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Apolipoprotein E and Low-Density Lipoprotein Binding and Internalization in Primary Cultures of Rat Astrocytes: Isoform-Specific Alterations

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Abstract: Apolipoprotein (apo) E is likely involved in redistributing cholesterol and phospholipids during compensatory synaptogenesis in the injured CNS. Three common isoforms of apoE exist in human (E2, E3, and E4). The apoE4 allele frequency is markedly increased in both late-onset sporadic and familial Alzheimer's disease (AD). ApoE concentration in the brain of AD subjects follows a gradient: ApoE levels decrease as a function of E2 > E3 ≫ E4. It has been proposed that the poor reinnervation capacity reported in AD may be caused by impairment of the apoE/low-density lipoprotein (LDL) receptor activity. To understand further the role of this particular axis in lipid homeostasis in the CNS, we have characterized binding, internalization, and degradation of human ^{125}I-LDL to primary cultures of rat astrocytes. Specific binding was saturable, with a K_D of 1.8 n*M* and a B_{max} of 0.14 pmol/mg of proteins. Excess unlabeled human LDL or very LDL (VLDL) displaced 70% of total binding. Studies at 37°C confirmed that astrocytes bind, internalize, and degrade ^{125}I-LDL by a specific, saturable mechanism. Reconstituted apoE (E2, E3, and E4)-liposomes were labeled with ^{125}I and incubated with primary cultures of rat astrocytes and hippocampal neurons to examine specific binding. Human LDL and VLDL displaced binding and internalization of all apoE isoforms similarly in both astrocytes and neurons. ^{125}I-ApoE2 binding was significantly lower than that of the other ^{125}I-apoE isoforms in both cell types. ^{125}I-ApoE4 binding was similar to that of ^{125}I-apoE3 in both astrocytes and neurons. On the other hand, ^{125}I-apoE3 binding was significantly higher in neurons than in astrocytes. These isoform-specific alterations in apoE-lipoprotein pathway could explain some of the differences reported in the pathophysiology of AD subjects carrying different apoE alleles. **Key Words:** Alzheimer's disease—Low-density lipoprotein receptor—Cholesterol—Astrocyte—Plasticity—CNS.
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Previous reports concerning the neuropathological changes that occur during the course of Alzheimer's disease (AD) implied that relative amounts of cholesterol may influence the structure of brain cortical membranes (Mason et al., 1992) and that phospholipid alterations correlate very well with the formation of senile plaques in AD (Kaufer et al., 1993). Impaired cholinergic functions in AD have been attributed to impaired choline and phospholipid homeostasis (Wurtman, 1992). Recently, links have been made between the pathology of AD and apolipoprotein (apo) E. ApoE, a lipid-binding glycoprotein, plays a key role in the transport and metabolism of triglyceride-rich lipoprotein particles and in cholesterol homeostasis (for a review, see Poirier, 1994). Lipoproteins containing apoE interact with the low-density lipoprotein (LDL) receptor [apoE/B receptor (Goldstein et al., 1983)], the LDL receptor-related protein [LRP (Beisiegel et al., 1989; Kowal et al., 1989)], the very low-density lipoprotein (VLDL) receptor (Takahashi et al., 1992), and perhaps others (Pedreno et al., 1992) to modulate the catabolism of triglyceride-rich lipoprotein particles. The LDL receptor pathway consists of cell surface binding of apoE- or apoB-containing lipoproteins followed by internalization and degradation via a lysosomal pathway. As the incoming cholesterol concentration increases, intracellular cholesterol synthesis is depressed, synthesis of LDL receptors is decreased, and cholesterol esterification and storage are increased (Goldstein et al., 1983).

In nervous tissue, apoE is involved in the transport of cholesterol in repair, growth, and maintenance of

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Abbreviations used: AD, Alzheimer's disease; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein; LRP, low-density lipoprotein receptor-related protein; VLDL, very low-density lipoprotein.

membranes during development or after injury (Boyles et al., 1989; Poirier et al., 1991, 1993*a*). In response to PNS injury, macrophages synthesize and release apoE to scavenge the lipids released from degenerating membranes and myelin. In the CNS apoE, released from astrocytes in response to injury, plays a pivotal role in the redistribution of cholesterol and phospholipids during membrane remodeling associated with synaptic plasticity and compensatory synaptogenesis (Poirier et al., 1991, 1993*a*). The importance of apoE in the brain is further underscored by the absence of other plasma apos such as apoA1 and apoB (Roheim et al., 1979). Recently, apoE knockout mice were shown to fail to induce compensatory synaptogenesis in response to hippocampal deafferentation (Masliah et al., 1995) and to exhibit marked cognitive impairment in the Morris swim maze test (Poirier et al., 1996). Owing to the critical role played by apoE and the LDL receptor in compensatory reactive synaptogenesis, we hypothesised that the poor reinnervation capacity observed in most AD patients could be caused by a selective impairment of the apoE/LDL receptor axis (Poirier, 1994).

