Expression of ABCG1, but Not ABCA1, Correlates with Cholesterol Release by Cerebellar Astroglia*

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Central nervous system lipoproteins mediate the exchange of cholesterol between cells and support synaptogenesis and neuronal growth. The primary source of lipoproteins in the brain is astroglia cells that synthesize and secrete apolipoprotein (apo) E in high density lipoprotein-like particles. Small quantities of apoA1, derived from the peripheral circulation, are also present in the brain. In addition to the direct secretion of apoE-containing lipoproteins from astroglia, glia-derived lipoproteins are thought to be formed by cholesterol efflux to extracellular apolipoproteins via ATP-binding cassette (ABC) transporters. We used cultured cerebellar murine astroglia to investigate the relationship among cholesterol availability, apoE secretion, expression of ABCA1 and ABCG1, and cholesterol efflux. In many cell types, cholesterol content, ABCA1 expression, and cholesterol efflux are closely correlated. In contrast, cholesterol enrichment of glia failed to increase ABCA1 expression, although ABCG1 expression and cholesterol efflux to apoA1 were increased. Moreover, the liver X receptor (LXR) agonist TO901317 up-regulated ABCA1 and ABCG1 expression in glia without stimulating cholesterol efflux. Larger lipoproteins were generated when glia were enriched with cholesterol, whereas treatment with the LXR agonist produced smaller particles that were eliminated when the glia were loaded with cholesterol. We also used glia from ApoE-/- mice to distinguish between direct lipoprotein secretion and the extracellular generation of lipoproteins. Our observations indicate that partially lipiddated apoE, secreted directly by glia, is likely to be the major extracellular acceptor of cholesterol released from glia in a process mediated by ABCG1.

The human brain contains 15–20% of total body cholesterol but represents only ~5% of total body weight (1). Cholesterol is a key component of all membranes, including myelin, in the central nervous system (CNS),4 and cholesterol is synthesized continuously, albeit at a low rate, in the adult brain (1). All cholesterol in the CNS is synthesized within the CNS rather than being imported from the periphery (2). As a mechanism for maintaining cholesterol homeostasis in the CNS, cholesterol can be converted into the more polar 24-hydroxycholesterol, which is released into the circulation by a subset of neurons (3, 4). Because cholesterol metabolism and distribution are not uniform across all cell types of the brain, an efficient system is necessary for the transport of cholesterol, and probably other lipids, among cells of the CNS. In the CNS, as in the periphery, cholesterol exchange between cells is mediated by lipoproteins. Lipoproteins in the CNS are derived from glia (astrocytes and microglia), which synthesize and secrete apolipoprotein (apo) E and apo (5–7) as well as apoD (8). Unlike the plasma, in which apoA1 is the most abundant apolipoprotein, apoE is the major apolipoprotein in the CNS (9, 10). Glia-derived lipoproteins play important roles in the brain by enhancing synaptogenesis and synaptic efficacy (11–13) and by promoting axonal growth (14). Glial lipoproteins have been proposed to be taken up by axons of neighboring neurons so that the lipids, particularly cholesterol, can be used in membranes during axon repair or remodeling (15). The importance of cholesterol homeostasis in the CNS is underscored by the link between disturbances in CNS cholesterol metabolism and several neurodegenerative diseases such as Niemann-Pick disease type C and Alzheimer’s disease (reviewed in Ref. 16). Moreover, imbalances in cholesterol homeostasis in the CNS have been proposed to cause synaptic dysfunction (17).

Glia lipoproteins are thought to be formed by two poorly characterized processes: the direct secretion of lipiddated apolipoproteins, and the efflux of lipids from glial cells to lipid-free or lipid-poor extracellular apolipoproteins. Lipoprotein particles in the brain and cerebrospinal fluid are the size and density of plasma high density lipoproteins (HDLs) (reviewed in Refs. 9 and 18). Whereas all apoE in the CNS is derived from cells within the CNS, some plasma apoA1 crosses the blood-brain barrier by an unknown mechanism (19) and is incorporated into CNS lipoproteins. ApoA1 is also synthesized by endothelial cells of the blood-brain barrier (20, 21). Nascent discoidal lipoproteins are transformed into spherical particles in cerebrospinal fluid by the action of lecithin: cholesterol acyltransferase (5).

Lipid efflux from cells to apolipoproteins is thought to be mediated by members of the ATP-binding cassette (ABC) transporter family. During HDL formation in the plasma ABCA1 is required for transferring phospholipids and/or cholesterol to lipid-free, or lipid-poor extracellular apolipoproteins. Lipoprotein particles in the brain and cerebrospinal fluid are the size and density of plasma high density lipoproteins (HDLs) (reviewed in Refs. 9 and 18). Whereas all apoE in the CNS is derived from cells within the CNS, some plasma apoA1 crosses the blood-brain barrier by an unknown mechanism (19) and is incorporated into CNS lipoproteins. ApoA1 is also synthesized by endothelial cells of the blood-brain barrier (20, 21). Nascent discoidal lipoproteins are transformed into spherical particles in cerebrospinal fluid by the action of lecithin: cholesterol acyltransferase (5).

