Astrogial Regulation of Apolipoprotein E Expression in Neuronal Cells

IMPLICATIONS FOR ALZHEIMER'S DISEASE*

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Although apolipoprotein (apo) E is synthesized in the brain primarily by astrocytes, neurons in the central nervous system express apoE, albeit at lower levels than astrocytes, in response to various physiological and pathological conditions, including excitotoxic stress. To investigate how apoE expression is regulated in neurons, we transfected Neuro-2a cells with a 17-kilobase human apoE genomic DNA construct encoding apoE3 or apoE4 along with upstream and downstream regulatory elements. The baseline expression of apoE was low. However, conditioned medium from an astrocytic cell line (C6) or from apoE-null mouse primary astrocytes increased the expression of both isoforms by 3–4-fold at the mRNA level and by 4–10-fold at the protein level. These findings suggest that astrocytes secrete a factor or factors that regulate apoE expression in neuronal cells. The increased expression of apoE was almost completely abolished by incubating neurons with U0126, an inhibitor of extracellular signal-regulated kinase (Erk), suggesting that the Erk pathway controls astroglial regulation of apoE expression in neuronal cells. Human neuronal precursor NT2/D1 cells expressed apoE constitutively; however, after treatment of these cells with retinoic acid to induce differentiation, apoE expression diminished. Cultured mouse primary cortical and hippocampal neurons also expressed low levels of apoE. Astrocyte-conditioned medium rapidly up-regulated apoE expression in fully differentiated NT2 neurons and in cultured mouse primary cortical and hippocampal neurons. Thus, neuronal expression of apoE is regulated by a diffusible factor or factors released from astrocytes, and this regulation depends on the activity of the Erk kinase pathway in neurons.

The e4 allele of the gene encoding apolipoprotein (apo) E has been genetically linked to late-onset familial and sporadic Alzheimers disease (AD) and has a gene-dose effect on the risk and age of onset of the disease (1–5). Individuals with two copies of the e4 allele have a 50–90% chance of developing AD by the age of 85, and those with one copy have about a 45% chance (1, 6). Only about 20% of the general population develops AD by the age of 85 (1).

ApoE is found in amyloid plaques and neurofibrillary tangles, two neuropathological hallmarks of AD (7–13), but its role in their pathogenesis is unclear. ApoE4 has several adverse effects that might explain the association between AD and the e4 allele. It modulates the deposition and clearance of amyloid beta peptides and plaque formation (14–21), impairs the antioxidative defense system (22), dysregulates neuronal signaling pathways (23), disrupts cytoskeletal structure and function (24, 25), and alters the phosphorylation of tau and the formation of neurofibrillary tangles (26–30). However, the mechanisms of these effects are still largely unknown, and it is not known which are the primary effects and which are subsequent or downstream effects.

Initially, apoE was thought to be synthesized in the brain only by astrocytes, oligodendrocytes, and ependymal layer cells (31, 32). However, under diverse physiological and pathological conditions, central nervous system (CNS) neurons also express apoE, albeit at lower levels than astrocytes (33–40). ApoE protein and mRNA are found in cortical and hippocampal neurons in humans (39) and in transgenic mice expressing human apoE under the control of the human apoE promoter (41). In rats treated with kainic acid to induce excitotoxic stress, apoE expression is induced in surviving hippocampal neurons, as determined by in situ hybridization and anti-apoE immunohistochemistry (42). Neuronal expression of apoE can be induced in human brains after cerebral infarction (43). ApoE is also expressed in primary cultured human and rat CNS neurons (44) and in many human neuronal cell lines, including SY-5Y, Kelly, and NT2 cells (32, 45–47).

The cellular origin of apoE appears to influence its effects on AD pathology. Astrocyte-derived apoE3 and apoE4 have different effects on the production, deposition, and clearance of amyloid beta (17–20, 48, 49) and on cholesterol efflux (50, 51). Neuron-derived apoE3 and apoE4 differ in their susceptibility to proteolysis (28, 30) and in their effects on tau phosphorylation (27, 28, 30, 52), lysosomal leakage (53), neurodegeneration (54, 55), androgen receptor deficiency (56), and cognitive decline (56–58). Thus, a better understanding of the regulation of neuronal production of apoE is important for unraveling the mechanisms underlying apoE4-related neurodegenerative disorders.

In this study we used Neuro-2a cells transfected with human apoE genomic DNA, human NT2/D1 neuronal precursor cells, and mouse primary cortical or hippocampal neurons to investigate how apoE expression is regulated in neurons. Here we...
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Demonstrate that neuronal expression of apoE can be induced by astrocyte-conditioned medium and that the astroglial regulation of apoE expression in neurons is controlled by the extra-cellular signal-regulated kinase (Erk) pathway.

