Phosphatidylethanol Accumulation Promotes Intestinal Hyperplasia by Inducing ZONAB-Mediated Cell Density Increase in Response to Chronic Ethanol Exposure

Julie Pannequin,[1] Nathalie Delaunay,[1] Charbel Darido,[1] Tangui Maurice,[2] Philippe Crespy,[1] Michael A. Frohman,[4] Maria S. Balda,[5] Karl Matter,[5] Dominique Joubert,[1] Jean-François Bourgaux,[3] Jean-Pierre Bali,[1] and Frédéric Hollande[1]

[1]CNRS UMR5203; INSERM U661, University of Montpellier I, and University of Montpellier II, Cellular and Molecular Oncology Department, Institut de Génomique Fonctionnelle, Montpellier Cedex, France; [2]INSERM U710; EPHE; Université de Montpellier II, F-34095, Montpellier, France; [3]Service d'Hépato-Gastroentérologie, CHU Carémeau, Nîmes, France; [4]Department of Pharmacology and Center for Developmental Genetics, Stony Brook University, New York, New York; and [5]Department of Cell Biology, University College London, London, United Kingdom

Abstract

Chronic alcohol consumption is associated with increased risk of gastrointestinal cancer. High concentrations of ethanol trigger mucosal hyperregeneration, disrupt cell adhesion, and increase the sensitivity to carcinogens. Most of these effects are thought to be mediated by acetaldehyde, a genotoxic metabolite produced from ethanol by alcohol dehydrogenases. Here, we studied the role of low ethanol concentrations, more likely to mimic those found in the intestine *in vivo*, and used intestinal cells lacking alcohol dehydrogenase to identify the acetaldehyde-independent biological effects of ethanol. Under these conditions, ethanol did not stimulate the proliferation of nonconfluent cells, but significantly increased maximal cell density. Incorporation of phosphatidylethanol, produced from ethanol by phospholipase D, was instrumental to this effect. Phosphatidylethanol accumulation induced claudin-1 endocytosis and disrupted the claudin-1/ZO-1 association. The resulting nuclear translocation of ZONAB was shown to mediate the cell density increase in ethanol-treated cells. *In vivo*, incorporation of phosphatidylethanol and nuclear translocation of ZONAB correlated with increased proliferation in the colonic epithelium of ethanol-fed mice and in adenomas of chronic alcoholics. Our results show that phosphatidylethanol accumulation after chronic ethanol exposure disrupts signals that normally restrict proliferation in highly confluent intestinal cells, thus facilitating abnormal intestinal cell proliferation.

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Introduction

Ethanol is one of the multiple exogenous compounds thought to have harmful effects on the gastrointestinal mucosa, in particular because the gastrointestinal epithelium is directly and regularly in contact with ethanol in chronic alcohol consumers. High concentrations are in contact with epithelial cells in the oral cavity, the esophagus and, to a lesser degree, the stomach, whereas the intestinal and colonic mucosas are exposed to lower doses. Chronic ethanol consumption is associated with an increased risk for cancers of the digestive tract. In particular, a causal relationship is thought to exist between alcohol consumption and colorectal cancer (1-3), and ethanol was shown to promote tumor development in a mouse model of intestinal tumorigenesis (2). High ethanol concentrations are known to induce significant mucosal damage (4), thereby allowing the paracellular passage of luminal substances favoring cytotoxicity and inflammation (5), and several studies show the induction of a strong proliferative response in the colon and rectum following alcohol abuse, resulting in mucosal hyperregeneration (6, 7). Yet, the molecular mechanisms underlying the tumor-promoting action of alcohol are only partially understood. Ethanol was previously shown to disrupt the organization of intestinal cell cytoskeleton proteins and to induce a translocation of the tight junction (TJ) protein ZO-1 away from TJ complexes through an activation of myosin light chain kinase activity (8). Despite the well reported role for proteins of the TJ cytoplasmic plaque (such as ZO-1) in cell signaling and regulation of gene expression (9), the main consequence described to date for these disruptions concerns paracellular permeability alterations (8).

The effects of alcohol on mucosal integrity and cell proliferation are generally attributed to the genotoxic effects of acetaldehyde (10), an ethanol metabolite produced by specific isoforms of alcohol dehydrogenases (ADH) present in the colorectal mucosa and intestinal bacteria (11, 12). Yet,

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Requests for reprints: Frédéric Hollande, Laboratoire de Biochimie, Faculté de Pharmacie, Institut de Génomique Fonctionnelle, Bâtiment E, 15 Avenue Charles Flahault, 34093 Montpellier Cedex 5, France. Phone: 33-467-66-8144; Fax: 33-467-66-8144. E-mail: fhollande@univ-montp1.fr

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some of these effects have been described using the Caco-2 colorectal carcinoma cell line (8), which do not express any ADH (13), suggesting that one or several other metabolic pathways could play a role in the tumor-promoting activity of ethanol. As an example, free radicals generated from ethanol metabolism are thought to play a role in the pathogenesis of alcohol-associated colorectal hyperproliferation (14). Furthermore, ethanol could also undergo chemical coupling to membrane phospholipids via the action of phospholipase D (PLD; refs. 15, 16), resulting in the conversion of phosphatidylcholine into phosphatidylethanol (17-19). In the presence of high ethanol concentrations, this transphosphatidylation reaction dominates over the hydrolytic reaction done by PLD in the absence of alcohol, which generates phosphatidic acid, and the resulting loss of phosphatidic acid can interfere with signal transduction in hepatocytes and glia (20-22). However, lower concentrations of ethanol are found in the intestine, where the accumulation of phosphatidylethanol has previously been detected following chronic ethanol exposure (17, 18, 23). Although these low concentrations should not significantly disrupt the production of phosphatidic acid, little is known concerning the effect of low-level phosphatidylethanol accumulation on intestinal cell physiology.

In the present study, we asked whether phosphatidylethanol accumulation had a role to play in the ethanol-induced alterations of intestinal cell proliferation and/or TJ organization, and we sought to characterize the molecular mechanisms leading to these biological disruptions. Finally, we attempted to determine whether activation of the identified mechanism was detected *in vivo*, in the intestinal mucosa of ethanol-fed mice and of chronic alcoholics.

Results

Low Concentrations of Ethanol Increase Maximal Cell Density in Intestinal Cells Lacking ADH

The Caco-2 colorectal carcinoma cell line was previously shown not to express any of the ADH isoforms usually found

