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*Published in:*  
The Journal of Biological Chemistry

*DOI:*  
[10.1074/jbc.M601019200](https://doi.org/10.1074/jbc.M601019200)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2006

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Abildayeva, K., Jansen, P. J., Hirsch-Reinshagen, V., Bloks, V. W., Bakker, A. H. F., Ramaekers, F. C. S., ... Mulder, M. (2006). 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. *The Journal of Biological Chemistry*, 281(18), 12799-12808. DOI: 10.1074/jbc.M601019200

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# 24(S)-Hydroxycholesterol Participates in a Liver X Receptor-controlled Pathway in Astrocytes That Regulates Apolipoprotein E-mediated Cholesterol Efflux\*

Received for publication, February 2, 2006 Published, JBC Papers in Press, March 8, 2006, DOI 10.1074/jbc.M601019200

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Both apolipoprotein E (apoE) and 24(S)-hydroxycholesterol are involved in the pathogenesis of Alzheimer disease (AD). It has been hypothesized that apoE affects AD development via isoform-specific effects on lipid trafficking between astrocytes and neurons. However, the regulation of the cholesterol supply of neurons via apoE-containing high density lipoproteins remains to be clarified. We show for the first time that the brain-specific metabolite of cholesterol produced by neurons, *i.e.* 24(S)-hydroxycholesterol, induces apoE transcription, protein synthesis, and secretion in a dose- and time-dependent manner in cells of astrocytic but not of neuronal origin. Moreover, 24(S)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE. Similar results were obtained using the synthetic liver X receptor (LXR) agonist GW683965A, suggesting involvement of an LXR-controlled signaling pathway. A 10–20-fold higher basal LXR $\alpha$  and - $\beta$  expression level in astrocytoma compared with neuroblastoma cells may underlie these differential effects. Furthermore, apoE-mediated cholesterol efflux from astrocytoma cells may be controlled by the ATP binding cassette transporters ABCA1 and ABCG1, since their expression was also up-regulated by both compounds. In contrast, ABCG4 seems not to be involved, because its expression was induced only in neuronal cells. The expression of sterol regulatory element-binding protein (SREBP-2), low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, and SREBP-1c was transiently up-regulated by GW683965A in astrocytes but down-regulated by 24(S)-hydroxycholesterol, suggesting that cholesterol efflux and synthesis are regulated independently. In conclusion, evidence is provided that 24(S)-hydroxycholesterol induces apoE-mediated efflux of cholesterol in astrocytes via an LXR-controlled pathway, which may be relevant for chronic and acute neurological diseases.

Disturbances in brain cholesterol homeostasis are associated with the onset of severe neurological diseases (1) and have recently been suggested to play a key role in the development of Alzheimer disease (AD)<sup>4</sup> (2, 3). The brain, although composing just 2% of the total body mass, contains about a quarter of an individual's whole body unesterified cholesterol. Brain cholesterol originates almost exclusively from *in situ* neosynthesis (1); circulating cholesterol is prevented from entering the brain by the blood-brain-barrier (4). Because cholesterol cannot be degraded and is neurotoxic at high levels, excess cholesterol is secreted from the brain into the circulation (5). Cholesterol is removed from the brain predominantly (about 60%) in the form of the relatively polar brain-specific metabolite, 24(S)-hydroxycholesterol, formed by the enzyme cholesterol 24(S)-hydroxylase (CYP46) (1). The remaining 40% of cholesterol is secreted from the brain via an unknown pathway that may involve apoE (6). CYP46 is expressed predominantly by neurons (7, 8). Several studies have suggested a role for 24(S)-hydroxycholesterol in the pathogenesis of AD (9–11). Polymorphisms of CYP46 have been linked to AD, and the expression of this enzyme appeared to be shifted from neurons to glia in AD patients (12). Finally, increased levels of 24(S)-hydroxycholesterol levels have been detected in cerebrospinal fluid of AD patients (13).

24(S)-Hydroxycholesterol is a natural ligand of the liver X receptors (LXR), which have recently been identified as central players in the regulation of cholesterol metabolism (14, 15). LXR belong to the nuclear hormone receptor superfamily, and two isoforms, *i.e.* LXR $\alpha$  and LXR $\beta$ , have been identified that are activated by oxysterols. Both isoforms of LXR are expressed in the central nervous system (16) and are thought to be involved in the regulation of brain cholesterol metabolism. LXR $\alpha$ / $\beta$ -null mice show a variety of central nervous system defects upon aging, including lipid accumulation, astrocyte proliferation, and disorganized myelin sheaths (17). The synthetic LXR ligand T0901317 was found to induce the expression of apoE and of the ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1) in astrocytes (18). However, reported *in vivo* effects of T0901317 on apoE expression in mouse brain are inconsistent (16, 19, 20).

The strongest genetic risk factor known for sporadic AD is apolipoprotein E4 (apoE4), one of the three common apoE variants (apoE2,

\* This work was supported in part by the Internationale Stichting Alzheimer Onderzoek Grant 03516 and in part by Netherlands Brain Foundation Grant H00.15. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by the Canadian Institutes of Health Research.

<sup>2</sup> Supported by the Alzheimer Society of Canada.

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<sup>4</sup> The abbreviations used are: AD, Alzheimer disease; HMG, hydroxymethylglutaryl; LDLR, low density lipoprotein receptor; ABC, ATP binding cassette; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; QRT-PCR, real-time quantitative PCR; LXR, liver X receptor.

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apoE3, apoE4) in humans (21, 22). ApoE is a key player in the transport of cholesterol in the circulation (23) and is also thought to fulfill such a role within the brain (24). Astrocytes are the predominant source of apoE in the brain. These cells secrete apoE in association with cholesterol and phospholipids in the form of small, high density lipoprotein-like particles (25). It has been suggested that these particles provide neurons with cholesterol required for the formation of new membranes, e.g. during development, regeneration after injury, or during the formation of new synaptic contacts (26). It has been hypothesized that apoE may affect the pathogenesis of AD by isoform-specific effects on lipid trafficking between astrocytes and neurons (27). Indeed, apoE in combination with cholesterol induces the outgrowth of neurites in an isoform-specific manner in neuronal cultures (28). However, factors that regulate the supply of glial-derived apoE-containing lipoproteins are poorly understood.

In this study we tested the hypothesis that 24(S)-hydroxycholesterol represents a natural brain-specific LXR ligand that is involved in the regulation of the apoE-mediated lipid supply. For this purpose the effects of 24(S)-hydroxycholesterol and the synthetic LXR agonist GW683965A on the expression of apoE and additional LXR target genes involved in cholesterol efflux were compared using human neuroblastoma and astrocytoma cell lines as well as primary astrocytes. We found that 24(S)-hydroxycholesterol, like GW683965A, is able to induce the expression of ABCA1, ABCG1, and apoE in astrocytes and to elevate apoE-mediated cholesterol efflux in astrocytoma but not in neuroblastoma cells. Our observations support the hypothesis that 24(S)-hydroxycholesterol participates in an LXR-controlled pathway that regulates cholesterol availability in the brain.

### EXPERIMENTAL PROCEDURES

**Chemical Reagents**—24(S)-Hydroxycholesterol was a kind gift from Dr. D. Lutjohann (Bonn University, Germany), and 22(R)-hydroxycholesterol was a kind gift from Dr. J. Plat (Maastricht University, The Netherlands). GW683965A was provided by GlaxoSmithKline. The following reagents were purchased from Sigma: 9-*cis*-retinoic acid, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, bovine insulin, human transferrin, putrescine, sodium selenite, and progesterone. ApoA-I and apoE were purchased from Calbiochem. Stocks of 24(S)-OH cholesterol (10 mM), 22(R)-OH cholesterol (10 mM), and cholesterol (10 mM) were dissolved in ethanol. GW683965A (2 mM) and 9-*cis*-retinoic acid (10 mM) were dissolved in dimethyl sulfoxide.

