Ethanol Induces Cholesterol Efflux and Up-regulates ATP-binding Cassette Cholesterol Transporters in Fetal Astrocytes*

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Cholesterol plays an important role during brain development, since it is involved in glial cell proliferation, neuronal survival and differentiation, and synaptogenesis. Astrocytes produce large amounts of brain cholesterol and produce and release lipoproteins containing apoE that can extract cholesterol from CNS cells for elimination. We hypothesized that some of the deleterious effects of ethanol in the developing brain may be due to the disruption of cholesterol homeostasis in astrocytes. This study investigates the effect of ethanol on cholesterol efflux mediated by ATP-binding cassette (ABC) cholesterol transporters. In fetal rat astrocytes in culture, ethanol caused a concentration-dependent increase in cholesterol efflux and increased the levels of ABCA1 starting at 25 mM. Similar effects of ethanol on cholesterol efflux and ABCA1 were also observed in fetal human astrocytes. In addition, ABCA1 levels were increased in the brains of 7-day-old pups treated for 3 days with 2, 4, or 6 g/kg ethanol. Ethanol also increased apoE release from fetal rat astrocytes, and conditioned medium prepared from ethanol-treated astrocytes extracted more cholesterol than conditioned medium from untreated cells. In addition, ethanol increased the levels of another cholesterol transporter, ABCG1. Ethanol did not affect cholesterol synthesis and reduced the levels of intracellular cholesterol in rat astrocytes. Retinoic acid, which induces teratogenic effects similar to ethanol, also caused up-regulation of ABCA1 and ABCG1.

Offspring of mothers who abuse alcohol during pregnancy are often diagnosed with fetal alcohol syndrome (FAS) and have phenotypic features similar to those born with syndromes linked to genetic defects in cholesterol biosynthesis, such as Smith-Lemli-Opitz Syndrome (SLOS) (1–4). Similarities include facial dysmorphism, microcephaly, holoprosencephaly, mental retardation, and limb malformations, which have been reported in humans as well as in animal models of both FAS and SLOS (5–9).

In the developing CNS, cholesterol is essential for the activation and propagation of hedgehog signaling that is responsible for patterning and development of the CNS (10–12), for proliferation of glial cells (13), for synaptogenesis (14), for neuronal survival (15), and for neuronal development (16–18). All of these functions are inhibited by ethanol (19–25).

The brain has a high rate of cholesterol synthesis, since the uptake of cholesterol from the circulation is low even during brain development (26, 27). Major producers of cholesterol in the brain are glial cells, namely oligodendrocytes, which immobilize it into the myelin, microglia, and astrocytes. Astrocytes also produce and release apolipoprotein E (apoE)-containing high density lipoprotein (HDL)-like particles that can extract cholesterol from CNS cells (28).

The first step in lipoprotein formation consists in cholesterol and phospholipid efflux from cells to apolipoproteins; this is mediated by members of the ATP-binding cassette (ABC) transporter family, namely ABCA1, ABCG1, and ABCG4. During HDL formation in the plasma, ABCA1 is required for transferring phospholipids and/or cholesterol to lipid-free or lipid-poor apoAI or apoE (29). The half transporters ABCG1 and ABCG4 (which may function as homodimers or heterodimers) are responsible for the further lipidation of particles that have been partially lipidated by ABCA1 (30). ABCA1, ABCG1, and ABCG4 are highly expressed in the brain (31). In astrocytes, ABCA1 and ABCG1 have been implicated in cholesterol efflux to lipid-poor apolipoproteins and HDL and in lipoprotein formation (32–35). ABCA1 has been involved in regulating apoE release; indeed, in astrocytes derived from ABCA1 null mice, cholesterol accumulates intracellularly as its efflux is inhibited, apoE secretion is reduced, and lipoprotein particles contain less cholesterol and apoE and are smaller than those present in wild type astrocytes (33, 35). Neurons also express ABCA1 and ABCG1 cholesterol transporters that induce cholesterol efflux to lipid-poor apolipoproteins and apoE discs (36, 37).
Based on the observed similarities between FAS and SLOS, we hypothesized that ethanol may affect cholesterol homeostasis in the developing brain and that such an effect may contribute to FAS (38). We found that ethanol increased the levels of the cholesterol transporters ABCA1 and ABCG1 and cholesterol efflux in fetal rat and human astrocytes, thus decreasing cholesterol levels.

