*E* and *G*) and center (*F* and *H*) of cells incubated with β-VLDL and either apoE3 (*E* and *F*) or apoE4 (*G* and *H*) also are shown. *Scale bar* = 7 μm.

labeled apoE and β-VLDL at 37 °C for 48 h, and the amount of cell-associated apoE (bound and internalized) was quantitated. A differential accumulation of ^{125}I-apoE was observed, with twice as much ^{125}I-apoE3 as ^{125}I-apoE4 being associated with the cells at the end of the incubation period (Fig. 4).

Effects of ApoE3 and ApoE4 on the Microtubular Architecture of Neuro-2a Cells—Microtubules and their associated proteins play a crucial role in neurite outgrowth (32–37). Because apoE accumulates in the neurons and affects neurite outgrowth, we hypothesized that it also might affect tubulin polymerization and microtubule formation. Immunocytochemistry and electron microscopy were used to examine the cells incubated in medium alone, in medium containing β-VLDL, and in medium containing β-VLDL together with either apoE3 or apoE4. In cells grown in medium alone (Fig. 5*A*), with β-VLDL alone (Fig. 5*B*), or with β-VLDL and apoE3 (Fig. 5*C*), a well-formed network of microtubules was observed by immunocytochemistry. In contrast, in cells incubated with apoE4 and β-VLDL, few well-organized microtubules were present (Fig. 5*D*). The tubulin immunoreactivity in the apoE4-treated cells was diffuse throughout the cell body and in numerous cell-surface projections (consistent with the presence of monomeric tubulin) (38).

The photomicrographs in Fig. 5 (*A-D*) are composites of all optical sections. However, we also analyzed individual optical sections visualizing various levels through the cells (Fig. 5, *E-H*) to ensure that the presence of microtubules in the apoE4-treated cells was not obscured by the diffuse tubulin staining. In cells treated with β-VLDL and apoE4 (Fig. 5, *G* and *H*), none of the optical sections contained well-formed microtubules, whereas in cells incubated with β-VLDL and apoE3 (Fig. 5, *E*

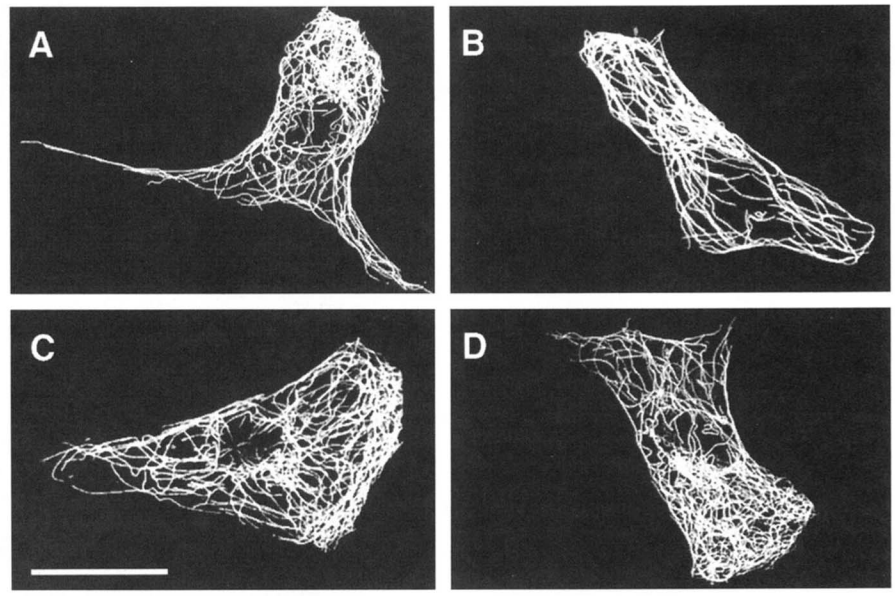

FIG. 6. **Immunocytochemical localization of tubulin in fibroblasts.** BALB/c fibroblasts were incubated for 2 days in medium alone (*A*), in medium containing β-VLDL (40 μg cholesterol/ml) (*B*), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 (*C*) or human apoE4 (*D*). Immunocytochemistry for β-tubulin was performed as described in the legend to Fig. 5. *Scale bar* = 7 μm.