The abbreviations used are: CNS, central nervous system; apo, apolipoprotein; ABC, ATP-binding cassette transporter; DMEM, Dulbecco’s modified Eagle’s medium; HDL, high density lipoproteins; LDL, low density lipoproteins; LXR, liver X receptor; PBS, phosphate-buffered saline; qPCR, quantitative PCR.
lipidation of apoE (synthesized by glia) and apoA1 (derived from the plasma) mediated by one or more of these transporters. Recently, the secretion and lipidation of apoE by astrocytes were shown to depend, at least in part, on ABCA1 (28, 29). However, these studies revealed that astrocytes and microglia also operate pathways for the release of cholesterol to apoE that are independent of ABCA1 (28). The currently emerging model for the formation of plasma HDL is that apoA1 and apoE bind to ABCA1 (30–33), most likely via a direct protein-protein interaction that results in the transfer of phospholipids and cholesterol from cells to the acceptor apolipoprotein. Once apoE associates with lipids, however, the ability of ABCA1 to interact with apoE is reduced (33). These findings support the idea that ABCA1 is responsible for the initial lipidation of apoA1/apoE, whereas another mechanism, perhaps involving ABCG1, provides additional lipids for complete lipidation of the particle.

To gain a better understanding of the mechanisms involved in lipoprotein formation in the CNS, we have investigated the regulation of expression of three key components of lipoprotein formation by cerebellar astroglia, apoE, ABCA1, and ABCG1, in relation to cholesterol release. Our data demonstrate that the expression of apoE, ABCA1, and ABCG1 in astroglia is differentially regulated by cholesterol and that cholesterol efflux from cerebellar astroglia, in contrast to peripheral cells, does not correlate with the expression of ABCA1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) and phospholipid C (from Clostridium welchii) were purchased from Sigma. DNase I was from Cedarlane (Hornby, Ontario, Canada). All other materials for cell culture and PCR were from Invitrogen. Cholesterol was purchased from Sigma. Supplies for polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Mississauga, Ontario, Canada). The LXr agonist T901317 was from Key Biosciences (Camelford, UK). The inhibitor of acyl-CoA:cholesterol acyltransferase Sandoz 58-035 was purchased from Sigma. Nile Red was from Invitrogen Molecular Probes (Burlington, Ontario, Canada). [1-14C]Acetic acid (57 mCi/mmol) was from Amersham Biosciences. Silica gel G60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). A rabbit anti-human ABCA1 antibody, that recognizes mouse ABCA1, was from Novus Biologicals (Littleton, CO) and the goat anti-human apoE antibody that recognizes mouse apoE was from Biodesign (Saco, ME). A rabbit polyclonal anti-dog calnexin antibody was a generous gift from Dr. G. Fran

**Cell Culture**—Primary cerebellar astroglia were cultured as described previously (34). Briefly, cerebella were dissected from 1- to 2-day-old Balb/cCrAltBM or B6.6H2P–apoem1Unc mice (stock 002052, Jackson Laboratories, Bar Harbor, ME). Meninges and surface blood vessels were removed and discarded, and then the tissue was finely chopped, briefly digested with trypsin and DNase I, and dissociated by gentle trituration through a Pasteur pipette. Cells were plated in DMEM containing 10% fetal bovine serum at a density of one cerebellum per 25-mm² flask. Upon reaching confluence (~7 days after plating) the cells were washed three times with PBS, trypsinized, and replated at a density of 1:3. All experiments were performed with confluent cells.

Under these conditions, 90–95% of cells were astroglia as assessed by immunoreactivity of glial fibrillary acidic protein (34). All procedures were approved by the Health Sciences Animal Welfare Committee of the University of Alberta.

**Efflux of Endogenously Synthesized Cholesterol**—Upon reaching ~60% confluency, glial cells were incubated with 1 µCi/ml [14C]sodium acetate in DMEM containing 5% fetal bovine serum for ~3 days until confluency was attained. The cellular concentration of cholesterol was then raised by incubation of glia for 24 h in serum-free DMEM with or without 30 µg/ml cholesteryl (added from a 10 mg/ml stock solution in ethanol) in the absence of radiolabel to minimize the presence of radio-labeled cholesterol precursors. In some experiments, glia were incubated with the LXr agonist T901317 (2 µM) for 24 h. The cells were washed three times with PBS then incubated in serum-free DMEM, with or without 10 µg/ml apoA1, for 24 h. Medium was collected and centrifuged for 10 min at 1000 × g to remove cell debris. The supernatant (glia-conditioned medium) was used for immunoblotting, cholesterol analysis by gas-liquid chromatography, and fast protein liquid chromatography over a gel-filtration column, as indicated.