Three major isoforms of apoE (E2, E3, and E4) differ by a single unit of net charge (Davignon et al., 1988). The apoE4 allele frequency is markedly increased in sporadic (Poirier et al., 1993*b*; Saunders et al., 1993) and late-onset familial (Corder et al., 1993; Payami et al., 1993) AD. In vitro, amyloid β peptide was shown to bind to apoE; whether apoE4 binds more avidly to apoE3 (Strittmatter et al., 1993) or the reverse (Ladu et al., 1994) is still an open question. Increased β-amyloid peptide deposition has been reported in the brain (Rebeck et al., 1993; Schmechel et al., 1993; Beffert and Poirier, 1996) of late-onset sporadic AD patients carrying the apoE4 allele. Furthermore, a reduction in choline acetyltransferase activity (a classical hallmark of AD) in AD patients has been shown to correlate with increased apoE4 allele copy number (Poirier, 1994; Soininen et al., 1995). We showed recently that apoE levels in the hippocampus and cortical areas of AD subjects are reduced according to the apoE4 allele dose, i.e., as apoE4 allele copy number increases, apoE levels decrease (Bertrand et al., 1995). These results parallel the low levels of apoE reported in the CSF of nongenotyped AD subjects when compared with age-matched controls (Blennow et al., 1994).

Despite numerous studies delineating the LDL pathway in cultured fibroblasts and hepatocytes, information on lipoprotein metabolism in the CNS is scarce. The presence of LDL receptors has been reported in the brains of rats (Poirier et al., 1993*a*), monkeys (Pitas et al., 1987*b*), and rabbits (Swanson et al., 1988). LDL receptors have been shown to be present and functional on the human malignant glioma cell lines U-251MG and KMG-5 (Murakami et al., 1990; Rudling et al., 1983), primary cultures of rat brain astrocytes (Pitas et al., 1987*a*) and neurons (Poirier et al., 1992), and rat glial cells (Jung-Testas et al., 1992). Recently, in normal human brain, LRP immunoreactivity was demonstrated by both neurons (strong) and astrocytes (light), whereas the neuropil of astrocytes demonstrated LDL receptor immunoreactivity (Rebeck et al., 1993).

To gain a better understanding of basic lipoprotein pathways in cultured astrocytes, we examined binding, internalization, and degradation properties of human ^{125}I-LDL to primary cultures of rat astrocytes. To examine potential isoform-specific differences in apoE-lipoprotein metabolism, functional liposomes, constructed from purified apoE (E2, E3, and E4), were labeled with ^{125}I and used to study binding and internalization characteristics in both cultured rat astrocytes and neurons.

MATERIALS AND METHODS

Cells

Cell cultures solutions and supplies were purchased from Gibco (Burlington, Ontario, Canada). Primary cultures of type 1 astrocytes were derived from the cortex of 1–2-day-old Sprague–Dawley rats (Charles River, St. Constant, Quebec, Canada) as described by Intebi et al. (1990). In brief, brains were removed, whole cortex was dissected out, meninges were removed, and tissue was minced in ice-cold dissection medium [Dulbecco's modified Eagle's medium (DMEM) with 20 m*M* HEPES and gentamicin (50 ng/ml)]. Tissue was then incubated for 15 min at 37°C in dissection medium containing dispase (0.5 mg/ml). DNase I (Sigma, St. Louis, MO, U.S.A.) was then added (final concentration, 0.1 mg/ml), tissue was filtered through nylon mesh (Tekto; size 40 mesh), and the cell suspension was washed three times with astrocyte growth medium [50:50 DMEM:Ham's F-12 nutrient mix with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (Fungizone; 2.5 μg/ml), and gentamicin (50 ng/ml)]. Cells were plated in poly-D-lysine-coated 75-cm^2 tissue culture flasks and maintained at 37°C in a humidified incubator. The next day each culture was washed twice with Hanks' balanced salt solution. When confluent, cells were vigorously rinsed and shaken with phosphate-buffered saline until >95% type 1 astrocytes remained (confirmed by glial fibrillary acidic protein immunoreactivity). All cells were used within 3 weeks of primary culture. Primary cultures of hippocampal neurons were isolated from the brains of embryonic Long-Evans rats exactly as described before by Alonzo et al. (1994). Before experiments, all cells were plated in 16-mm-diameter wells, grown to 80% confluence, and maintained for an additional 48 h in serum-free DMEM/F-12 medium.