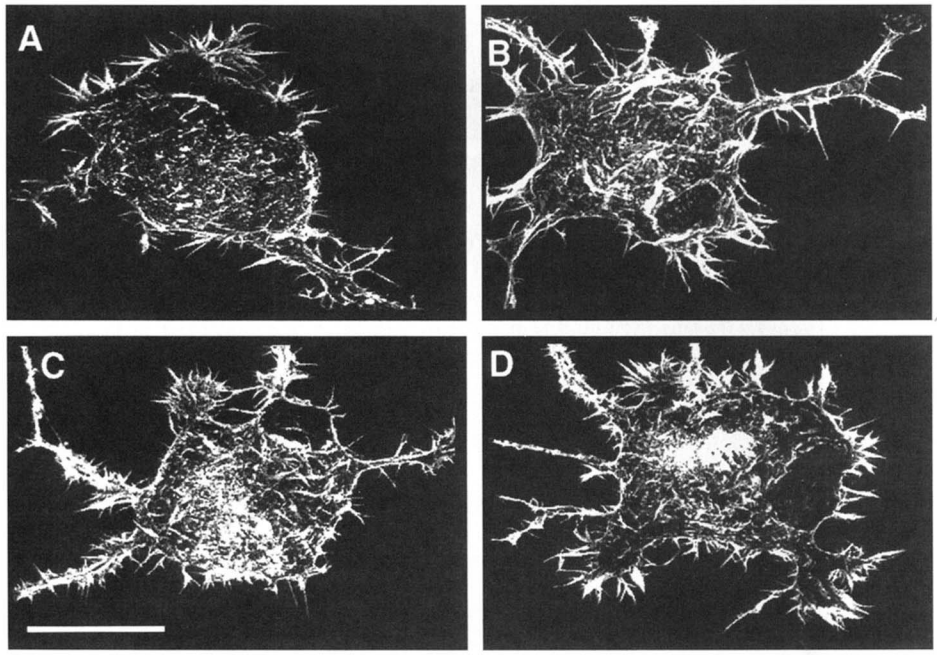

FIG. 7. **Localization of actin in Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Actin was detected using rhodamine-phalloidin. Optical sections (1 μm in thickness) were made using a confocal microscope, and sections were overlaid to obtain a reconstructed image. Reconstructed images of cells incubated in medium alone (*A*), in medium containing β-VLDL (40 μg cholesterol/ml) (*B*), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 (*C*) or human apoE4 are shown (*D*). *Scale bar* = 8 μm.

and *F*), numerous microtubules were seen in nearly all of the sections. When similar experiments were performed with murine fibroblasts, no significant difference in microtubular morphology was observed among cells treated with medium alone (Fig. 6*A*), medium containing β-VLDL (Fig. 6*B*), or medium containing β-VLDL together with either apoE3 (Fig. 6*C*) or apoE4 (Fig. 6*D*).

Additional experiments were performed to determine if apoE4 disrupts other cytoskeletal structures, such as actin filaments. As shown in Fig. 7, no significant difference in actin morphology was evident among Neuro-2a cells from the four treatment conditions when actin was detected using rhodamine-phalloidin.

To confirm the fluorescence microscopy data and to examine the effect of apoE on the microtubular architecture in the neurites of Neuro-2a cells, we performed electron microscopic studies. Long parallel arrays, identified by morphologic appearance and size (~22 nm in width) as microtubules, were present in cells grown in medium alone (Fig. 8*A*), β-VLDL alone (Fig. 8*B*), and β-VLDL and apoE3 (Fig. 8*C*). In contrast, in cells treated with apoE4 and β-VLDL, only a few fragments of microtubules were observed both in the cell body and neurites (Fig. 8*D*). All of these data taken together demonstrate that the differences in neurite outgrowth induced by apoE3 and apoE4 are associated with differences in microtubular formation and suggest that apoE4 depolymerizes microtubules in neuronal cells.