**Cell Culture Experiments**—The human astrocytoma cell line CCF-STTG1 and human neuroblastoma cell line SH-SY-5Y were purchased from European Collection of Cell Cultures (Salisbury, UK). CCF-STTG1 and SH-SY-5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. At 80–90% confluency cells were washed with phosphate-buffered saline (PBS) and treated with different reagents in DMEM/Ham's F-12 medium (1:1) with 10% fetal calf serum for various periods of time as indicated. SH-SY-5Y cells were preincubated for 24 h in the DMEM-Ham's F-12 (1:1) medium without serum containing the N2 supplement (2.5 mg/ml bovine insulin, 10 mg/ml human transferrin (iron-saturated), 0.52  $\mu$ g/ml sodium selenite, 1.61 mg/ml putrescine, and 0.63  $\mu$ g/ml progesterone in PBS, pH 7.3). At the end of the treatments conditioned media were collected, and the cells were either lysed in radioimmune precipitation assay buffer (Santa Cruz, CA), or total RNA was isolated as described below for subsequent analyses.

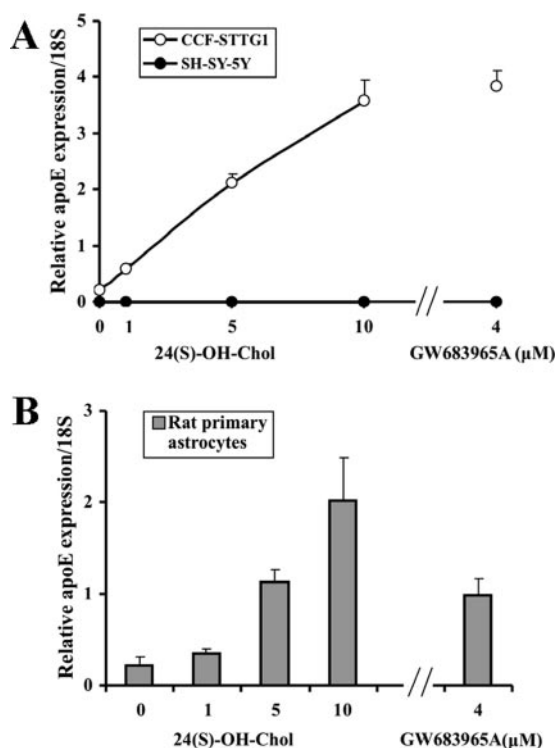
The method of rat primary astrocytes culturing was similar to published procedures (29, 30). Neonatal (postnatal day 1) Lewis rat pups, bred in the animal facilities of Maastricht University, were decapitated,

and the neocortex was dissected and cleared of meninges. The tissue was diced in small fragments and incubated in trypsin (0.05% in phosphate-buffered saline) at 37 °C for 15 min. Trypsinization was stopped by adding culture medium, and the tissue was gently centrifuged. The supernatant was discarded, and the pellet was resuspended in 1 ml of culture medium. Single cell dissociation was achieved by 3–5 passes through a 5-ml pipette (Greiner, Germany) and 10–20 passes through a 1-ml pipette (Greiner). Then the tissue was centrifuged very briefly to separate cells from tissue debris. The supernatant containing the cells was then plated into 25-cm<sup>2</sup> cell culture flasks (Corning, NY) at a density of 10<sup>6</sup> cells per flask. Culture medium was refreshed after 4–5 days and every 2 days thereafter. At DIV12 (days *in vitro*) the cultures reached confluence, and contaminating cells were shaken off on a rotary shaker (Rotofix 32, Hettich Zentrifugen). This involves shaking of the flasks for 48 h and refreshment of the medium. At DIV14 the purification is complete and renders >95% glial fibrillary acid protein (GFAP)-immunopositive astrocyte cultures.

Primary murine mixed glial cultures were prepared from postnatal day-1–2 C57Bl/6 mice. Brains from individual animals were placed into ice-cold Hanks'-buffered salt solution (Canadian Invitrogen) containing 6 mg/ml glucose and 10 mM HEPES. Meninges were removed, frontal cortices were dissected, and cells were dissociated by repeated passage through a series of wide to fine bored pipettes. Dissociated cells were plated in DMEM (Invitrogen) with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen) and 100 units/ml penicillin-streptomycin (Invitrogen) at one 24-well plate per mouse. Cells were cultured in the presence of 5% CO<sub>2</sub> for 12 days when cells were confluent and contained at least 80% astrocytes. Cells were treated with either vehicle-only (ethanol) or increasing concentrations of 24(S)-hydroxycholesterol for 24 h. Subsequently, cells were washed once with phosphate-buffered saline, harvested, and lysed using a buffer containing 10% glycerol, 1% Triton X-100, and protease inhibitor (Roche Applied Science) in PBS. Protein concentration was determined by a Dc protein assay (Bio-Rad).

**Western Blot Analysis**—Cell lysates (25  $\mu$ g of protein/lane) or conditioned media concentrates (concentrated using Microcon<sup>®</sup> centrifugal filter device, Millipore, Billerica, MA) were subjected to dodecyl sulfate-10 (SDS) or 12% PAGE and then transferred to Protran nitrocellulose transfer membranes (Schleicher & Schuell) or to polyvinylidene membranes (Millipore). After blocking with 5% nonfat dry milk (Protifar-plus, Nutricia Netherland B.V.) in washing buffer (PBS with 0.5% Triton-X100), the membranes were incubated with antibodies against human apoE (1:500, DAKO A/S, Denmark), murine apoE (Santa Cruz), human glutamine synthetase (1:500, BD Transduction Laboratories), glyceraldehyde-3-phosphate dehydrogenase (Chemicon), or a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) (31) overnight at 4 °C. The membranes were then incubated with peroxidase-conjugated secondary antibodies, after which the results were visualized using ECL reagents (Amersham Biosciences) and autoradiography (LAS 3000, Fuji Photo Film Co., Ltd., Japan). Bands were quantitated by densitometry using NIH Image J.

**RNA Isolation and Real-time Quantitative PCR (QRT-PCR) Procedures**—Total RNA was isolated using the Trizol method (Invitrogen) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (PerkinElmer Life Sciences) as previously described (32) with modifications (33). Primer sequences are available upon request. Primers were obtained from Invitrogen. Fluorogenic probes labeled with 6-carboxyfluorescein



**FIGURE 1.** Concentration-dependent effect of 24(S)-hydroxycholesterol (Chol) on apoE mRNA levels in CCF-STTG1 and SH-SY-5Y cells and primary rat astrocytes. Cells were incubated with increasing concentrations of 24(S)-hydroxycholesterol and 4  $\mu\text{M}$  GW683965A compound for a period of 72 h. Total RNA was prepared from cells. Expression levels of apoE in astrocytoma and neuroblastoma cells (A) and rat primary astrocytes (B) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Values represent the mean  $\pm$  S.D.,  $n = 4$  in all groups.

