Reactivity of Apolipoprotein E4 and Amyloid β Peptide

LYSOSOMAL STABILITY AND NEURODEGENERATION*

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We previously demonstrated that apolipoprotein E4 (apoE4) potentiates lysosomal leakage and apoptosis induced by amyloid β (Aβ) peptide in cultured Neuro-2a cells and hypothesized that the low pH of lysosomes accentuates the conversion of apoE4 to a molten globule, inducing reactive intermediates capable of destabilizing cellular membranes. Here we report that neutralizing lysosomal pH with bafilomycin or NH₄Cl abolished the apoE4 potentiation of Aβ-induced lysosomal leakage and apoptosis in Neuro-2a cells. Consistent with these results, apoE4 at acidic pH bound more avidly to phospholipid vesicles and disrupted them to a greater extent than at pH 7.4. Comparison of “Arctic” mutant Aβ, which forms multimers, and GM6 mutant Aβ, which remains primarily monomeric, showed that aggregation is essential for apoE4 to potentiate Aβ-induced lysosomal leakage and apoptosis. Both apoE4 and Aβ1-42 had to be internalized to exert these effects. Blocking the low density lipoprotein receptor-related protein with small interfering RNA abolished the enhanced effects of apoE4 and Aβ on lysosomes and apoptosis. In cultured Neuro-2a cells, Aβ1–42 increased lysosome formation to a greater extent in apoE3- or apoE4-transfected cells than in Neuro-transfected cells, as shown by immunostaining for lysosome-associated membrane protein 1. Similarly, in transgenic mice expressing apoE and amyloid precursor protein, hippocampal neurons displayed increased numbers of lysosomes. Thus, apoE4 and Aβ1–42 may work in concert in neurons to increase lysosome formation while increasing the susceptibility of lysosomal membranes to disruption, release of lysosomal enzymes into the cytosol, and neuronal degeneration.

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2 The abbreviations used are: AD, Alzheimer disease; apo, apolipoprotein; Aβ, amyloid β; APP, amyloid precursor protein; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine; LRP, low density lipoprotein receptor-related protein; MEM, minimum essential medium; NSE, neuron-specific enolase; Tricine, N-[-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; siRNA, small interfering RNA.

Alzheimer disease (AD) is a debilitating neurodegenerative disease. With no effective treatment available, the incidence of AD is likely to increase as the population continues to age. Although in most cases the exact cause is unknown, two proteins have been implicated in its pathogenesis: apolipoprotein E4 (apoE4) and amyloid β (Aβ). ApoE has important functions in lipid transport in the blood and in the redistribution of lipids among cells in the brain (1). One of its three common isoforms (2, 3), apoE4, is the major genetic risk factor for AD (4–6). Moreover, the e4 allele is associated with impaired central nervous system repair after injury and in other neurodegenerative diseases (7–22).

The Aβ peptide, typically 39–43 amino acids, is derived from the proteolytic cleavage of the amyloid precursor protein (APP) (23, 24). Amyloid plaques, a pathological hallmark of AD, are extracellular deposits of Aβ (25, 26). Several findings suggest that apoE, another constituent of plaques, may modulate plaque formation or alter the availability of Aβ for plaque formation. Lipid-free apoE4 avidly complexes with Aβ (27), possibly inducing plaques, whereas lipidated apoE3 facilitates Aβ clearance (28–32) and may reduce plaque formation. ApoE also modulates the cleavage of APP by γ-secretase (33).

Soluble multimeric or aggregated forms of Aβ are neurotoxic and appear to be important in the pathogenesis of AD (34–38). They are taken up by neurons and accumulate in lysosomes (39), where they cause lysosomal proteases and cathepsins to leak into the cytosol, leading to apoptosis and necrosis (40). One receptor involved in this uptake is the low density lipoprotein receptor-related protein (LRP). Most studies have shown that to be taken up by the LRP, Aβ must form a complex with an LRP ligand, such as apoE or α₂-macroglobulin, both of which bind to the LRP and other low density lipoprotein receptors (41–43). Aβ can bind directly and with high affinity to immobilized LRP clusters II and IV, possibly facilitating Aβ clearance (44).

The cellular uptake and lysosomal degradation of Aβ have been thought to reduce the concentration of Aβ in interstitial fluids. However, Aβ1–42 is degraded poorly by lysosomes (45), and its accumulation in lysosomes of cultured neurons causes leakage of lysosomal enzymes and ultimately cell death (39). Activation of endocytic pathways and lysosomal dysfunction in neurons are involved in neuronal degeneration and are the earliest changes seen in AD patients (for a review, see Ref. 46). Central nervous system neurons of AD patients have an increased number of structurally abnormal endosomes and lysosomes (39, 45, 47, 48), a 3-fold increase in endosomal volume (49), and an increase in gene expression and synthesis of lysosomal hydrolases, including cathepsins that can cause apoptotic cell death (40, 50). We have shown that apoE4 potentiates Aβ1–42-induced lysosomal leakage and apoptosis in neuronal cells in culture (51).