**Visualization of Neutral Lipids with Nile Red**—Glia were grown to confluence in DMEM containing 10% fetal bovine serum. The cells were then incubated in serum-free DMEM with or without 30 µg/ml cholesteryl for 24 h, washed three times with PBS, and fixed for 15 min in 3% (w/v) paraformaldehyde in PBS. The cells were permeabilized by treat-
ment for 20 min with 50 µg/ml saponin in PBS and then stained with Nile Red (0.1 µg/ml in PBS) for 10 min. The cells were washed and examined in a Leica DM IRE2 digital microscope (Leica Microsystems, Wetzelar, Germany) equipped with an N PLAN L 20×0.40 objective and a Leica epifluorescence lamp.

Lipoprotein Size—The size of glia-derived lipoprotein particles was evaluated by fast protein liquid chromatography. Glia-conditioned medium was concentrated 50-fold using an Amicon Ultra Filter (30-kDa molecular mass cut-off) and applied to a Superose 6 gel filtration column (Amersham Biosciences) attached to a Beckman Systems Gold or Nouveau Gold apparatus. The cholesterol content of the eluate was monitored by an in-line detection assay (Infinity Cholesterol Reagent, Sigma).

RNA Isolation and Real-time qPCR—Total RNA was isolated from cultured glia by extraction with TRIzol (Invitrogen), cDNA was synthesized from 1.5 µg of total RNA using oligo(dT)12-18 random primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time qPCR was performed using Platinum® Quantitation PCR supermix (Invitrogen), SYBR Green I (Molecular Probes), and intron-spanning, gene-specific oligonucleotides (250 nM of each primer) in a total volume of 25 µl. Transcripts were detected by real-time qPCR with a Rotor-Gene 3000 instrument (Montreal Biotech, Montreal, Quebec, Canada). Data were analyzed using the Rotor-Gene 6.0.19 program. A standard curve was used to calculate mRNA level relative to that of a control gene, cyclophilin. The specificity of products was confirmed by agarose gel electrophoresis and sequence analysis. All primers were synthesized at the DNA Core Facility of the University of Alberta with sequences as follows: cyclophilin, 5′-TCC AAA GAC AGC AGA AAA CTT TCG (sense), 5′-TCT TCT TGC TGG TCT TGC CAT TCC (antisense); ABCA1, 5′-TTG GAT GGA TTA GAT TGG AC (sense), 5′-ATG CCT GTG AAG AGC ATG (antisense); ABCG1, 5′-TGA CAC ATC TGC GAA TCA C (sense), 5′-AGG GGA AAG GTC AGA ACA (antisense).

**RESULTS**

Cholesterol Loading of Astroglia Increases Cholesterol Efflux to Exogenous apoA1—Astroglia release cholesterol in the form of apoE-containing lipoproteins in the absence of any exogenously added apolipoprotein acceptor. Astroglia can also release cholesterol to extracellular acceptors such as apoA1 in a process mediated by ABC transporters (28, 29). To examine cholesterol efflux mediated by ABC transporters, we measured cholesterol output into the medium of cerebellar glial cells in the absence and presence of apoA1. We also investigated cholesterol efflux from glia in which the intracellular cholesterol content had been raised by pre-treatment with cholesterol, because the amount of radiolabeled cholesterol in the medium does not necessarily reflect cholesterol mass. In these experiments, as an alternative method for increasing the cholesterol content, glia were preincubated for 24 h in serum-free medium containing low density lipoproteins (LDL), which increased the cholesterol content of the glia from 39.9 ± 2.7 µg/mg of protein to 115.5 ± 6.2 µg/mg of protein. As shown in Fig. 1A, the efflux of cholesterol synthesized endogenously from [14C]acetate was not increased by the addition of apoA1 alone, or by cholesterol loading alone. However, when glia were enriched with cholesterol, apoA1 promoted [14C]cholesterol efflux.

We also measured the mass of cholesterol in the culture medium, because the amount of radiolabeled cholesterol in the medium does not necessarily reflect cholesterol mass. In these experiments, as an alternative method for increasing the cholesterol content, glia were preincubated for 24 h in serum-free medium containing low density lipoproteins (LDL), which increased the cholesterol content of the glia from 39.9 ± 2.7 µg/mg of protein to 57.3 ± 6.0 µg/mg of protein. The cholesterol-enriched cells were incubated for 3 days in serum- and LDL-free medium, containing or lacking apoA1, and the amount of cholesterol in cells and medium was determined (Fig. 1B). A longer conditioning period, compared with that used for the experiments in Fig. 1A, was necessary to attain sufficient cholesterol concentrations in...
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the medium for analysis by gas-liquid chromatography. In agreement with the data shown in Fig. 1A, the release of cholesterol was enhanced only when the cholesterol content of the cells was increased and, in addition, apoA1 was added exogenously (Fig. 1B). The amount of apoE in the medium was not changed by these treatments (Fig. 1C).