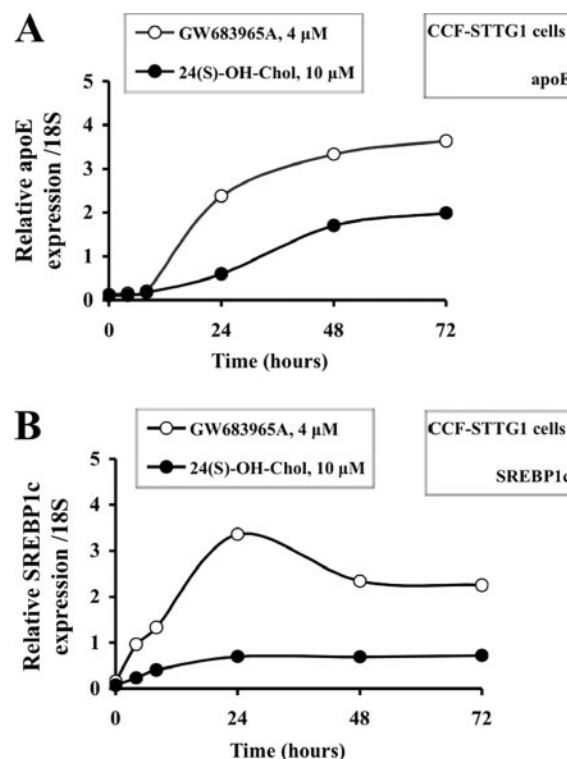
(FAM) and 6-carboxytetramethylrhodamine (TAMRA) were made by Eurogentec (Seraing, Belgium).

**Efflux Studies**—CCF-STTG1 or SH-SY-5Y cells were cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum. After washing with DMEM/Ham's F-12 medium (1:1), cells were loaded with 30  $\mu\text{g}/\text{ml}$  [ $^3\text{H}$ ]cholesterol (38 Ci/mmol) in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum for 24 h in the presence or absence of 24(S)-hydroxycholesterol or GW683965A. After cells were washed 5 times with PBS-bovine serum albumin 0.2% (w/v), the efflux assay was started by adding 2.5  $\mu\text{g}/\text{ml}$  apoE or 5  $\mu\text{g}/\text{ml}$  apoA-I in DMEM/Ham's F-12 medium (1:1) to the wells. After 20 h of incubation at 37  $^{\circ}\text{C}$ , the medium was collected and centrifuged. Subsequently, [ $^3\text{H}$ ]cholesterol was quantified in the supernatant by liquid scintillation counting. Total cellular [ $^3\text{H}$ ]cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract.

**Statistical Analysis**—Values are presented as the mean  $\pm$  S.D. Statistical significance was determined by comparing means using an unpaired the Student's  $t$  test, the Mann-Whitney  $U$  test, and one-way analysis of variance with Newman-Keul's post-test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

**The Natural LXR Ligand 24(S)-Hydroxycholesterol Induces ApoE Gene Expression and Protein Levels in Astrocytoma Cells and in Primary Astrocytes but Not in Neuroblastoma Cells**—To determine the effect of the natural LXR ligand 24(S)-hydroxycholesterol on apoE expression in astrocytoma and neuroblastoma cells and in primary astrocytes, cells

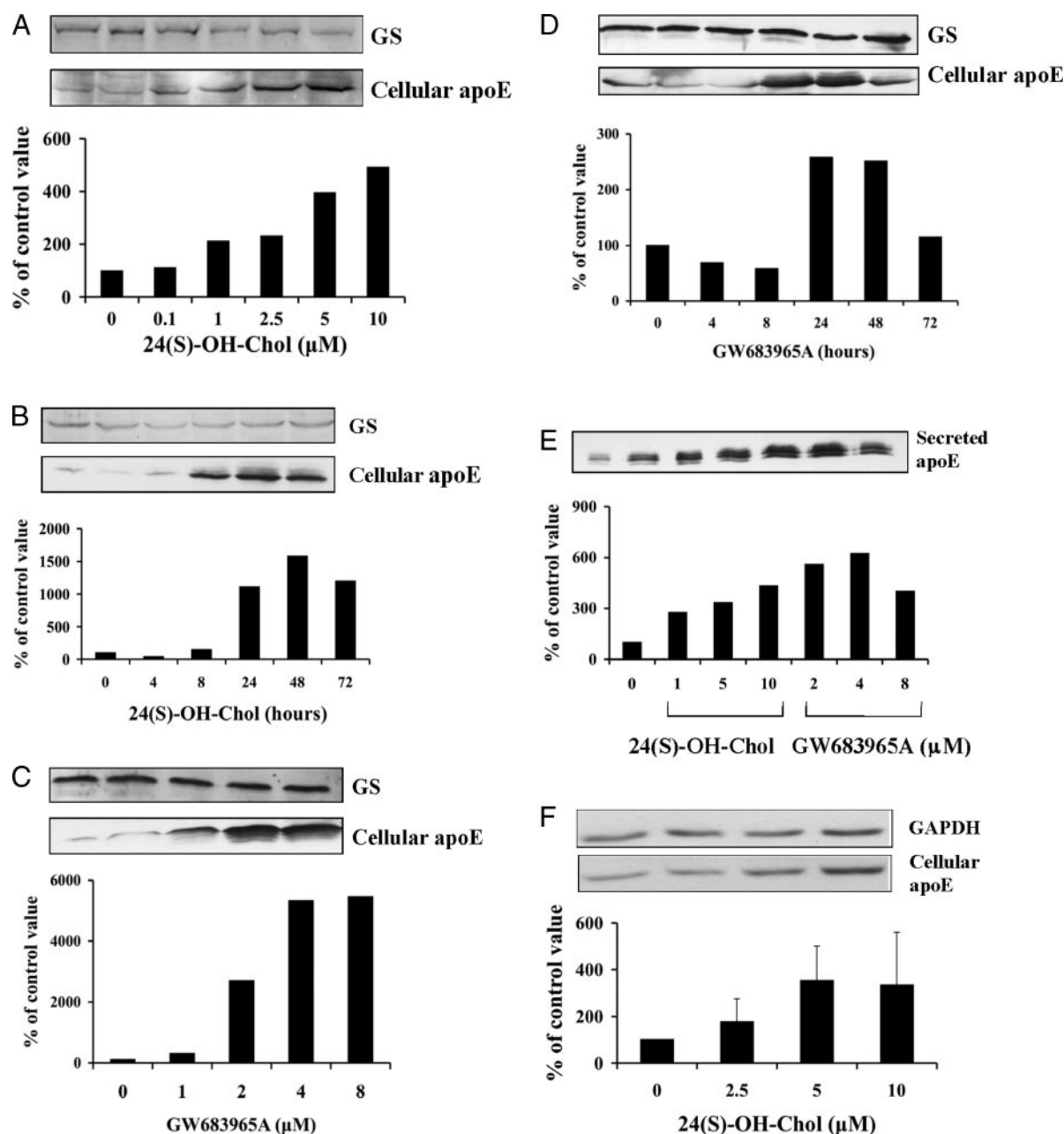


**FIGURE 2.** Time-dependent effect of 24(S)-hydroxycholesterol (Chol) and GW683965A on apoE and SREBP1c expression in CCF-STTG1 cells. Cells were incubated in the presence of 10  $\mu\text{M}$  24(S)-hydroxycholesterol (●) or 4  $\mu\text{M}$  GW683965A (○) for increasing periods of time. Total RNA was prepared from cells. Expression levels of apoE (A) and SREBP1c (B) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Representative examples of three independent experiments are shown.

were incubated with increasing amounts of the compound. We found that the incubation with 24(S)-hydroxycholesterol resulted a dose-dependent increase of APOE mRNA levels in astrocytoma cells (CCF-STTG1) but failed to induce APOE expression in neuroblastoma cells (SH-SY-5Y) even after 72 h of incubation (Fig. 1A). The synthetic LXR agonist GW683965A (4  $\mu\text{M}$ ) also clearly induced APOE expression in astrocytoma cells but not in neuroblastoma cells (Fig. 1A), strongly suggesting that 24(S)-hydroxycholesterol exerts its effects via the LXR pathway. Importantly, 24(S)-hydroxycholesterol also induced APOE gene expression in primary rat astrocytes in a dose-dependent manner (Fig. 1B), supporting the physiological relevance of this process.

