ALCOHOLISM CLINICAL & EXPERIMENTAL RESEARCH

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Effect of Lipid Raft Disruption on Ethanol Inhibition of L1 Adhesion

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Background: Alcohol causes fetal alcohol spectrum disorders in part by disrupting the function of the neural cell adhesion molecule L1. Alcohol inhibits L1-mediated cell–cell adhesion in diverse cell types and inhibits L1-mediated neurite outgrowth in cerebellar granule neurons (CGNs). A recent report indicates that ethanol (EtOH) induces the translocation of L1 into CGN lipid rafts and that disruption of lipid rafts prevents EtOH inhibition of L1-mediated neurite outgrowth. The same butanol–pentanol cutoff was noted for alcohol-induced translocation of L1 into lipid rafts that was reported previously for alcohol inhibition of L1 adhesion, suggesting that EtOH might inhibit L1 adhesion by shifting L1 into lipid rafts.

Methods: The NIH/3T3 cell line, 2A2-$L1_s$, is a well-characterized EtOH-sensitive clonal cell line that stably expresses human L1. Cells were treated with 25 mM EtOH, 5 μM filipin, or both. Lipid rafts were enriched in membrane fractions by preparation of detergent-resistant membrane (DRMs) fractions. Caveolin-1 was used as a marker of lipid rafts, and L1 and Src were quantified by Western blotting in lipid-raft-enriched membrane fractions and by immunohistochemistry.

Results: EtOH (25 mM) increased the percentage of L1, but not Src, in 2A2-$L1_s$ membrane fractions enriched in lipid rafts. Filipin, an agent known to disrupt lipid rafts, decreased the percentage of caveolin and L1 in DRMs from 2A2-$L1_s$ cells. Filipin also blocked EtOH-induced translocation of L1 into lipid rafts from 2A2-$L1_s$ cells but did not significantly affect L1 adhesion or EtOH inhibition of L1 adhesion.

Conclusions: These findings indicate that EtOH does not inhibit L1 adhesion in NIH/3T3 cells by inducing the translocation of L1 into lipid rafts.

Key Words: Fetal Alcohol Spectrum Disorders, L1 Neural Cell Adhesion Molecule, Lipid Raft, Ethanol.

ALCOHOL EXPOSURE DURING pregnancy may cause fetal alcohol spectrum disorders, in part, by disrupting the function of the neural cell adhesion molecule L1. L1 mutations and prenatal alcohol exposure cause similar brain dysmorphology in children (Ramanathan et al., 1996; Wilkemeyer et al., 2003), and some of these L1 mutations disrupt L1-mediated cell–cell adhesion (L1 adhesion) or L1-mediated neurite outgrowth (De Angelis et al., 2002; Schultheis et al., 2007). Concentrations of ethanol (EtOH) attained after just 1 or 2 drinks—5 to 10 mM—inhibit L1 adhesion in cerebellar granule neurons (CGNs), neural cell lines, and fibroblasts and decrease L1-mediated neurite outgrowth in CGNs (Bearer et al., 1999; Charness et al., 1994; Ramanathan et al., 1996). Drugs that block EtOH inhibition of L1 adhesion also prevent EtOH teratogenesis in mice (Chen et al., 2001, 2005; Wilkemeyer et al., 2003, 2004). Finally, differential activity of kinases that regulate L1 sensitivity to EtOH may account for genetically determined differences in susceptibility to EtOH teratogenesis in clonal cell lines and in mice (Dou et al., 2013).

L1 is a transmembrane protein comprising 6 Ig-like domains, 5 fibronectin type III repeats, a transmembrane region, and a highly conserved cytoplasmic domain (Maness and Schachner, 2007). The Ig domains fold into a horseshoe configuration that favors L1 adhesion (Haspel and Grumet, 2003), and an alcohol binding pocket has been identified at the domain interface between Ig1 and Ig4 (Arevalo et al., 2008). Small amino acid substitutions within this alcohol binding pocket markedly alter the pharmacology of alcohol inhibition of L1 adhesion (Dou et al., 2011). These findings support the hypothesis that EtOH disrupts L1 adhesion by interacting with a locus on the L1 extracellular domain (L1-ECD).