Preparation of apoE-liposomes

Lipoproteins used for the preparation of apoE-containing liposomes were isolated from the plasma of apoE-homozygous, pregenotyped patients. ApoE was purified as previously described (Brissette et al., 1986). In brief, phenylmethylsulfonyl fluoride (10 μM), EDTA (0.3 mM), and sodium azide (3 μM) were added to all sera, and human plasma was ultracentrifuged at 8,500,000 *g* for 30 min and then twice at 1,800,000 *g* for 1 h to isolate and wash the

VLDL. An equal volume of isopropanol was added to a 1.5 mg/ml VLDL solution while agitating vigorously and centrifuging at 10,000 g for 10 min. Isopropanol was evaporated from the supernatant, and acetone (1.2 times the volume) was added to the remaining solution while agitating and centrifuging at 20,000 g for 20 min. The apoE pellet was resuspended in 150 mM NaCl, and the acetone extraction was repeated. Solvent-extracted apos were then delipidated by extracting twice with chloroform/methanol (2:1 vol/vol); each extraction was followed by a 20-min centrifugation at 20,000 g. Delipidated proteins were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.3 mM EDTA, and 5.0 M urea. The solutions were centrifuged at 10,000 g for 10 min to remove undissolved material and then dialyzed against the same buffer containing no urea.

Liposomes were prepared according to the method of Matz and Jonas (1982) with the following conditions per mole of protein: 100 mol of phosphatidylcholine, 100 mol of cholate, and 25 mol of cholesterol. The lipid/cholate/protein mixtures were incubated for 12 h at 4°C and then dialyzed for 2 days against 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.3 mM EDTA. The liposome-reconstituted proteins were isolated by a centrifugation of 3,000,000 g for 1 h at a density of 1.125 g/ml, and the isolated fraction was dialyzed against a 150 mM NaCl solution (pH 7.5) containing 0.3 mM EDTA and 3 mM sodium azide.

Radioiodination of lipoproteins

All lipoproteins were labeled according to the Bolton–Hunter procedure (Innerarity et al., 1979). Benzene was evaporated with a gentle stream of nitrogen, the lipoprotein, in potassium phosphate buffer (pH 7.5), was added, and the mixture was incubated on ice for 5 h with gentle agitation every 30 min. The reaction was terminated with 0.2 ml of glycine (1 mg/ml in phosphate buffer), and the mixture was incubated an additional 15 min and then applied to a preequilibrated column (Sephadex G-25 equilibrated with 0.1% gelatin-phosphate buffer). Radiolabeled protein fractions were pooled.

Measurement of binding, internalization, and degradation of ^{125}I-LDL

Binding assays of ^{125}I-labeled lipoproteins were measured as described by Innerarity et al. (1986). For 4°C studies, cells were first placed on ice in a 4°C cold room, and medium was replaced with ice-cold DMEM containing 0.2% bovine serum albumin. Fresh DMEM/0.2% bovine serum albumin was then added containing the indicated amount of ^{125}I-LDL in the presence or absence of a 500-fold excess of unlabeled LDL (to estimate nonspecific binding), and cells were incubated for 4 h at 4°C. Incubation medium was then removed and used to for quantifying degradation products. The cells were washed rapidly three times with ice-cold phosphate-buffered saline containing 0.2% bovine serum albumin and then twice for 10 min with phosphate-buffered saline. To displace LDL bound to its receptor, each well then received phosphate-buffered saline containing dextran sulfate (10 mg/ml), sodium suramin (25 mM), or a mixture of proteases (50 μg/ml of trypsin, 50 μg/ml of proteinase K, and 5 mM EDTA) and then incubated on ice for 1 h in a rotary shaker (60 rpm). The solution was then counted for radioactivity to determine ^{125}I-LDL released. The cells were then rinsed with phosphate-buffered saline and detached with 0.1 M NaOH to determine the amount of radioactivity still associated with the cells. An aliquot of this solution was used to determine the cellular protein concentration. For experiments at 37°C, the medium was replaced with DMEM containing 0.2% bovine serum albumin containing the indicated amount of ^{125}I-LDL in the presence or absence of a 500-fold excess of unlabeled LDL. Cells were incubated for 5 h at 37°C, placed at 4°C for 15 min, and processed exactly the same as for the 4°C studies. Specific binding and internalization are represented by total cell-associated radioactivity that is sensitive or resistant, respectively, to release by 25 mM suramin minus values obtained in the presence of excess unlabeled LDL. Results are expressed as nanograms of ^{125}I-LDL per milligram of cellular protein unless otherwise specified. K_D data are expressed as mean ± SE values. The degradation rate of ^{125}I-LDL was determined by measuring the amount of specific ^{125}I-labeled trichloroacetic acid-soluble material in the medium after the incubation as previously described (Goldstein et al., 1983).

Data analysis

Binding parameters for the various peptides were calculated from the competition binding analyses by the Lundon Software Receptor Fit Competition (Lundon Software, Chagrin Falls, OH, U.S.A.). Scatchard plots were constructed, and the K_D and B_{max} values were computed using weighted nonlinear least-squares regression analysis. All results are reported as mean ± SE values. The statistical significance of differences between binding and internalization mean values was evaluated using t test analysis.