ApoE4 is more unstable than apoE3 under various conditions, including low pH (52), and it forms a reactive intermediate or molten globule that has increased binding to phospholipids, alters membrane stability, and affects translocation across membranes (53). We will show that increasing lysosomal pH blunts the enhanced lysosomal leakage induced by apoE4 in the context of Aβ1–42. ApoE4 avidly interacts with phospholipid vesicles and disrupts them more readily at pH 4 than at pH 7.4. Likewise, the physical state of Aβ is altered at low pH, stimulating aggregation and enhancing membrane interactions (54–57). We examined the effect of Aβ aggregation and multimer formation on apoE4 enhancement of lysosomal leakage and apoptosis and will show that multimers are required for the apoE4 effects to be observed. The
ApoE4/Aβ Destabilize Membranes

naturally occurring "Arctic" mutation (E693G), which is associated with early onset AD and enhances aggregation in vitro (58, 59) and in vivo (60), and the GM6 mutation (F690S/L705P), which was engineered to resist aggregation (61), were used. Furthermore, we will demonstrate that apoE in the context of Aβ1–42 stimulates the appearance of the endosomal/lysosomal pathway in cultured neuronal cells. Likewise, coexpression of APP in apoE transgenic mice increases this pathway in hippocampal neurons.

EXPERIMENTAL PROCEDURES

Reagents—Heparinase I, Lucifer Yellow, bafilomycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma. 1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) was from Avanti (Alabaster, AL). Aβ1–42 from Bachem (Torrance, CA), and LysoSensor DND-160 was from Molecular Probes, Inc. (Eugene, OR). Rabbit anti-LRP antibody was a gift from Dr. E. Koo (University of California, San Diego, CA). LAMP1 (lysosome-associated membrane protein 1) monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human apoE3 and apoE4 (provided by Karl H. Weisgraber) were prepared as described (52).

Cell Culture—Neuro-2a cells and astroglial cells (C6 cells) were transfected with human apoE3 or apoE4 cDNA ligated into the CMV3 vector and selected in medium containing G418 (400 μg/ml). Colonies of transfected Neuro-2a cells were maintained in minimum essential medium (MEM) containing 10% fetal bovine serum and 400 μg/ml G418. C6 cells were grown in Dulbecco’s MEM containing 10% fetal bovine serum and 400 μg/ml G418. Cell colonies were further selected that secreted about 50 ng/ml apoE3 or apoE4 (Neuro-2a cells) and 120 ng/ml (C6 cells) per 24 h as estimated by apoE immunoblots (62).

siRNA Preparation and Transfection—Double-stranded siRNAs specific for LRP were designed with two-base overhangs on each strand. They were chemically synthesized by Dharmacon (Lafayette, CO), according to the following sequences: si-LRP6600, sense 5′-UGGCAU-CUCAGUAACAUUUU-3′ and antisense 5′-AUAGUCUACUGAG-AUGCCAU-3′; si-LRP12348, sense 5′-UGUGUAGUCCAGGAUC-UCAUU-3′ and antisense 5′-UGAAGUCCUCAGACACAU-3′. For transfection, Neuro-2a cells were seeded in 6-well plates and allowed to reach 60% confluence. After 24 h, cells were transfected with siRNA (1.5 μg/ml) with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The transfection complex was diluted in Opti-MEM (final volume, 1 ml), which was replaced after 4 h. Aβ1–42 (20 μM) was added after 48 h after transfection. Cells were lysed for immunoblotting at 72 h. After blocking of LRP expression with siRNA, other cells were homogenized, and the cytosol was isolated by centrifugation and assayed for hexosaminidase activity. Some cells were also examined for apoptosis by measuring cellular DNA fragmentation with cell death detection enzyme-linked immunosorbent assay kits (Roche Applied Science). For LAMP1 immunocytochemistry, cells were fixed in ethyl alcohol.

Measurement of Lyosomal Membrane Stability—The stability of lysosomal membranes was assessed by measuring the leakage of the lysosomal enzyme β-hexosaminidase or the concentrated lysosomal fluorescent dye Lucifer Yellow into the cytosol of Neuro-2a cells. The cells were incubated with Lucifer Yellow (100 μg/ml) for 16 h, washed three times, and incubated with Aβ1–42 at 37°C for 8–40 h. After incubation, the cells were examined by confocal microscopy (Radiance 2000 KR3, Bio-Rad) for Lucifer Yellow in the cytosol. The percentage of cells showing diffuse fluorescence indicative of lysosomal leakage was determined by counting 10–20 cells in each of 12–20 fields at ×60 magnification. β-Hexosaminidase was measured as described (63).