**EXPERIMENTAL PROCEDURES**

*Rat Astrocyte Cultures*—Primary cultures of cortical astrocytes, prepared from fetuses at gestational day 21 as previously described (39), were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), low glucose, supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM orthovanadate) (9-cis-RA), and then chased for 5–6 h in fresh DMEM/BSA in the presence or in the absence of the cholesterol acceptors apoA1, apoE, and HDL (purchased from Calbiochem) or astrocyte-conditioned medium (prepared by incubating separate astrocyte cultures in serum-free DMEM, 0.1% BSA for 48 h). The medium was collected and centrifuged to remove detached cells, and [3H]cholesterol content in the medium and in the cellular lipids, extracted in hexane/isopropl alcohol (3:2, v/v), was measured using a scintillation counter (Beckman LS 6000SC). Cholesterol efflux was expressed as a percentage of total cholesterol.

**Western Blot Analyses**—At the end of each incubation, cells were scraped in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0% Triton X-100, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1.5 mM sodium orthovanadate) supplemented with a protease inhibitor mixture (Roche Applied Science), as previously described (42). Samples were sonicated, boiled for 40 min at 4 °C, and centrifuged at 14,000 × g for 15 min, and the supernatant was collected. Intracellular proteins were quantified by the Bradford method, and 25 or 50 μg of proteins were loaded on a 7.5% SDS-PAGE to detect ABCA1, ABCG1, and ABCG4. To detect secreted apoE levels, 30 μl of medium were loaded on a 4–10% gel. After separation, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA); labeled with polyclonal antibodies against ABCA1 (Chemicon, Tamecula, CA), ABCG1, ABCG4 (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or ApoE (Biodesign International, Saco, Maine) followed by the appropriate horseradish peroxidase-conjugated IgG; and detected by chemiluminescence. Membranes from cytosolic extracts were reprobed for β-actin, whereas membranes from medium–derived proteins were reprobed for biglycan, a proteoglycan secreted by astrocytes whose release is not affected by ethanol,3 to ensure equal loading.

*Animal Treatments and Tissue Preparation*—Time-pregnant Sprague-Dawley rats (Charles River, Hollister, CA) were housed in individual cages with free access to food (Harlan 8604, Madison, WI) and water and were on a 12 h/12 h light/dark cycle. The day of birth was considered postnatal day (PND) 0 for the pups. Each litter was adjusted within 3 days to eight pups, and rats were housed with their dams until weaning (PND 21). For measurement of developmental changes, 4–6 pups were sacrificed on PNDs 1, 4, 7, 10, 15, 21, and 60. For ethanol treatments, pups were matched by weight between treatment groups and controls. Ethanol was diluted to 20% (w/v) in water and was administered by intragastric intubation at the dose of 2, 4, or 6 g/kg of body weight, as described previously (43). Each dose was divided into two equal doses given 3 h apart. A control group received a 11.4% sucrose solution. During treatments, pups were separated from their dams for 3 h and kept under a heating lamp. Animals were treated with ethanol or sucrose from postnatal day 4 to day 7 and were sacrificed 45 min after the second dose of PND 7. Cerebral cortices were removed, placed in lysis buffer supplemented with the protease inhibitor mixture, sonicated, and centrifuged at 15,000 × g for 15 min. Proteins were quantified and analyzed by Western blot.