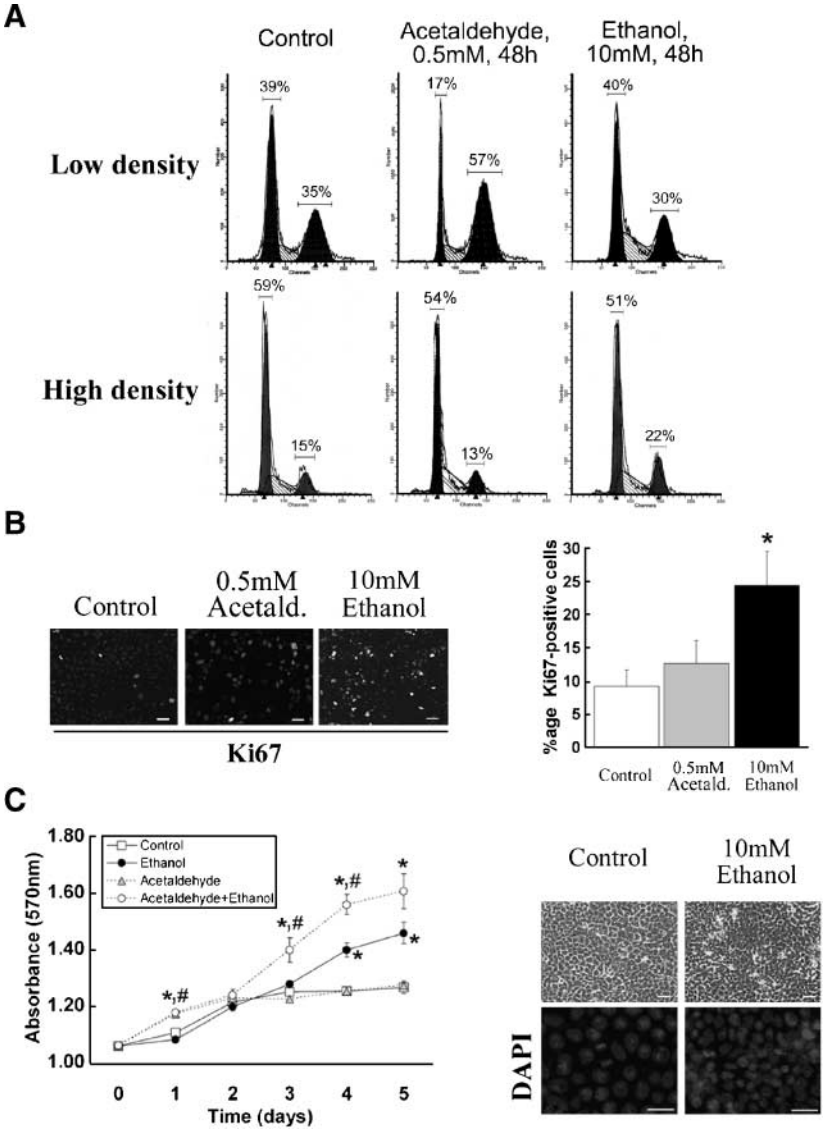

FIGURE 1. Low concentrations of ethanol block the decrease of proliferation in confluent Caco-2/TC7 cells and increase maximal cell density. **A.** Caco-2/TC7 cells, grown for 2 d after reaching 50% (*Low density*) or 100% confluency (*High density*), were treated once daily for 48 h with 10 mmol/L of ethanol or 0.5 mmol/L of acetaldehyde, and incubated with propidium iodide as indicated in Materials and Methods. The proportions of cells in the different cell cycle phases were quantified by flow cytometry (read-outs from one representative experiment). Percentages of cells in G_0/G_1 and G_2-M phases of the cell cycles are shown above the first and second peak, respectively. Columns, mean from three similar but independent experiments; bars, SE (Supplementary Fig. S2). **B.** Caco-2/TC7 cells were grown for 2 d after reaching 100% confluency, and treated once daily for 48 h with 10 mmol/L of ethanol or 0.5 mmol/L of acetaldehyde. Ki67 antigen was then detected by immunostaining (*left*), and the percentages of Ki67-positive cells were quantified from >500 cells in each experiment (*right*). Columns, mean from three independent experiments; bars, SE. *, $P < 0.05$ compared with control cells, Student's *t* test. **C.** Confluent Caco-2/TC7 cells were treated once daily with 10 mmol/L of ethanol, 0.5 mmol/L of acetaldehyde, or a combination of the two for the indicated number of days (*left*). On the indicated days, they were stained with crystal violet, lysed in PBS + 1% SDS, and the absorbance was measured at 570 nm. Points, mean from three independent experiments; bars, SE. *, $P < 0.05$ compared with controls; #, $P < 0.05$ compared with ethanol-treated cells, Student's *t* test. Caco-2/TC7 cells were treated for 5 days with 10 mmol/L of ethanol from the moment they reached confluency, then fixed with 3% PFA in PBS, photographed and stained with 4′,6-diamidino-2-phenylindole to localize cell nuclei (*right*). Representative fields from three independent experiments. Bars, 20 μmol/L.

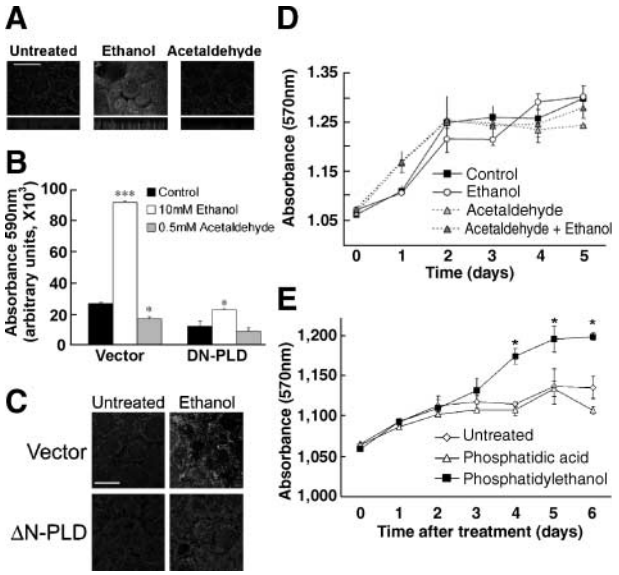

FIGURE 2. PLD-mediated phosphatidylethanol production is essential for the maximal cell density increase induced by chronic exposure of Caco-2/TC7 intestinal cells to low ethanol concentrations. **A.** Confluent Caco-2/TC7 cells were treated once daily for 48 h with 0.5 mmol/L of ethanol or 500 μmol/L of acetaldehyde, and the localization of phosphatidylethanol along the *XY*- and *XZ*-axis was detected by confocal microscopy as indicated in Materials and Methods. Note that membrane staining is more visible on the *XZ* image (*bars,* 10 μm). **B.** PLD activity was quantified in protein lysates from Caco-2/TC7 cells electroporated with dominant-negative PLD or with control constructs, plated at high density, and treated for 48 h with 10 mmol/L of ethanol or 500 μmol/L of acetaldehyde. **C.** Phosphatidylethanol localization was analyzed by immunofluorescent staining in Caco-2/TC7 cells electroporated with dominant-negative PLD (*ΔN-PLD*) or with control constructs (*Vector*), plated at high density, and treated or not for 48 h with 0.5 mmol/L of ethanol. **D.** Caco-2/TC7 cells electroporated with dominant-negative PLD were plated to confluency, and treated once daily with 10 mmol/L of ethanol, 0.5 mmol/L of acetaldehyde, or a combination of the two for the indicated number of days. On the indicated days, the cells were stained with crystal violet, lysed in PBS + 1% SDS, and the absorbance at 570 nm was measured. **E.** Confluent Caco-2/TC7 cells were treated once daily with 20 μmol/L of phosphatidic acid or phosphatidylethanol liposomes for the indicated number of days. On the indicated days, they were stained with crystal violet, lysed in PBS + 1% SDS, and the absorbance at 570 nm was measured. Points, mean from three independent experiments; bars, SE. *, $P < 0.05$ compared with untreated and phosphatidic acid–treated cells, Student's *t* test.

in the intestine (ADH 1, 3, and 4; ref. 13). In order to rule out any indirect effect due to acetaldehyde and to directly study the effect of ethanol, we first confirmed that the Caco-2/TC7 clone used in this study did not express any ADH mRNAs either (Supplementary Fig. S1). The effect of low concentrations (0.05%, 10 mmol/L) of ethanol on the cell cycle was then compared with that of acetaldehyde, a known mitogen for these cells (24), using Caco-2/TC7 cells at various levels of confluence. When Caco-2/TC7 cells were grown at low density (40-60%), ethanol treatment did not affect the cell cycle, whereas 0.5 mmol/L of acetaldehyde significantly reduced the number of resting cells and increased the proportion of cells going through the G_2-M transition (Fig. 1A; Supplementary Fig. S2). When cell cycle variables were analyzed on cells several days after they reached confluence, only 10.2 ± 0.9% of untreated cells were found in the G_2-M phases, whereas a large proportion (59.7 ± 3.1%) were quiescent. Under these conditions, treatment with 10 mmol/L of ethanol for 2 days reduced the number of resting cells and doubled the proportion of cells undergoing the G_2-M transition, whereas treatment with 0.5 mmol/L of acetaldehyde had no effect (Fig. 1A; Supplementary Fig. S2). Accordingly, a 3-day treatment with 10 mmol/L of ethanol was found to markedly increase the number of postconfluent cells displaying positive staining for Ki67, a proliferation marker expressed throughout the cell cycle. In contrast, acetaldehyde had no effect (Fig. 1B).

