Apolipoprotein E Is Synthesized in the Retina by Müller Glial Cells, Secreted into the Vitreous, and Rapidly Transported into the Optic Nerve by Retinal Ganglion Cells*

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We have investigated the synthesis and transport of apoE, the major apolipoprotein of the central nervous system, in the retina of the living rabbit. Four hours after the injection of [35S]methionine/cysteine into the vitreous, 44% of [35S]Met/Cys-labeled apoE is in soluble and membrane-enclosed retinal fractions, while 50% is in the vitreous. A significant amount of intact [35S]Met/Cys-labeled apoE is rapidly transported into the optic nerve and its terminals in the lateral geniculate and superior colliculus within 3–6 h in two distinguishable vesicular compartments. Müller glia in cell culture also synthesize and secrete apoE. Taken together, these results suggest that apoE is synthesized by Müller glia and secreted into the vitreous. ApoE is also internalized by retinal ganglion cells and/or synthesized by these cells and rapidly transported into the optic nerve and brain as an intact molecule. We discuss the possible roles of retinal apoE in neuronal dynamics.

Apolipoprotein E, a 36-kDa glycoprotein, is a component of a number of circulating plasma lipoproteins, including very low density lipoproteins, high density lipoproteins, and chylomicron remnants (1). Its primary function appears to be that of a recognition ligand for the receptor-specific removal of cholesteryl ester-rich lipoproteins from the circulation (2, 3). It also plays a role in local transport of cholesterol, as seen in nerves of both peripheral (4, 5) and central nervous systems (reviewed in Ref. 6) and has been implicated in mediating immune responses and cell proliferation, processes that may not be related to its role in local transport of cholesterol, as seen in nerves of both peripheral (4, 5) and central nervous systems (reviewed in Ref. 6). It is highly likely that apoE plays a major role in cholesterol and lipid transport in the central nervous system (reviewed in Ref. 6), and we have employed the in vivo rabbit retina to probe the synthesis, intracellular transport, and metabolism of central nervous system proteins including kinesin, the motor for anterograde transport (12, 13), and the β-amyloid precursor protein (APP) (14, 15). Since it has been shown recently that individuals having the e4 allele of apoE are at high risk for Alzheimer’s disease (16), we decided to use this widely accepted model of normal adult retinal protein synthesis and metabolism (e.g. Refs. 17 and 18) to examine the synthesis and transport of apoE. In this report, we present evidence, obtained by the injection of [35S]methionine/cysteine into the vitreous chamber that overlies the retina, that apoE is synthesized in significant quantities in vivo by rabbit Müller cells, the predominant glial cell of the retina. ApoE is then transferred, presumably after secretion, into the vitreous. A substantial quantity of newly synthesized apoE is also subsequently transported as an intact molecule into the optic nerve by retinal ganglion cells (RGCs) through vesicular anterograde transport. We discuss the possible role of this pathway in axonal lipid metabolism and the possible interactions of apoE with APP or its metabolites.

MATERIALS AND METHODS

Preparation of [35S]Met/Cys-labeled Gradient Fractions—The methods of isotope injection, membrane isolation, and density gradient analysis of retinal protein and retina have been used in our laboratory for several years and are detailed in several reports (e.g. Refs. 14 and 19). Adult male albino rabbits (6 pounds) were anesthetized with 1 mL of 5% sodium pentobarbital (intravenous), and 2 drops of local anesthetic (0.5% proparacaine HCl) were put in each eye. One hundred-unit insulin syringes with 28-gauge needles were used to introduce 0.5 μCi (50 μl) of [35S]Met/Cys into the vitreous of one or both eyes. The animals awakened within 1–2 h and showed no signs of discomfort or redness of the eye. At the various times following injection of labeled, subcellular fractionation was done using a modification of the method of Lorenz and Willard (20). Within 10 min of sacrifice by intravenous injection of 100 mg/kg sodium pentobarbital, optic nerves and optic tracts (ON/OT) were rapidly dissected and placed in 7 ml of ice-cold homogenization buffer (1 mM triethanolamine, 320 mM sucrose containing 30 units/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin. Homogenization consisted of three strokes in a motor-driven Teflon/glass homogenizer (0.005-inch clearance) in a volume of 7 ml, followed by three strokes in a glass Dounce homogenizer in a volume of 10 ml. Homogenates were diluted to 40 ml with homogenization buffer.
and spun at 1200 \times g for 7 min. The resulting supernatant was then spun at 100,000 \times g for 60 min. The resulting pellet was resuspended in 2 ml of homogenization buffer and either used for immunoprecipitations directly or loaded onto a discontinuous gradient consisting of 2 ml each of 20/26/32/37/45% sucrose (w/w) in homogenization buffer. These gradients were spun at 150,000 \times g for 1 hr. Twenty-four 0.5-ml fractions were collected from each gradient. A very similar protocol was employed for the retina (21).