**Biotinylation of Surface Proteins and Immunoprecipitation**—For selective labeling of plasma membrane proteins, astrocytes were incubated for 30 min at room temperature with PBS containing 1 mg/ml sulfo-N-hydroxysuccinimide-biotin (Pierce) as previously described (41). Cells were then solubilized in the presence of 1% Nonidet P-40, and protease inhibitors and proteins were quantified. In one set of experiments, cell lysates were incubated with a polyclonal antibody to ABCA1 for 1 h at 4 °C under constant mixing by inversion. 50 μl of protein A-agarose beads (Roche Applied Science) were then added to the mixture at 4 °C overnight. After several washes, the samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The total ABCA1 and the biotinylated ABCA1 were detected using an ABCA1 polyclonal antibody followed by horseradish peroxidase-conjugated secondary antibody and a streptavidin-horseradish peroxidase conjugate, respectively. In another set of experiments, cell lysates were incubated with 200 μl of streptavidin beads

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3 N. H. Moore, M. Guizzetti, and C. G. Costa, unpublished observation.
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FIGURE 1. Effect of ethanol on cholesterol efflux from fetal rat astrocytes. Fetal rat astrocytes labeled with 1 μCi/ml [3H]cholesterol for 24 h were incubated for an additional 24 h in the absence or presence of different concentrations of ethanol. Ethanol was subsequently washed away and replaced by fresh serum-free medium. In the 6 h following ethanol treatment, [3H]cholesterol released in the medium was quantified in the absence (A) or in the presence (B) of astrocyte conditioned medium, apoA1 (10 μg/ml), or HDL (50 μg/ml). C, after [3H]cholesterol labeling, [3H]cholesterol efflux was measured from astrocytes incubated with 100 mM ethanol in the presence or absence of cholesterol acceptors for 6 h. Each value is the mean ± S.E. of triplicate incubations. The relative rate of cholesterol synthesis was expressed as the sum of the counts/min associated with intracellular and medium cholesterol divided by the [3H] radioactivity associated with the total lipid mixture.

(Pierce) and mixed by inversion at 4 °C for 1 h. The precipitated proteins were washed several times, pelleted, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Biotinylated ABCA1 was detected with the polyclonal antibody. Biotinylated ABCA1 was detected with the polyclonal antibody.

RNA Extraction and Real Time PCR Analysis—RNA was extracted using the TRIzol reagent (Invitrogen) following the directions of the manufacturer. Total RNA and its purity were quantified by spectrophotometry. Electrophoresis in 1.5% agarose gels was used to ensure good mRNA integrity. Levels of mRNA were determined by real time PCR by the DNA Sequencing and Gene Analysis Center (Department of Pharmaceutics, University of Washington) using TaqMan® reverse transcription kits and an ABI 7900 HS Sequence Detection System (Applied Biosystems, Foster City, CA). β-Actin was used as reference gene in all of the experiments, since preliminary observations demonstrated that β-actin expression is not affected by ethanol treatments.

Cholesterol Synthesis—Cholesterol synthesis was measured as previously described (44) with some modifications. After 24 h in serum-free medium, astrocytes were labeled with 25 μCi/ml [3H]acetate (PerkinElmer Life Sciences) for 4 h, followed by a 24-h incubation in the presence or in the absence of 75 mM ethanol. Lipids were then extracted in hexane/isopropyl alcohol (3:2, v/v); after evaporation, the organic phase was dissolved again in 55 μl of chloroform. 5 μl of each sample were counted on a scintillation counter to determine the total [3H] radioactivity incorporated into cell lipids, whereas 50 μl were spotted on activated silica gel TLC plates (Macherey-Nagel Inc., Easton, PA) and developed in hexane/diethyl ether/methanol/acetic acid (120:30:10:1.5). The plates were allowed to dry and stained with iodine vapor. The cholesterol and cholesterol ester bands, determined by the co-migration of cholesterol and cholesterol oleate standards, were excised from the plates, and the associated radioactivity was quantified. The cholesterol synthesized and released in the medium during the 24-h incubation was also quantified: the medium was collected and centrifuged at 800 × g for 10 min to remove any cells, and lipids were extracted in hexane/isopropyl alcohol (3:2, v/v) and analyzed by TLC as described above. The relative rate of cholesterol synthesis was expressed as the sum of the counts/min associated with intracellular and medium cholesterol synthesis was measured.

Measurement of Cholesterol Mass—At the end of astrocyte incubation, total cholesterol was extracted with hexane/propyl alcohol (3:2, v/v). The solvent was subsequently evaporated, and the lipids were resuspended in 1.5% Triton X-100. Cholesterol was measured by an enzymatic method using the cholesterol kit Amplex Red cholesterol assay (Molecular Probe, Eugene, OR) according to the direction of the manufacturer, and read with a fluorescence microplate reader (563-nm absorbance and 587-nm emission).