We therefore hypothesized that, if ethanol is able to inhibit signals arresting the cell cycle once cells reached confluence, ethanol-treated cells should be able to reach a higher maximal density than controls. To test this hypothesis, we used crystal violet incorporation to quantify the time course of ethanol's effect on cell numbers, in the presence or absence of acetaldehyde.

In the absence of treatment, cell numbers ceased to increase progressively after reaching confluence, whereas ethanol-treated cells did not stop growing and reached significantly higher cell numbers after a 5-day treatment (Fig. 1C, *left*). In contrast, cells treated with acetaldehyde were found to reach confluency faster, but their total numbers were similar to those observed in untreated cells. However, in the presence of ethanol, the stimulatory effect of acetaldehyde was maintained after cells reached confluence, demonstrating the additive effect of both compounds on postconfluent cell growth (Fig. 1C, *left*). Finally, bright-field microscopy and 4′,6-diamidino-2-phenylindole nuclear staining confirmed that maximal cell density was increased after treatment of Caco-2/TC7 with 10 mmol/L of ethanol (Fig. 1C, *right*).

Taken together, our results strongly suggest that, contrary to acetaldehyde, ethanol does not stimulate proliferation per se, but inhibits signals arresting the cell cycle in highly confluent cells, leading to an increase in maximal cell density.

PLD-Dependent Incorporation of Phosphatidylethanol Is Essential for the Cell Density Increase Induced by Chronic Ethanol Exposure

Because previous studies showed that phosphatidylethanol could be produced and incorporated into cell membranes in the blood and various organs including the intestine (17, 19), we analyzed whether this accumulation had a role to play in the modification of maximal cell density detected in ethanol-treated Caco-2/TC7 cells. In order to detect phosphatidylethanol accumulation in live cells, we first generated and characterized the specificity of a polyclonal rabbit antibody against phosphatidylethanol. This antibody displayed moderate avidity but significant specificity towards phosphatidylethanol (Supplementary Fig. S3). Although diffuse background staining was observed in all cells when this antibody was used for immunofluorescent detection, staining was markedly increased in the cytoplasm as well as along the lateral cell membrane in ethanol-treated cells, reaching a maximum after a 48-h treatment with 10 mmol/L of ethanol (Fig. 2A). In contrast, no signal was detected after incubation of those cells with acetaldehyde. Similarly, the formation of phosphatidylethanol was detected in the perinuclear area as well as in the membranes of the human colon cancer cell line HT-29 and nontumoral colon cells

obtained from H-2Kb-tsA58 (YAMC) and APC$^{+/+}$ mice (25, 26) after treatment with 10 mmol/L of ethanol, demonstrating that this pathway is not activated in Caco-2/TC7 cells only because of their inability to metabolize ethanol into acetaldehyde (Supplementary Fig. S3).

Aiming to gain insights into the biological role of phosphatidylethanol accumulation in ethanol-treated cells, we inhibited its production by transfecting Caco-2/TC7 cells with a construct encoding a dominant-negative form of PLD (27), the enzyme primarily involved in the production of phosphatidylethanol from ethanol (28, 29). As expected, expression of this construct markedly reduced the increase in PLD activity (Fig. 2B) and the production of phosphatidylethanol in cell membranes (Fig. 2C) otherwise induced by a 48-h treatment with 10 mmol/L of ethanol. Interestingly, we found that down-regulation of PLD completely prevented the biological effects of ethanol on maximal cell density increase (Fig. 2D). A similar effect was obtained when Caco-2/TC7 cells were pretreated with 1-butanol (Supplementary Fig. S4), previously shown to inhibit the ability of PLD to metabolize ethanol (30). Finally, we showed that a 16-h treatment of Caco-2/TC7 cells with phosphatidylethanol liposomes was able to mimic the maximal cell density increase observed after ethanol treatment (Fig. 2E).

Taken together, these results show that the PLD-mediated formation of phosphatidylethanol is essential for the disruption of cell density induced by chronic ethanol exposure of intestinal cells.

Disrupted Association between ZO-1 and the Y-Box Transcription Factor ZONAB Leads to Nuclear Translocation of ZONAB after Chronic Exposure of Intestinal Cells to Ethanol

The results described above indicated that phosphatidylethanol accumulation in ethanol-treated cells was capable of maintaining proliferation in highly confluent cells. Because high doses of ethanol or acetaldehyde were previously reported to induce strong mucosal damage and disruption of cell/cell adhesion complexes (8, 31), we assessed whether β-catenin localization at the adherens junction was disrupted after treatment with low doses of ethanol.

Indeed, although these colorectal carcinoma cells bear a mutation of the *APC* tumor suppressor gene, confluent Caco-2 cells display a strongly adhesive phenotype and the large majority of β-catenin is recruited at adherens junctions through its interaction with E-cadherin (32). Consequently, any significant disruption of this interaction would be expected to result in the nuclear translocation of β-catenin and the activation of the β-catenin/Tcf-4 transcriptional program, thus inducing the expression of numerous genes involved in proliferation events (33, 34). However, our results clearly showed that neither β-catenin localization, nor the transcriptional activity of Tcf-4 (quantified using the TOP/FOP reporter assay; ref. 33), were affected by low doses of ethanol in confluent Caco-2/TC7 cells (Supplementary Fig. S5).