Alteration of Lysosomal pH—The cells were prelabeled with LysoSensor DND-160 (2 μM) for 16 h at 37°C. LysoSensor DND-160 accumulates in lysosomes and, at the low pH in lysosomes, is robustly fluorescent with an emission peak of 540 nm and an excitation wavelength of 365 nm. The labeled cells were incubated with bafilomycin or NH4Cl to raise the pH of endosomes and lysosomes (64–66). As the pH of the lysosomes approaches neutral levels, the fluorescence intensity of the LysoSensor DND-160 is greatly reduced (67). Fluorescence intensity was monitored with a BD FACS Vantage SE flow cytometer (BD Biosciences).

Turbidity Clearance of DMPC—DMPC was dissolved in benzene, lyophilized, resuspended in 10 mM Tris-HCl, 5.5% KBr, 1 mM EDTA (pH 7.4 or 4.0), and preincubated in water at 23.9°C. DMPC multilamellar vesicles (0.5 ml, 500 μg/ml) were added to a cuvette, and 250 μg of apoE was added to the solution. The decrease in turbidity was monitored and recorded with a Hitachi F-2000 spectrofluorometer; excitation and emission were set at 600 nm.

Aβ Preparation—Except where noted, Aβ1–42 was solubilized in Me2SO and water and incubated at 37°C for 24–48 h, as described (51). In studies that compared Arctic and GM6 Aβ, the Aβ was prepared as described for Aβ-derived diffusible ligands (34). Briefly, each form of Aβ was dissolved in cold hexafluorooctanoic acid, incubated at room temperature for 1 h, and centrifuged to remove hexafluorooctanoic acid. The Aβ was dried, resuspended in a small amount of Me2SO, diluted in water, and incubated at 4°C for 24 h. The Aβ solution was centrifuged at 14,000 × g for 10 min, the supernatant was collected, and the protein concentration was determined. To assess Aβ aggregation, each Aβ sample (100 ng) was separated on Tris-Tricine (Bio-Rad) gels and transferred to a nitrocellulose membrane. The membrane was incubated with anti-Aβ monoclonal antibody (3D6). Aβ was visualized by horseradish peroxidase-conjugated secondary antibody and ECL reagent (Amersham Biosciences).

Internalization of 125I-Aβ in Neuro-2a Cells—Neo-, apoE3-, and apoE4-transfected cells were grown to about 90% confluence in MEM containing 10% fetal bovine serum, washed with serum-free medium, and incubated with 125I-Aβ (3 μg/ml) at 37°C for 24 h. The cells were then plated on ice. After five washes with phosphate-buffered saline containing 0.2% bovine serum albumin and two with phosphate-buffered saline, the cells were washed with 10 ml of 10 mM suramin at 4°C for 30 min, and radioactivity was counted with a γ-counter.

Wild type and mutant Aβ were iodinated with Iodogen (Pierce). Aβ (0.5 mg) was incubated with Iodogen beads on ice for 15 min, and the 125I-labeled Aβ was dialyzed against phosphate-buffered saline. Nonspecific cell association was determined by adding a 50-fold excess of unlabeled Aβ.

DNA Fragmentation Assay—Apoptotic cell death was measured with a cellular DNA fragmentation assay as described (51).

LAMP1 Immunoblotting and Immunostaining—Neuro- and apoE-transfected cells were incubated with or without Aβ for 0, 8, or 16 h. The cells were then lysed for LAMP1 immunoblotting or fixed in 100% cold ethyl alcohol for 15 min for immunostaining. Proteins (40 μg) from lysed cells were separated on SDS gels and transferred to nitrocellulose membranes, and the LAMP1 immunoreactive bands were visualized by horse-radish peroxidase-conjugated secondary antibody and ECL reagent (Amersham Biosciences).

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LAMP1 quantitation, as determined by immunoreactivity, was measured with a BioQuant image analysis system (R&M Biometrics, Nash-
vil-le, TN). Briefly, the pixel number (intensity) of immunofluorescence in each cell from Neo- and apoE-transfected cells was measured. The mean pixel number of immunofluorescence was obtained from 10 cells randomly chosen for each condition.

LAMP1 immunocytochemistry was also performed on brain sections of Apoe knockout mice and transgenic mice expressing apoE3 or apoE4 under control of the neuron-specific enolase (NSE) promoter (68), APP (69), or APP and either apoE3 or apoE4 (70, 71). All of the mice were on an Apoe knockout background and studied at ~12 months of age. The construct for the APP mice was platelet-derived growth factor-APP with the Swedish and Indiana mutations (K670N/M671L and V717F, respectively) (69). Immunocytochemistry was performed on 50-μm free floating vibratome sections of brains fixed in paraformaldehyde. The staining procedure is described above. The sections were examined under a BX-60 fluorescence microscope (Olympus, Melville, NY). The intensity of LAMP1 immunofluorescence in the CA3 region of the hippocampus was quantitated with a BioQuant image analysis system, which measures the area of the CA3 region (183,302–197,677 μm²) and determines the mean number of pixels of LAMP1 immunofluorescence in each area.