Incubations with Ethanol—In order to reduce ethanol evaporation, cultures were treated as previously described (40, 45). In some ethanol experiments, cultures were placed in sealed chambers (Billups-Rothenberg Inc.) together with a reservoir tray containing an ethanol solution at the same concentration as the ethanol in the culture medium. Control cultures were placed in identical chambers in which the reservoir tray contained only water. Ethanol concentrations were determined at the beginning and at the end of some experiments in vitro and in the blood of ethanol-fed pups by an enzymatic method using...
a Roche Applied Science/R-Biopharm kit. Ethanol losses were usually maintained below 15% after 24-h incubations and 20% after 48-h incubations.

Statistical Analysis—All statistical tests were carried out using a Kaleidagraph 4.0 software on a Macintosh personal computer. One-way analysis of variance followed by Fisher’s least significance difference (LDS) test were used to determine significant (\(p < 0.05\), \(p < 0.01\), or \(p < 0.001\), as indicated) differences from controls.

RESULTS
To determine the effect of ethanol on cholesterol efflux from fetal rat astrocytes, \[^{3}H\text{-}	ext{cholesterol}-	ext{labeled}\] cells were incubated with 25–100 mM ethanol for 24 h. Cholesterol efflux was measured during the subsequent 6-h incubations in the absence (Fig. 1A) and in the presence of HDL, apoA1, or astrocyte-conditioned medium as cholesterol acceptors (Fig. 1B). The rationale for using conditioned medium as a cholesterol acceptor lies in the fact that astrocytes release HDL-like particles containing apoE (28, 46); indeed, we found that, under the described conditions, medium conditioned for 48 h by separate astrocyte cultures induced cholesterol release (Fig. 1B). Ethanol, starting at 25 mM, induced a dose-dependent increase in cholesterol release from astrocytes in the absence of acceptors (Fig. 1A) and enhanced the efflux mediated by apoA1, HDL, and conditioned medium (Fig. 1B). This effect was not due to increased permeability to cholesterol of the plasma membrane by residual ethanol, since the presence of 100 mM ethanol during cholesterol efflux measurement did not affect cholesterol release (Fig. 1C).

The cholesterol transporter ABCA1 plays a pivotal role in cholesterol efflux in many cell types, including astrocytes. Ethanol increased the levels of ABCA1 protein, measured in whole cell lysate, in a concentration-dependent manner starting at 25 mM (Fig. 2A). To verify that levels of active ABCA1 were increased, we measured the levels of ABCA1 in the plasma membrane, where it can directly interact with apolipoproteins for cholesterol removal (29). After biotinylation of membrane proteins, ABCA1 was immunoprecipitated and detected by streptavidin chemiluminescence. As shown in Fig. 2B, ethanol increased levels of biotinylated ABCA1. Similar results were obtained by immunoprecipitating all biotinylated proteins and measuring ABCA1 with a polyclonal antibody (Fig. 2C). It should be noted that we and others (37) detected multiple bands by immunoblot, suggesting the presence of post-translational modifications or different isoforms of ABCA1 in these cells. We also assessed whether ethanol increased ABCA1 transcription by real time PCR.

**FIGURE 2. Effect of ethanol on ABCA1 protein levels and localization.** A, fetal rat astrocytes were incubated for 24 h in the absence or presence of different concentrations of ethanol. ABCA1 protein levels were assessed by immunoblot (IB) analysis. Results are representative of five similar experiments. B and C, membrane-bound ABCA1 was determined by labeling membrane proteins with sulfo-N-hydroxysuccimide-biotin followed by ABCA1 immunoprecipitation (IP) and streptavidin detection (B) or by streptavidin precipitation and ABCA1 immunoblot (C). The results are representative of two or three experiments.