Recent data suggest that EtOH might also disrupt L1 function by altering protein trafficking. EtOH treatment of CGNs induced the translocation of L1 into lipid rafts and the translocation of Src out of lipid rafts (Tang et al., 2011). L1-mediated neurite outgrowth in CGNs requires L1 activation of Src signaling (Ignelzi et al., 1994); therefore, EtOH

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might inhibit L1-mediated neurite outgrowth by translocating L1 and Src to separate membrane microdomains, thereby reducing their normal interactions. Consistent with this hypothesis, disruption of CGN lipid rafts with methyl-beta-cyclodextrin prevented EtOH inhibition of L1-mediated neurite outgrowth (Tang et al., 2011). Translocation of L1 into lipid rafts also occurred after treatment of CGNs with methanol, 1-propanol and 1-butanol—alcohols of 1 to 4 carbons—but not with 1-pentanol, a 5-carbon 1-alcohol. This same butanol–pentanol cutoff was also observed for EtOH inhibition of L1 adhesion in transfected fibroblasts and neural cell lines (Charness et al., 1994; Dou et al., 2011; Wilkemeyer et al., 2002). These observations suggest that translocation of L1 into lipid rafts is necessary for EtOH inhibition of L1-mediated neurite outgrowth and might also account for EtOH inhibition of L1 adhesion.

Lipid rafts are major components of plasma membranes (Edidin, 2003). Many protein ligands, including L1, reversibly associate with lipid rafts to regulate intracellular physiological processes and extracellular interactions (Kamiguchi, 2006). EtOH-induced translocation of L1 into lipid rafts might alter the conformation of the L1-ECD in ways that reduce L1 adhesion. Here, we use a well-characterized NIH/3T3 cell line stably transfected with human L1, 2A2-L1$_s$, to test the hypothesis that EtOH inhibits L1 adhesion by inducing the translocation of L1 into lipid rafts. We confirm that EtOH induces the translocation of L1 into lipid rafts in NIH/3T3 cells; however, filipin, a drug that disrupts lipid rafts (Schnitzer et al., 1994), prevents EtOH-induced translocation of L1 into lipid rafts, but does not alter L1 adhesion or its inhibition by EtOH.

MATERIALS AND METHODS

Cell Culture, Adhesion Assay

NIH/3T3 cells expressing human L1 (2A2-L1$_s$) were cultured as described (Dou et al., 2011). L1-mediated cell–cell adhesion (L1 adhesion) was assayed by separating cells into single-cell suspensions, agitating, and measuring the percentage of adherent cells using phase contrast microscopy, as described (Dou et al., 2011).

Immunohistochemistry

2A2-L1$_s$ cells were plated in T75 flasks in DMEM supplemented with 10% bovine serum (BS) and grown to 75 to 85% confluence (Dou et al., 2011). Cells were treated with 25 mM EtOH, 5 μM filipin, or both for 1 hour. Cells were harvested in phosphate buffered saline (PBS) plus 2 mM EDTA, fixed in 4% paraformaldehyde for 30 minutes, blocked with PBS supplemented with 5% BS, and incubated with L1 mAb 5G3 (Dou et al., 2011), caveolin-1 polyclonal antibody (AB18199; Abcam), or Src polyclonal antibody (Ab47405; Abcam, Cambridge, MA) in PBS/BS at room temperature for 2 hours. Cells were washed 3 times with PBS and incubated with goat anti-mouse IgG conjugated with Alexa Fluor-488 and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (Invitrogen, Grand Island, NY) in PBS/BS. Cells were washed again with PBS and fixed in paraformaldehyde. Images were captured using a Zeiss Multiphoton microscope LSM T-PMT system and Zen 2009 software from Carl Zeiss (Carl Zeiss International, Jena, Germany).

Detergent-Resistant Membrane Preparation

Lipid rafts are normally detergent resistant and therefore localize predominantly to detergent-insoluble fractions during separation at low temperature (Magee and Parmryd, 2003). These are referred to as detergent-resistant membrane (DRM) fractions. 2A2-L1$_s$ cells were cultured in DMEM supplemented with 10% BS. At 70 to 80% confluence, cells were treated for 1 hour in DMEM with drugs and collected with PBS plus 2 mM EDTA. Whole cell lysates were prepared with NP-40 cell lysis buffer plus 1% Triton X-100 and Halt protease/phosphatase inhibitors on ice for 5 minutes and then centrifuged at 10,000$\times g$ for 5 minutes to remove cell debris. The supernatant was then centrifuged at 34,800$\times g$ at 4°C for 2 hours in a TLA120.2 rotor (Beckman, Indianapolis, IN). The resulting pellet and supernatant were dissolved in equal volume of 1$\times$ SDS sample buffer (Boston Bioproduct, Ashland, MA). L1, Src, and caveolin in DRM fractions were analyzed with Western blot and densitometric analysis of protein bands from scanned images of PVDF membranes using NIH Image J software (Abramoff et al., 2004).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical differences in means were compared using the *t*-test (Prism 5; GraphPad Software, La Jolla, CA.). Statistical significance was defined as $p < 0.05$.