Biochemical and Immunochromical Methods—Protein determinations were carried out by the method of Bradford (22). SDS gel electrophoresis was performed on 10 or 10–20% gels by the method of Laemmli (23). Gels were prepared for autoradiography by immersing them in a PhosphorImager (24), or that of films using a Molecular Dynamics laser densitometer. Immunoprecipitations were performed as described previously (19). Briefly, samples were dissolved in 1% SDS, 1% Triton X-100 in phosphate-buffered saline, and undissolved material was removed by centrifugation at 15,000 \times g for 1 min in an Eppendorf centrifuge. Samples were incubated overnight at 4°C with agitaton with the appropriate primary antibody previously bound to Sepharose bead-linked anti-IgG. The beads were then centrifuged in an Eppendorf centrifuge and washed six times in phosphate-buffered saline containing 1% Triton X-100 and 1 M NaCl. The final samples were then subjected to SDS-polyacrylamide gel electrophoresis. Quantitation of gels was performed using a Molecular Dynamics PhosphorImager, or that of films using a Molecular Dynamics laser densitometer.

Müller Glial Cell Culture—Müller glial cells were cultured from adult white rabbits following the procedure described previously by Edwards et al. (24), which was modified from the method of Lewis et al. (25). Animals were killed by intravenous sodium pentobarbital (100 mg/kg). Eyes were enucleated, and the anterior half and vitreous were discarded. The retinas were removed, rinsed, cut into 4 × 4-mm fragments, and incubated for 30 min in 0.5 mg/ml protease Nagarse (Sigma) in Ca2+/Mg2+-free balanced salt solution at 37°C. The tissue was rinsed, resuspended in balanced salt solution containing 0.1 mg/ml DNAse and 0.5 mg/ml bovine serum albumin, and dissociated into a single-cell suspension by passing the retinal fragments through the mouth of a 10-ml plastic syringe 10 times. The cells were gently centrifuged; resuspended in culture medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum); seeded in one-25 cm² culture flask per two retinas; and maintained at 37°C in a 5% CO2, 95% air. The medium was changed every 3–4 days. Retinal membranes were then collected, and the cells were rinsed one time with Hanks’ balanced saline and dissolved in immunoprecipitation buffer.

RESULTS

ApoE Synthesis in the Retina and Rapid Transport in the ON/OAT—We injected 0.5 mCi of [35S]Met/Cys into each vitreous. Three hours later, we removed the retinas, vitreous, ON, OT, and lateral geniculate nuclei (LGNs). Membrane and soluble fractions from the retina and ON/OT and the vitreous were subjected to immunoprecipitation with a specific antibody against rabbit apoE (26). The results are shown in Fig. 1. We found a major polypeptide of 36 kDa specifically immunoprecipitated by the apoE antiserum from both the retinal membrane and soluble fractions. We also found a significant amount of the immunoprecipitated 36-kDa polypeptide in the ON/OT membrane fraction, but very little in the soluble fraction. We also detected the 36-kDa band in the LGN. A large quantity of the 36-kDa polypeptide was also specifically immunoprecipitated from the vitreous.

We repeated the experiment using a 6-h interval between [35S]Met/Cys injection and sacrifice. A similarly significant quantity of specifically immunoprecipitated 36-kDa polypeptide was still found in the ON/OT membrane fraction and in the LGN, in addition to a significant amount of the immunoprecipitated 36-kDa polypeptide in the retinal fractions (Fig. 2). These results indicate that there is a very active synthesis of apoE in the retina, and some of this apoE is rapidly transported down the ON/OT in transport vesicles. We still did not, however, find a detectable quantity of soluble apoE in the ON/OT. This suggests that apoE does not gain access to the axonal cytoplasm or the extracellular milieu of the ON/OT, at least for 6 h following synthesis and transport.