Cholesterol Efflux to Exogenous ApoA1 Is Not Increased by the LXR Agonist TO901317—LXR agonists increase the expression of ABCA1 and other ABC transporters in fibroblasts and macrophages (37–40) and, correspondingly, enhance cholesterol efflux to apoA1. In the following experiments, we determined whether or not the LXR agonist, TO901317, also stimulated cholesterol efflux from astroglia. Surprisingly, treatment of glia with TO901317 did not increase the efflux of either radiolabeled cholesterol (Fig. 2A), or cholesterol mass (Fig. 2B). Nor did a combination of apoA1 and TO90137 increase cholesterol efflux (Fig. 2, A and B). Fig. 2C shows that apoE secretion was independent of the presence of apoA1, whereas the LXR agonist (2 μM) did increase apoE secretion. Treatment of glia with a higher concentration of TO901317 (10 μM) also failed to stimulate cholesterol efflux but increased apoE secretion to a greater extent than did 2 μM TO901317 (not shown). Thus, in cerebellar glia, in contrast to other types of cells, the LXR agonist TO901317 does not enhance the release of cholesterol to apoA1. When cholesterol-loaded cells were incubated with TO901317, cholesterol efflux to apoA1 was greater than that induced by cholesterol loading or the LXR agonist alone (Fig. 2D).

ABCA1 Expression Is Increased by the LXR Agonist But Not by Cholesterol Loading—We next examined the regulation of expression of two ABC transporters, ABCA1 and ABCG1, in cerebellar glia. We also determined whether or not cholesterol efflux from astroglia to exogenous apoA1 correlated with ABCA1 expression, because ABCA1 is thought to be a key factor in mediating cholesterol efflux to exogenous apoA1, and in regulating plasma levels of HDL (30, 31). ABCA1 protein and mRNA levels were measured under the same conditions that were used for the cholesterol efflux experiments described in Figs. 1 and 2. TO901317 markedly increased the amount of ABCA1 protein in astroglia (Fig. 3A). The magnitude of the increase in ABCA1 expression after treatment with 2 μM and 10 μM TO90137 was similar (not shown). In contrast, enrichment of glia with cholesterol did not increase the level of ABCA1 (Fig. 3A). A similar pattern was seen in ABCA1 mRNA levels: the abundance of ABCA1 mRNA increased 1.9-fold after treatment of the glia with TO901317, whereas cholesterol loading did not significantly increase the level of ABCA1 mRNA (Fig. 3B). In parallel experiments, in which the glia were enriched with cholesterol by incubation with LDL, the amount of ABCA1 protein was not increased, but was modestly decreased (not shown).

To test whether the increased amount of ABCA1 induced by the LXR agonist resulted in an increase in the amount of ABCA1 on the cell surface, we biotinylated proteins on the surface of cells that had been either loaded with cholesterol or treated with TO901317. As shown in Fig. 3C, essentially all ABCA1 in control cells and in cells that had been incubated with either cholesterol or the LXR agonist was biotinylated and was, therefore, on the cell surface. Almost no ABCA1 was detectable in the non-biotinylated (intracellular) fraction. Consistent with the increased amount of total ABCA1 in TO901317-treated cells, the amount of ABCA1 on the cell surface was also increased by the LXR agonist, whereas cholesterol loading did not alter the amount of ABCA1 on the cell surface or the total amount of ABCA1 in the lysate. Calnexin, an integral protein of the endoplasmic reticulum, was, as expected, largely restricted to the non-biotinylated fraction. Thus, in agreement with the finding that cholesterol loading of glia does not increase cholesterol efflux or the amount of ABCA1 protein, cholesterol loading also does not alter the amount of ABCA1 on the cell surface. However the LXR agonist increased cholesterol efflux and increased the total amount of ABCA1 as well as ABCA1 on the cell surface.

These observations demonstrate that enrichment of glia with cholesterol increases cholesterol efflux to apoA1 (Fig. 1, A and B) but does not increase ABCA1 expression (Fig. 3, A and B). On the other hand, treatment of glia with the LXR agonist increases both the total amount of
ABC1 (Fig. 3, A and B) as well as the amount of ABCA1 on the cell surface (Fig. 3C), without stimulating cholesterol efflux (Fig. 2, A and B). Thus, neither in cholesterol-loaded astroglia nor astroglia treated with the LXR agonist does the level of ABCA1 mRNA or protein on the cell surface correlate with the release of cholesterol into the medium.