**FIGURE 3. Effect of ethanol and 22(R)-HC/9-cis-RA on ABCA1 mRNA levels.** Astrocytes were incubated in the presence or absence of 100 mM ethanol for different times (A) for 24 h in the presence or absence of different concentrations of ethanol (B) or for different times in the presence of 10 \(\mu M\) 22(R)-HC and 10 \(\mu M\) 9-cis-RA (C). Messenger RNA was quantified by real time PCR as described under “Experimental Procedures.” Each value represents the mean ± S.E. of three experiments. *, \(p < 0.05\); ***, \(p < 0.001\) by Fisher’s LDS test.
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quantification of ABCA1 mRNA. In time course experiments, we observed a significant increase in ABCA1 mRNA after 18-, 24-, and 32-h incubation with 100 mM ethanol (Fig. 3A); concentration-response experiments showed a small ABCA1 mRNA induction by ethanol at lower ethanol concentrations (Fig. 3B). By comparison, treatment of astrocytes with the nuclear liver X receptor agonist 22(R)-HC plus the retinoid X receptor agonist 9-cis-RA, known to induce ABCA1 expression in these cells (37), induced a robust and rapid increase in ABCA1 mRNA (Fig. 3C). These finding suggest that, although ethanol may affect ABCA1 transcription, additional mechanisms should also be involved in ethanol-induced increase of ABCA1.

It has been recently reported that activation of phospholipase D2 (PLD2) induces ABCA1 degradation in macrophages and in ABCA1-expressing BHK cells (47) and that ethanol, starting at 25 mM, inhibits PLD signaling in astrocytes (48). Fig. 4A shows that PLD2 is indeed the main isofrom present in cortical primary astrocytes, whereas PLD1 is hardly detected. We investigated the involvement of PLD in the up-regulation of ABCA1 by incubating astrocytes with a short chain primary alcohol (1-butanol), which, like ethanol, is an inhibitor of PLD signaling, and its analogue tert-butanol. Short chain alcohols, including ethanol and 1-butanol, are competitive substrates for a PLD-catalyzed transphosphatidylation reaction, leading to the formation of phosphatidylethanol or phosphatidylbutanol instead of phosphatidic acid, thereby inhibiting PLD signaling. Short chain secondary and tertiary alcohols (such as tert-butanol) have been used as “inactive analogues,” since they are not PLD substrates. We found that 1-butanol, but not tert-butanol, increased ABCA1 protein levels (Fig. 4B); the relative optical density of ABCA1 bands in 1-butanol- and tert-butanol-treated astrocytes were (-fold increase versus control) 1.97 ± 0.2 and 0.98 ± 0.09, respectively, indicating that, indeed, inhibition of PLD signaling induces ABCA1 up-regulation in astrocytes.

To substantiate our results with ethanol on ABCA1 levels and on cholesterol efflux, we tested whether up-regulation of ABCA1 by 8-Br-cAMP, a cAMP analogue that induces ABCA1 and cholesterol efflux in other cell types (29), or by 22(R)-HC/9-cis-RA, would also increase cholesterol efflux. Both treatments increased apoA1- and HDL-mediated cholesterol efflux from astrocytes, and 22(R)-HC/9-cis-RA increased cholesterol efflux even in the absence of exogenously added acceptors (Fig. 5A). As expected, both 22(R)-HC/9-cis-RA and 8-Br-cAMP increased the levels of ABCA1 in astrocytes (Fig. 5B).

Mechanisms of cholesterol transport may differ among species (29). We thus verified our findings in fetal human astrocytes. Ethanol (50 mM) increased cholesterol efflux also from these cells, both in the presence and in the absence of cholesterol acceptors (Fig. 6A). Pretreatment with 22(R)-HC/9-cis-RA or 8-Br-cAMP increased cholesterol efflux to HDL and apoA1, whereas 22(R)-HC/9-cis-RA increased the efflux also in the absence of acceptors (Fig. 6B). Treatment of fetal human astrocytes with 22(R)-HC/9-cis-RA, 8-Br-cAMP, or ethanol increased ABCA1 levels (Fig. 6C). Some differences between the responses of human and rat astrocytes were, however, identified. Cholesterol efflux in the presence of cholesterol acceptors was generally higher in human astrocytes than in rat astrocytes. In human astrocytes, the enhancement of cholesterol efflux to apoA1 and HDL after pretreatment with 8-Br-cAMP was significantly lower than the enhancement
after 22(R)-HC/9-cis-RA pretreatment, whereas these two treatments elicited an equal efflux from rat astrocytes. These observations suggest that species differences exist in brain cholesterol transport.