These data provide strong evidence for the rapid transport of apoE into the optic nerve since detectable apoE reached the termination of the 3-cm long optic nerve within 3 h after synthesis, conforming to the known speed of rapid transport, 1

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cm/h. Because we found little apparent quantitative difference in the amounts of apoE transported into the ON/OT at 3 and 6 h after [35S]Met/Cys injection, we chose an intermediate time point (4 h) to carry out a quantitative distribution of apoE in the various retinal and ON/OT fractions. Table I shows the results. As can be seen, over 50% of the total newly synthesized apoE was in the vitreous, while a slightly smaller quantity was in the retinal membranes, presumably from either the Müller apoE was in the vitreous, while a slightly smaller quantity was in the various retinal and ON/OT fractions. Table I shows the results. As can be seen, over 50% of the total newly synthesized apoE was in the vitreous, while a slightly smaller quantity was in the retinal membranes, presumably from either the Müller apoE was in the vitreous, while a slightly smaller quantity was in the various retinal and ON/OT fractions. Table I shows the results. As can be seen, over 50% of the total newly synthesized apoE was in the vitreous, while a slightly smaller quantity was in the retinal membranes, presumably from either the Müller apoE was in the vitreous, while a slightly smaller quantity was in the various retinal and ON/OT fractions. Table I shows the results. As can be seen, over 50% of the total newly synthesized apoE was in the vitreous, while a slightly smaller quantity was in the retinal membranes, presumably from either the Müller  

| Compartment | Total apoE | %   |
|-------------|------------|-----|
| Retina      | 38.7       |     |
| Membrane    | 5.4        |     |
| Soluble     | 4.1        |     |
| Vitreous    | 50.6       |     |
| ON/OT       | 0.3        |     |
| Membrane    | 0.9        |     |

When we immunoprecipitated apoE from the three peaks described above, we obtained the results shown in Fig. 3. At 3 h after labeling, all the apoE was found in peak 2, while after 6 h, radioactive apoE was found in both peaks 2 and 3. The most reasonable interpretation of these results is that apoE is carried into the axon in two distinct transport vesicles with different kinetic and biochemical properties.

ApoE Synthesis in the Müller Cell—Given the evidence that, in other portions of the central nervous system, apoE is synthesized in astrocytes (10), a reasonable interpretation of the data presented above is that the Müller glial cell, a bipolar glial cell that is the major glial cell of the retina, is a strong candidate cell type for apoE production. Its cell body is in the inner nuclear layer, and one process reaches the vitreal surface, while the other reaches the outer discs of the photoreceptors on the outside of the retina. These cells take up a significant quantity of vitreally injected [35S]Met/Cys and utilize it to synthesize proteins, including APP (14).

We therefore cultured Müller cells from adult rabbits by the technique described by one of us (24). These cells at early stages of growth have the bipolar appearance of Müller cells in vivo (24). They also stain positively for GFAP, a widely accepted marker for these cells. Routinely, our cultures are over 95% positive for this marker (Fig. 4). They are also positive for retinal-binding protein (24).

We labeled confluent Müller cells for 4 h with [35S]Met/Cys and carried out immunoprecipitation with anti-apoE with both cells and medium. The cells synthesized a 36-kDa polypeptide and secreted it into the medium (Fig. 5). The cells also produced a significant quantity of two high molecular mass isoforms of APP, which is also characteristic of Müller cells in vivo (14). 

**FIG. 3.** Subcellular location of apoE processing in the optic nerve. Three- and 6-h post [35S]Met/Cys-labeled optic nerve membranes were subjected to sucrose density gradient separation (18). Fractions from peak 1 (light membranes), peak 2 (intermediate membranes), and peak 3 (heavy membranes) were separately pooled and subjected to immunoprecipitation using a goat anti-rabbit apoE antibody. Immunoprecipitates were separated on 10% acrylamide gels, followed by autoradiography. Lane 1, peak 3; lane 2, peak 2; lane 3, peak 3. The location of apoE is indicated by arrows. Bars denote the positions of molecular mass protein markers: 80, 49, 32, and 18 kDa.

**FIG. 2.** ApoE synthesis in the retina and rapid axonal transport into the optic nerve at 6 h. Rabbits were given vitreal injections of [35S]Met/Cys and sacrificed 6 h later. A retinal membrane fraction (R-M), optic nerve membranes (ON-M), a soluble fraction (ON-S), and LGN were immunoprecipitated with apoE antibody (I) and with normal goat serum (P), followed by electrophoresis and autoradiography as described for Fig. 1. The location of apoE (~36 kDa) is indicated by arrows. Bars denote the positions of molecular mass protein standards: 106, 80, 49, and 32 kDa.