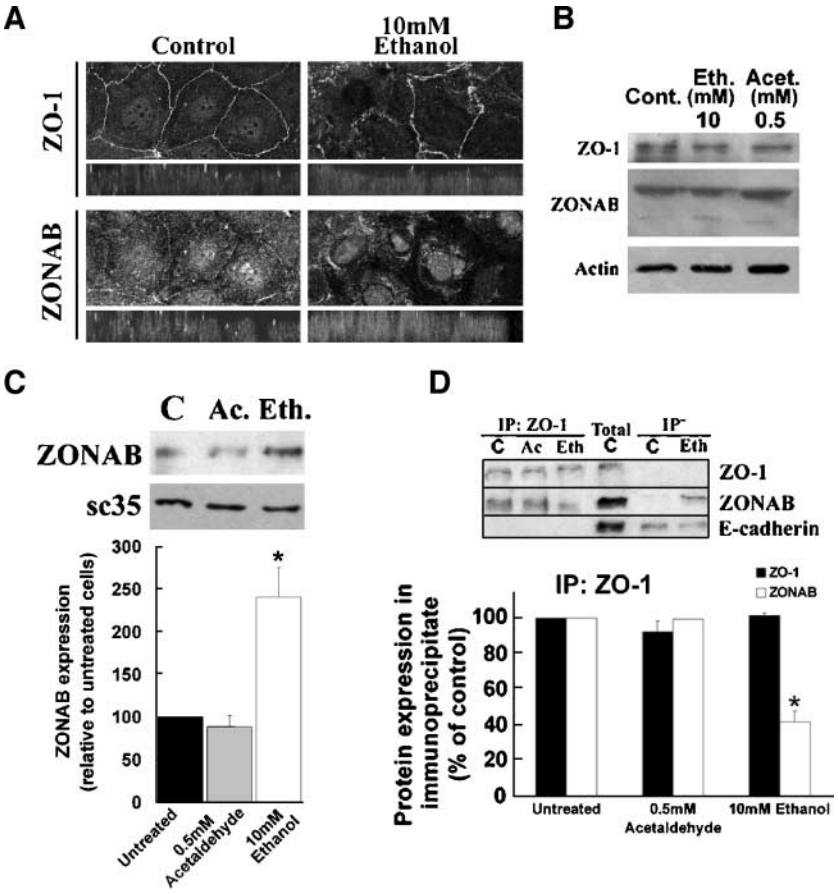

FIGURE 3. Low concentrations of ethanol induce a partial dissociation from ZO-1 of the Y-box transcription factor ZONAB, and increase its nuclear translocation and its transcriptional activity. **A** and **B.** Confluent Caco-2/TC7 cells were treated once daily for 48 h with 10 mmol/L of ethanol, and the localization of ZO-1 and ZONAB along the *XY*- and *XZ*-axis were analyzed by confocal microscopy (*bars,* 10 μmol/L; **A**), whereas the expression of ZO-1 and ZONAB was quantified by Western blotting in comparison with cells treated with 0.5 mmol/L of acetaldehyde (**B**). **C.** Caco-2/TC7 cells were grown and treated as described above, lysed, and their nuclei purified as described in Materials and Methods. Nuclear proteins were analyzed by Western blotting for the expression of ZONAB and the nuclear protein sc35. Top, one representative experiment; bottom, the quantification of ZONAB nuclear expression from three similar experiments (*, $P < 0.05$, Student's *t* test). **D.** ZO-1 immunoprecipitates from total cell lysates of cells treated as above, along with the immunoprecipitation supernatant (IP^-) and a total lysate from untreated control cells (*Total C*), were analyzed by immunoblotting using antibodies against ZO-1, ZONAB, or E-cadherin. Top, one of three similar experiments; bottom, summary of the quantification of ZO-1 (*black columns*) and ZONAB (*white columns*) in the immunoprecipitate of all three experiments (*, $P < 0.05$, Student's *t* test).

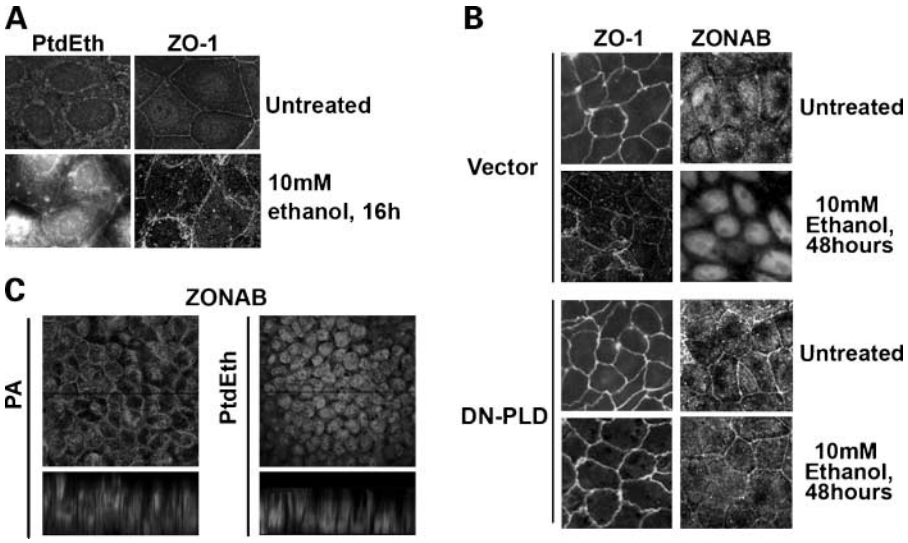

FIGURE 4. Accumulation of phosphatidylethanol after chronic ethanol exposure is responsible for the nuclear translocation of ZONAB. **A.** Confluent Caco-2/TC7 cells were incubated for 16 h with 10 mmol/L of ethanol, and the localization of phosphatidylethanol (*PtdEth*), ZO-1, and ZONAB was detected by standard immunofluorescence microscopy. **B.** The localization of ZO-1 and ZONAB was analyzed by immunofluorescent staining in Caco-2/TC7 cells electroporated with dominant-negative PLD (*DN-PLD*) or with control constructs (*Vector*), plated at high density, and treated or not for 48 h with 10 mmol/L of ethanol. **C.** Caco-2/TC7 cells were treated once daily for 48 h with 20 μmol/L of phosphatidic acid (*PA*) or phosphatidylethanol (*PtdEth*) liposomes (*bars,* 5 μm). Confocal microscopy was then used to detect the localization of ZONAB along the *XY*- and *XZ*-axes.

Alternatively, because ethanol treatment was also reported to disrupt ZO-1 localization in Caco-2 cells (8) and because the increase of maximal cell density described above was reminiscent of that detected upon overexpression of the ZO-1–associated Y-box transcription factor ZONAB in other models (35), we analyzed whether a mechanistic link existed between a potential disruption of TJ and the lack of growth arrest in highly confluent intestinal cells.

To do so, we first compared the localization of ZONAB on highly confluent Caco-2/TC7 cells before and after a 48-h treatment with 10 mmol/L of ethanol. ZONAB was partly colocalized with ZO-1 at the TJ cytoplasmic plaque in untreated samples, but most of the transcription factor shifted to the cytoplasm or the nucleus of ethanol-treated cells, concomitantly with a partial shift of ZO-1 along the lateral membrane and into the cytoplasm (Fig. 3A). In contrast, the overall expression of both proteins was unaffected by this treatment (Fig. 3B). Purified nuclear fractions were then prepared to assess whether the apparent increase in ZONAB nuclear localization detected by immunofluorescent staining reflected the accumulation of this transcription factor in the nucleus. The nuclear amount of ZONAB was markedly increased after treatment with ethanol, whereas acetaldehyde had no effect (Fig. 3C). Accordingly, the amount of ZONAB found to be associated with ZO-1 was greatly reduced in ethanol-treated cells, as determined by coimmunoprecipitation (Fig. 3D).

These results show that chronic exposure to low doses of ethanol are sufficient to disrupt the association between the TJ molecule ZO-1 and the transcription factor ZONAB, resulting in the cytoplasmic and nuclear translocation of the latter.

Phosphatidylethanol Production Is Essential for the Functional Activation of ZONAB after Chronic Ethanol Exposure

Because the appearance of phosphatidylethanol in the membranes of Caco-2/TC7 cells was first detected after about 16 h of ethanol exposure, concomitantly with modifications of ZO-1 staining (Fig. 4A), we assessed whether the formation of phosphatidylethanol was indeed necessary for ZONAB translocation to the nucleus.

To do so, we determined whether the down-regulation of PLD in these cells was preventing the biological effects of ethanol on ZONAB translocation. We found that both ethanol-induced ZO-1 disruption and ZONAB nuclear translocation were not detectable in cells expressing dominant-negative PLD constructs (Fig. 4B), which are unable to metabolize phosphatidylethanol from ethanol (Fig. 2C). In addition, treatment of Caco-2/TC7 cells with phosphatidylethanol liposomes was sufficient to induce a nuclear translocation of ZONAB (Fig. 4C).

To determine whether the nuclear translocation of ZONAB, induced by phosphatidylethanol after chronic exposure to low ethanol concentrations, had a functional effect on the activity of this transcription factor, the transcriptional activity of ZONAB was quantified using a luciferase reporter gene assay in which ZONAB acts as a repressor of the reporter gene (36, 37). A strong down-regulation of luciferase expression was found only in ethanol-treated Caco-2/TC7 cells, confirming that this treatment significantly increases ZONAB activity (Fig. 5A). Concomitantly, ErbB2 expression, which is negatively regulated by ZONAB (35), was significantly reduced in ethanol-treated but not acetaldehyde-treated cells (Fig. 5B). In addition, we found that long-term treatment with 10 mmol/L of ethanol up-regulated the expression of proliferating cell nuclear antigen and of cyclin D1 (Fig. 5C), two cell cycle–related genes recently identified as ZONAB targets (37). Acetaldehyde was also found to increase cyclin D1 expression, whereas it had no effect on that of proliferating cell nuclear antigens (Fig. 5C). This is in agreement with results showing that a higher number of acetaldehyde-treated cells seem to enter the S phase without being able to progress through the G_2-M transition when they are highly confluent (see Supplementary Fig. S2). Along with the lack of effect of acetaldehyde on ZONAB localization and activity, and on β-catenin/Tcf-4 transcriptional activity

(Fig. 5A; Supplementary Fig. S5, respectively), these results indicate that acetaldehyde is able to modulate cyclin D1 levels through a pathway that is different from that induced by ethanol, but that acetaldehyde alone cannot drive confluent intestinal cells through the G_2-M phases of the cell cycle.