To investigate the developmental expression of ABCA1 in the brain, we quantified ABCA1 immunoreactivity in cerebral cortex from rats at PND 1, 4, 7, 10, 15, 21, and 60. Levels of ABCA1 were low at birth and increased with age, reaching a maximum at PND 21, whereas levels of the reference protein, β-actin, decreased with age (Fig. 7A). In order to verify the effect of ethanol on ABCA1 in vivo, rats were treated with 2, 4, and 6 g/kg ethanol from PND 4 to 7. 30 min after the last ethanol administration, animals were sacrificed, and levels of ABCA1 were determined by Western blot in cerebral cortex homogenates. Ethanol increased ABCA1 levels (Fig. 7B). Blood alcohol concentrations following each dose level were 125 ± 9, 321 ± 7, and 462 ± 6 mg/dl (n = 4), corresponding to 27.2, 69.8, and 100.4 mM ethanol, respectively.

It has been reported that in astrocytes and in macrophages, ABCA1 facilitates the secretion of apoE without affecting its transcription or its intracellular levels (33, 35, 49). Based on these observations, we hypothesized that ethanol, by inducing ABCA1 levels, may also induce apoE release. Indeed, a 48-h incubation with ethanol increased the release from astrocytes of apoE in a concentration-dependent manner (Fig. 8A); intracellular levels of apoE were slightly decreased by ethanol treatments (Fig. 8B), whereas apoE mRNA levels were not significantly affected by up to 100 mM ethanol (not shown). Proteins in the medium were normalized for biglycan, a proteoglycan whose secretion by astrocytes is not affected by ethanol3 (Fig. 8A). Since apoE is a major lipid acceptor in the ABCA1 pathway (29), astrocyte-conditioned medium, prepared in the presence of ethanol, would be expected to extract more cholesterol from cells. Indeed, astrocyte-conditioned
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A

IB: Apo E

IB: Biglycan

B

IB: Apo E

IB: β-Actin

C

D

FIGURE 8. Effect of ethanol on apoE expression and release. Fetal rat astrocytes were incubated for 24 h in the absence or presence of different concentrations of ethanol. ApoE levels were determined by immunoblot (IB) in the medium (A) and in the cytosolic extract (B). Cholesterol efflux was measured in [3H]cholesterol prelabeled astrocytes exposed to serum-free medium, astrocyte conditioned medium, or astrocyte conditioned medium prepaired in the presence of 50 or 100 mM ethanol (C) or 10 µg/ml apoE (D). Each value is the mean ± S.E. of three determinations. **, p < 0.01; ***, p < 0.001 by Fisher’s LSD test.

medium prepared in the presence of 50 and 100 mM ethanol, extracted more cholesterol than conditioned medium prepared in the absence of ethanol (Fig. 8C). Exogenous apoE (10 µg/ml) induced cholesterol efflux in fetal rat astrocytes similarly to apoAI and to the astrocyte-conditioned medium (Fig. 8D).

Two additional ABC cholesterol transporters, ABCG1 and ABCG4, have been identified in astrocytes, and ABCG1 has been involved in cholesterol efflux in these cells (32, 34). We found that ethanol increased ABCG1 protein and mRNA levels (Fig. 9, A and C) without affecting ABCG4 (Fig. 9B).

The increase in cholesterol transporters and cholesterol efflux induced by ethanol may result in a compensatory up-regulation of cholesterol synthesis. To assess this possibility, astrocytes labeled for 4 h with the cholesterol precursor [3H]acetate were incubated for 24 h with ethanol in the presence or in the absence of apoAI to maximize the effect of ethanol on cholesterol efflux through the ABCA1 pathway. Ethanol did not affect cholesterol synthesis under any of the tested conditions (Fig. 10A). As a positive control, we used a medium containing 10% lipoprotein-deficient serum, which greatly increased cholesterol synthesis in these cells. Lipoprotein-deficient serum-induced cholesterol synthesis was not significantly affected by ethanol (Fig. 10A). Additionally, ethanol did not affect the intracellular relative abundance of free cholesterol versus esterified cholesterol, and esterified cholesterol in astrocytes was less than 1% of total cholesterol (not shown).