**Table I**

Percentage of the total apoE radioactivity in each compartment of the retina, ON/OT, and LGN. Four hours after a vitreal injection of 0.5 μCi of [35S]Met/Cys in one eye, membrane and soluble fractions were prepared from retina and ON/OT as described under "Materials and Methods." LGN membranes were prepared as well. Each fraction was immunoprecipitated with anti-apoE and subjected to SDS-polyacrylamide gel electrophoresis. Radioactivity in each fraction was quantitated in a Molecular Dynamics PhosphorImager. No radioactive apoE was found in any fraction obtained from the uninjected eye.

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**DISCUSSION**

The in vivo data presented here suggest that retinal apoE is synthesized at least in part by Müller glial cells and secreted into the vitreous and extracellular compartment of the retina. The apoE synthesized in the retina is also internalized by Müller cells and carried into the axon in two distinct transport vesicles with different kinetic and biochemical properties. 

The bipolar shape of the Müller cell, with a long process extending in each direction, allows it to secrete apoE at essentially any point in the retina, and Müller cells directly contact...
all nerve and photoreceptor cells in the retina. It is therefore ideally situated to supply apoE and any lipid moieties it associates with to all the cells of the retina including the RGCs. Since there appears to be a large pool of newly synthesized, soluble apoE in the retina itself and an even larger pool in the vitreous, it is likely that apoE is released directly into the vitreous as well as into the extracellular space of the retina. The axons of the RGCs, which are non-myelinated until they pass through the retina, lie in the perfect position to internalize the vitreal apoE, via surface receptors. Müller cells synthesize and secrete apoE in cell culture. Also in other regions of the central nervous system as well as in the periphery, apoE is synthesized mainly by glial cells and to some degree by macrophages, but not by neurons (6).

However, there is still the possibility that the apoE we detected in the optic nerve in the brain has been synthesized by the RGCs themselves, although our data provide no evidence for this possibility. We hope to resolve this question by use of in situ RNA hybridization. We should also note that some of the apoE could be synthesized by retinal astrocytes that are associated with the RGC axons located near the center of the retina (27). These cells are, however, much less abundant than Müller cells.

There are three possible sites of uptake for apoE on the retinal ganglion cell: the dendrites, the cell body, and the proximal axons of the RGCs, which are non-myelinated. It is very likely that apoE is taken up via a high affinity receptor, either the LDL receptor or LRP. While the distribution of these two receptors in the retina is not known, it is known that in the brain, the LRP is present on neuronal cell bodies, dendrites, and proximal axons (11). LDL receptors are found on astrocytes, but not on neurons (11).

We are now determining the distribution of the LRP and LDL receptor in the retina. We have also attempted to obtain direct evidence for receptor-mediated uptake of apoE into retinal ganglion cells by the injection of radiolabeled apoE into the vitreous with and without excess unlabeled apoE. These experiments have been unsuccessful, probably because of the large quantity of endogenous apoE in the vitreous.

Whatever site(s) of uptake are utilized, the vesicular transport pathway utilized by apoE (and potentially by its receptor) appears likely to be one of two types that are not mutually exclusive. One is a transcytotic pathway involving internalization into endosomal vesicles in either dendrites and/or the cell soma and transfer to axonally bound transport vesicles. This pathway is analogous to the basolateral-to-apical transcytosis pathway utilized by epithelial cells (28). There is recent evidence, utilizing the transfected IgA receptor, for the existence of a dendritic/cell body-to-axonal transcytotic pathway in cultured neurons (29). Also very recently, evidence for APP transcytosis has been obtained in cultured hippocampal neurons (30).

The delayed appearance of radioactive apoE in peak 3 of the sucrose density gradient-separated transport vesicles may be

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**Fig. 4. Immunostaining of cultured adult rabbit Müller glia with anti-GFAP.** Cultures of Müller glia were immunostained with anti-GFAP serum followed by a horseradish peroxidase-labeled second antibody as described under "Materials and Methods." a, cells incubated with anti-GFAP and viewed with a phase-contrast microscope; b, cells incubated with anti-GFAP and viewed with bright-field optics to accentuate the peroxidase-labeled filamentous network; c, cells stained with nonimmune IgG and viewed with phase-contrast optics, showing no reaction product. Magnification x 200.