Co-culture of ApoE-transfected C6 Cells and Neuro-2a Cells—Neotransfected Neuro-2a cells were grown in 6-well culture plates or chamber slides. ApoE-transfected and nontransfected C6 astrocytic cells were grown in separate cell culture inserts with pores (0.3 μm in diameter) in the bottom, which allows free exchange of culture medium through the pores (BD Biosciences). The inserts were placed in culture dishes containing Neuro-2a cells. Aβ1–42 (20 μM) was added to the Neuro-2a cells, and incubation was continued for 24 h. The cells were fixed in cold 100% ethyl alcohol for LAMP1 immunostaining as described above.

RESULTS

Low pH Required for ApoE4 Potentiation of Aβ1–42-induced Lysosomal Leakage and Apoptosis—To assess the effect of pH on lysosomal leakage and apoptosis, lysosomal pH was neutralized with NH4Cl or bafilomycin, a specific inhibitor of the transmembrane component of ATPases, the H⁺ pump responsible for the acidification of late endosomes and lysosomes (72). Incubating Neuro-2a cells (Neo-transfected controls and apoE3- and apoE4-transfected cells) with 5 nM bafilomycin for 8 h resulted in optimal neutralization of lysosomal pH and minimal cellular toxicity (88–95% survival of all three cell lines by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). Neutralization was determined by prelabeling Neuro-2a cells with LysoSensor DND-160, a pH-sensitive fluorescent dye, and the change in fluorescence intensity was measured by flow cytometry. In the absence of bafilomycin, Neuro-2a cells displayed robust LysoSensor fluorescence, indicating a pH of 3–4. This fluorescence intensity was set at 100%. The addition of 1.25, 2.5, and 5.0 nM bafilomycin for 8 h markedly decreased fluorescence intensity (89, 80, and 56%, respectively), indicating that the pH was increased toward neutral.

To examine the effect of increased lysosomal pH on the potentiation of Aβ1–42-induced lysosomal leakage by apoE4, we measured the release of β-hexosaminidase into the cytosol in Neo-, apoE3-, and apoE4-transfected Neo-2a cells in the presence or absence of bafilomycin. Bafilomycin alone did not affect lysosomal leakage of β-hexosaminidase. Compared with apoE3, apoE4 expression significantly increased Aβ1–42-induced lysosomal leakage, as previously shown (51), and bafilomycin treatment resulted in a pronounced decrease in lysosomal leakage, markedly reducing the apoE4 potentiation of lysosomal leakage (Fig. 1). Increasing lysosomal pH with bafilomycin had little effect on Aβ1–42-induced lysosomal leakage and apoptosis in apoE3- and Neo-transfected cells (Fig. 1). These results were confirmed by demonstrating Lucifer Yellow leakage into the cytosol (data not shown).

To assess the effect of neutralizing lysosomal pH and reducing lysosomal leakage on DNA fragmentation, transfected Neuro-2a cells were treated with Aβ1–42 in the presence or absence of bafilomycin. DNA fragmentation in apoE4-transfected cells was markedly enhanced by Aβ1–42, but in the presence of bafilomycin, fragmentation was reduced nearly to the levels seen in Neo- and apoE3-transfected cells treated with Aβ1–42 alone (Fig. 2). The apoE3-transfected cells appeared to be protected against the Aβ1–42 effects of DNA fragmentation. Thus, potentiation of both lysosomal leakage and apoptosis by apoE4 can be blocked significantly by neutralizing lysosomal pH, which may interfere with the formation of reactive apoE4 intermediates in late endosomes and lysosomes.

To confirm that bafilomycin reduced lysosomal leakage by neutralizing lysosomal pH, the cells were treated with NH4Cl (5 mM; 8 h) to increase lysosomal pH. Results identical to those obtained with bafilomycin were observed. In apoE4-transfected Neuro-2a cells, Aβ1–42 increased cytosolic β-hexosaminidase activity by about 5-fold compared with values in Aβ-treated Neo- and apoE3-transfected cells. NH4Cl treatment of the apoE4-transfected cells decreased lysosomal leakage induced by Aβ1–42 to the range seen for Aβ1–42-treated Neo-transfected cells.