An increase in cholesterol efflux without an increase of cholesterol synthesis would be expected to lead to a depletion of intracellular cholesterol. Indeed, after a 72-h treatment with 75 mM ethanol in the presence of apoAI, cholesterol levels in astrocytes were significantly decreased (Fig. 10B).

Our initial hypothesis was that disruption of cholesterol homeostasis in astrocytes by ethanol may be involved in its teratogenic effects. We thus sought to determine whether 13-cis-RA (isotretinoin), a derivative of vitamin A used for treatment of severe cystic acne known to be a teratogen in a number of experimental animals, including rats and mice, and in humans (50), would cause similar alteration in cholesterol transporters. We found that 13-cis-RA increased ABCA1 and ABCG1 protein levels in astrocytes (Fig. 11, A and B), adding support to the hypothesis that up-regulation of cholesterol transporters may represent a more general mechanism of teratogenesis. It should be noted in this regard that in rodent models, malformations induced by 13-cis-RA and ethanol are quite similar (51).

DISCUSSION

Ethanol abuse by pregnant women has deleterious effects on the development of the fetus and can lead to long lasting cognitive and behavioral deficits known as alcohol-related neurodevelopmental disorders, the most severe of which is FAS (1, 4). Cholesterol is essential during brain development, and lack of cholesterol due to a genetic defect in cholesterol synthesis results in a severe syndrome, SLOS, which presents several phenotypic similarities with FAS (5–9). Lack of cholesterol indeed results in an impairment of several neuronal and glial cell functions (13–17), most of which are also affected by ethanol (19–
These observations, together with the notion that ethanol increases HDL in the cardiovascular system (51, 52), led to the hypothesis that alcohol may alter cholesterol homeostasis in the developing brain and that such effects may be involved in its neurodevelopmental toxicity.

Astrocytes are major producers of cholesterol in the brain and are also involved in cholesterol redistribution and/or elimination, since they produce nascent lipoproteins containing apoE that can extract cholesterol from brain cells (28). The cholesterol transporters ABCA1 and ABCG1 have been implicated in removing cholesterol from astrocytes and neurons through the interaction of these transporters with nascent lipoproteins released by astrocytes (28, 32–36).

In the present study, we have found that ethanol (25–50 mM) increased cholesterol efflux and the levels of active ABCA1 in fetal rat astrocytes. Such an effect was mediated by an increase in ABCA1 transcription and by inhibition of the PLD signaling. PLD2 has been shown to induce ABCA1 degradation (47), and ethanol, starting at 25 mM, inhibits PLD signaling (48). These findings were also confirmed in fetal human astrocytes.

The role of ABCA1 in the developing brain had not been previously investigated. Our results show that ABCA1 expression is very low at birth and increases with age. Low levels of ABCA1 in the immature brain may reflect the need to prevent cholesterol loss at a time when the blood-brain barrier is not yet fully developed; indeed, the presence of a mature blood-brain barrier prevents the exit of cholesterol-rich endogenous lipoproteins from the adult brain (53). In vivo exposure to ethanol from PND 4 to 7, a period of high susceptibility to ethanol developmental neurotoxicity (54, 55), increased expression of ABCA1 in the rat cortex, thus substantiating the in vitro findings. Since cortical homogenates also contain other cell types expressing ABCA1, namely microglia and neurons (37), effects of ethanol on ABCA1 expression in these cells may also have contributed to the observed in vivo effect.

The response of these cell types to ethanol in relationship to ABCA1 up-regulation will need to be further investigated in in vitro systems. Of note, however, is that the blood ethanol concentrations, which presumably reflect brain concentrations, obtained in the in vivo study, are comparable with those shown to up-regulate ABCA1 in vitro.