**ABCG1 mRNA Expression Is Increased by Both Cholesterol Loading and the LXR Agonist**—Recent studies indicate that ABCA1 is required for the initial lipiddation of extracellular apoA1, whereas ABCG1, an ABC transporter that is abundant in the brain, lungs, and macrophages (26, 41), is required for the release of cholesterol to HDL, and other partially lipidated apoA1- or apoE-containing lipoprotein particles. Because our data revealed a lack of correlation between the efflux of cholesterol and ABCA1 expression, we next examined the expression of ABCG1 mRNA in astroglia cultured under the same conditions that were used for the cholesterol efflux experiments depicted in Figs. 1 and 2. Treatment of astroglia with TO901317 increased ABCG1 mRNA 2.4-fold (Fig. 4), and cholesterol loading of the glia increased ABCG1 mRNA 1.6-fold (Fig. 4). Thus, enrichment of the glia with cholesterol enhances cholesterol efflux in concert with an increased expression of ABCG1 mRNA but not ABCA1 mRNA or protein.

**Cholesterol Release by ApoE<sup>−/−</sup> Glia**—To distinguish between the two pathways of cholesterol release from glia (i.e. a direct secretion in conjunction with apoE compared with efflux to extracellular apolipoproteins via ABC transporters) we cultured cerebellar astroglia from ApoE<sup>−/−</sup> mice and determined the relationship between cholesterol efflux to apoA1 and the expression of ABCA1 and ABCG1. We reasoned that cholesterol released from ApoE<sup>−/−</sup> glia to an extracellular acceptor would primarily originate from an ABC transporter-mediated pathway, because the secretion of cholesterol with endogenously synthesized apoE was eliminated. ApoE<sup>−/−</sup> glia were, therefore, radiolabeled with [<sup>14</sup>C]acetate, and the appearance of radiolabeled cholesterol in the medium was monitored. ApoE<sup>−/−</sup> glia released only negligible amounts of endogenously synthesized [<sup>14</sup>C]cholesterol in the absence of exogenously added apolipoprotein (Fig. 5). However, when ApoE<sup>−/−</sup> glia were incubated with apoA1, the efflux of endogenously synthesized [<sup>14</sup>C]cholesterol was markedly enhanced (Fig. 5), such that 29% of cellular cholesterol was released during the 24-h incubation period. In contrast, in wild-type glia only 8–12% of cellular cholesterol is typically released to apoA1 under the same conditions (Figs. 1A and 2A). Sucrose density gradient ultracentrifugation showed that the size of the lipoproteins generated by ApoE<sup>−/−</sup> glia was similar to that of lipoproteins produced by wild-type glia in the absence of exogenous apoA1 (data not shown). Thus, the ABC transporter-mediated pathway for cholesterol efflux to an exogenous acceptor apolipoprotein, such as apoA1, is functional in ApoE<sup>−/−</sup> glia. Nevertheless, despite an active cholesterol efflux pathway, enrichment of ApoE<sup>−/−</sup> glia with cholesterol did not enhance the release of [<sup>14</sup>C]cholesterol to apoA1 (Fig. 5). These observations differ from the situation in wild-type glia in which cholesterol loading significantly increased the release of cholesterol to apoA1 (Fig. 1, A and B).

**Cholesteryl Ester Levels of ApoE<sup>−/−</sup> Glia Are Increased by Cholesterol Loading**—These observations raised the question of why cholesterol loading of wild-type, but not ApoE<sup>−/−</sup>, glia increased cholesterol efflux to apoA1. Wellington and coworkers (28) have reported that in cortical glia from Abca1<sup>−/−</sup> mice, apoE secretion is reduced and neutral lipids accumulate within the cells. We hypothesized, therefore, that in cerebellar glia from ApoE<sup>−/−</sup> mice neutral lipids (primarily cholesteryl esters) would similarly accumulate, because the secretion of cholesterol with apoE had been eliminated. ApoE<sup>−/−</sup> glia were, therefore, incubated with cholesterol and stained with Nile Red to visualize neutral lipids. Fig. 6A shows that neutral lipids accumulate markedly within all ApoE<sup>−/−</sup> glia upon cholesterol loading. In contrast, far less neutral lipid accumulates in cholesterol-enriched wild-type glia. Analysis of cellular lipids of wild-type and ApoE<sup>−/−</sup> glia by gas-liquid chromatography verified this finding and showed that the amount of cholesteryl esters in cholesterol-enriched ApoE<sup>−/−</sup> glia is approximately double that in wild-type glia loaded with cholesterol (Fig. 6B). These data suggest that in ApoE<sup>−/−</sup> glia excess cholesterol is shunted into the esterification pathway, because the normal route of cholesterol release with apoE has been eliminated.