Low pH Enhances Destabilization of Phospholipid Vesicles by ApoE4—Previously, we demonstrated that apoE4 has a greater ability than apoE3 to interact with and destabilize phospholipid membranes in vitro, resulting in the release of fluorescent dye trapped within phospholipid vesicles (51). In the present study, large multimellar vesicles composed of DMPC, which scatter visible light, were incubated with human recombinant apoE3 and apoE4 at low (4.0) and neutral (7.4) pH. ApoE4 interacts with such vesicles, disrupts their structure, and forms small apoE4-DMPC complexes that reduce light scattering (turbidity). At pH 7.4, apoE4 was more active than apoE3 in interacting with and reorganizing the phospholipid membranes, as reflected by a reduction of turbidity (~10% difference between apoE3 and apoE4 compared with the buffer control) (Fig. 3). At pH 4, both apoE3 and apoE4 had a greater ability to interact with the phospholipid membranes; however, apoE4 was more reactive (a 25% difference). At a low pH, in two additional separate studies, apoE4 was more effective than apoE3 in

FIGURE 1. Effect of bafilomycin on apoE4 and Aβ1–42-induced lysosomal leakage. Neo-, apoE3-, and apoE4-secreting Neuro-2a cells were incubated with MEM with or without bafilomycin (5 nM). After 20 min, 20 μM Aβ1–42 was added to the culture medium, and cells were incubated for 8 h. Cytosolic β-hexosaminidase activity was determined as described under “Experimental Procedures.” The control value represented the cytosolic hexosaminidase activity in cells incubated without Aβ1–42 or bafilomycin. Values are mean ± S.D. of two independent experiments performed in duplicate, * p < 0.027 for apoE4-transfected cells incubated with Aβ in the absence or presence of bafilomycin.
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To determine whether mutant forms of Aβ can also be internalized by Neuro-2a cells, we used two mutant forms of Aβ1–42: the Arctic mutant, which is prone to aggregation and fibril formation (58, 59), and the GM6 mutant, which is highly resistant to multimerization or aggregation (61). In vitro, wild type and Arctic Aβ1–42 readily formed high molecular weight forms, whereas GM6 Aβ1–42 remained monomeric (Fig. 4).

To determine how these Aβ1–42 mutants affect lysosomal leakage, Neo-, apoE3-, and apoE4-transfected Neuro-2a cells were preincubated with Lucifer Yellow to label lysosomes, treated with 10 μM Aβ1–42 (wild type, Arctic, or GM6) for 40 h at 37 °C, and examined by confocal microscopy. Intact lysosomes are revealed by a bright and punctate fluorescence pattern, whereas lysosomal leakage results in diffuse labeling of the cytosol (51). For quantitation, the percentage of cells in multiple fields displaying a diffuse pattern of fluorescence indicative of lysosomal leakage was determined. Lysosomal leakage was greater in cells treated with wild type or Arctic Aβ1–42, especially those expressing apoE4, than in cells treated with GM6 (Fig. 5).

The aggregation state of Aβ also affected apoptosis. Wild type and Arctic Aβ1–42 enhanced DNA fragmentation in all Neuro-2a cell lines, particularly those expressing apoE4 (Fig. 6). GM6 Aβ1–42 caused little, if any, increase in DNA fragmentation in any of the cell lines. Thus, aggregated Aβ appears to be more cytotoxic, as reported (34–37), and is required for the potentiating effects of apoE4.

Previously, we demonstrated that lysosomal leakage induced by apoE4 and Aβ1–42 required that both proteins be internalized (51). To determine whether mutant forms of Aβ can also be internalized by Neuro-2a cells, we incubated the cells with 125I-labeled Aβ1–42 for 24 h at 37 °C and measured internalized 125I-Aβ. Internalization of GM6 mutant and Arctic mutant Aβ by Neuro-transfected cells was similar to that of wild type Aβ and was 30% greater in apoE3- and apoE4-transfected cells than in Neo-transfected cells (Fig. 7), demonstrating that apoE facilitated the uptake of all forms of Aβ.

These results indicate that lysosomal leakage and apoptosis induced by wild type and Arctic Aβ1–42 and, to a lesser extent, by GM6 did not reflect the internalization of different amounts of mutant Aβ.

Exogenous ApoE4 Incubated with Neo-transfected Neuro-2a Cells Potentiates Aβ-induced Lysosomal Leakage—Recombinant human apoE4 and apoE3 were added to Neuro-2a cells in the presence of Aβ1–42. ApoE4 plus Aβ1–42 significantly enhanced DNA fragmentation as levels of apoE4 were increased; however, apoE3 plus Aβ1–42 did not (Fig. 8). It is likely that the recombinant apoE scavenged lipids from the cells and was at least partially lipidated. Thus, exogenous apoE behaves similarly to apoE secreted by transfected cells.

Dose-dependent Protective Effect of ApoE3 on ApoE4 Potentiation of Aβ-induced Lysosomal Leakage—ApoE4 (7.5 μg/ml) plus Aβ increased DNA fragmentation 2.25-fold compared with Aβ alone (Fig. 9). At increasing concentrations of apoE3 added along with apoE4 plus Aβ, there was a dose response with a decrease in DNA fragmentation, suggesting a dose-dependent protective effect of apoE3 on apoE4 potentiation of Aβ-induced lysosomal leakage. However, even at a 1:1 ratio of apoE4 and apoE3 (7.5 μg/ml), DNA fragmentation remained 1.6-fold
higher than with Aβ alone (p < 0.003). Only when apoE3 was added in a 2:1 excess was the apoE4 potentiation of Aβ-induced apoptosis negated.