The induction of APOE expression (Fig. 2A) by 24(S)-hydroxycholesterol and GW683965A in astrocytoma cells also appeared to be time-dependent, with strong induction already occurring after 24 h and a steady increase up to 72 h of incubation. The established LXR target gene SREBP-1c was also induced by both compounds but displayed a different induction profile compared with apoE (Fig. 2B). The expression of APOD, another LXR-target gene that has been suggested to compensate cholesterol transport functions in the absence of APOE (34), was not affected by the natural or synthetic LXR ligand in either cell line (data not shown). Next we determined if induction of APOE mRNA up-regulation resulted in increased protein levels. As shown in Fig. 3A, cellular apoE protein levels in astrocytoma cells were also clearly up-regulated by 24(S)-hydroxycholesterol in a concentration-dependent manner. Upon incubation of the cells for increasing periods of time in the presence of 10  $\mu\text{M}$  24(S)-hydroxycholesterol, apoE protein levels increased up to 48 h and remained fairly constant thereafter up to 72 h (Fig. 3B). Similar to 24(S)-hydroxycholesterol, the synthetic LXR agonist GW683965A

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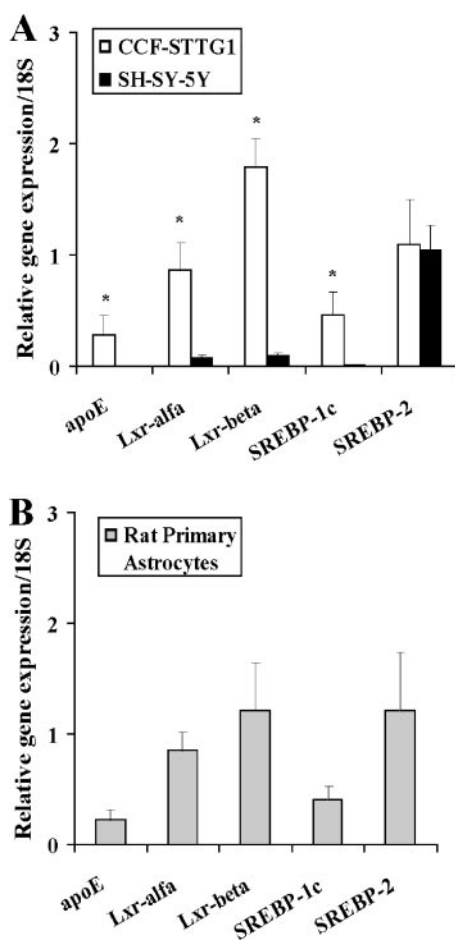
**FIGURE 3. 24(S)-Hydroxycholesterol (Chol) and GW683965A increase apoE protein levels and apoE secretion in CCF-STTG1 cells.** 24(S)-hydroxycholesterol up-regulates apoE protein levels in murine primary glial cultures. CCF-STTG1 cells were treated either with increasing concentrations of 24(S)-hydroxycholesterol (A) and GW683965A (C) for a period of 72 h or with 10  $\mu\text{M}$  24(S)-hydroxycholesterol (B) and 4  $\mu\text{M}$  GW683965A (D) for increasing periods of time. Conditioned medium was collected after the treatment of CCF-STTG1 cells with different concentrations of 24(S)-hydroxycholesterol and GW683965A for a period of 48 h (E). Murine primary glial cultures were treated with increasing concentrations of 24(S)-hydroxycholesterol for 24 h (F). Cell lysates and medium concentrates were subjected to Western blot analysis as described under "Experimental Procedures." ApoE expression was measured using a polyclonal antibody against apoE. Bands were quantified by densitometry, normalized to glutamine synthetase (GS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Representative experiments of at least three independent experiments are shown.

increased apoE protein levels in astrocytoma cells in a concentration- and time-dependent manner (Fig. 3, C and D). Secretion of apoE by astrocytoma cells into the medium was also strongly induced by 24(S)-hydroxycholesterol and GW683965A in a concentration-dependent manner (Fig. 3E). 24(S)-Hydroxycholesterol also up-regulated cellular apoE protein levels in murine primary mixed glial cultures containing >80% astrocytes (Fig. 3F), further substantiating our findings.

Because differences in expression of LXRs may underlie the differential effects of 24(S)-hydroxycholesterol and GW683965A in the two cell types, we next determined their basal expression levels. Significantly higher mRNA levels of LXR $\alpha$  and LXR $\beta$  were found in astrocytoma

cells compared with neuroblastoma cells (Fig. 4). Also SREBP-1c, an established LXR target gene involved in cholesterol and fatty acid metabolism (35), was expressed predominantly in astrocytoma cells. In contrast, mRNA levels of SREBP-2, another transcription factor involved in control of cholesterol metabolism, were similar in both cell types (Fig. 4A). Primary rat astrocytes also displayed a similar transcription profile for these genes (Fig. 4B).

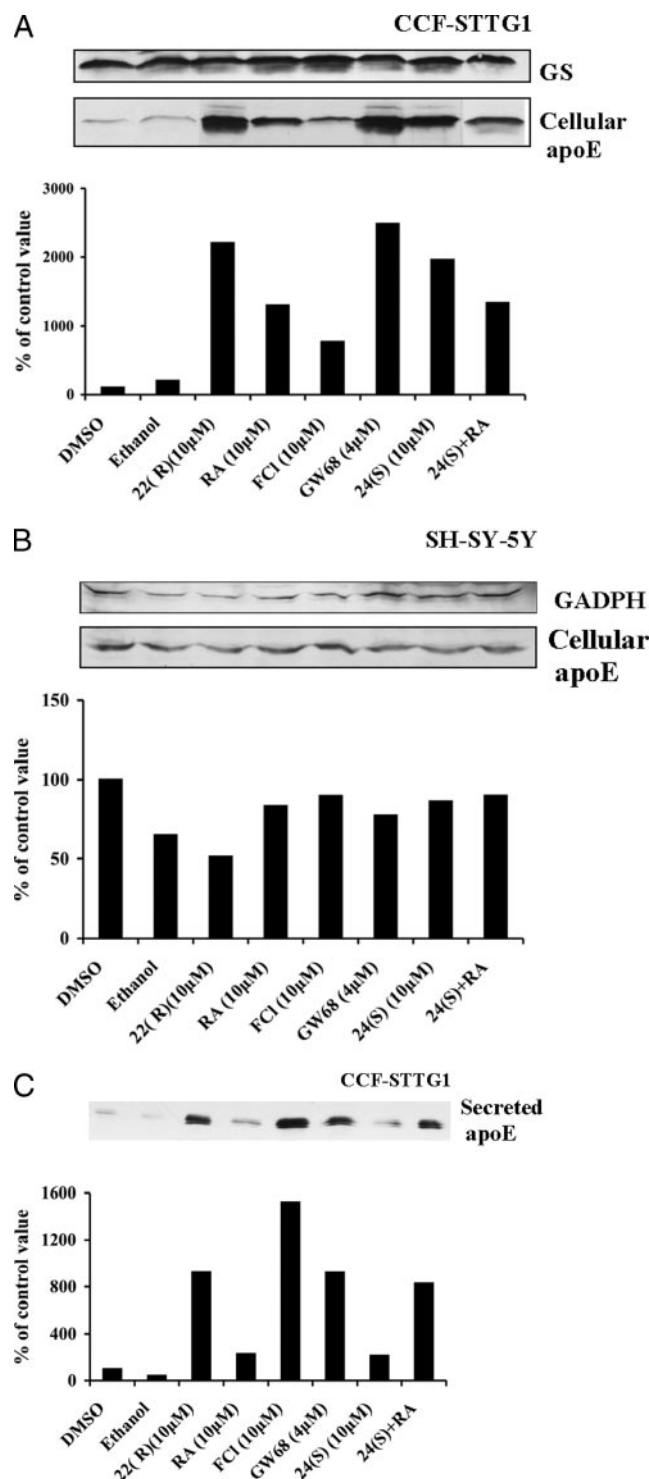
*24(S)-Hydroxycholesterol Is a Stronger Regulator of Intracellular ApoE Protein Levels in Astrocytoma Cells than Retinoic Acid and Free Cholesterol*—24(S)-Hydroxycholesterol was compared with other natural LXR/retinoid X receptor (RXR) ligands with respect to its effect on