Effects of ApoE on the Polymerization State of Tubulin in Neuro-2a Cells—Further studies were performed to determine whether apoE associates with microtubules and to determine by biochemical assays if the amount of polymerized tubulin differed in the cells treated with apoE3 or apoE4. To determine if apoE associates with microtubules, total tubulin was extracted from the cells grown in medium alone and polymerized *in vitro*. This partially purified preparation, which contained microtubules and microtubule-associated proteins, was tested for its ability to bind apoE3 and apoE4. When ^{125}I-apoE was incubated with the partially purified microtubules from Neuro-2a cells, both ^{125}I-apoE3 and ^{125}I-apoE4 bound to the microtubules and/or their associated proteins (Fig. 9). However, an isoform-specific difference was

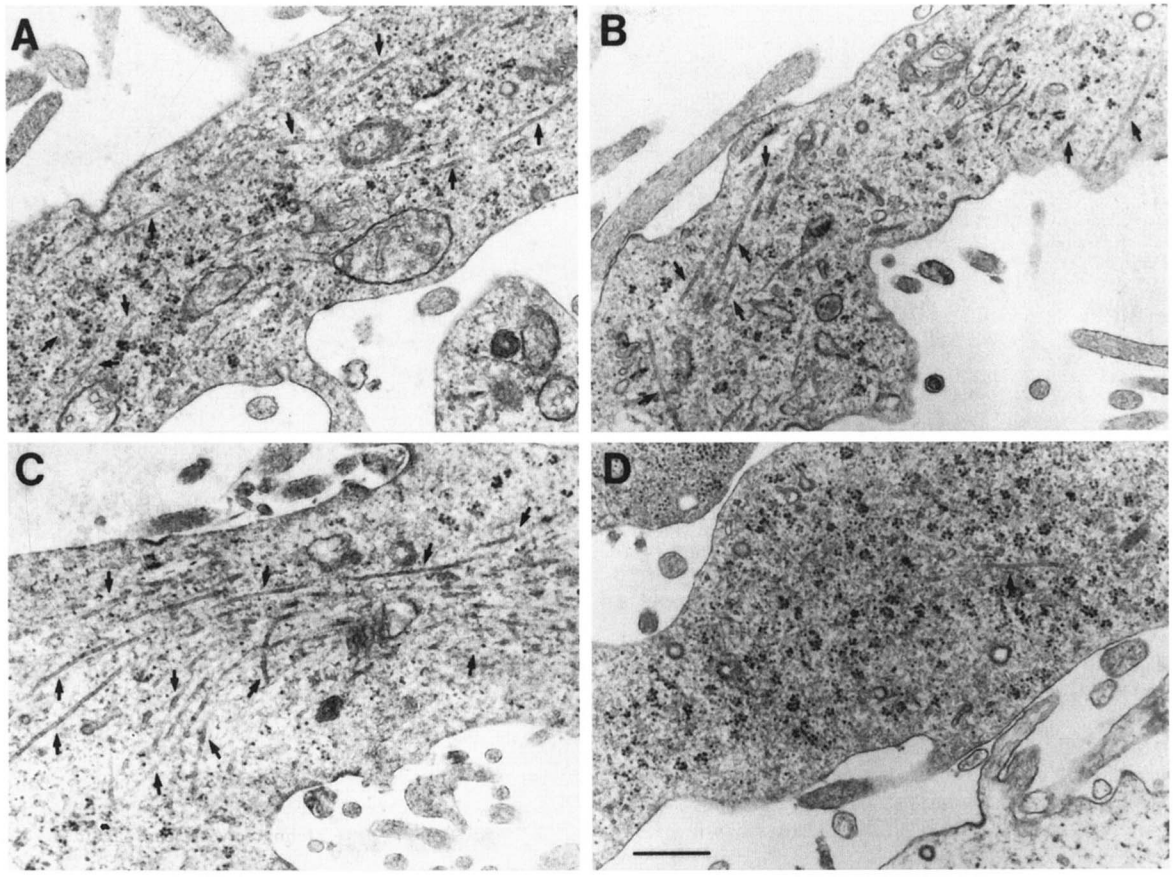

FIG. 8. **Electron microscopy of microtubules in Neuro-2a cells.** Neuro-2a cells were incubated for 2 days in medium alone (*A*), in medium containing β-VLDL (40 μg cholesterol/ml) (*B*), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 (*C*) or human apoE4 (*D*). Following incubation, electron microscopy was performed, as described under "Experimental Procedures," to detect microtubules (*arrows*) in the neurites. *Scale bar* = 0.5 μm.

observed, with more ^{125}I-apoE3 than ^{125}I-apoE4 bound to the crude microtubule preparation.

The effect of apoE on the ratio of polymerized to monomeric tubulin was examined biochemically. Total tubulin, monomeric tubulin, and polymerized tubulin were extracted from Neuro-2a cells incubated under the various conditions and then were quantitated by Western blotting and densitometry. The amounts of total tubulin in extracts from the cells grown under the four treatment conditions were similar (Fig. 10*A*). Incubation of cells with N2 medium containing β-VLDL did not significantly affect the quantity of monomeric or polymeric forms of tubulin as compared with cells grown in N2 medium alone (Fig. 10*A*). However, a dramatic isoform-specific difference in the polymerization state of the microtubules was observed when the cells were grown in medium containing β-VLDL and apoE. In contrast to cells treated with apoE3 and β-VLDL, incubation of the cells with apoE4 and β-VLDL resulted in an increase in the amount of monomeric tubulin and a decrease in the amount of polymeric tubulin (microtubules).