**LRP Involved in ApoE4 Potentiation of Aβ-Induced Lysosomal Leakage**—The LRP and cell-surface heparan sulfate proteoglycans act in concert to internalize apoE-containing lipoproteins in the liver (73, 74), and the LRP has been implicated in the uptake of Aβ (41–44). To examine the uptake process in neuronal cells, we treated transfected Neuro-2a cells with heparinase (20 units/ml) or LRP siRNA and assessed lysosomal leakage by measuring cytosolic hexosaminidase activity, siRNA decreased Aβ42-treated apoE4-secreting cells versus Aβ1–42-treated apoE3-secreting cells or Neo cells. p < 0.05, Aβ1–42 and Arctic mutant Aβ versus GM6 mutant Aβ for all three cell types.

Pretreatment of Neuro-2a cells with siRNA that blocks LRP mRNA significantly reduced the level of LRP protein (Fig. 11A). As measured by cytosolic hexosaminidase activity, siRNA decreased Aβ-induced lysosomal leakage in Neo- and apoE3-transfected cells (Fig. 11B). Notably, LRP siRNA very significantly reduced apoE4 potentiation of Aβ-induced lysosomal leakage (Fig. 11B). Aβ-induced apoptosis, determined by DNA fragmentation, was also significantly reduced in all three cell lines, especially in the apoE4-transfected cells (Fig. 11C). Thus, the LRP is critical in Aβ-induced lysosomal leakage and apoptosis and in the potentiation of Aβ-induced effects of apoE4. In studies without siRNA, similar amounts of Aβ (~215 ng/mg of cell protein) were internalized by both apoE3- and apoE4-transfected cells, suggesting that differences in internalized Aβ levels do not account for the apoE4 potentiation of Aβ-induced lysosomal leakage. Furthermore, siRNA decreased Aβ

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**FIGURE 5.** Aβ-induced Lucifer Yellow leakage in Neuro-2a cells. Neo-, apoE3-, and apoE4-secreting cells were grown in chamber slides to ~60% confluence, switched to serum-free medium, and incubated with Lucifer Yellow for 22 h. The cells were washed with serum-free medium to remove excess fluorescent dye, and Aβ1–42 (10 μM) was added at 37 °C for 40 h. After incubation, DNA fragmentation of the cells was determined as described in the legend to Fig. 6, and the cells were placed on ice, and the medium was removed. The cells were incubated with 10 mM sodium citrate to remove Aβ bound to the cell surface, washed, dissolved in 0.1 N NaOH, and counted with a Cytofluorometer. Values are the mean ± S.D. calculated from four wells in a single study.

**FIGURE 6.** Aβ-induced DNA fragmentation in Neuro-2a cells. Neo-, apoE3-, and apoE4-secreting cells were grown to ~90% confluence in MEM containing 10% fetal bovine serum, transferred to serum-free medium, and incubated with Aβ1–42 (10 μM) at 37 °C for 40 h. After incubation, DNA fragmentation of the cells was determined as described in the legend to Fig. 5. Values are the mean ± S.D. calculated from 6 wells in one representative study.

**FIGURE 7.** Internalization of 125I-labeled Aβ by Neuro-2a cells. Neo-, apoE3-, and apoE4-secreting cells were grown as described in the legend to Fig. 6, and the cells were incubated with 125I-labeled Aβ (3 μg/ml) at 37 °C for 24 h. After incubation, the cells were placed on ice, and the medium was removed. The cells were incubated with 10 mM sodium citrate to remove Aβ bound to the cell surface, washed, dissolved in 0.1 N NaOH, and counted with a Cytofluorometer. Values are the mean ± S.D. calculated from two independent studies performed in duplicate, p < 0.05, wild type, GM6 mutant, and Arctic mutant Aβ uptake by apoE3- or apoE4-transfected cells versus Aβ uptake by Neuro-transfected cells (wild type Aβ1–42, APP residue numbers 672–713; GM6 Aβ, APP residue numbers F6905 and L705P; Arctic Aβ, APP residue number E693G).

**FIGURE 8.** Effect of increasing concentrations of exogenous human apoE3 and apoE4 incubated with Neo-transfected Neuro-2a cells with Aβ1–42 (20 μM) on DNA fragmentation. Exogenous apoE (0–15 μg/ml) was added to serum-free medium and incubated for 24 h at 37 °C. The control represents DNA fragmentation with no additions (medium alone). Values are the mean ± S.D. calculated from four wells in a single study. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus Aβ alone without exogenous apoE4.
internalization equally in both apoE3- and apoE4-transfected cells by 30%.