RESULTS

Filipin Disrupts Lipid Rafts in 2A2-L1s Cells

Caveolin is a major component of lipid rafts that localizes to DRMs and is commonly used as a lipid raft marker (Parton and Simons, 2007; Pike, 2009). We refer to detergent-resistant, caveolin-enriched fractions as DRMs or lipid rafts. Filipin disrupts lipid rafts by depleting membrane cholesterol, leading to the redistribution of caveolin out of lipid rafts (Kim et al., 2004; Marwali et al., 2003; Schnitzer et al., 1994). 2A2-L1$_s$ cells were incubated for 1 hour at 37°C in the absence and presence of 5 μM filipin and 25 mM EtOH, and cell lysates were separated into detergent-soluble (supernatant) and DRM fractions (pellet) using ultracentrifugation. Western blot analysis showed that in control cells, 82.4 $\pm$ 4.4% of caveolin was distributed in the DRM fraction; EtOH treatment did not alter this distribution (Fig. 1) ($n = 9$, $p = 0.175$). Filipin significantly decreased the percentage of caveolin in the DRM fraction (49.3 $\pm$ 1.3%; $n = 9$, $p < 0.001$) (Fig. 1*C*), and EtOH did not modify this effect of filipin ($n = 9$, $p = 0.591$). These results indicate that under our experimental conditions, filipin, but not EtOH, disrupts lipid rafts in NIH/3T3 fibroblasts.

Ethanol Induces the Translocation of L1 into Lipid Rafts

The effects of EtOH and filipin on L1 lipid raft localization were evaluated using immunohistochemistry and confocal microscopy. Immunolabeling with antibodies against L1 and caveolin showed a homogeneous pattern in the plasma membrane of control and EtOH-treated 2A2-L1$_s$ cells. EtOH treatment increased the co-localization of L1 and caveolin (Fig. 1*A*). Western blot analysis showed that treatment of

2A2-$L1_s$ cells with 25 mM EtOH significantly increased the association of L1 with DRMs from 56.5 ± 6.4% to 71.2 ± 4.7% ($n = 9$, $p = 0.002$). In contrast, EtOH did not alter the co-localization of Src and caveolin (Fig. 2). Importantly, filipin treatment significantly reduced the proportion of L1 associated with DRMs (35.6 ± 4.5%; $n = 9$, $p = 0.046$), and EtOH did not significantly increase this proportion (Fig. 1*D*). These findings indicate that EtOH induces the translocation of L1 into lipid rafts in NIH/3T3 cells, and filipin prevents this action by disrupting lipid rafts.

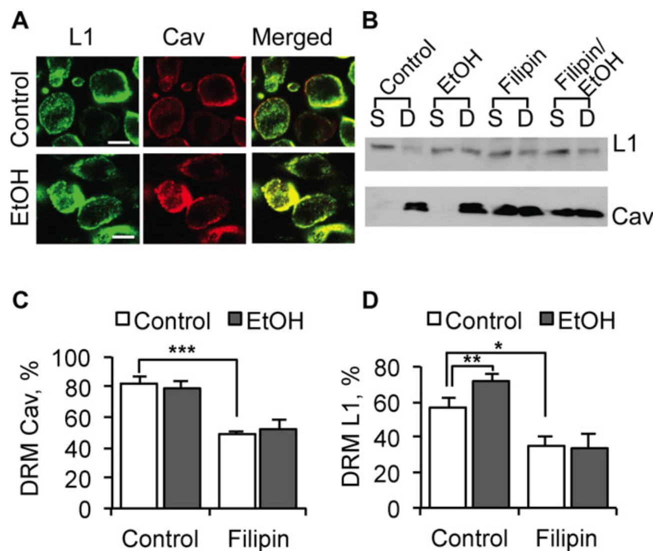

Fig. 1. Disruption by filipin of ethanol (EtOH)-induced translocation of L1 into lipid rafts. NIH/3T3 cells transfected stably with human L1 (2A2-$L1_s$) were incubated for 1 hour in the absence and presence of 25 mM EtOH and 5 μM filipin. (**A**) Immunofluorescence labeling of L1 (green) and caveolin (Cav) (red) under the indicated conditions; bar = 10 μm. Yellow color in merged panels indicates co-localization of L1 and caveolin, a marker of lipid rafts. (**B**) Representative Western blot shows caveolin distribution in detergent-soluble (S) and detergent-resistant membrane fractions (D or DRM) of total cell lysates. Densitometric analysis of percentage of caveolin (**C**) and L1 (**D**) in DRM from experiments shown in (**B**). Data shown are mean ± SEM % from 9 independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