Quantitation of the immunoblots from three independent experiments by densitometry revealed that the incubation of Neuro-2a cells with β-VLDL resulted in a slight increase in the amount of monomeric and polymeric tubulin (Fig. 10*B*) as compared with cells grown in N2 medium alone. However, cells incubated with β-VLDL plus apoE3 had a significant decrease in monomeric tubulin and a significant increase in polymeric tubulin as compared with cells grown in β-VLDL alone ($p < 0.001$) (Fig. 10*B*). On the other hand, the opposite effect was observed in cells treated with apoE4 and β-VLDL, as monomeric tubulin increased significantly and polymeric tubulin decreased ($p < 0.001$). These biochemical results confirm and extend the ultrastructural studies and suggest that apoE alters the state of tubulin polymerization in an isoform-specific manner.

DISCUSSION

The present study demonstrates that the isoform-specific effect of apoE in association with a lipid source on neurite outgrowth that was previously seen in rabbit DRG neurons also is observed in Neuro-2a cells, a murine neuroblastoma cell line derived from the central nervous system (19, 20). Furthermore, this study demonstrates that the isoform-specific effect of apoE on neurite outgrowth correlates with a differential accumulation of apoE3 *versus* apoE4 within the neurons and with a differential effect of the apoE3 and apoE4 on cellular microtubules.

Previous studies of nonneuronal cells have shown that apoE-containing lipoproteins are taken up and degraded by receptor-mediated endocytosis. Apolipoprotein E3- and apoE4-containing lipoproteins have a similar binding affinity and cause a similar degree of lipoprotein internalization (1, 22, 30). The LDL receptor and the LRP have been implicated in this process (1–3, 29). Neuronal cells possess both of these major receptors (19, 39, 40), and apoE3-induced neurite extension in DRG neurons has been suggested to be mediated, at least in part, by the LRP (19).