Finally, similar to what was found in Caco-2/TC7 cells (Figs. 1B and 3), nuclear translocation of ZONAB was rapidly followed by cell cycle activation (visualized here by Ki67 staining) in nontumoral mouse colon cells (Supplementary Fig. S6).

Taken together, these results suggest that phosphatidylethanol produced after chronic exposure to low concentrations of ethanol, is able to induce the translocation of ZONAB from the TJ cytoplasmic plaque towards the nucleus in highly confluent cells, thereby increasing its transcriptional activity in cells in which it is normally inactive, and resulting in the activation of proliferation in otherwise quiescent cells.

Phosphatidylethanol Induces Claudin-1 Endocytosis and Disrupts the Claudin-1/ZO-1 Association

We then sought to further our understanding of molecular mechanisms leading to the dissociation of ZO-1 from TJs in ethanol-treated Caco-2 cell monolayers. Previous reports showed that the activation of myosin light chain kinase is essential for ethanol-induced ZO-1 translocation and increased TJ permeability (8), but the effect of alcohol on TJ transmembrane proteins, in particular on claudins, is partially unclear. We were not able to detect quantitative alterations in the expression of tight or adherens junction proteins in ethanol-treated cells (Fig. 6A), and immunofluorescent detection showed that the localization of E-cadherin, occludin, claudin-2, and claudin-4 were not affected after several days of ethanol treatment (Fig. 6B). In contrast, whereas claudin-1 expression was restricted to a thin apical ring at the membrane of untreated Caco-2/TC7 cells, partial cytoplasmic relocation of this protein was detected after ethanol treatment (Fig. 6B). When highly confluent Caco-2/TC7 cells were treated for 48 h with 10 mmol/L of ethanol, claudin-1 staining was found to colocalize with the late endosome marker CD63 (Fig. 6C), suggesting that this protein is targeted for recycling or degradation. In addition, this change in claudin-1 localization was paralleled by a disruption of its association with ZO-1, as seen by a strong decrease in the amount of claudin-1

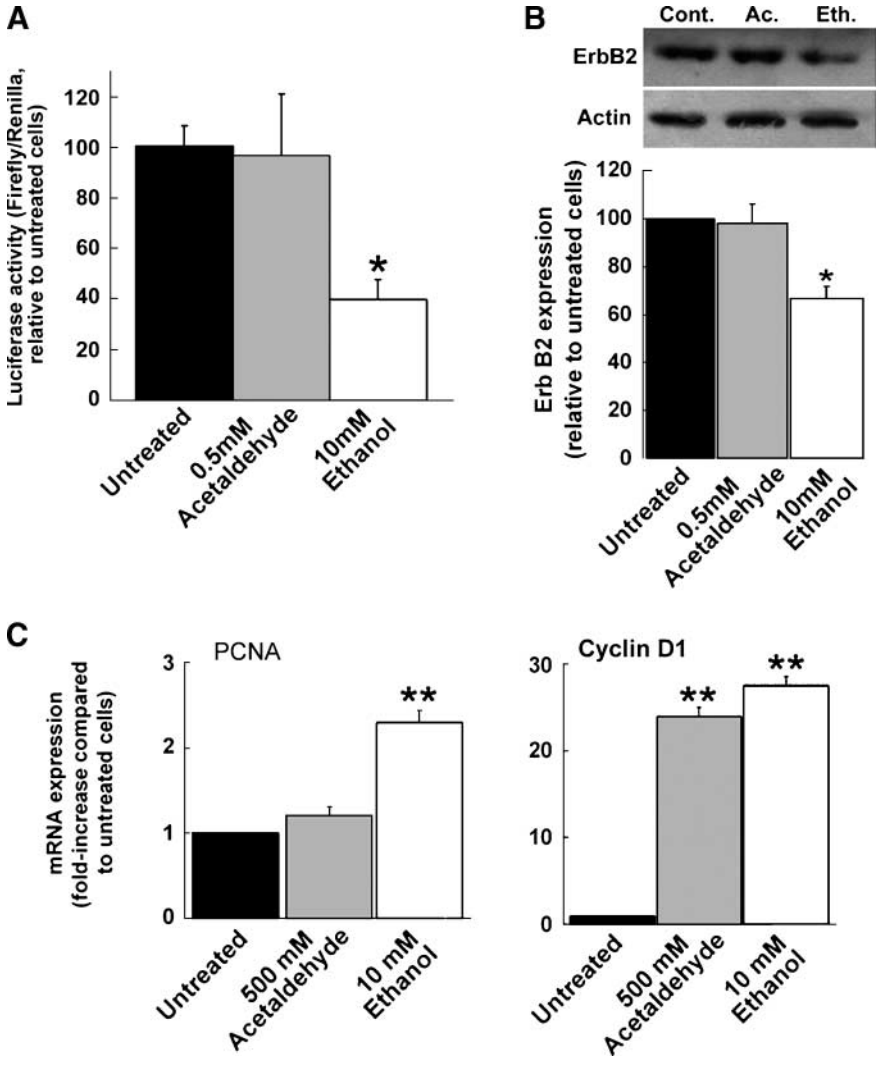

FIGURE 5. Chronic exposure to low concentrations of ethanol increases the transcriptional activity of ZONAB and results in increased expression of proliferating cell nuclear antigen and cyclin D1 in postconfluent Caco-2/TC7 cells. **A.** Caco-2/TC7 cells were electroporated with reporter constructs containing the native or mutated ZONAB binding sites coupled to Firefly or Renilla luciferase, respectively, then grown and treated as described above and lysed. ZONAB transcriptional activity was quantified as described under Materials and Methods, and is expressed as a percentage of the activity measured in untreated cells. Because ZONAB is a transcriptional repressor in this assay, the decrease detected after ethanol treatment reflects a stimulation of ZONAB activity. **B.** Protein lysates from Caco-2/TC7 cells grown and treated as described were analyzed by immunoblotting for the expression of the ZONAB target ErbB2. Top, a representative experiment; bottom, quantification of ErbB2 expression in three similar experiments. **C.** Expression of proliferating cell nuclear antigen (*left*) and cyclin D1 (*right*) mRNAs was quantified using quantitative reverse transcription-PCR in Caco-2/TC7 cells treated as described. *, $P < 0.05$ and **, $P < 0.02$ compared with untreated cells, Student's *t* test ($n = 3$ in **A, B,** and **C**).