RESULTS

Binding, internalization, and degradation of human ^{125}I-LDL by primary cultures of rat astrocytes

The ability of polysulfonated compounds such as heparin, dextran sulfate, and suramin to displace LDL from its receptor has been well established in many cell types (Goldstein et al., 1976; Innerarity al., 1986; Salter et al., 1986). We first examined the ability of these compounds to displace ^{125}I-LDL that had been bound to primary cultures of rat astrocytes at 4°C. Cultured astrocytes deprived of fetal bovine serum for 48 h were incubated with ^{125}I-LDL for 4 h at 4°C, washed, and incubated with buffer containing dextran sulfate, suramin, or proteases at 4°C as described in Materials and Methods. Figure 1 illustrates the proportion of cell-associated radioactivity released by dextran sulfate, suramin, or a mixture of proteases in these conditions. Excess dextran sulfate (100 mg/ml) released ~50% of the ^{125}I-LDL associated with primary cultures of rat astrocytes. Suramin displaced slightly more of the radioactivity associated with the cells (57%), whereas protease treatment released 48% of the bound ^{125}I-LDL. Increasing the incubation time to 2 h had no detectable effect on the amount of ^{125}I-LDL released with any of the above-mentioned agents (data not shown). For all subsequent experiments, 25 mM suramin was used to release the ^{125}I-LDL bound to the cells.

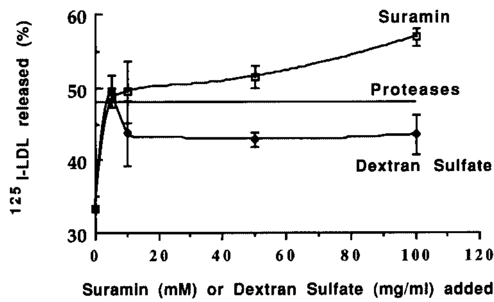

FIG. 1. Release of bound ^{125}I-LDL from rat astrocytes induced by polysulfonated compounds. Primary cultures of rat astrocytes were plated and deprived of serum for 48 h as described and then incubated at 4°C for 4 h with ^{125}I-LDL (10 μg/ml of protein). The cells were then incubated for 1 h with buffer containing various concentrations of suramin or dextran sulfate or with a fixed concentration of proteases (50 μg/ml of trypsin, 50 μg/ml of proteinase K, and 5 mM EDTA). Data are mean ± SE (bars) values of triplicate dishes.

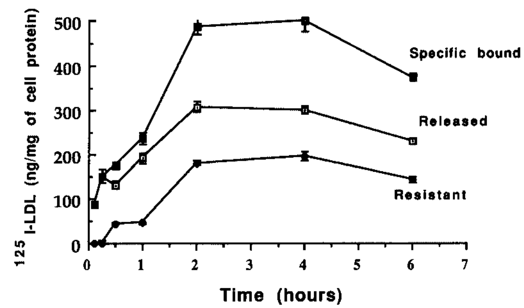

FIG. 3. Time course of specific ^{125}I-LDL binding to rat astrocytes. Primary cultures of rat astrocytes were plated and deprived of serum for 48 h as described. Cells were then incubated at 4°C with ^{125}I-LDL (10 μg/ml) in the presence or absence of unlabeled LDL (500 μg/ml) (to estimate nonspecific binding). At each indicated time, the assay was terminated, and wells were rinsed and then incubated for 1 h at 4°C with 25 mM suramin. Data are mean ± SE (bars) values of triplicate dishes, after subtracting the values for nonspecific binding for suramin-releasable binding (released), suramin-resistant uptake (resistant), and the sum of these (specific bound).

LDL receptor activity is known to increase in the absence of exogenous cholesterol in many cell types (Goldstein et al., 1983; Innerarity et al., 1986). We examined the effect of serum deprivation on ^{125}I-LDL binding and internalization in rat astrocytes. As shown by Fig. 2, at 37°C, both binding and internalization of ^{125}I-LDL by rat astrocytes are markedly increased within 48 h after changing to serum-free conditions. A 2.8-fold increase in binding and a 2.2-fold increase in internalization are obtained after 48 h of serum deprivation. For all subsequent experiments, cells were deprived of serum for 48 h.

Figure 3 demonstrates the time course of specific ^{125}I-LDL binding at 4°C. Specific binding reached saturation at 2 h for both the suramin-releasable component (representing ~65% of the specific ^{125}I-LDL associated with the cells) and the suramin-resistant portion (representing ~35% of the specific ^{125}I-LDL associated with the cells).