**FIGURE 4. Comparison of the basal expression of apoE, LXR $\alpha$ , LXR $\beta$ , SREBP1c, and SREBP-2 in CCF-STTG1 and SH-SY-5Y cells and in rat primary astrocytes.** Cells were incubated for 72 h in DMEM/Ham's F-12 (1:1) medium containing 10% fetal calf serum. Total RNA was prepared from cells. Expression levels of apoE, LXR $\alpha$ , LXR $\beta$ , SREBP1c, and SREBP2 in CCF-STTG1 and SH-SY-5Y cells (A) and rat primary astrocytes (B) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Values represent the mean  $\pm$  S.D.,  $n = 4$  in all groups. The Mann-Whitney  $U$  test was used to determine significant differences in gene expression. The asterisk represents  $p < 0.01$ .

intracellular apoE levels. In accordance with the observation that brain apoE is predominantly synthesized by astrocytes (36, 37), we observed that the basal expression of the APOE gene in astrocytoma cells was substantially higher than in neuroblastoma cells (Fig. 4A). In astrocytoma cells, 24(S)-hydroxycholesterol and GW683965A are as potent as 22(R)-hydroxycholesterol in inducing intracellular and secreted apoE and were considerably more effective than retinoic acid or free cholesterol (Fig. 5, A and C). Neither 24(S)-hydroxycholesterol nor GW683965A or any of the other LXR/retinoid X receptor (RXR) agonists affected apoE synthesis in neuroblastoma cells (Fig. 5B), demonstrating that LXR-mediated up-regulation of apoE synthesis and secretion is a pathway specific to astrocytes.

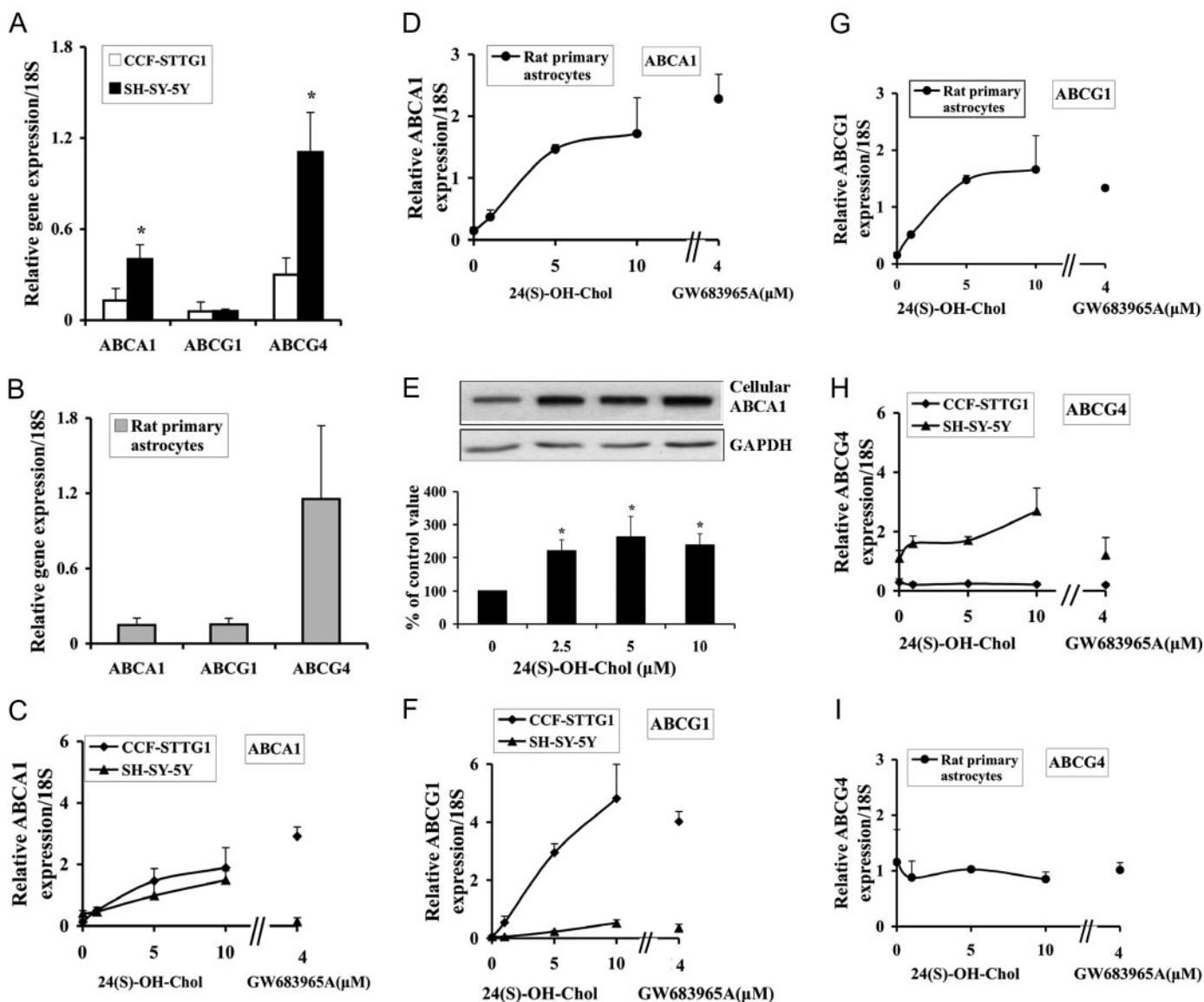
**24(S)-Hydroxycholesterol and GW683965A Regulate the Expression of ABC Transporters and Other Genes Related to Cholesterol Metabolism in a Cell Type-specific Manner**—ABCA1, ABCG1, and ABCG4 are transporters of cholesterol and/or phospholipids from cells to extracellular acceptors that may also be involved in intercellular lipid transport within the brain (38, 39). Because all three transporters have been identified as LXR target genes present in the brain (40, 41), we examined the effects of 24(S)-hydroxycholesterol and GW683965A on their expression. The results presented in Fig. 6A show that basal expression of ABCG1 mRNA was comparable in astrocytoma cells and in neuroblas-