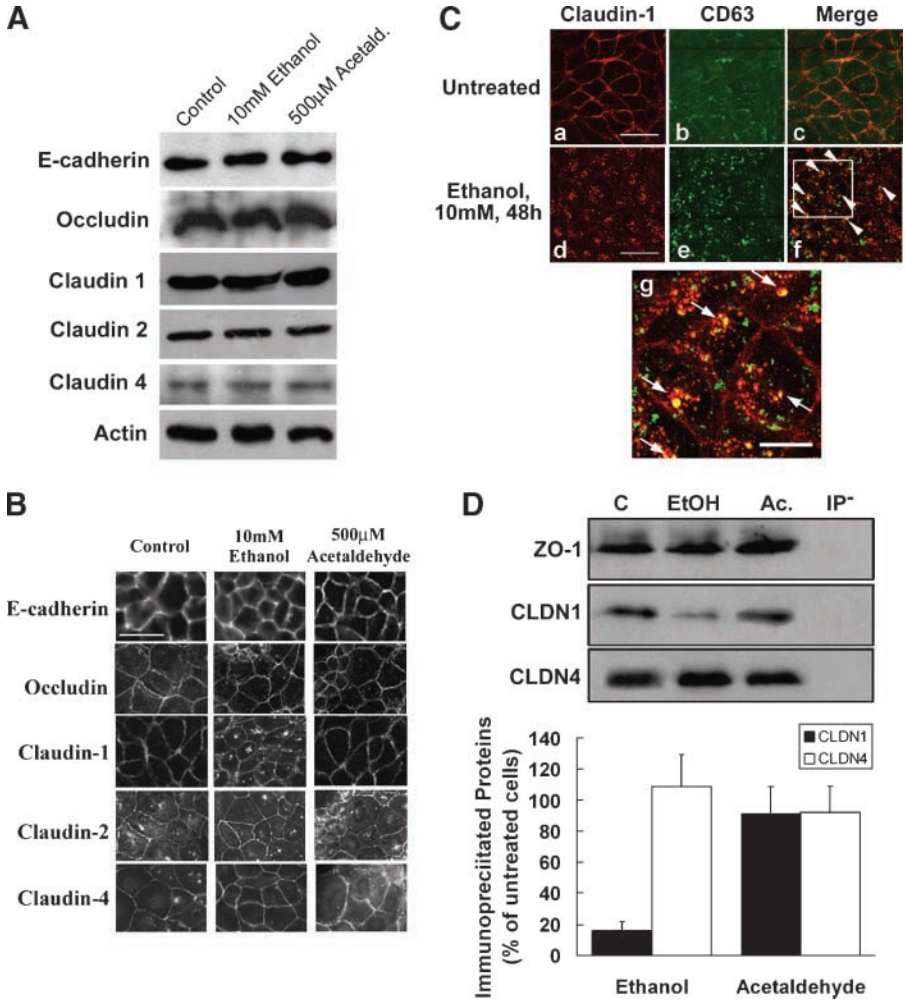

FIGURE 6. Low concentrations of ethanol induce claudin-1 endocytosis and disrupt its association with ZO-1. **A** and **B.** Confluent Caco-2/TC7 cells were treated once daily with 10 mmol/L of ethanol or 500 μmol/L of acetaldehyde for 48 h, and the expression of adherens and TJ proteins quantified using Western blotting (**A**), whereas their localization was analyzed by immunofluorescence (**B**). **C.** The localization of claudin-1 (*red, a* and *d*) and CD63 (*green, b* and *e*) along the *XY*-axis was detected using confocal microscopy. Yellow fluorescent staining (*Merge; c, f,* and *g*) of a colocalization of the two proteins after ethanol treatment (*arrowheads* in *f, arrows* in *g*). Inset in *f* is magnified in *g*. **D.** ZO-1, claudin-1, and claudin-4 were detected by immunoblotting in ZO-1 immunoprecipitates or immunoprecipitation supernatant (IP^-) from lysates of cells treated as above. Quantification of the amounts of claudin-1 and claudin-4 coimmunoprecipitated with ZO-1 (*bottom*). Columns, mean from three similar experiments, and are expressed relative to the amount of ZO-1 present in each sample; bars, SE [*bars,* 10 μm in **B** and **C** (*a-f*), and 5 μm in **C** (*g*)].

coimmunoprecipitating with ZO-1 after ethanol treatment, whereas the interaction between ZO-1 and claudin-4 was unaffected (Fig. 6D).

Incorporation of Membrane Phosphatidylethanol and Disruption of ZO-1 and ZONAB Localization Correlate with Increased Proliferation in the Colonic Epithelium of Ethanol-Fed Mice and in the Adenomas of Chronic Alcohol Consumers

To determine whether the ethanol-induced formation of phosphatidylethanol and modulation of ZO-1 staining were detectable *in vivo*, and in order to correlate their status with the proliferation index in the colonic epithelium, we analyzed the expression of phosphatidylethanol, ZO-1, and Ki67 in the colonic mucosa of mice chronically consuming a 2-mol/L (10%) ethanol solution for 4 months in their drinking water. Although phosphatidylethanol immunoreactivity was not detectable in the colonic epithelium of control mice, accumulation of phosphatidylethanol was detected in the epithelial cells from the Lieberkühn crypts of ethanol-fed animals. This staining pattern was only seen in a small proportion of crypts in the colon, particularly in areas displaying smaller and abnormal crypts, which were never seen in controls (Fig. 7A). In these regions, Ki67-positive cells were detected in cells located in the upper half of Lieberkühn crypts, in areas normally devoid of proliferative cells in control mice (Fig. 7A). In addition, ZO-1 staining along the cell membranes was irregular, consistent with a partial dissociation of this protein from TJ in these areas (Fig. 7A). Unfortunately, we were unable to obtain reproducible staining for ZONAB in mouse tissues, using any of the three antibodies available to us.

Finally, accumulation of phosphatidylethanol and partial mislocalization of ZO-1 correlated with strong disruptions of ZONAB staining in six colon adenoma samples collected from patients consuming at least 30 g/d of ethanol, whereas adenomas from nondrinkers did not exhibit any phosphatidylethanol staining and displayed regular membrane staining for ZO-1 and ZONAB (Fig. 7B).

Discussion

The results of the present study show that chronic exposure to low doses of ethanol induces an increase of the maximal intestinal cell density. Indeed, whereas basal proliferation of Caco-2/TC7 cells decreases sharply when they form a polarized monolayer, the present study shows that treatment with low ethanol concentrations partially prevented the cell cycle from

slowing down when they reached confluency. The mechanism leading to this enhanced cell density seems to be distinct from other previously described biological effects of ethanol, which generally involve its metabolism into acetaldehyde (10). Indeed, Caco-2/TC7 cells are unable to metabolize ethanol into acetaldehyde and, unlike acetaldehyde, ethanol did not stimulate the proliferation of nonconfluent cells. In turn, we found that acetaldehyde was capable of stimulating the proliferation of subconfluent Caco-2 cells, as reported previously (24), but that it was unable to stimulate the proliferation of these cells once they reached confluency. Thus, the present work identifies a novel biological effect of ethanol on intestinal cells, independent from the common metabolic pathway thought to mediate the mitogenic effects of this alcohol.

This effect was detected when cells were treated with doses of ethanol (10 mmol/L) lower than those known to induce important disruptions of the cytoskeleton and TJ barrier (200 mmol/L-2 mol/L; ref. 8). In view of the distal position of the colon and of the important secretion of mucus in the colonic mucosa (34), it is reasonable to think that concentrations of ethanol reaching the crypts of Lieberkühn should be low, and that the doses used in the present study better reflect the situation found *in vivo*.

In addition, we found that the continued proliferation of highly confluent cells under ethanol treatment was due to a disruption of the association between the TJ molecule ZO-1 and the Y-box transcriptional regulator ZONAB, allowing the nuclear translocation of the latter and the activation of cell cycle–related genes in otherwise quiescent cells. The role of ZONAB in the modulation of cell proliferation and maximal density *in vitro* was previously described (36), and another mechanism leading to the activation of ZONAB transcriptional activity in response to cellular stress was also described in Madin-Darby canine kidney cells, in which the heat shock protein Apg-2 competes with ZONAB for binding to ZO-1 (38). Although ethanol treatment also represents a type of cellular stress, we were unable to detect modifications in the cellular localization of Apg-2 in Caco-2 cells treated with ethanol (data not shown). In contrast, we showed that the partial relocation of ZO-1 from the TJ to the lateral membrane after ethanol treatment was induced by a decreased association between ZO-1 and claudin-1 at the TJ, resulting from claudin-1 endocytosis. Endocytosis of various claudin isoforms is thought to play a crucial role in the remodeling of TJ (39). This is however, to our knowledge, the first time that selective endocytosis of one claudin isoform in response to an exogenous modulator has been detected. Interestingly, claudin-1 was colocalized with late endosome markers in ethanol-treated cells, implying that the endocytotic pathway identified here is different from that affecting adhesion proteins in inflammatory bowel disease (40). The nature of the molecular switch determining which endocytotic pathway TJ proteins are targeted towards remains unknown, but the results presented here clearly identified decreased interaction with ZO-1 as a major consequence of claudin-1 endocytosis.