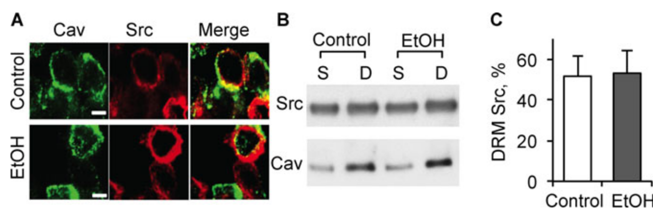

Fig. 2. Effect of ethanol (EtOH) on the co-localization of Src and caveolin. (**A**) Immunofluorescence labeling of Cav (green) and Src (red) under the indicated conditions, as described in Fig. 1; bar = 10 μM. Yellow color in merged panels indicates co-localization of Src and caveolin. (**B**) Representative Western blot shows Src distribution in detergent-soluble (S) and DRM fractions (D) of total cell lysates. (**C**) Densitometric analysis of percentage of Src in DRM fractions from experiments shown in (**B**). Data shown are mean ± SEM % from 5 independent experiments; $n = 5$; $p = 0.10$. DRM, detergent-resistant membrane.

Filipin Disruption of Lipid Rafts Does Not Affect L1 Adhesion or Ethanol Inhibition of L1 Adhesion

Filipin disrupted lipid rafts and blocked EtOH-induced translocation of L1 into lipid rafts. If EtOH inhibits L1 adhesion by inducing its movement into lipid rafts, then EtOH should not inhibit L1 adhesion in filipin-treated cells. 2A2-$L1_s$ cells were treated with 5 μM filipin for 1 hour and harvested for cell adhesion assays. As reported previously (Dou et al., 2013; Wilkemeyer and Charness, 1998), 25 mM EtOH significantly reduced L1 adhesion in 2A2-$L1_s$ cells (Fig. 3) (control 26.6 ± 3.1%; EtOH 14.0 ± 2.7%; $n = 8$, $p < 0.001$). Filipin treatment had no significant effect on L1 adhesion ($n = 8$, $p = 0.232$) or EtOH inhibition of L1 adhesion ($n = 8$, $p = 0.814$).

DISCUSSION

We conducted these studies to test the hypothesis that EtOH inhibits L1 adhesion by shifting L1 into lipid rafts, thereby reducing its adhesivity. Our work confirms the recent report of Tang and colleagues (2011) that EtOH induces the translocation of L1 into lipid rafts. The fact that EtOH alters L1 trafficking in both neuronal and mesenchymal cells suggests that EtOH is modulating molecular processes that are common to both cellular lineages. Targeting of L1 to lipid rafts is believed to occur through palmitoylation of the L1 membrane spanning region (Ren and Bennett, 1998); hence, it is possible that EtOH alters palmitoylation of both the neuronal isoform of L1 expressed in CGNs and the non-neuronal isoform stably expressed in our 2A2-$L1_s$ NIH/3T3 clonal cell line (Wilkemeyer and Charness, 1998).

The magnitude of L1 translocation was lower in NIH/3T3 cells than in CGNs, perhaps because under differing experimental conditions, L1 was more highly localized to lipid rafts in untreated NIH/3T3 cells than in untreated CGNs, providing less opportunity for EtOH to translocate L1. The