**FIGURE 5. 24(S)-Hydroxycholesterol is a stronger regulator of apoE synthesis in CCF-STTG1 cells than retinoic acid and cholesterol.** Shown are Western blot analyses of CCF-STTG1 (A) and SH-SY-5Y (B) cell lysates and conditioned medium from CCF-STTG1 cells (C) that were incubated for 72 h in the presence of either 10  $\mu$ M 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 9-cis-retinoic acid, free cholesterol (FCI), or 4  $\mu$ M GW683965A. Bands were quantified by densitometry, normalized to glutamine synthetase (GS) and glyceraldehyde-3-phosphate dehydrogenase (GADPH). Representative experiments are shown; at least three independent experiments were performed for all conditions. DMSO, Me<sub>2</sub>SO.

toma cells. The relative expression of ABCA1 mRNA was higher than that of ABCG1 and also 3-fold higher in neuroblastoma than in astrocytoma cells. Likewise, the basal relative expression of ABCG4 mRNA

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**FIGURE 6. Effect of 24(S)-hydroxycholesterol (Chol) and GW683965A on mRNA levels of ATP binding cassette transporters in CCF-STTG1 and SH-SY-5Y cells (A) and in rat primary astrocytes.** This panel represents a basal gene expression of ATP binding cassette transporters in CCF-STTG1 and SH-SY-5Y cells (A) and in rat primary astrocytes (B). Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression relative to 18S was determined by QRT-PCR. The Mann-Whitney *U* test was used to determine significant differences in gene expression. An asterisk indicates that  $p < 0.01$ . Astrocytoma and neuroblastoma cells (C, F, and H) and rat primary astrocytes (D, G, I) were incubated for 72 h in the presence of 1, 5, or 10  $\mu\text{M}$  24(S)-hydroxycholesterol or in the presence of 4  $\mu\text{M}$  GW683965A. Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression of ABCA1 (C and D), ABCG1 (F and G), and ABCG4 (H and I) relative to 18S was determined by QRT-PCR. Protein levels of ABCA1 in whole lysates of primary mixed glial cultures treated with increasing concentrations of 24(S)-hydroxycholesterol for a period of 24 h were determined by Western blot and quantified by densitometry. Intracellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal protein loading control (E). Data represent the mean and S.D. of cellular ABCA1 levels. One-way analysis of variance with Newman-Keul's post-test was used to determine significant differences (the asterisk represents  $p < 0.01$  compared with vehicle-only).

was significantly higher in SH-SY-5Y cells than in astrocytoma cells (Fig. 6A). A pattern of relative ABC transporter expression similar to that in astrocytoma cells was found in primary rat astrocytes (Fig. 6B). Incubation with 24(S)-hydroxycholesterol resulted in a dose-dependent up-regulation of the expression of ABCA1 mRNA in astrocytoma, in neuroblastoma cells (Fig. 6C), and in primary astrocytes (Fig. 6D). In contrast, GW683965A induced ABCA1 mRNA only in astrocytoma cells and rat primary astrocytes (Fig. 6, C and D). Furthermore, 24(S)-hydroxycholesterol increased ABCA1 protein levels in murine primary glia (Fig. 6E).

ABCG1 mRNA was strongly induced by both 24(S)-hydroxycholesterol and GW683965A in astrocytoma cells (Fig. 6F), and primary astrocytes also demonstrated a significant induction of ABCG1 mRNA (Fig.

6G). In contrast, ABCG1 was unresponsive to either compound in neuroblastoma cells (Fig. 6F). Interestingly, ABCG4 was slightly up-regulated by 24(S)-hydroxycholesterol but not by GW683965A in neuroblastoma cells, whereas this gene was not affected or even slightly down-regulated in astrocytoma cells and in primary astrocytes (Fig. 6, H and I). Effects of 24(S)-hydroxycholesterol and GW683965A on the expression of the ABC transporters mentioned were maximal at 24 h of incubation in astrocytoma cells (data not shown).

Next we determined the effect of 24(S)-hydroxycholesterol or GW683965A on mRNA levels of other genes involved in cholesterol and/or fatty acid metabolism. Incubation of astrocytoma cells with 24(S)-hydroxycholesterol resulted in a transient down-regulation of SREBP-2, LDLR, and HMG-CoA reductase mRNA levels. In contrast,

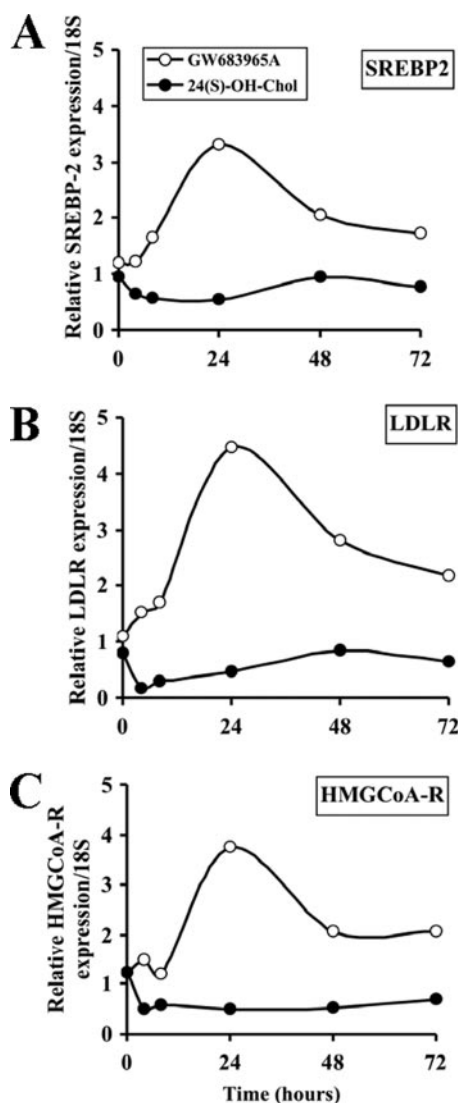


FIGURE 7. Time-dependent effect of 24(S)-hydroxycholesterol and GW683965A on expression of SREBP2 (A), LDLR (B), HMG-CoA reductase (C) in CCF-STTG1 cells. CCF-STTG1 cells were incubated in the presence of 10  $\mu$ M 24(S)-hydroxycholesterol (●) or 4  $\mu$ M GW683965A (○) for increasing periods of time. Total RNA was prepared from cells, and gene expression relative to 18S was determined by QRT-PCR.

GW683965A induced a transient increase in the expression of these genes with a maximal effect at 24 h (Fig. 7). SR-BI expression in astrocytoma cells was not affected by either compound (data not shown). Additionally, neither 24(S)-hydroxycholesterol nor GW683965A detectably affected the expression of SREBP-2, HMG-CoA reductase, and LDLR in neuroblastoma cells (data not shown).

*24(S)-Hydroxycholesterol Enhances ApoE- and ApoA-I-mediated Cholesterol Efflux from Astrocytoma Cells, whereas Only the ApoA-I-mediated Cholesterol Efflux from Neuroblastoma Is Enhanced*—We observed that basal cholesterol efflux from astrocytoma cells was higher than from neuroblastoma cells (~4% compared with 0.5%, respectively; Fig. 8). The addition of 24(S)-hydroxycholesterol induced apoE- and apoA-I-mediated cholesterol efflux from astrocytoma cells to equivalent levels (Fig. 8A), and similar effects were observed with synthetic LXR agonist GW683965A. Interestingly, neither compound affected apoE-mediated cholesterol efflux from neuroblastoma cells (Fig. 8B) but, rather, specifically induced apoA-I-mediated cholesterol efflux from neuroblastoma cells (Fig. 8).