**Aβ1–42 Treatment of Cells and APP Expression in Transgenic Mice Enhances LAMP1 Immunoreactivity**—Immunocytochemical analysis and quantification of LAMP1 have been used to detect increases in the size and number of lysosomes and in the activity of the endosome/lysosome pathway (75). In neurons of patients with AD, lysosomes are increased in size and number (46, 48, 49). To examine the effects of apoE and Aβ1–42 on neuronal lysosomes, we used LAMP1 (a specific lysosomal marker) for quantitation and immunostaining in Neo-, apoE3-, and apoE4-transfected Neuro-2a cells. After 8–16 h of treatment with Aβ1–42, all three cell lines showed increases in LAMP1 (Fig. 12, A–C).

However, Aβ increased LAMP1 immunoreactivity to a greater extent in both apoE3- and apoE4-transfected cells than in Neo-transfected cells (2.5–3-fold), indicating that both isoforms of apoE enhance the response of lysosomes to Aβ.

Examination of brain sections from mice demonstrated significant LAMP1 immunofluorescence in the CA3 region of the hippocampus (Fig. 13A). The intensity of the immunofluorescence was greater in APP, NSE-apoE3/APP, and NSE-apoE4/APP transgenic mice than in Apoe−/−, NSE-apoE3, or NSE-apoE4 mice (Fig. 13B) and was 70–80% greater in the doubly transgenic mice expressing NSE-apoE3 and NSE-apoE4 than in singly transgenic APP mice (Fig. 13, C and D). Thus, apoE increases LAMP1 immunoreactivity in the presence of Aβ in cultured neuronal cells and in the hippocampus of transgenic mice, producing increased amounts of Aβ.

**Astroglia-secreted ApoE Stimulates LAMP1 Immunoreactivity**—Astrocytic C6 cells were transfected with apoE3 or apoE4 cDNA. Transfected cells secreting similar amounts of apoE (120 ng/ml/24 h) into culture medium were co-cultured with Neo-transfected Neuro-2a cells treated with or without Aβ. The studies were performed using inserts that physically separated the two cell types. The apoE3 and apoE4 secreted by transfected astroglial cells significantly enhanced LAMP1...
immunoreactivity in the Neuro-2a cells treated with Aβ1–42 (Fig. 14). Therefore, in the presence of Aβ, apoE secreted by either neurons or astrocytes can increase LAMP1 immunoreactivity and enhance the lysosomal pathway.

DISCUSSION

Previously, we showed that apoE4 potentiates Aβ1–42-induced lysosomal leakage, leading to apoptotic cell death, and destabilizes phospholipid bilayers to a greater extent than apoE3 (51). The present study demonstrates that these effects of apoE4 on lysosomal and phospholipid membrane stability require an acidic pH. Increasing lysosomal pH with bafilomycin, which inhibits the V-type ATPase responsible for lysosomal acidification (64), or NH4Cl markedly reduced the apoE4 potentiation of lysosomal leakage and apoptosis in apoE4-transfected Neuro-2a cells. Although lower levels of Aβ1–42-induced lysosomal leakage and apoptosis were also seen in Neo- and apoE3-transfected cells, blockage of lysosomal acidification did not significantly reduce lysosomal leakage and apoptosis in these cells. These results suggest that alteration in protein conformation, which is modulated by pH, contributes significantly to the effects of both Aβ and apoE on membrane stability, especially in the case of apoE4.

Why does apoE4, but not apoE3, work in concert with Aβ1–42 to enhance the disruption of lysosomal membranes? The answer may lie in the conformational difference between apoE4 and apoE3. ApoE4 is more unstable than apoE3 and can exist uniquely in a molten globular state (52). Many proteins exist naturally in a distinct molten globular state (53). Interestingly, low pH, which occurs in late endosomes and lysosomes, favors the molten globular structure in proteins susceptible to forming reactive intermediates. Importantly, one of the distinctive properties of reactive intermediates is avid binding to phospholipids and membranes and the ability to alter and disrupt membrane structure (52, 53).

We speculate that apoE4 more readily forms these reactive intermediates in lysosomes at low pH, thereby enhancing its ability to interact with and destabilize lysosomal membranes. Aβ can also bind to phospholipid membranes (55, 57), and apoE4 may act in concert with Aβ to destabilize them. Both apoE and Aβ1–42 have a lipid-binding amphipathic α-helical structure. The hydrophilic face of these molecules could interact with the membrane, and the hydrophobic face could penetrate the membrane. This is the mechanism proposed for the interaction of apoproteins with the lipids of lipoproteins (53).