Saturation and competition curves representing the specific binding of human ^{125}I-LDL to primary cultures of rat astrocytes at 4°C are shown in Fig. 4a. The specific binding exhibited a typical saturation profile, indicating that human ^{125}I-LDL binds to a limited number of sites. Scatchard analysis revealed an average

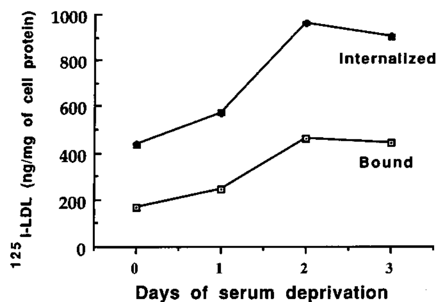

FIG. 2. Effect of serum deprivation on rat astrocyte-associated ^{125}I-LDL. Primary cultures of rat astrocytes were plated as described. After 48 h in culture, cells were rinsed, subjected to serum-free conditions for 0, 1, 2, or 3 days, and then incubated at 37°C for 5 h with ^{125}I-LDL (10 μg/ml) in the presence or absence of 500 μg of unlabeled LDL/ml to estimate nonspecific binding. Cells were then cooled to 4°C, rinsed, and incubated for 1 h with 25 mM sodium suramin as described in Materials and Methods. Specific binding and internalization are represented by total cell-associated radioactivity that is sensitive or resistant, respectively, to 25 mM suramin minus values obtained in the presence of excess unlabeled LDL. Data are mean ± SE (bars) values of triplicate dishes.

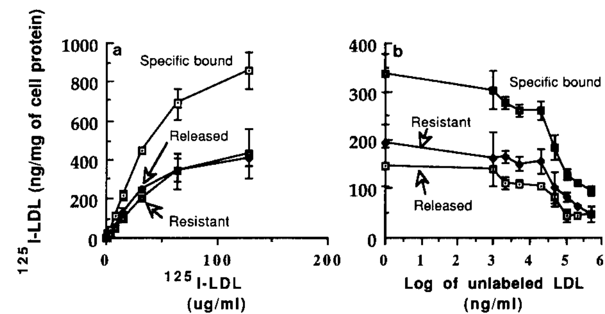

FIG. 4. Saturation (**a**) and competition (**b**) curves representing specific binding of human ^{125}I-LDL to rat astrocytes. Primary cultures of rat astrocytes were plated and serum-deprived for 48 h as described. In (a), cells were incubated at 4°C for 4 h with the indicated concentration of ^{125}I-LDL in the presence or absence of a 500-fold excess of unlabeled LDL (to estimate nonspecific binding). In (b), cells were incubated at 4°C for 4 h with ^{125}I-LDL (2 μg/ml) and increasing concentrations of unlabeled LDL. In both experiments, specific bound represents the total of suramin-releasable and suramin-resistant binding after subtracting the values for nonspecific binding. Data are mean ± SE (bars) values of triplicate dishes. No degradation products were detected in either experiment.

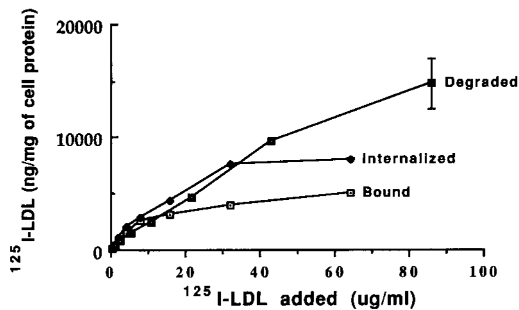

FIG. 5. Saturation curves representing specific binding, internalization, and degradation of human ^{125}I-LDL by rat astrocytes. Primary cultures of rat astrocytes were plated and serum-deprived for 48 h as described. Cells were then incubated at 37°C for 4 h with the indicated concentration of ^{125}I-LDL in the presence or absence of a 500-fold excess of unlabeled LDL (to estimate nonspecific binding). Specific binding and internalization are represented by total cell-associated radioactivity that is sensitive or resistant, respectively, to 25 mM suramin minus values obtained in the presence of excess unlabeled LDL. Specific degradation products were determined as described in Materials and Methods. Data are mean ± SE (bars) values of triplicate dishes after subtracting nonspecific values.

K_D of 1.8 ± 0.14 nM for the higher-affinity binding component and a B_{max} of 0.14 ± 0.02 pmol/mg.

Specificity of ^{125}I-LDL binding to rat astrocytes was determined by competitive binding assays performed at 4°C. As shown by Fig. 4b, >70% of the specific ^{125}I-LDL (an apoB-containing lipoprotein) associated with the cells at 4°C was displaced by 500 μg/ml of unlabeled LDL. ^{125}I-LDL binding could also be displaced, to a similar extent, by 500 μg/ml of β-VLDL, an apoE-containing lipoprotein (data not shown).

Specific binding, internalization, and degradation of human ^{125}I-LDL to rat astrocytes at 37°C are shown in Fig. 5. B_{max} values for binding and internalization were 3.8 ± 0.87 and 5.9 ± 0.89 pmol/mg, respectively. At 37°C, the average K_D values of the ^{125}I-LDL high-affinity binding site was 4.5 ± 0.9 nM.