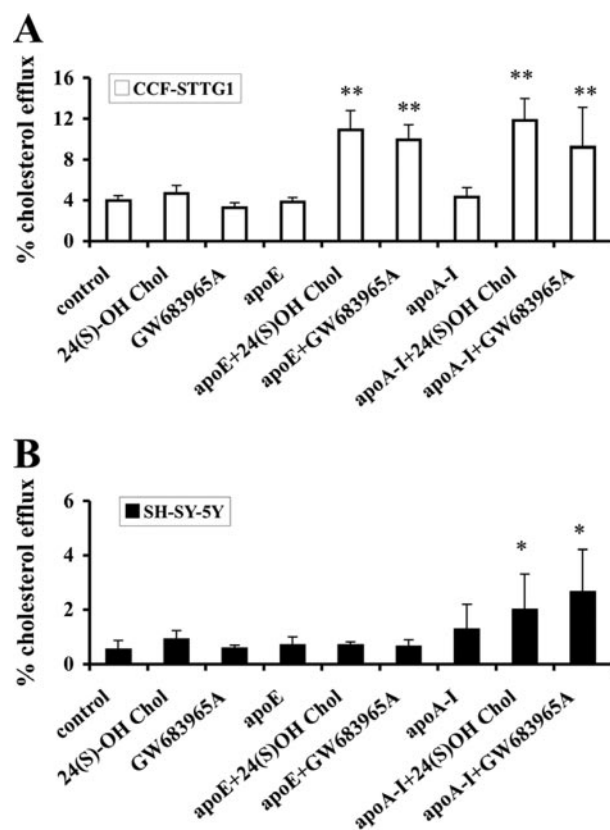


FIGURE 8. 24(S)-Hydroxycholesterol (Chol) and GW683965A induce apoE- and apoA-I-mediated cholesterol efflux from CCF-STTG1 cells but not from SH-SY-5Y cells. After loading the cells with [ $^3$ H]cholesterol for 24 h, the apoE- or apoA-I-mediated cholesterol efflux over a period of 20 h from CCF-STTG1 cells (A) and SH-SY-5Y cells (B) was determined. [ $^3$ H]cholesterol was quantified in medium and in cell extracts. Values are the mean  $\pm$  S.D. of three independent experiments, each measured in duplicate. Student's *t* test was used to determine significant differences of cholesterol efflux levels. The single asterisk represents  $p < 0.05$ , and the double asterisk represents  $p < 0.001$ .

## DISCUSSION

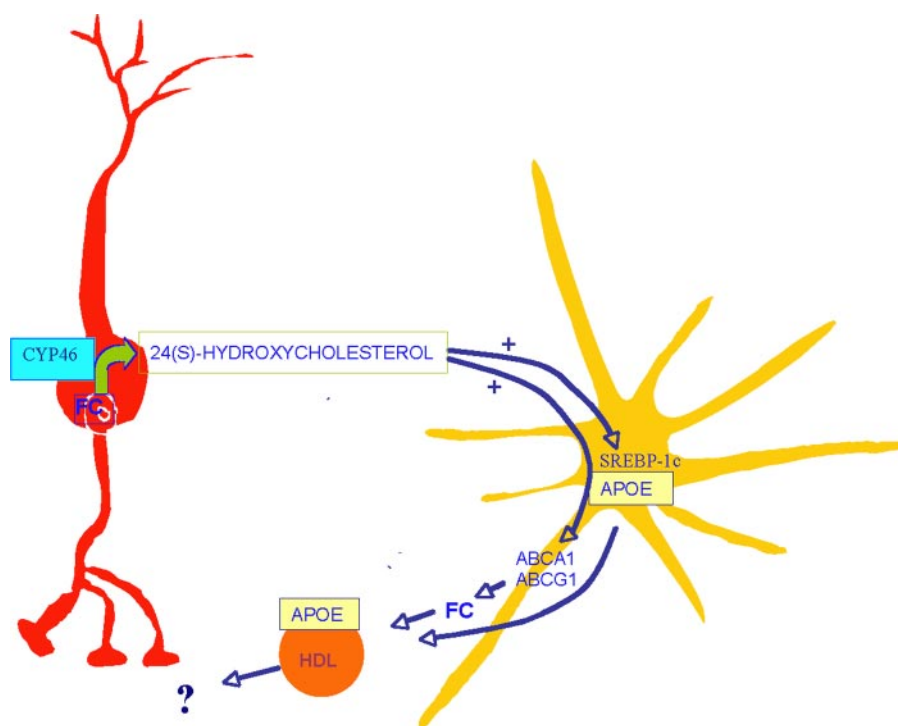
In this paper we addressed the regulation of genes involved in apoE-mediated cholesterol trafficking between astrocytes and neurons, an important process during regeneration and synaptic plasticity (25). We observe that 24(S)-hydroxycholesterol is capable of inducing apoE and ABC transporter expression in astrocytic cells as well as in primary rat and murine astrocytes. In contrast, 24(S)-hydroxycholesterol did not induce expression of these genes in SHSY5Y neuronal cells. Notably, 24(S)-hydroxycholesterol is a natural LXR ligand found in the brain, and elevated levels of 24(S)-hydroxycholesterol levels are often associated with neuronal injury. Our results are consistent with a model in which release of 24(S)-hydroxycholesterol from neurons can induce the secretion of apoE-associated cholesterol from astrocytes. This glial-derived cholesterol would then be available for neuronal uptake during the process of dendritic and axonal extension and regeneration of synapses (26).

Because the potent synthetic LXR agonist GW683965A also induced apoE, ABCA1, and ABCG1 mRNA expression in astrocyte-derived cells, our results suggest that 24(S)-hydroxycholesterol mediates its action through LXR activation. Furthermore, differential expression of the nuclear hormone receptor LXR isoforms, *i.e.* LXR $\alpha$  and - $\beta$ , between astrocytoma and neuroblastoma cells may also account for the difference in sensitivity to these agonists between astrocytic and neuronal cells. Our results suggest that 24(S)-hydroxycholesterol-mediated induction of ABCG1 and ABCA1 primes the astrocytes to deliver cholesterol to apoE or apoA-I, since their expression was robustly induced



## 24(S)-Hydroxycholesterol Regulates ApoE Metabolism in Brain

**FIGURE 9. Schematic presentation of the differential effects in astrocytes and neurons of 24(S)-hydroxycholesterol on the expression of apoE and ABC transporters expression as well as apoE-mediated cholesterol efflux.** 24(S)-Hydroxycholesterol up-regulates apoE synthesis as well as apoE-mediated cholesterol efflux in astrocytes but not in neurons. Up-regulation of ABCA1 and ABCG1 in astrocytes by 24(S)-hydroxycholesterol suggests involvement of these transporters in the efflux of cholesterol. HDL, high density lipoprotein.



by both compounds. In neuroblastoma cells, neither of the two compounds stimulated apoE-mediated cholesterol efflux, but both compounds induced apoA-I-mediated cholesterol efflux, probably involving ABCA1 actions.

It has been proposed that, after differentiation of astrocytes, neurons reduce their endogenous cholesterol synthesis and rely predominantly on cholesterol delivery by astrocytes via lipoprotein-like particles that contain astroglia-derived apoE (47). Cholesterol delivery may at least in part participate in regulating the number of synapses formed (42). A continuous turnover of cholesterol in neurons facilitates cell ability for efficient and quick adaptation of cholesterol homeostasis required for dynamic structural changes of neurons, their extensions, and their synapses during synaptic plasticity (43). Conversion of cholesterol into 24(S)-hydroxycholesterol, which is also a brain-specific LXR ligand, by CYP46 represents a major route for cholesterol turnover in neurons (44).