In the present study, using immunocytochemistry of apoE, we have observed a differential accumulation of apoE3 and apoE4 in Neuro-2a cells. Apolipoprotein E3, incubated with the cells together with β-VLDL, accumulated widely throughout the cell bodies and the neurites in a diffuse pattern, suggesting that the internalized apoE3 is not restricted to a specific organelle. Confocal microscopy revealed that the apoE3 is intra-

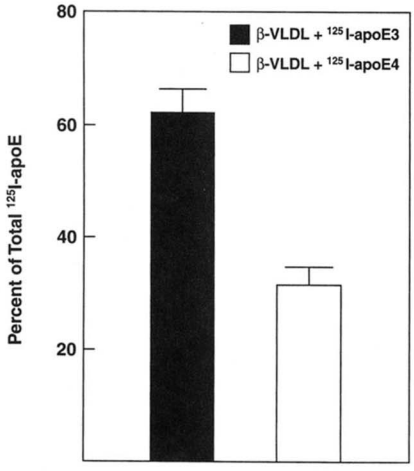

FIG. 9. **Binding of ^{125}I-apoE to partially purified microtubules *in vitro*.** Partially purified microtubules were prepared from Neuro-2a cells grown in N2 medium, as described under "Experimental Procedures." Aliquots of microtubular preparations were incubated with ^{125}I-apoE3 or ^{125}I-apoE4 (2 μg/ml), followed by centrifugation to separate the free ^{125}I-apoE from that bound to microtubules. The radioactivity associated with the supernatant and pellet was determined, and the percentage of total radioactivity in the pellet was calculated. The data are the mean ± S.E. for three independent experiments.

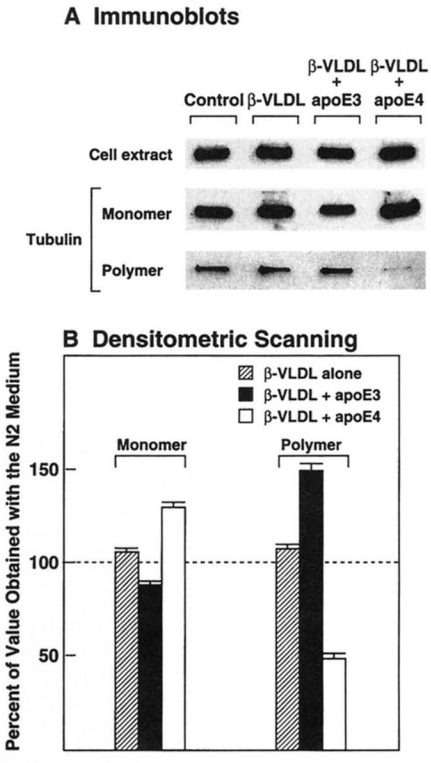

FIG. 10. **Immunoblotting of total, polymeric, and monomeric forms of tubulin from Neuro-2a cells.** Neuro-2a cells were incubated for 2 days in medium alone (control), in medium containing β-VLDL (40 μg cholesterol/ml), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 or human apoE4. Following incubation, cell extract was prepared as described under "Experimental Procedures." *A*, an aliquot of the cell extract containing 50 μg of total protein from each treatment condition was immunoblotted using a monoclonal antibody to α-tubulin. Monomeric and polymeric forms of tubulin were separated from the cell extract by centrifugation, as described under "Experimental Procedures," and immunoblotted for α-tubulin as described above. *B*, densitometric scanning of immunoblots obtained from three independent experiments performed as described in *A*. The value for the N2 medium alone was set at 100% (*dashed line*), and data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. Data are presented as the mean ± S.E.

cellular, an observation confirmed by the fact that the pattern of staining did not change when the cells were treated with suramin to remove surface-bound apoE and by the fact that the immunoreactivity was observed only after membrane permeabilization to allow the antibodies to enter the cells. In comparison to apoE3, less apoE4 accumulates within the Neuro-2a cells, even though equal amounts of lipoprotein-derived lipid are delivered to the cells. The data suggest a differential processing of apoE3 and apoE4, resulting in a greater accumulation of apoE3. How apoE escapes lysosomal degradation remains to be determined. The ability of apoE to escape lysosomal degradation is supported by the recent observations of Han *et al.* (31), which suggest that apoE is present in the cytoplasm of human neurons.