Binding of human ^{125}I-apoE-liposomes by primary cultures of rat astrocytes and neurons

The second series of experiments examined potential isoform-specific differences in apoE binding in both primary rat cultures of astrocytes and neurons. Because purified apoE does not bind to its receptor, liposomes were constructed from purified apoE that had been isolated from the serum of pregenotyped apoE-homozygous patients. The resulting apoE-liposomes were then labeled with ^{125}I and incubated with primary rat cultures of cortical astrocytes or hippocampal neurons as described in Materials and Methods.

To examine the ability of LDL (containing primarily apoB) and VLDL (containing primarily apoE) to inhibit binding and internalization of ^{125}I-apoE-liposomes, cultured rat astrocytes and neurons were incubated at 37°C with ^{125}I-apoE liposomes in the presence or absence of 500 μg/ml of either human unlabeled LDL or VLDL. Figure 6 demonstrates that both LDL and VLDL displace ^{125}I-apoE-liposome binding to a similar extent (^{125}I-labeled apoE2 and apoE4 yielded results identical to those with ^{125}I-apoE3; data not shown).

To examine isoform-specific differences in ^{125}I-apoE-liposome binding, primary cultures of rat astrocytes and neurons were incubated at 37°C with ^{125}I-apoE (E2, E3, or E4)-liposomes in the presence or absence of 500 μg/ml of unlabeled human LDL (to estimate specific binding). Experiments were performed in parallel for the three ligands and for both astrocytes and neurons. Figure 7 represents specific binding of ^{125}I-apoE-liposomes at 37°C. Binding of ^{125}I-apoE2-liposomes was significantly lower than that of the other ^{125}I-apoE-liposome isoforms in both cultured astrocytes and neurons. ^{125}I-ApoE4-liposome binding was similar to that of ^{125}I-apoE3-liposomes in astrocytes but quite reduced in neurons.

DISCUSSION

Cells requiring cholesterol for membrane synthesis are known to take up lipoproteins via receptor-mediated endocytosis or to initiate the de novo synthesis of cholesterol (Goldstein et al., 1983). Because the brain lacks key plasma apos such as apoB and apoA1 (Roheim et al., 1979), apoE is the lipid carrier of choice in the CNS, playing a pivotal role in the hippocampal response to injury (Poirier et al., 1991, 1993*a,b*). The coordinated expression of apoE and its receptor appears to regulate the transport of cholesterol and phospholipids during the early and intermediate phases of reinnervation following injury (for a review, see Poir-

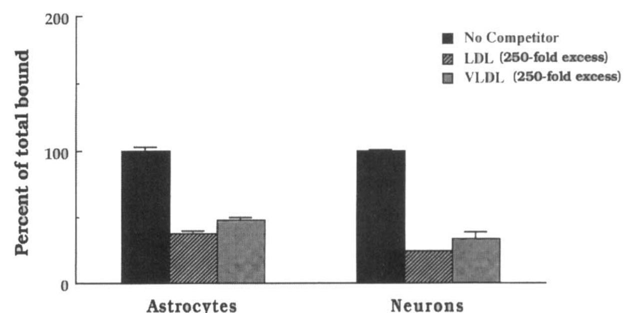

FIG. 6. Effect of excess unlabeled LDL and VLDL on ^{125}I-apoE liposome binding in cultured rat astrocytes and neurons. Primary cultures of rat cortical astrocytes and hippocampal neurons were plated and serum-deprived for 48 h as described. Cells were incubated at 37°C for 4 h, with 2 μg/ml of ^{125}I-apoE3 liposomes and 500 μg/ml of unlabeled LDL or VLDL. Specific binding is represented by total cell-associated radioactivity that is sensitive to 25 mM suramin minus values obtained in the presence of 500 μg/ml of unlabeled LDL or VLDL. The percentage of total bound is shown in conditions with no competitor present, excess LDL, and excess VLDL. Data are mean ± SE (bars) values of triplicate dishes.