A common feature that astrocytes share with macrophages and adipocytes is their large content of free cholesterol (45, 46). In agreement with what has been reported for macrophages (47), we found that activation of LXR by 24(S)-hydroxycholesterol or GW683965A stimulates cellular cholesterol efflux through the coordinated regulation of ABCA1, ABCG1, and apoE. Some aspects of these pathways may be specific to astrocytes, however, because apoD was unresponsive to LXR agonists in astrocytes, whereas it is induced in adipocytes (48).

GW683965A up-regulated the expression of HMG-CoA reductase, LDLR, and SREBP2 in astrocytoma cells, supposedly to maintain cellular cholesterol homeostasis during excessive loss by efflux. However, 24(S)-hydroxycholesterol down-regulated the expression of these genes. It is long since known that oxysterols reduce the activity of HMG-CoA reductase and are more potent inhibitors of cholesterol synthesis than cholesterol (49). However, the mechanisms by which cholesterol and oxysterols reduce cholesterol synthesis differ (50). Cholesterol directly interferes with SREBP cleavage-activating protein, inducing a conformational change that prevents the processing of SREBP to its active form. Oxysterols have similar effects without directly

interacting with SREBP cleavage-activating protein. Thus, 24(S)-hydroxycholesterol may not exert its effect exclusively via the LXR pathway. The observation that 24(S)-hydroxycholesterol, but not GW683965A, enhanced the expression of ABCG4 in neurons suggests that LXR-independent pathways may be involved. Apparently, cholesterol efflux from astrocytes is not directly driven solely by the rate of cholesterol biosynthesis, because efflux was induced to a similar level by 24(S)-hydroxycholesterol and GW683965A. A concomitantly enhanced cholesterol efflux, up-regulation of ABCA1 and ABCG1, and impaired synthesis of cholesterol has recently also been reported as a consequence of statin treatment in macrophages (47).

Numerous studies have demonstrated that ABCA1 is necessary for the efflux of cellular cholesterol to lipid-poor apoA-I (51). Recently, ABCA1 was found to facilitate the efflux of central nervous system cholesterol to apoE as the absence of ABCA1 compromised apoE secretion from both astrocytes and microglia. In addition, apoE that is present in the cerebrospinal fluid of ABCA1-deficient animals is poorly lipidated (38, 52). In contrast to ABCA1, ABCG1 and ABCG4 are thought to facilitate the efflux of cholesterol to high density lipoprotein rather than to lipid-poor apolipoproteins (53, 54). A relationship between ABCG1 and the secretion of apoE was suggested by the observation that treatment of macrophages with antisense oligonucleotides to ABCG1 decreased the efflux of cholesterol and phospholipids to high density lipoprotein and, surprisingly, also the secretion of apoE (53, 55). Although ABCG1 and ABCG4 may function both as homodimers and heterodimers (56, 57), expression of ABCG1 and ABCG4 overlaps in some but not all tissues assayed (54), which may indicate different functions in different tissues. The expression of ABCG4 appears to be largely restricted to nervous tissue (56). Our results strongly suggest that apoA-I-mediated cholesterol efflux from astrocytes involves ABCA1 and that apoE mediates cholesterol efflux via ABCG1 and possibly also ABCA1, but not via ABCG4. Although 24(S)-hydroxycholesterol also stimulated apoE synthesis and secretion from astrocytic cells, it remains to be established why 24(S)-hydroxycholesterol treated resulted in observable cholesterol efflux only in the presence of exogenous apoE or

apoA-1. A possible explanation may be that the levels of endogenous apoE secreted from 24(S)-hydroxycholesterol-treated cells is >100-fold lower than the concentrations of exogenous apoE added as a lipid acceptor under our experimental conditions. Alternatively, endogenously secreted apoE may already be lipidated and thereby act a less efficient cholesterol acceptor.

Neurons are thought to dispose their cholesterol by conversion into 24(S)-hydroxycholesterol, which is more polar than cholesterol and, as a result, may easily traverse the blood-brain barrier and perhaps the neuronal plasma membrane itself (4). However, how oxysterols are transported across membranes and through the intracellular water phase is not yet known. The selective up-regulation of ABCG4 in neuroblastoma cells by 24(S)-hydroxycholesterol suggests a possible role for this transporter in oxysterol transport. Both 24(S)-hydroxycholesterol and also GW683865A enhanced apoA-I-mediated cholesterol efflux from neuronal cells, suggesting this is another neuronal pathway to dispose of cholesterol. However, GW683965A had only a limited effect on ABCA1 expression in these cells. Rebeck *et al.* (57) recently reported up-regulation of neuronal ABCA1 expression by the synthetic LXR ligand T0901317. A role for apoA-I in the disposal of cholesterol from neurons is in line with its well known role in so called "reverse cholesterol transport." ApoA-I is present in brain and in cerebrospinal fluid and has been detected in senile plaques in AD patients (58, 59). So far apoA-I synthesis within the brain has only been ascribed to endothelial cells of the blood brain barrier (60). It remains to be established why 24(S)-hydroxycholesterol does not alone, but only in concert with apoE or apoAI increase cholesterol efflux from astrocytes. A possible explanation may be the relatively small amounts (<100-fold) of apoE that are secreted by the cells in comparison with the amount of apoE that is added as cholesterol acceptor. Alternatively, secreted apoE may be lipidated and thereby become a less efficient cholesterol acceptor.

LXR isoforms differ in their pattern of expression (61). In the brain LXR $\beta$  levels are 2–5-fold higher than in the liver, whereas LXR $\alpha$  levels are 3.5–14-fold lower than in the liver (62–64). However, 24(S)-hydroxycholesterol and GW683965A up-regulated LXR $\alpha$  but not LXR $\beta$  expression in astrocytoma cells (data not shown), similar to what has been reported for macrophages and adipocytes, but not liver and muscle (65, 66). These results suggest the possibility that the autoregulation of LXR $\alpha$  that has been suggested to occur in adipocytes to coordinate expression of target genes such as APOE (66) may also occur in brain.

In conclusion, our results provide evidence indicating that 24(S)-hydroxycholesterol acts as a signaling molecule that induces the apoE-mediated cholesterol efflux from astrocytes but not from neurons (Fig. 9). Our findings also suggest a role for ABCA1 and ABCG1 in mediating cholesterol efflux from astrocytes. Thus, in the intact brain, 24(S)-hydroxycholesterol derived from neurons may signal astrocytes to increase production of lipidated apoE particles in order to supply neurons with additional cholesterol during synaptogenesis or neuritic remodeling. Moreover alterations in the transcriptional regulation role of 24(S)-hydroxycholesterol on apoE-mediated cholesterol efflux may affect the progression of neurodegenerative diseases including AD.

*Acknowledgments*—We thank Dr. Dieter Lütjohann from the University of Bonn for providing the 24(S)-hydroxycholesterol, Dr. Jogchum Plat from the University of Maastricht for providing 22(R)-hydroxycholesterol, and Dr. P. Groot (GSK, Stevenage, UK) for the kind gift of GW683965A. We also thank Mieke Henfling from the University of Maastricht for the assistance in tissue culturing and Dr. Vadim Tchaikovsky from the University of Maastricht for help in Western blotting.

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**24(S)-Hydroxycholesterol Participates in a Liver X Receptor-controlled Pathway in Astrocytes That Regulates Apolipoprotein E-mediated Cholesterol Efflux**

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*J. Biol. Chem.* 2006, 281:12799-12808.

doi: 10.1074/jbc.M601019200 originally published online March 8, 2006

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