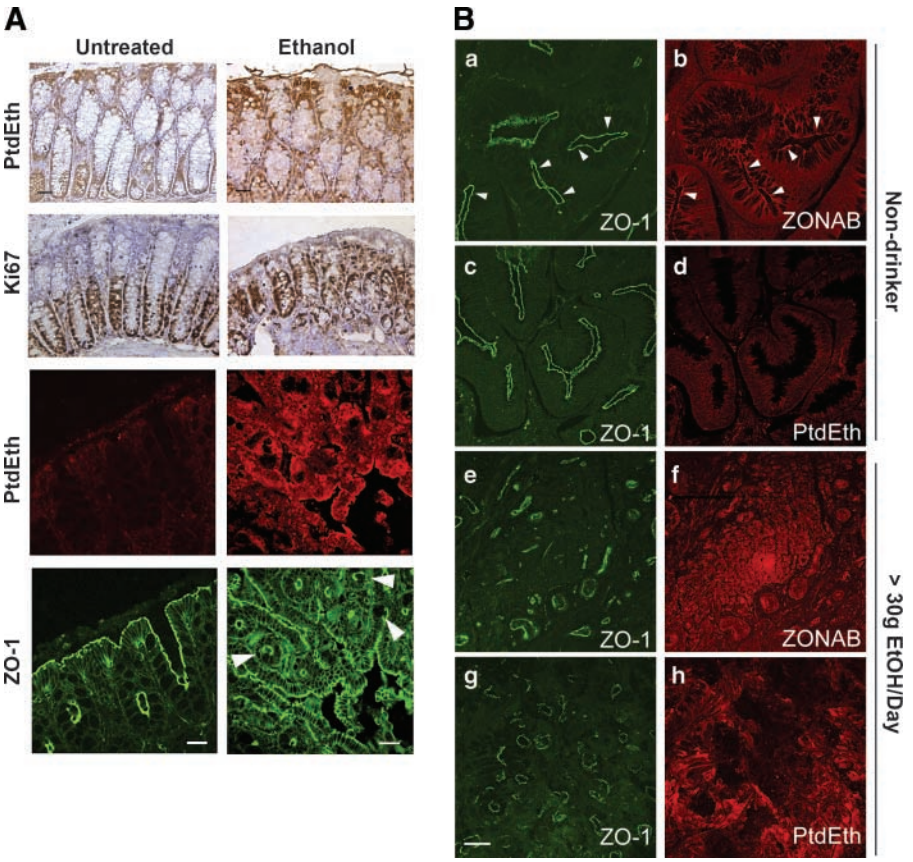

FIGURE 7. Accumulation of membrane phosphatidylethanol and disruption of ZO-1 and ZONAB staining patterns in the colonic epithelium of ethanol-fed mice and human chronic alcohol consumers. **A.** Colon tissue sections were prepared from mice who ingested 2 mol/L of ethanol (*Ethanol*) or saline (*Untreated*) *ad libitum* for 4 mo, and the expression of phosphatidylethanol (*PtdEth*) and Ki67 analyzed using immunohistochemistry, whereas ZO-1 expression was detected using immunofluorescent staining, also in correlation with phosphatidylethanol. Fluorescence images are confocal stacks spanning the whole epithelium thickness. In ethanol-fed mice only, membrane staining for ZO-1 was not detected in discrete areas (*white arrowheads*) located within phosphatidylethanol-positive, disorganized epithelium; an overall increase of cytoplasmic ZO-1 staining was also detected in these areas (n = 6 mice/group). **B.** Sections of human colonic adenomas obtained from a nondrinking patient (*a-d*) or from chronic alcohol consumers (*e-h*) were costained using selective antibodies for ZO-1 and ZONAB (*a* and *b, e-f*) or ZO-1 and phosphatidylethanol (*c* and *d, g-h*), and observed using confocal microscopy (*bars,* 50 μm). Areas in which localization of ZO-1 and ZONAB is clearly similar at the cell membrane (*white arrowheads* in *a* and *b*).

Another important finding of our study was the demonstration that the resulting loss of association between ZO-1 and ZONAB was due to the accumulation of phosphatidylethanol in the membrane of ethanol-treated cells, which may induce a disruption of the local phospholipid environment, as previously reported (41). The production of phosphatidylethanol, mediated by ethanol-induced PLD activation, was shown for the first time to be essential for the increase of proliferation and maximal cell density in Caco-2/TC7 cells. It is noteworthy that the production of phosphatidylethanol was also detected in other human colorectal cancer cells and in a nontumoral murine intestinal cell type, as well as in the colonic epithelium of mice and human after chronic alcohol intake. In contrast with Caco-2/TC7 cells, these different models all express ADH and are thus capable of metabolizing ethanol into acetaldehyde. Therefore, this result strongly suggests that the PLD-mediated formation of phosphatidylethanol from ethanol is a physiologically relevant pathway, activated concomitantly with ADH-mediated metabolism.

Finally, our results show that the production of phosphatidylethanol and alterations of ZO-1 distribution could also be detected *in vivo* in the colonic epithelium of mice chronically exposed to ethanol, as well as in chronic alcohol consumers. The affected areas are located in the upper half of Lieberkühn crypts, which is of no surprise because these areas are likely to be the most exposed to the residual ethanol present in the colon. Interestingly, only selected areas of the mucosa were found to be affected. This could be due to altered protection of the mucosa in these regions, to the presence of locally higher concentrations of ethanol, to preexisting local inflammatory processes, or to other unidentified mechanisms. In the human colon, phosphatidylethanol accumulation, as well as mislocalization of ZO-1 and ZONAB, was detected in sections of colonic polyps from chronic alcohol consumers, but not in healthy mucosa biopsies from the same patients or in polyps from nonalcoholics. The presence of proliferative cells in areas of the crypts where they should be absent was detected only in phosphatidylethanol-positive areas, suggesting that phosphatidylethanol-induced disruption of the ZO-1/ZONAB relationship could release a brake normally applied on the proliferation of epithelial cells once they have left an area located at the bottom-third of the crypts of Lieberkühn (34). To our knowledge, this is the first report of the involvement of ZONAB in a pathologic process *in vivo*. Because the same effect was reproduced *in vitro* in cells lacking ADH by ethanol and phosphatidylethanol micelles, whereas acetaldehyde had no effect on ZONAB translocation and cell density changes, we conclude that the alterations detected *in vivo* are at least partially triggered by the presence of ethanol itself in the colonic mucosa.

It is of note that the production of acetaldehyde by intraluminal bacteria is also likely to play a role in the activation of proliferation seen in the mouse and human colonic mucosa after ethanol intake, as shown in other studies (10-12). Yet, because acetaldehyde alone was unable to induce a nuclear translocation of ZONAB *in vitro*, and because ethanol and acetaldehyde were shown to act in a synergistic manner to increase maximal cell density, we believe that the conclusions of our study do not conflict with the generally accepted view that acetaldehyde is essential to activate cell proliferation in the gastrointestinal epithelium. Instead, they strongly suggest that the PLD-mediated formation of phosphatidylethanol in intestinal cell membranes plays a permissive role in mucosal hyperregeneration, by disrupting physiologic signals sent by TJ complexes to the cell nucleus in order to slow down the proliferation of highly confluent cells. The disruption of these signals might facilitate the stimulatory role of ethanol metabolites such as acetaldehyde on the proliferation of cells within intestinal crypts, thereby participating in the well established cocarcinogenic role of alcohol consumption in the colon.

Materials and Methods

Cells and Cell Culture Conditions

The colorectal cancer cell line Caco-2 cloneTC-7 (42) was maintained at 37°C with 5% CO_2 in DMEM (Cambrex) with 10% heat-inactivated fetal bovine serum, 2 mmol/L of L-glutamine, and 1% nonessential amino acids (Sigma-Aldrich). The percentage of dead cells before and after treatment was quantified by trypan blue exclusion.