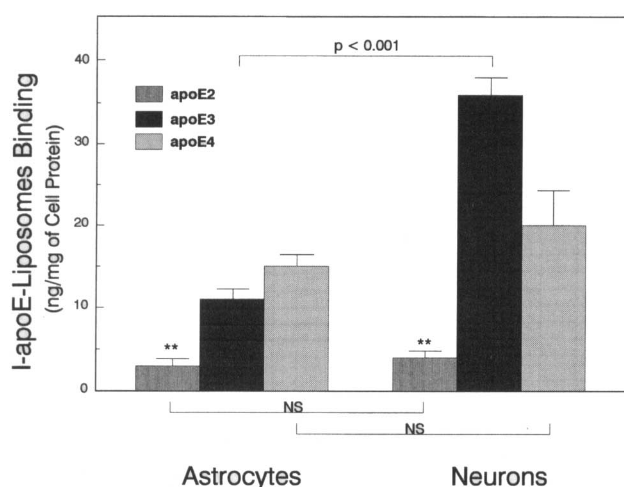

FIG. 7. Effect of apoE isoform on ^{125}I-apoE-liposome binding in rat astrocytes and neurons. Primary cultures of rat cortical astrocytes and hippocampal neurons were plated and serum-deprived for 48 h as described. Cells were incubated at 37°C for 4 h, with 2 μg/ml of ^{125}I-apoE (E2, E3, or E4) liposomes and 500 μg/ml of unlabeled LDL to estimate nonspecific binding. Specific binding is represented by total cell-associated radioactivity that is sensitive or resistant, respectively, to 25 m*M* suramin minus values obtained in the presence of excess unlabeled LDL. Data are mean $\pm$ SE (bars) values of triplicate dishes after subtracting the values for nonspecific binding. $^{**}p < 0.01$. NS, not significant.

ier, 1994). As neurons undergoing dendritic remodeling and synaptogenesis favor cholesterol internalization through the apoE/LDL receptor pathway, they progressively inhibit cholesterol synthesis (Poirier et al., 1991, 1993*a*). The recent discoveries that apoE4 is strongly linked to sporadic and familial late-onset AD and that this particular apoE isoform is associated with low levels of apoE in the brain (Bertrand et al., 1995) raise the possibility that a dysfunction of the lipid transport system associated with compensatory sprouting and synaptic remodeling could be central to the AD process (Poirier, 1994).

We demonstrate here that primary cultures of rat astrocytes bind, internalize, and degrade ^{125}I-LDL by a specific, saturable, high-affinity process and that uptake is markedly increased within 48 h after changing to serum-free conditions (low lipoprotein concentrations). This confirms a previous report on the ability of cultured astrocytes to metabolize apoE-containing lipoproteins (Pitas et al., 1987*a,b*), and confirms in brain cells the previous observations in fibroblasts of LDL receptor up-regulation in response to lipoprotein deprivation (Goldstein et al., 1983; Innerarity et al., 1986). This is also consistent with previous reports suggesting that the LDL (apoB/E) receptor is present and functional on astrocytes (Pitas et al., 1987*a,b*; Jung-Testas et al., 1992) and is responsible for at least a portion of our observed ^{125}I-LDL binding in astrocytes.

Only half of the ^{125}I-LDL associated with the cultured rat astrocytes could be released by excess polysulfonated compounds (Figs. 1, 3, and 4). Suramin was more efficient at displacing ^{125}I-LDL than dextran sulfate. The suramin-resistant ^{125}I-LDL associated with the cells at 4°C probably reflects a small degree of internalization, occurring at low temperatures (Hara and Howard, 1990). Alternatively, it may suggest the presence of two distinct binding sites, as has been reported in hepatocytes (Salter et al., 1986). Polysulfonated compounds, such as suramin, interact with the same apoB amino acid residues involved in LDL binding to the LDL receptor (Mahley et al., 1979). The inability of suramin to release part of the ^{125}I-LDL specifically bound to astrocytes implies that these residues are not involved in binding or that these residues are not accessible to suramin when ^{125}I-LDL is bound. This could be explained by (a) ^{125}I-LDL binding to two distinct sites, the LDL receptor (releasable) and another site representing an interaction unaffected by polysulfonated compounds (resistant), or alternatively (b) by partial internalization of the bound ^{125}I-LDL. The fact that protease treatment induced release of less than half of the ^{125}I-LDL associated with the cells supports the concept of internalization at 4°C by astrocytes rather than dual binding sites. Further studies are in progress to determine if, in fact, two different LDL binding sites are present and functional on astrocytes. Although the LRP (which binds apoE) has been shown to be present on astrocytes (Rebeck et al., 1993), it cannot bind apoB (the major apo present in human LDL) and therefore is unlikely to be responsible for any ^{125}I-LDL binding observed in our studies.

Scatchard analysis of the specific ^{125}I-LDL bound at 4°C revealed a K_D of 1.8 n*M* for the higher-affinity binding component, which is very similar to the value of 3.5 n*M* reported by Pitas et al. (1987*a*) using rat LDL rather than human LDL. Competitive binding assays confirmed the specific nature of LDL binding to rat astrocytes (Fig. 4b). The observation that an excess of human unlabeled VLDL (containing apoE) was able to displace as much ^{125}I-LDL binding as an excess of human LDL (containing primarily apoB) further supports the notion that the LDL (apoB/E) receptor is responsible for the specific ^{125}I-LDL binding.