Experimental Procedures

Materials—Minimum essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), N2-medium supplements, and fetal bovine serum (FBS) were purchased from Invitrogen. The ECL chemiluminescence detection kit for Western blots was from Amersham Biosciences. Recombinant human apoE3 and apoE4 were prepared as described (28). Polyclonal goat anti-human apoE and inhibitors of the Erk (U0126, c-Jun-N-terminal kinase (JNK) (c-Jun-N-terminal kinase inhibitor 1), and p38 (SB203580) pathways were from Calbiochem. Horseradish peroxidase-coupled anti-goat IgG was from Dako (Carpinteria, CA).

Cell Cultures—Neuro-2a cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C in a humidified 5% CO₂ incubator in MEM containing 10% FBS supplemented with nonessential amino acids, penicillin, and streptomycin. Human neuronal precursor NT2/D1 cells were kindly provided by Dr. Virginia M.-Y. Lee (University of Pennsylvania School of Medicine, Philadelphia, PA) and maintained in Opti-MEM-I (Invitrogen) containing 5% FBS and penicillin/streptomycin (59). The cells were treated with retinoic acid to induce differentiation into NT2 neurons (59, 60). Primary cultures of cortical or hippocampal neurons were prepared from embryonic day 17 wild-type or apoE-null mice (61, 62). As determined by immunostaining with cell-specific antibodies, >95% of cells in 6-day-old cortical cultures in vitro are positive for neuron-specific enolase (63).

Primary astrocytes were prepared from embryonic day 17 wild-type or apoE-null mice (64). As determined by immunostaining with cell-specific antibodies, >95% of cells in 6-day-old astrocyte cultures in vitro are positive for glial fibrillary acidic protein (63).

Preparation of Astrocyte-conditioned Medium—Rat astrocyte C6 cells were grown to 80% confluence in DMEM containing 20% FBS in T175 flasks, washed 3 times with serum-free DMEM, and incubated with 10 ml of serum-free DMEM for 24 h. The medium was collected, centrifuged to remove cellular debris, and stored at -80°C.

The apoE-null mouse primary astrocytes were grown in DMEM containing 20% FBS in 20-cm dishes, washed 3 times with serum-free DMEM, and incubated with 10 ml of serum-free DMEM for 24 h. The conditioned medium was collected, centrifuged, and stored at -80°C.

Preparation of C6 Neurons—Rat astrocyte C6 cells were cotransfected with a neomycin resistance gene by using LipofectAMINE (Invitrogen) (65). The construct consisted of 5 kilobases of 5′flanking region of the neomycin resistance gene by using LipofectAMINE (Invitrogen) (65). The construct consisted of 5 kilobases of 5′flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases of 3′-flanking region, four exons, three introns, and 8 kilobases (65). The construct consisted of 5 kilobases of 5′flanking region, four exons, three introns, and 8 kilobases of 3′flanking region of the neomycin resistance gene by using LipofectAMINE (Invitrogen) (65). The construct consisted of 5 kilobases of 5′flanking region, four exons, three introns, and 8 kilobases of 3′flanking region of the neomycin resistance gene by using LipofectAMINE (Invitrogen) (65). The construct consisted of 5 kilobases of 5′flanking region, four exons, three introns, and 8 kilobases of 3′flanking region of the neomycin resistance gene by using LipofectAMINE (Invitrogen) (65). The construct consisted of 5 kilobases of 5′flanking region, four exons, three introns, and 8 kilobases of 3′flanking region of the neomycin resistance gene by using LipofectAMINE (Invitrog...

Preparation of Cell Lysates and Western Blotting—Neuro-2a cells were transfected with apoE, NT2/D1 neuronal precursor cells, fully differentiated NT2 neurons, and mouse primary cortical and hippocampal neurons were grown to 80% confluence in six-well plates and then incubated with medium conditioned by C6 or primary astrocytes (1:1 dilution in serum-free MEM or Opti-MEM) in the presence or absence of an inhibitor of the Erk (U0126, 5 μM), JNK inhibitor 1 (5 μM), or p38 inhibitor-1 and SB203580 (2.5 μM) pathway at noncytotoxic doses for 24 h. The cells were harvested, lysed in ice-cold lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet p-40, 0.5% sodium deoxycholate, and a mixture of protease inhibitors) for 30 min, and centrifuged at 13,000 rpm for 15 min. Proteins in the supernatant were subjected to SDS-PAGE and detected by anti-apoE Western blotting (28). The full-length apoE bands were scanned, normalized to cellular proteins, and reported as arbitrary units.

In some experiments 95% primary cortical neurons from apoE-null mice were mix-cultured with 5% primary astrocytes from wild-type mice in serum-free medium for 24 h. ApoE in the cell lysate and medium was then determined as described above.