The differential effect of the apoE isoforms on neurite extension and their differential intracellular accumulation suggested that apoE might alter the neuronal cytoskeleton, specifically the microtubular system. Microtubules have been shown to have several important functions in neurons, including the development and maintenance of neuronal polarity, neurite extension, and retraction, the transport of macromolecules, and the release of neurotransmitters (32–37, 41, 42). In fact, microtubular stability is linked to several neurodegenerative disorders, including Alzheimer's disease (43). We have shown by three different criteria that apoE3 and apoE4, when incubated with the cells together with β-VLDL, have differential effects on microtubular structure. The apoE3 clearly supports microtubule formation in the Neuro-2a cells, whereas apoE4 is associated with microtubular depolymerization. By immunocytochemistry, the cells incubated with apoE3 along with β-VLDL displayed an extensive microtubular system as visualized using an anti-tubulin antibody. In the apoE4-treated cells, the microtubules were poorly formed and revealed a diffuse immunoreactivity to tubulin, suggesting the depolymerization of the microtubules. These observations were confirmed by electron microscopy of the Neuro-2a cells. Furthermore, quantitation of monomeric and polymeric tubulin extracted from the Neuro-2a cells revealed that incubation of the cells with apoE3 along with β-VLDL resulted in a reduction in monomeric tubulin and an increase in polymeric tubulin, whereas the opposite results were observed with apoE4.

The mechanism whereby apoE may alter the microtubular system is unclear. However, apoE3 binds to crude microtubule preparations from Neuro-2a cells to a greater extent (2-fold) than does apoE4. These results are consistent with those of Huang *et al.* (44) and Strittmatter *et al.* (45), who demonstrated that apoE3 bound much more avidly to tau and MAP2c, two microtubule-associated proteins, than did apoE4. Tau, MAP2c, or other microtubule-associated proteins may be mediating the binding of apoE to the crude microtubule preparations observed in our studies. In fact, the accumulation and retention of apoE3 in the Neuro-2a cells may reflect an interaction of apoE3 with the microtubules. Based on their biochemical studies, Roses (46) and Strittmatter *et al.* (45) postulated that the interaction of apoE3 with tau might support and stabilize microtubule formation and prevent hyperphosphorylation of tau. Hyperphosphorylated tau is a major component of neurofibrillary tangles, one of the characteristic lesions of Alzheimer's disease, suggesting a role for the microtubular system in the pathogenesis of the disease.

Even though apoE4 does not support neurite outgrowth, it is important to note that apoE4 does not have a general toxic effect on the Neuro-2a cells. Removal of the apoE4 from the cells allows neurite extension to occur. Furthermore, the effect of apoE4 on microtubules does not reflect a general disruption of the cytoskel-

eton. The apoE4 did not affect actin stability, and actin filaments appeared identical in cells incubated with β-VLDL and either apoE3 or apoE4. Furthermore, apoE4 did not effect cell replication, as determined by thymidine incorporation.

In conclusion, these studies suggest that apoE4 might play a role in the pathogenesis of Alzheimer's disease by destabilizing microtubules. In the aging brain, it is known that tubulin concentrations are low, favoring microtubular disassembly (47, 48). In combination with a low tubulin level, the expression of apoE4, which appears to stimulate microtubular depolymerization, may prevent normal neuronal remodeling from occurring later in life, when the disease process occurs. Ongoing studies aimed at elucidating the mechanism responsible for the apoE4-mediated inhibition of neurite extension and the possible involvement of the microtubular system in this inhibition may shed light on the pathogenesis of Alzheimer's disease and other neurodegenerative disorders.

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