Studies performed at 37°C revealed that binding and internalization of ^{125}I-LDL reached saturation at low ^{125}I-LDL concentrations (Fig. 5). Degradation, on the other hand, exhibits a more linear relationship at low concentrations, which is consistent with the proposed notion of receptor-dependent and -independent catabolism of lipoprotein complexes. At 37°C, the affinity of the ^{125}I-LDL high-affinity binding site was less than that at 4°C (K_D values were 1.8 and 4.5 n*M* at 4 and 37°C, respectively), whereas the B_{max} was much greater at 37°C. This is consistent with temperature-

dependent differences in K_D and B_{max} values obtained with fibroblasts (Goldstein et al., 1983).

To examine the role of different apoE isoforms in CNS lipoprotein metabolism, we constructed liposomes from purified apoE that had been isolated from pregenotyped, apoE-homozygous patients. The apoE-liposomes were then labeled with ^{125}I and incubated at 37°C with primary cultures of rat cortical astrocytes or hippocampal neurons as described in Materials and Methods.

Both apoB-containing LDL and apoE-containing VLDL were found to displace, at 37°C, binding and internalization of ^{125}I-apoE-liposomes to a similar extent. This was true of all isoforms of ^{125}I-apoE tested (E2, E3, and E4) in both astrocytes and neurons.

Our studies revealed isoform-specific differences in ^{125}I-apoE-liposome binding in primary cultures of both rat astrocytes and neurons at 37°C. Binding of ^{125}I-apoE2-liposomes was significantly lower than that of the other ^{125}I-apoE-liposome isoforms in cultured astrocytes and neurons. These results are consistent with data examining binding of apoE-phospholipid complexes to LDL receptors of fibroblasts and HeLa cells [apoE2 binding was <2% of apoE3 binding (Utermann, 1987). ^{125}I-ApoE4-liposome binding was similar to ^{125}I-apoE3-liposome binding in astrocytes, consistent with a previous study comparing apoE4 and apoE3 binding in fibroblasts in culture (Davignon et al., 1988). ^{125}I-ApoE4-liposome binding is similar in both primary neurons and astrocytes, whereas ^{125}I-apoE3-liposome binding in neurons was found to be markedly increased when compared with ^{125}I-apoE4-liposome binding. This is quite interesting in view of the recent demonstration that β-amyloid, a peptide that accumulates preferentially in the brain of apoE4 carriers with AD, is a powerful regulator of apoE internalization and degradation in astrocyte cells in culture (Beffert et al., 1995). In evolutionary terms, apoE4 is considered to be the ancestral form of apoE, which is found in both rodents and monkeys (Mahley, 1988). This could explain why rodent cells better handle human apoE4-liposomes when compared with apoE3-liposomes. Alternatively, this phenomenon could represent an intrinsic characteristic of the apoE4/neuron interaction.

Cultured astrocytes, on the other hand, failed to exhibit this preferential binding for apoE3 liposomes. At least two different explanations can be proposed to explain this phenomenon: First, astrocytes may express a different type of LDL receptor or a different combination of cell surface receptors that recognize the apoE3-liposomes (LDL receptor, LRP, or VLDL receptor). Alternatively, apoE synthesized endogenously in astrocytes (but not in neurons) could affect the binding kinetic of the apoE3 isoform without compromising the apoE2 and E4 binding profiles. These different possibilities are now currently being explored in our laboratory.

These results clearly indicate that there are cell-specific differences in the binding properties of the different human apoE isoforms in different brain cells. ApoE concentrations in the brain of AD decrease according to the following gradient: E2 > E3 ≫ E4 (Bertrand et al., 1995). A similar profile was reported for serum apoE levels in young healthy subjects (Utermann, 1987). A possible interpretation of these results is that apoE2 catabolism, which depends on binding and internalization by scavenging cells such as astrocytes, is significantly reduced when compared with that of apoE3 and apoE4. Alternatively, the catabolism (degradation) of apoE4 could proceed at a higher rate when compared with apoE3, leading to lower steady-state levels of apoE in apoE4 carriers. This mechanism is consistent with the fate of serum apoE4-lipoproteins, which are catabolized more rapidly than apoE3-containing lipoproteins in vivo in humans (Gregg et al., 1986).

Recently, it was shown that apoE-liposomes (E3 and E4) can bind selectively to soluble β-amyloid (1–40) and catalyze the internalization of the β A4 peptide into neurons and astrocytes through the LDL receptor pathway (Beffert and Poirier, 1996). This process can be completely blocked by the presence of an LDL receptor-specific monoclonal antibody raised against the apoE binding site of the receptor. It is thus conceivable that the internalization of β-amyloid by astrocytes and neurons may proceed at different rates depending on the concentration and type of apoE isoform present in the CNS. Additional work is now required to determine the relevance of these isoform-specific interactions of apoE with its receptor and β-amyloid peptide in vivo, as the relationship between these two key markers may represent an important clue in the understanding of AD pathophysiology.

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