If increased cholesteryl ester formation were the reason why cholesterol efflux from cholesterol-loaded ApoE<sup>−/−</sup> glia was not stimulated by cholesterol loading (Fig. 5), one would predict that inhibition of acyl-CoA:cholesterol acyltransferase, the enzyme that catalyzes cholesterol esterification, might stimulate cholesterol efflux to apoA1 in cholester-
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FIGURE 5. Cholesterol efflux from ApoE<sup>−/−</sup> astroglia to apoA1 is not increased by cholesterol loading or TO901317. Cerebellar glia from ApoE<sup>−/−</sup> mice were incubated with [<sup>14</sup>C]acetate for 3 days during the growth phase. Confluent glia were then incubated in serum-free DMEM with or without 30 μg/ml cholesterol (cholesterol) or 2 μM TO901317 for 24 h after which serum-free DMEM, with or without 10 μg/ml apoA1, was added for 24 h. Lipids were extracted from the cells and medium and separated by thin-layer chromatography, and radioactivity in cholesterol was measured. Data are expressed as [<sup>14</sup>C]cholesterol in the medium as a percentage of [<sup>14</sup>C]cholesterol plus [<sup>14</sup>C]cholesterol ester in medium and cells combined. Data are means ± S.D. of two independent experiments, each performed in triplicate.

ol-loaded glia. Fig. 6C shows that this is, indeed, the case. When an inhibitor (S58-035) of cholesterol esterification was added to cholesterol-enriched glia during the efflux period, the amount of cholesterol released from cholesterol-loaded ApoE<sup>−/−</sup> glia was significantly increased. The addition of S58-035 to glia not enriched with cholesterol did not increase cholesterol efflux to apoA1 (Fig. 6C). These observations support the hypothesis that increased formation of cholesteryl esters in cholesterol-loaded ApoE<sup>−/−</sup> glia limits the availability of unesterified cholesterol for efflux.

Regulation of Expression of ABCA1 and ABCG1 in ApoE<sup>−/−</sup> Glia—One possible reason why cholesterol loading fails to increase cholesterol efflux to apoA1 in ApoE<sup>−/−</sup> glia is that ABCG1 expression might not have been up-regulated. We, therefore, determined if the expression of ABCA1 and ABCG1 was differently regulated in wild-type glia and ApoE<sup>−/−</sup> glia. The amount of ABCA1 protein in ApoE<sup>−/−</sup> glia was similar to that in wild-type glia (not shown). Consistent with our observation that cholesterol efflux to apoA1 was not increased upon cholesterol enrichment of ApoE<sup>−/−</sup> glia (Fig. 5), the amounts of ABCA1 protein and mRNA were not increased by cholesterol enrichment. Cholesterol enrichment of the glia even caused a slight reduction in the amount ABCA1 protein (Fig. 7A). In contrast, the LXR agonist increased ABCA1 mRNA abundance by 2.5-fold (Fig. 7D), an increase that is similar in magnitude to that in wild-type glia (Fig. 4). The simplest interpretation of these observations is that cholesterol loading of ApoE<sup>−/−</sup> glia does not increase cholesterol efflux to apoA1, because the expression of ABCG1 is not up-regulated. These data support the idea that ABCG1 plays a key role in lipoprotein formation by glia. We speculate that even though ABCG1 mRNA is increased by TO901317 in ApoE<sup>−/−</sup> glia, cholesterol efflux is not enhanced because the conversion of cholesterol to cholesteryl esters is increased and the availability of unesterified cholesterol for efflux is reduced.

Lipoprotein Size—In view of previous studies indicating that ABCA1 is required for the initial addition of cholesterol and phospholipids to lipid-free/lipid-poor apolipoproteins, whereas ABCG1 facilitates the transfer of lipids to pre-formed lipoprotein particles (27, 37, 39, 41), we analyzed the size of lipoproteins in glia-conditioned medium. We hypothesized that if ABCA1 were the key regulator of glial lipoprotein formation, and if the amount of cellular cholesterol limited cholesterol efflux, up-regulation of ABCA1 (for example, by the LXR agonist) would

FIGURE 6. Cholesteryl esters accumulate in cholesterol-enriched ApoE<sup>−/−</sup> glia. Confluent wild-type (WT) and ApoE<sup>−/−</sup> glia were incubated for 24 h in serum-free DMEM with (cholesterol) or without (SF) 30 μg/ml cholesterol, then stained with Nile Red to visualize neutral lipids (A) or harvested and analyzed for cholesteryl ester content by gas-liquid chromatography (B). Data in B are expressed as micrograms of cholesteryl esters/mg of cell protein and are means ± S.D. of two independent experiments performed in triplicate.

ABC1 expression (Fig. 4). Nevertheless, the LXR pathway was intact in ApoE<sup>−/−</sup> glia, because incubation with TO901317 increased ABCG1 mRNA abundance by 2.5-fold (Fig. 7D), an increase that is similar in magnitude to that in wild-type glia (Fig. 4). The simplest interpretation of these observations is that cholesterol loading of ApoE<sup>−/−</sup> glia does not increase cholesterol efflux to apoA1, because the expression of ABCG1 is not up-regulated. These data support the idea that ABCG1 plays a key role in lipoprotein formation by glia. We speculate that even though ABCG1 mRNA is increased by TO901317 in ApoE<sup>−/−</sup> glia, cholesteryl efflux is not enhanced because the conversion of cholesterol to cholesteryl esters is increased and the availability of unesterified cholesterol for efflux is reduced.
result in the formation of more, but smaller, particles. On the other hand, if the amount of ABCG1 regulated cholesterol efflux into glial lipoproteins, increased expression of ABCG1 would result in generation of larger lipoprotein particles, provided that sufficient cholesterol was available for efflux.