Consistent with the formation of reactive intermediates of apoE4, we previously showed that apoE4 interacts with phospholipid (DMPC) vesicles more readily than apoE3 and disrupts the vesicular membranes,
releasing an intravesicular fluorescent dye and forming a modified apoE/H18528 DMPC disk (51). After treatment with A/H9252 1–42, the vesicles are more sensitive to disruption by apoE4 than by apoE3 (51). In the present study, we showed that pH affected the ability of apoE to alter multilamellar phospholipid vesicles; apoE4 was more active than apoE3 at both neutral and acidic pH, but apoE4 was most active at pH 4. These findings are consistent with the greater ability of apoE4 to form reactive intermediates that can interact with and destabilize membranes. In lysosomes, these effects require A/H9252, because apoE4 alone does not cause lysosomal leakage. When both proteins are present in lysosomes, apoE4 has a potentiating effect that may occur by delaying the degradation of these reactive proteins or by allowing them to interact with each other to form a more highly reactive molecule capable of destabilizing membranes. In contrast, apoE3 protects the cells from the A/H9252 effects relative to apoE4. Regardless of the mechanism, apoE4 enhanced lysosomal leakage and apoptotic cell death.

Blocking the acidification of lysosomes in PC12 cells also inhibited the toxicity of A/B (64), suggesting that A/B toxicity requires endosomal/lysosomal-mediated uptake and modification. Furthermore, acidic pH promotes aggregation of A/B and inhibits its degradation (54, 76). Taking advantage of two A/B 1–42 mutants, one with an enhanced ability to aggregate (Arctic) (58–60) and the other with a greatly reduced ability to aggregate (GM6) (61), we demonstrated that the formation of aggregates is required for apoE4 to enhance A/B-induced lysosomal leakage and apoptosis. Our study also shows that a small portion of the apoE4 potentiation of...
Aβ1–42-induced lysosomal leakage requires heparan sulfate proteoglycans, whereas the LRP is critically important for the Aβ1–42-induced lysosomal leakage and apoptosis in Neo- and apoE3-transfected cells and for a significant portion of the apoE4-enhanced effects. Most studies show that Aβ must form a complex with an LRP ligand, such as apoE or α2-macroglobulin, to be internalized by neuronal cells (41–43), consistent with a role for the LRP in Aβ uptake (42). Our finding that Aβ1–42 is internalized to a greater extent in both apoE3- and apoE4-transfected Neuro-2a cells than in Neo-transfected cells suggests that at least some of the Aβ is taken up as an apoE complex (Fig. 7).

Several lines of evidence have indicated that Aβ stimulates an increase in the number and size of lysosomes in neurons (48). Our studies confirm and extend these observations. In cultured Neuro-2a cells, Aβ1–42 treatment for 8–16 h significantly increased the level of LAMP1, as determined by immunoblots of cellular extracts and by immunocytochemical localization in the cells. ApoE3- and apoE4-transfected cells treated with Aβ1–42 had a similar 4-fold increase in immunocytochemical labeling in the cells. Again, this result probably reflects increased uptake of Aβ1–42 by apoE-secreting cells. Consistent with the role of Aβ in modulating LAMP1 expression in vitro, transgenic mice expressing APP have higher LAMP1 levels by immunocytochemistry in the CA3 region of the hippocampus than Apoe−/−, NSE-apoE3, and NSE-apoE4 transgenic mice. Furthermore, transgenic mice expressing both APP and apoE3 or apoE4 had a 2-fold increase in immunocytochemical LAMP1 labeling in CA3 hippocampal neurons compared with singly transgenic APP mice. These findings are consistent with the notion that APP transgenic mice produce increased amounts of Aβ as evidenced by an increased amyloid plaque burden (77) and that Aβ facilitates the uptake of Aβ, thereby stimulating the lysosomal pathway.

Importantly, we showed that apoE synthesized by neurons (apoE-transfected Neuro-2a cells) or secreted by transfected C6 astroglial cells had the same effect on enhancing the lysosomal pathway. LAMP1 immunofluorescence was increased in Neo-transfected neurons treated with Aβ1–42 in the absence of apoE and to a greater extent in the presence of either apoE3 or apoE4 secreted into the medium by transfected astrocytes.

In conclusion, the pathogenic synergism between apoE4 and Aβ1–42 working in concert to destabilize lysosomal membranes and cause apoptosis may contribute to AD pathology. Lysosomal pH is important in modulating the effects of Aβ1–42 and the effects of apoE4 potentiation of Aβ-induced lysosomal leakage and apoptosis. Consistent with biophysical data, it is reasonable to speculate that the enhanced reactivity of apoE4 reflects its reduced lysosomal leakage and apoptosis. Consistent with biophysical data, it is reasonable to speculate that the enhanced reactivity of apoE4 reflects its reduced lysosomal leakage and apoptosis. Consistent with biophysical data, it is reasonable to speculate that the enhanced reactivity of apoE4 reflects its reduced lysosomal leakage and apoptosis.
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