Preparation of apoE mRNA by Real-time RT-PCR—Total RNA from apoE3- or apoE4-transfected Neuro-2a cells treated with medium conditioned by C6 or primary astrocytes was isolated with the RNeasy mini kit (Qiagen). RT-PCR was performed with 10 ng of total RNA and the RT-PCR kit from Invitrogen. The RT-PCR product of apoE was 75 bp. The RT-PCR product of the glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal standard, was 112 bp. Different amounts of input RNA, cycling temperatures, and cycle numbers were evaluated to assure a linear response of the apoE and the glyceraldehyde-3-phosphate dehydrogenase signals. The RT-PCR products were measured in real time with an ABI-PRISM-7700 sequence detector from Applied Biosystems (Foster City, CA). The apoE mRNA levels were then calculated and normalized to the internal glyceraldehyde-3-phosphate dehydrogenase RNA standards.

Immunocytochemistry—Mouse primary cortical neurons were grown for 6 days on glass coverslips (Fisher) coated with polylysine in 24-well plates, washed 3 times with serum-free medium, and incubated for 24 h with serum-free medium conditioned by C6 astrocytes (1:1 dilution in serum-free Opti-MEM). After incubation the neurons were fixed, permeabilized, and stained with polyclonal anti-apoE and a fluorescein-coupled secondary antibody (Vector Laboratories) (28). The immunofluorescent-labeled slides were mounted in VectorShield (Vector Laboratories) and viewed with an MRC-1024 laser scanning confocal system (Bio-Rad) mounted on an Optiphot-2 microscope (Nikon).

Statistical Analysis—Results are reported as the mean ± S.D. Differences were evaluated by t test or analysis of variance.

Results

Astroglial Regulation of ApoE Expression in Neuro-2a Cells—To investigate how apoE expression is regulated in neurons, we transfected Neuro-2a cells with a large fragment of human apoE genomic DNA (Fig. 1A) (66) and selected stable cell lines expressing matched levels of apoE3 and apoE4 (Fig. 1B). All of the transfectants expressed low levels of apoE (<100 ng of apoE/mg of cell protein/24 h), suggesting that the apoE gene promoter is not very active in Neuro-2a cells. However, medium conditioned by an astrocytic C6 cell line or apoE-null mouse primary astrocytes increased apoE secretion 4–10-fold and apoE mRNA expression 3–4-fold, with no difference between the isoforms (Fig. 2, A–C). These results suggest that astrocytes secrete a factor or factors that regulate apoE expression in Neuro-2a cells. In preliminary studies, the putative astrocyte-derived factor was sensitive to heat or protease treatment, suggesting a protein (data not shown).

Astroglial Regulation of ApoE Expression in Neuro-2a Cells Is Dependent on the Erk Pathway—Next, we sought to identify the pathway involved in the astroglial regulation of apoE expression in Neuro-2a cells. At noncytotoxic levels, the Erk pathway inhibitor U0126 almost completely abolished the increased expression of apoE3 (Fig. 3, A and B) or apoE4 (data not shown) induced by astrocyte-conditioned medium. Inhibitors of other mitogen-activated protein kinase pathways, JNK inhibitor-1 and SB203580 (for the p38 pathway), had no significant effects on apoE expression (Fig. 3A), although they did inhibit the JNK and p38 pathways, respectively, in control...
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ApoE Expression in Human NT2 Neurons Is Regulated by Astroglia—To avoid a potential artificial effect of transgene integration on apoE expression in transfected Neuro-2a cells, we also studied human neuronal precursor NT2/D1 cells. These cells expressed apoE constitutively (Fig. 4, three far left lanes), as shown previously (47). When the cells were treated with retinoic acid to induce differentiation, apoE expression increased and then diminished (Fig. 4, six middle lanes), suggesting that it is regulated by signaling pathways related to cell proliferation or differentiation. Incubation of fully differentiated human NT2 neurons with medium conditioned by C6 astrocytes rapidly up-regulated apoE expression (Fig. 4, three far right lanes), suggesting that an astroglia-derived factor or factors can also induce apoE expression in fully differentiated human NT2 neurons. Similar results were also obtained from fully differentiated human NT2 neurons treated with medium conditioned by apoE-null mouse primary astrocytes (data not shown).