The sizes of lipoprotein particles produced by glia incubated with cholesterol alone, TO901317 alone, or a combination of both compounds were analyzed by gel filtration using fast protein liquid chromatography. Most of the lipoproteins derived from untreated glia eluted at 39.5 ± 0.5 min (Fig. 8A). Enrichment of glia with cholesterol resulted in many lipoprotein particles eluting earlier (after 36.5 min) (Fig. 8B), indicating that larger lipoproteins had been generated by glia that had been loaded with cholesterol. These data support our hypothesis, because cholesterol loading increased the expression of ABCG1 (Fig. 4) but not ABCA1 (Fig. 3). Thus, an increased cellular content of cholesterol and an increased expression of ABCG1 (Fig. 4) correlate with an increased size/ lipidation of the lipoprotein particles. Treatment of the glia with TO901317, in the absence of cholesterol loading, resulted in production of a population of smaller particles that eluted later (after 49.0 min) than those produced in the absence of the LXR agonist (Fig. 8, compare C with A). Because expression of both ABCA1 and ABCG1 was increased by the LXR agonist (Figs. 3 and 4), we speculate that under these conditions an increased level of ABCA1 results in a larger number of small particles, whereas a limited supply of cholesterol to the “efflux-accessible” pool restricts the amount of cholesterol available for release via ABCG1. In accordance with this concept, a combination of cholesterol loading and treatment with the LXR agonist (Fig. 8D) eliminated the population of small particles generated by glia treated with the LXR agonist in the absence of cholesterol loading (Fig. 8C).

DISCUSSION

We have investigated the generation of lipoproteins by cerebellar glia in relation to the expression of two ABC transporters, ABCA1 and ABCG1. Our experiments demonstrate that the amount of cholesterol released into the medium of cultured cerebellar glial cells does not correlate with the level of ABCA1 or the amount of cell-surface ABCA1. This conclusion is in marked contrast to the well established relationship between the ABCA1 expression and cholesterol efflux in other types of cells (reviewed in Refs. 30 and 31). Our major findings are: (i) cholesterol enrichment of glia does not increase the expression of ABCA1 mRNA or protein, but does increase the expression of ABCG1 mRNA, as well as cholesterol efflux to exogenously added apoA1; (ii) the LXR agonist, TO901317, increases the expression of both ABCA1 and

FIGURE 7. Regulation of expression of ABCA1 and ABCG1 in ApoE−/− glia by cholesterol loading and TO901317. Confluent ApoE−/− glia were incubated for 24 h in serum-free DMEM alone (control), or in DMEM containing 30 μg/ml cholesterol (chol), or 2 μM TO901317 (TO). Cell lysates were analyzed for ABCA1 protein by immunoblotting (A). Data are representative of three experiments with similar results. B, levels of ABCA1 mRNA relative to cyclophilin mRNA were assessed by real-time qPCR. Data are means ± S.D. of two independent experiments, each performed in triplicate. *, p < 0.00002 compared with control. C, surface expression of ABCA1 was analyzed by immunoblotting of cell surface proteins that had been biotinylated and isolated over a streptavidin column. For the lysate from untreated cells (con), cholesterol loaded cells (chol), or TO901317-treated cells, 45 μg of total cell lysate proteins were loaded on the gel. For the fraction corresponding to cell-surface biotinylated proteins (Surface) the amount of protein loaded on the gel was equivalent to 108 μg of total lysate protein. For the streptavidin column flow-through (intracellular proteins, not biotinylated) an amount of protein equivalent to 54 μg of total lysate protein was loaded on the gel (Intracellular). Calnexin was used as a marker of proteins associated with intracellular membranes. Data are representative of two independent experiments with similar results. D, the amount of ABCG1 mRNA was assessed relative to cyclophilin mRNA. Data are means ± S.D. of two independent experiments, each performed in triplicate. *, p < 0.00002 compared with control.

FIGURE 8. Cholesterol loading of glia increases lipoprotein size. Medium conditioned by cerebellar glia (A, untreated; B, incubated with 30 μg/ml cholesterol; C, treated with 2 μM TO901317; D, treated with 30 μg/ml cholesterol plus 2 μM TO901713) for 3 days was concentrated, then analyzed by gel filtration chromatography and post-column detection of cholesterol. Larger lipoprotein particles elute earlier than smaller particles. a.u. = arbitrary units. Data are averages of three independent experiments.