**Fig. 5. Immunoprecipitation of apoE and APP from rabbit Müller cells and medium.** Cultured Müller cells were metabolically labeled for 4 h by adding 0.2 mCi of [35S]Met/Cys per ml. Labeled cells (lanes 1, 2, 5, and 6) and medium (lanes 3, 4, 7, and 8) were immunoprecipitated for apoE (lanes 1–4) and APP (lanes 5–8). Precipitates were run on a 10–20% gradient acrylamide gel, followed by autoradiography to detect labeled proteins. A goat anti-rabbit apoE antibody (lanes 2 and 4) and a rabbit anti-APP C-terminal antibody (CB) (lanes 6 and 8) were used for detecting apoE and APP, respectively. Control immunoprecipitates were carried out using normal goat serum (lanes 1 and 3) and normal rabbit serum (lanes 5 and 7). Bars denote the positions of molecular mass protein markers: 205, 116, 80, 49, 32, 27, and 18 kDa. The locations of the apoE bands (lanes 2 and 4) and APP bands (lane 6) are indicated by arrows.
explained by the existence of such a transcytotic pathway. However, it also could be explained by the delayed export of peptide-containing secretory vesicle precursors that has been previously described in this system (19, 21). It is possible that all apoE molecules are taken up into the proximal axons and some are immediately transported in a forward direction, while the other portion is taken back to the Golgi apparatus and sorted into a peptide granule.

Another pathway would involve direct uptake of apoE into the proximal axon and anterograde transport toward the axon terminal. This is likely to be the pathway utilized by the plasma membrane-targeted transport vesicle found in peak 2 of these gradients. This pathway is very rapid as opposed to the peak 3 pathway, with detectable apoE in the LGN by 3 h after labeling. To our knowledge, there is no evidence at present for the existence of such a pathway in neurons. However, there are apoE receptors localized on the proximal axons in the brain (11).

Finally, apoE in either peak 2- or 3-containing transport vesicles could result from the synthesis of apoE in the RGCs, followed by packaging into secretory vesicles with different densities and kinetic behavior. We have recently found that newly synthesized apoE is rapidly transported in two discrete classes of secretory vesicle as well (15).

Irrespective of the pathway involved, what would be the function of such a pathway? One possibility would be the use of apoE as a lipid carrier to supply the axon’s needs for particular lipids. Consistent with this possibility, we have recently found that a high percentage of newly synthesized apoE is associated with lipids in the medium of cultured Müller cells as well as in the vitreous. There is recent evidence that while axons and nerve terminals in particular can re-synthesize phospholipids, they cannot synthesize cholesterol and glycolipids and therefore depend on transport from the cell body to supply these essential molecules (31), analogous to the situation with regard to proteins (32). There is evidence that apoE produced by astrocytes donates lipids and cholesterol to central nervous system neurons engaged in extensive axon growth and synaptic remodeling following lesioning (reviewed in Ref. 6). Also, it was very recently reported that homologous apoE-deficient mice display a significant loss of synapses and disruption of the dendritic cytoskeleton with age as well as decreased hippocampal compensatory synaptogenesis after lesioning of the projections from the entorhinal cortex (33). These changes would be consistent with long-term damage to these cells caused by a deficit in apoE-supplied lipid moieties, although other explanations cannot be ruled out.

Our finding that apoE is rapidly transported in the axon of a long projection neuron, the neuronal class that is selectively vulnerable to Alzheimer’s disease, may offer some insight into why the E4 isoform is a significant risk factor for Alzheimer’s disease (16, 34). If the E4 form of apoE is unable to supply lipid to neurons as well as the other two isoforms of apoE, as a recent experiment with cell-cultured dorsal root ganglia indicates (35), people with the E4 isoform may be more susceptible to the development of Alzheimer’s disease. Other evidence supporting this conjecture has recently been reviewed (6).

Our apoE data combined with our results demonstrating APP transport (14, 15) also suggest the possibility that apoE and APP could directly interact and be transported together in both Müller glia and RGCs. Since apoE4 and apoE3 appear to interact differently in vitro with the b-amyloid portion of APP (15), it is possible that they together perform some as yet unknown function in the central nervous system. In this regard, it has recently been demonstrated that the LRPs can internalize and mediate degradation of APP as well as apoE in the context of a lipoprotein particle, suggesting internalization of both molecules into the same compartment (36). We are presently carrying out experiments investigating possible interactions of these molecules in the in vivo retina.

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