Astroglial Regulation of ApoE Expression in Mouse Primary Neurons Is Also Dependent on the Erk Pathway—To study neuronal apoE expression in a more physiological scenario, we next assessed apoE expression in mouse primary neurons. In both cortical neurons (Fig. 5A) and hippocampal neurons (Fig. 5B), apoE was expressed at low levels; the levels increased in response to incubation with astrocyte-conditioned medium, and the increase was almost completely abolished by U0126 (Fig. 5, A and B), consistent with involvement of the Erk pathway. Similar results were obtained from mouse primary neurons treated with medium conditioned by apoE-null mouse primary astrocytes (data not shown). As shown by anti-glial fibrillary acidic protein Western blotting, the neuronal cultures contained undetectable levels of astrocytes (Fig. 5C). The absence of detectable apoE in the cell lysate and medium of a mixed culture containing 95% primary cortical neurons from apoE-null mice and 5% primary astrocytes from wild-type mice (our
primary neuronal cultures contained >95% neurons) further proved that the apoE detected in the primary neuronal cultures came from neurons but not the potentially contaminated astrocytes (data not shown). These results indicate that mouse primary cortical and hippocampal neurons also express low levels of apoE, which can be further stimulated by an astroglia-derived factor or factors through the Erk pathway. Interestingly, only ~10% of apoE generated in mouse primary cortical neurons was secreted into the culture medium during a 24-h incubation (Fig. 6A), whereas more than 60% of apoE generated in mouse primary astrocytes was secreted (Fig. 6B). Similar results were obtained during a 12-h incubation (data not shown). These results suggest that neuron-generated apoE tends to accumulate intracellularly, whereas astrocyte-generated apoE is more likely to be secreted. Anti-apoE immunofluorescent staining revealed that apoE generated in mouse primary neurons in response to treatment with medium conditioned by C6 astrocytes was packed into vesicles distributed along the neurites (Fig. 6C). This finding suggests that the neuron-generated apoE is transported from the soma to growth cones to support neurite outgrowth (24, 25, 67).

**DISCUSSION**

This study demonstrates that neuronal expression of apoE can be induced by a factor or factors secreted by astrocytes and that the astroglial regulation of apoE expression in neurons is controlled by the Erk pathway. The astroglial regulation of neuronal apoE expression may be relevant to neurodegeneration related to aging and AD. Glial fibrillary acidic protein, a marker of astroglial cells, increases with age (68), especially in the hippocampus and entorhinal cortex, which are susceptible to neurodegeneration in AD (69–72). Astrocytosis, the proliferation and activation of astrocytes (73), is more pronounced in AD brains than in normal brains and usually occurs in areas surrounding neuritic plaques (74, 75) and neurons bearing neurofibrillary tangles (75–78). Importantly, activated astrocytes in AD brains secrete many cytokines, including interleukins 1 and 6, tumor necrosis factor-α, and acute-phase inflammatory proteins, such as α2-macroglobulin and C-reactive protein (79–82). We speculate that in AD brains the astrocyte-derived factor or factors, whose effects were observed in the current study, induce neuronal expression of apoE, as observed in previous studies (35, 39). None of the astrocyte-derived cytokines mentioned above altered apoE expression in neuronal cells (data not shown). Additional studies are needed to identify the factor or factors that induce neurons to express apoE.

The involvement of the Erk pathway in astroglial regulation of apoE expression in neurons is intriguing. The Erk pathway plays a crucial role in cell proliferation and differentiation (83). In AD and stroke patients, significant numbers of neurons in the neocortex and hippocampus appear to reenter the cell cycle (84–92); in many of them, the Erk pathway was activated (93–97), perhaps by amyloid β peptides, oxidative stress, or astroglia-derived cytokines (94, 97, 98). Thus, it is reasonable to speculate that neuronal activation of the Erk pathway in brains of AD or stroke patients participates in the regulation of apoE expression in neurons. ApoE is expressed in some neurons in AD or stroke brains (39, 43). Interestingly, apoE can also regulate cell proliferation (99, 100) and activation of the Erk pathway in smooth muscle cells (101) and rat primary hippocampal neurons (102). Thus, there could be a feedback loop between Erk pathway activation and apoE expression in neurons.

Increased apoE levels have been demonstrated in the plasma...
that leads to activation of certain tissue-specific cis-acting regulatory elements of APOE that might be modified by APOE promoter polymorphisms.

Based on the current and previous studies (32–39, 41–47), we hypothesize that acute injury, such as head trauma, or chronic injury, such as damage related to amyloid β deposition or oxidative stress, of the CNS causes astrogliosis proliferation and activation (astrocytosis), leading to secretion of a factor or factors that activate the Erk pathway and induce neuronal expression of apoe (Fig. 7). Neuronal expression of apoe in response to brain injury probably promotes neuronal repair and remodeling and protects neurons from injury (42, 132–134). However, in apoe4 carriers, intraneuronal proteolytic processing of apoE4 and generation of neurotoxic apoE4 fragments (28, 30) may turn a neuroprotective response into a pathogenic process. In this scenario it may be advantageous to inhibit neuronal production of APOE. Our results suggest that this might be accomplished by inhibiting astrocytosis, blocking specific diffusible astroglial factors, or modulating the Erk pathway in neurons.

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