Ethanol also increased apoE release, in agreement with the observation made by others that ABCA1 levels regulate apoE release in astrocytes (33, 35). In addition, conditioned medium derived from ethanol-treated astrocytes extracted more cholesterol than conditioned medium derived from control cells. Recent studies indicate that ABCA1 mediates an initial lipida-
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![Figure 11](image-url)

**FIGURE 11. Effect of 13-cis-RA on ABCA1 and ABCG1 protein levels.** Fetal rat astrocytes were incubated for 24 h with 10 μM 13-cis-RA. ABCA1 (A) and ABCG1 (B) protein levels were assessed by immunoblot analysis; lower blots show β-actin levels. Results are representative of two or three similar experiments.

The up-regulation of lipid-poor or lipid-free apolipoproteins, whereas the half-transporters ABCG1 and ABCG4 are responsible for the further lipidation of particles that have been partially lipidated by ABCA1 (30). These observations are consistent with the involvement of ABCA1 in the increased cholesterol efflux to apoA1 observed after ethanol treatment. On the other hand, we also observed that ethanol enhanced cholesterol efflux to HDL, suggesting the involvement of other cholesterol transporters.

In agreement with this hypothesis, we found that ethanol increased the levels of ABCG1 but not of ABCG4.

Cholesterol efflux induced by astrocyte treatment with ethanol for 24 h was not compensated by increased cholesterol synthesis during the same time frame and led, after a 3-day ethanol treatment, to reduced cholesterol content in astrocytes. While we cannot rule out the possibility that astrocytes might compensate for the reduced levels of cholesterol by up-regulating cholesterol synthesis at this later time point, our data demonstrate that ethanol caused perturbations of astrocyte cholesterol levels by up-regulating mechanisms of cholesterol efflux mediated by ABC cholesterol transporters. This finding is substantiated by the observation that up-regulation of ABCA1 by liver X receptor/retinoid X receptor agonists (37), as well as a 5-day treatment with 50 mM ethanol (56), reduce cholesterol levels in neurons. A recent study also showed that ethanol dramatically decreases cholesterol content in the cerebellum of newborn rats prenatally exposed to ethanol (56). Further studies will investigate the effect of ethanol on astrocyte lipoprotein release, composition, and ability to extract cholesterol from brain cells.

Excess of vitamin A (retinol) and its metabolite, retinoic acid, are teratogenic in animals, and retinoic acid embroyopathy has been observed after topical use of 13-cis-RA (isotretinoin), a prescription drug used for the treatment of acne (57). The teratogenic effects of ethanol are similar to those of retinoic acid (58). It was recently reported that retinoic acid up-regulates ABCA1 levels in macrophages (59), and 9-cis-RA, in combination with 22(R)-HC, potently induces ABCA1 and ABCG1 in many cell types, including astrocytes. To explore the possibility that a common mechanism may underlie retinoid acid and ethanol teratogenesis, we investigated the effect of 13-cis-RA on ABCA1 and ABCG1 protein levels. We found that 13-cis-RA induced ABCA1 and ABCG1 protein levels, suggesting that the up-regulation of cholesterol transporters may, indeed, be a common mechanism of teratogenesis for retinoic acid and ethanol. Our hypothesis that ethanol may cause some of the reported neurodevelopmental abnormalities by up-regulating cholesterol transporters was further supported by other observations. For example, sonic hedgehog signaling is altered in retinoic acid embryopathy (60, 61). Cholesterol appears to have a role in sonic hedgehog signaling, and inhibition of cholesterol synthesis has been suggested to inhibit the cholesterol-dependent post translational modification of Shh, to disrupt cholesterol-rich plasma rafts involved in Shh signal transduction, and to interfere with the ability of target tissues to sense or transduce the Shh signaling (10–12).

Altogether, findings of this study indicate that ethanol causes disregulation of cholesterol homeostasis in the brain by affecting the levels of ABC cholesterol transporters in glial cells. The reported effects occurred in a range of ethanol concentrations (25–100 mM) physiologically relevant because they can be found in the blood of moderate to heavy drinkers (25 mM corresponds to 0.115% ethanol, 50 mM corresponds to 0.23%, and 100 mM corresponds to 0.46%). The effect of ethanol on cholesterol may be deleterious to the developing brain, where cholesterol is essential for its growth, the formation of synaptic contacts, and the activation of the hedgehog signaling pathway, all functions shown to be affected by in utero alcohol exposure.

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