Materials

The following primary antibodies were used: Ki67 monoclonal antibody from Neomarkers, polyclonal antibodies against ZO-1 and various claudin isoforms from Zymed, β-catenin and E-cadherin monoclonal antibodies from Transduction Laboratories, and sc-35 antibody from Sigma-Aldrich. Monoclonal ZO-1 antibody was a generous gift from Drs. S. Tsukita and M. Furuse (Department of Cell Biology, Kyoto University, Kyoto, Japan). ZONAB polyclonal antibody was described in ref. (35). ZONAB staining in human tissue sections was a rabbit polyclonal ZONAB antibody from Upstate-Cell Signaling. For mouse tissue sections, these two antibodies, along with the polyclonal ZONAB antibody from Zymed (InVitrogen), were used unsuccessfully.

Liposome Preparation

A mix of 3.3 mg of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol and 3.3 mg of 1,2-dioleyl-*sn*-glycero-3-phosphoethanol in chloroform and a mix of 3.3 mg of 1,2-dipalmitoyl-*sn*-glycero-3-phosphate and 3.3 mg of 1,2-dioleyl-*sn*-glycero-3-phosphate in chloroform were evaporated at 40°C and rehydrated overnight in filtered PBS. The next day, after a 1-min sonication, liposomes were ready to use.

Antiphosphatidylethanol Antibody Preparation

Three injections (300 μg/mL/rabbit) of phosphatidylethanol liposomes were carried out on days 1, 21, and 42. Sixty-two days after the first injection, animals were killed and serum collected and aliquoted for storage at −20°C.

ELISA for Antiphosphatidylethanol Antiserum Characterization

Phosphatidylethanol liposomes (10 μg) were coated in a 96-well plate in triplicate. After chloroform evaporation, antiserum (diluted five times) was added to wells for 90 min at

room temperature. After four PBS washes, secondary anti-rabbit IgG peroxidase was incubated for 1 h. Orthophenylenediamine was used as a substrate and, after the addition of 50 μL/well of 4 N sulfuric acid, absorbance was read at 490 nm.

Crystal Violet Staining

Cells were grown in 48-well plates until confluent, then treated or not with 10 mmol/L of ethanol, 0.5 mmol/L of acetaldehyde, or 20 μmol/L of phosphatidylethanol or phosphatidic acid liposomes, for the indicated time. After removing the medium and washing, 100 μL crystal violet solution was incubated for 10 min at room temperature. Cells were then washed twice in water by immersion in a large beaker, lysed in 1% SDS and homogenized. Absorbance was read at 570 nm using a 1:20 sample dilution.

Human Tissue Collection

Specimens of colon adenomas from three patients were obtained from the pathologist according to French government regulations and local committee guidelines. Informed consent was obtained from all patients. Tissue samples were immediately placed in liquid nitrogen–chilled isopenthane, then stored in liquid nitrogen until further use. Two patients were chronic alcohol consumers (~30 g ethanol/d), whereas the third one was a nondrinker.

Transepithelial Resistance Measurements

Caco-2 cells were grown in suspended wells until confluent and transepithelial resistance was measured at regular intervals as described in ref. (43), with or without treatment.

PLD Assay

PLD activity was measured using the Amplex Red Phospholipase D assay Kit (Molecular Probes), following the manufacturer's instructions.

Cell Cycle Analysis

The proportion of Caco-2/TC7 cells in various phases of the cell cycle were quantified using propidium iodide incorporation and flow cytometry. Briefly, confluent Caco-2/TC7 cells in six-well plates were treated with ethanol or acetaldehyde for the required amount of time, then washed twice in 3 mL of PBS containing 5 mmol/L of EDTA and 1% bovine serum albumin, then centrifuged for 5 min at 400 × *g*. The pellet was left on ice for 10 min, then resuspended in 500 μL of ice-cold sodium citrate solution (0.1% w/vol) containing 0.1% Triton X-100, 2 mmol/L of EDTA, 1 mmol/L of DTT, 50 μg/mL of propidium iodide, and 20 μg/mL of RNase A, and left overnight at 4°C under gentle shaking. The following day, samples were analyzed in a FACScan flow cytometer (Becton Dickinson).

Transient Transfections

Cells were transfected with dominant-negative constructs for PLD1 (PLD1-K898R) or control vectors (27), and 5 μL/well of Exgen 500 (Euromedex), according to the manufacturer's instructions. Forty-eight or 72 h after transfection, cells were lysed (for immunoblotting and PCR) or fixed for immunostaining.

Animal Studies

All animal procedures were conducted in strict adherence with the European Communities Council Directive of November 24, 1986 (86-609/EEC). Male Swiss mice, 1 month old and weighing 28 to 32 g, were purchased from Depré. Animals were housed in groups of 10 with access to food and water *ad libitum* and were kept in a temperature- and humidity-controlled animal facility on a 12:12 h light/dark cycle (lights off at 20:00). Mice were accustomed to consume a 2 mol/L (10%) ethanol, 30 g/L saccharose solution using a no-choice progressive procedure as previously described (44). The concentration of ethanol in the saccharose solution was initially increased every 4 days (0.4, 0.8, 1.6, then 2 mol/L). Thereafter, animals consumed solely a 2 mol/L ethanol, 30 g/L saccharose solution for 4 months. Colons were then collected and processed for immunostaining. Control groups consumed only the 30 g/L saccharose solution during the whole procedure.

Luciferase Reporter Gene Assay

The transcriptional activity of TCF/LEF-1 and the ZONAB reporter assay have been described elsewhere (33, 37). Briefly, 5×10^6 Caco-2/TC7 cells were electroporated with 2.5 μg of TCF/LEF-1 reporter (pTOP-FLASH) or control vector (pFOP-FLASH), and with 250 ng of pCMV-Renilla for the TCF/LEF-1 reporter assay. For the ZONAB reporter assay, cells were electroporated with a promoter containing a ZONAB binding site driving firefly luciferase expression, and with a promoter containing an inactivated binding site but with an otherwise identical sequence, used to express Renilla luciferase. Cells (4×10^5) were then plated into each well of a 24-well plate, and treated once daily with or without 10 mmol/L of ethanol or 500 μmol/L of acetaldehyde. Forty-eight hours after transfection, cells were highly confluent. They were lysed and luciferase activity was quantified using the Dual luciferase reporter assay system (Promega), and was normalized relative to Renilla activity.

Immunofluorescent Staining

Immunofluorescent staining was done as described previously (45). Slides were observed using laser scanning LEICA sp2 or Bio-Rad confocal microscopes, with a ×40 oil immersion lens.

Immunochemistry on Paraffin Tissue Sections

Paraffin tissue sections were prepared from the intestinal and colonic mucosa of control and ethanol-treated mice. Following dewaxing and hydration, sections were pretreated with peroxidase for 20 min at room temperature. Antigen was retrieved by boiling samples for 20 min in 10 mmol/L of Tris, 1 mmol/L of EDTA (pH 9). Slides were cooled to room temperature, and incubated overnight at 4°C with antibodies in PBS + 0.05% bovine serum albumin. The Envision+ kit (DAKO) was used as a secondary reagent, and slides were counterstained with hematoxylin and mounted.

Protein Lysates, Immunoprecipitation, SDS-PAGE, and Western Immunoblotting

Protein lysates, immunoprecipitations, and immunoblotting were done as described (45). Proteins were visualized using ECL Plus (Amersham Biosciences) and the bands were quantified by densitometry using ImageJ 1.32J (NIH, Bethesda, MD).

Statistical Analysis

All statistical analyses were done using SAS version 9.1 for Windows. Student's *t* test was used to determine the statistical significance of differences between controls and ethanol- or acetaldehyde-treated cells.

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