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ABC1 but does not enhance cholesterol efflux; (iii) cholesterol enrichment of glia results in formation of a population of larger lipoprotein particles, whereas TO901317 treatment produces smaller particles that are eliminated when the glia are loaded with cholesterol; (iv) in the absence of cholesterol enrichment, apoA1 promotes cholesterol efflux from ApoE−/− glia but not from wild-type glia; (v) cholesterol loading of ApoE−/− glia fails to increase cholesterol efflux to apoA1 and cholesterol esters accumulate; inhibition of cholesterol esterification restores cholesterol efflux; and (vi) treatment of ApoE−/− glia with TO901317 increases expression of both ABCA1 and ABCG1, whereas cholesterol enrichment of the cells does not increase the expression of either ABCG1 or ABCA1.

The increase in cholesterol efflux to apoA1 induced by cholesterol loading of glia is consistent with previous observations in fibroblasts (42). It is noteworthy, however, that the addition of apoA1, in the absence of cholesterol loading, fails to stimulate cholesterol release. These observations suggest that apoE that is secreted by glia acts as an acceptor for cholesterol and is so abundant that it saturates the ABC transporter-mediated pathway for cholesterol efflux. Thus, when the availability of cholesterol for efflux is limited, the addition of apoA1 is unable to stimulate cholesterol release. In contrast, when glia are loaded with cholesterol, apoA1 promotes cholesterol release.

Our observations raise the question of why the regulation of cholesterol efflux from CNS astroglia is different from that in peripheral cells in which ABCA1 appears to be the primary determinant of HDL formation. We speculate that one major difference between the cell types is that two distinct mechanisms are involved in the generation of lipoproteins by glia (Fig. 9). In one pathway, endogenously synthesized apoE is secreted in association with some lipid. This pathway is unlikely to generate significant amounts of lipoproteins in most non-CNS cells. In the second pathway, extracellular lipid-free, or lipid-poor, apoA1 and/or apoE acquire lipids via ABC transporter-mediated lipid efflux. Our data are consistent with a model in which glia secrete lipoprotein particles containing endogenously made apoE complexed with some lipids, including cholesterol. Consequently, the initial lipidation step, mediated by ABCA1 in the extracellular space, is bypassed (Fig. 9). Because partial lipidation of apoE inhibits its interaction with ABCA1 (33), the partially lipidated apoE-containing particles would be subject to further lipidation via ABCG1 but not ABCA1. The addition of glia-derived lipid to exogenously added apoA1 would be expected to occur by the same mechanism used by other types of cells in which apoA1 is lipidated by the sequential action of ABCA1 and ABCG1.

Interestingly, regulation of expression of the ABC transporters in ApoE−/− cerebellar glia appears to be different from that in wild-type glia. Our data show that cholesterol enrichment of wild-type but not ApoE−/− glia increases ABCG1 expression. A reason for, or a consequence of, the inability of cholesterol to up-regulate ABCG1 expression in ApoE−/− glia appears to be that cholesterol accumulates within the cells in the form of cholesterol esters. The accumulation of cholesterol esters has also been observed in Abca1−/− glia in which the absence of ABCA1, as well as an impairment of apoE secretion, would curtail both mechanisms of cholesterol release (28, 29).

The lack of correlation between ABCA1 expression and cholesterol release from glia appears to be partially dependent on the type of glia. In contrast to our results with cerebellar glia, Holtzmann and co-workers (29) and Wellington and co-workers (28) recently reported that addition of apoA1 to Abca1−/− cortical glia in the absence of cholesterol loading stimulates cholesterol efflux. Our experiments demonstrate that cholesterol efflux from cerebellar glia to apoA1 is increased only when the glia are enriched with cholesterol. Moreover, under these conditions cholesterol efflux is increased despite the lack of an increase in ABCA1 expression. The apparent differences between our studies in cerebellar glia and the previous studies in cortical glia are likely to be attributable to the different types of glia, because when we used cortical glia under our experimental conditions the results were similar to those previously reported for cortical glia. The glia in our cultures are likely to be primarily Bergmann glia, which not only guide migrating neurons during development (43), but also influence the shape of the dendritic tree of Purkinje cells in the mature cerebellum (44, 45).

In conclusion, our observations suggest that in cerebellar glia ABCG1 is the primary determinant of cholesterol release to apoE for lipoprotein formation. The major extracellular acceptor of glia-derived cholesterol is probably apoE that is secreted from the glia in a partially lipidated state. Our data suggest that ABCA1 mediates the initial lipidation of extracellular apoA1/apoE and that ABCG1 participates in the further lipidation of pre-formed apoE-containing lipoproteins secreted by the glia and of pre-formed apoA1-containing lipoproteins formed by the action of ABCA1 (Fig. 9). Our data also suggest that the ABCG1-dependent lipidation of lipoproteins is regulated by the availability of cholesterol, because increased expression of ABCG1 alone, in the absence of cholesterol loading, does not stimulate cholesterol efflux.

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