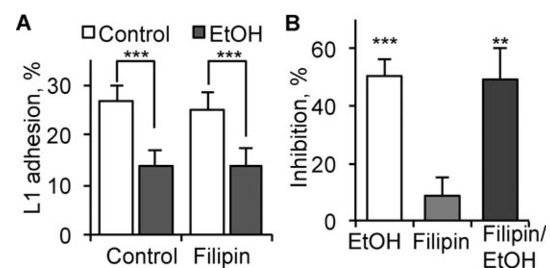

Fig. 3. Effect of filipin on L1 adhesion and ethanol (EtOH) inhibition of L1 adhesion. 2A2-$L1_s$ cells were incubated for 1 hour in the absence and presence of 25 mM EtOH and 5 μM filipin. Cells were harvested for cell adhesion assays. (**A**) L1 adhesion in the absence and presence of 25 mM EtOH and 5 μM filipin. Shown are the mean ± SEM levels of L1 adhesion for control and filipin-treated cells in the absence and presence of EtOH. (**B**) Inhibition of L1 adhesion by EtOH, filipin, and EtOH plus filipin. Data were derived from experiments shown in (**A**) in which the percent inhibition of control adhesion by EtOH, filipin, or filipin plus EtOH was calculated for paired experiments. Shown are the mean ± SEM % inhibition for each of the 3 conditions; $n = 8$; **$p < 0.01$, ***$p < 0.001$, indicating significant inhibition.

reported localization of L1 within CGN lipid rafts ranges from 10% to nearly 70% (Nakai and Kamiguchi, 2002; Tang et al., 2011), suggesting that results can be influenced by experimental conditions and methods for isolating lipid rafts. Furthermore, translocation of L1 to lipid rafts in CGNs is developmentally regulated, peaking between postnatal days 3 and 8 (Nakai and Kamiguchi, 2002).

EtOH did not alter the distribution of Src in lipid rafts in 2A2-L1$_s$ cells, in contrast to findings in CGNs (Tang et al., 2011). The absence of an EtOH effect on Src trafficking in NIH/3T3 cells might reflect intrinsic differences in the molecular apparatus that regulates the movement of Src in lipid rafts of mesenchymal and neuronal cells. Growth cone motility in CGNs results from dynamic remodeling of the growth cone in response to axon guidance molecules (Schmid and Maness, 2008; Vitriol and Zheng, 2012). Interactions of L1 and Src within lipid rafts are developmentally regulated and mediate the rapid dynamic remodeling of membrane and cytoskeletal elements that are integral to axon pathfinding in CGNs (Nakai and Kamiguchi, 2002). In contrast, fibroblasts lack growth cones and might also lack some of the regulatory elements that EtOH targets to alter Src trafficking in CGNs.

Disruption of lipid rafts in CGNs by methyl-beta4-dextran, a cholesterol-depleting agent, blocked EtOH-induced translocation of L1 into lipid rafts (Tang et al., 2011). Similarly, treatment of 2A2-L1$_s$ cells with filipin caused a significant shift in caveolin, a lipid raft marker, from the DRM fraction into the non-DRM fraction (detergent-soluble fractions) of 2A2-L1$_s$ cellular extracts. Our findings indicate that filipin was effective in disrupting lipid rafts in our NIH3/T3 cells. Although filipin did not completely shift caveolin from the DRM to the non-DRM fractions, the overall effect was sufficient to completely block EtOH-induced translocation of L1 into lipid rafts. Hence, we were able to test the hypothesis that EtOH inhibits L1 adhesion by promoting L1 translocation into lipid rafts. Filipin did not significantly modulate L1 adhesion or its inhibition by EtOH. These findings suggest that L1 adhesion is not reduced when L1 is localized to lipid rafts and that EtOH-induced translocation of L1 into lipid rafts does not underlie EtOH inhibition of L1 adhesion in NIH/3T3 cells. These observations are consistent with the hypothesis that EtOH inhibits L1 adhesion by interacting with an alcohol binding pocket at the Ig1–Ig4 interface of the L1-ECD (Arevalo et al., 2008; Dou et al., 2011).

It remains unclear why disruption of lipid rafts blocked EtOH inhibition of L1-mediated neurite outgrowth in CGNs, but had no effect on EtOH inhibition of L1 adhesion. In both model systems, EtOH induced a translocation of L1 into lipid rafts. One notable difference between our experiments and those of Tang and colleagues (2011) was that EtOH induced a redistribution of Src out of lipid rafts in CGNs, but did not do so in NIH/3T3 cells. Tang and colleagues (2011). speculated that EtOH disruption of CGN neurite outgrowth results from the physical separation of L1 and Src. Our results indicate that EtOH inhibition of L1 adhesion does not require this physical dissociation of L1 and Src, at least in NIH/3T3 cells, and previous experiments supported an extracellular site for EtOH's actions on L1 adhesion. Parallel experiments on EtOH inhibition of L1 adhesion and L1-mediated neurite outgrowth in CGNs would be required to definitively determine whether common or distinct mechanisms underlie the effects of EtOH on these 2 functions of L1.

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