Acetaldehyde accelerates HCV-induced impairment of innate immunity by suppressing methylation reactions in liver cells

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Acetaldehyde accelerates HCV-induced impairment of innate immunity by suppressing methylation reactions in liver cells

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Ganesan M, Zhang J, Bronich T, Poluektova L, Donohue TM Jr, Tuma DJ, Kharbanda KK, Osna NA. Acetaldehyde accelerates HCV-induced impairment of innate immunity by suppressing methylation reactions in liver cells. Am J Physiol Gastrointest Liver Physiol 309: G566–G577, 2015. First published August 6, 2015; doi:10.1152/ajpgi.00183.2015.—Alcohol exposure worsens the course and outcomes of hepatitis C virus (HCV) infection. Activation of protective antiviral genes is induced by IFN-α signaling, which is altered in liver cells by either HCV or ethanol exposure. However, the mechanisms of the combined effects of HCV and ethanol metabolism in IFN-α signaling modulation are not well elucidated. Here, we explored a possibility that ethanol metabolism potentiates HCV-mediated dysregulation of IFN-α signaling in liver cells via impairment of methylation reactions. HCV-infected HuH7.5 CYP2E1⁺ cells and human hepatocytes were exposed to acetaldehyde (Ach)-generating system (AGS) and stimulated with IFN-α to activate IFN-sensitive genes (ISG) via the Jak-STAT-1 pathway. We observed significant suppression of signaling events by Ach. Ach exposure decreased STAT-1 methylation via activation of protein phosphatase 2A and increased the protein inhibitor of activated STAT-1 (PIAS-1)-STAT-1 complex formation in both HCV⁺ and HCV⁻ cells, preventing ISG activation. Treatment with a promethylating agent, betaine, attenuated all examined Ach-induced defects. Ethanol metabolism-induced changes in ISGs are methylation related and confirmed by in vivo studies on HCV⁺ transgenic mice. HCV- and Ach-induced impairment of IFN signaling temporarily increased HCV RNA levels followed by apoptosis of heavily infected cells. We concluded that Ach potentiates the suppressive effects of HCV on activation of ISGs attributable to methylation-dependent dysregulation of IFN-α signaling. A temporary increase in HCV RNA sensitizes the liver cells to Ach-induced apoptosis. Betaine reverses the inhibitory effects of Ach on IFN signaling and thus can be used for treatment of HCV⁺ alcohol-abusing patients.

ethanol metabolism; IFN-α signaling; hepatitis C virus; hepatocytes; betaine

Alcohol consumption in patients infected with HCV exacerbates liver injury, leading to rapid progression to fibrosis, cirrhosis, and even hepatocellular carcinoma (34). Alcohol consumption significantly reduces responsiveness of patients with HCV to antiviral treatment. The mechanism by which alcohol consumption increases the severity of HCV infection is unclear. In this regard, the possibility of synergistic effects of HCV and alcohol on HCV spread and liver injury progression cannot be excluded because hepatocytes are primary sites for HCV replication and ethanol metabolism, both of which suppress innate immunity in liver cells.

Activation of an antiviral innate immune response is based on IFN signaling. Type 1 IFNs per se possess no antiviral properties; however, IFN-α signaling activates IFN-sensitive genes (ISGs) that encode the expression of antiviral proteins to control HCV replication. IFN-α signaling proceeds by endogenous IFN-α binding to membrane-bound receptors, which activate Janus kinases and then phosphorylate STAT-1 and -2. Activated STAT-1 and STAT-2 form a complex with IFN-regulated factor 9, which translocates from the cytosol to the nucleus. Following translocation, phosphorylated STAT-1 attaches to specific regions of DNA to activate antiviral ISGs.

HCV hijacks the innate immune responses by suppressing both upstream and downstream events of IFN signaling (12), including decreased STAT-1 methylation (7, 10), thereby interfering with ISG activation.

Ethanol also affects protein methylation by suppressing multiple methylation reactions in the liver (18) and reducing IFN-induced STAT-1 phosphorylation in hepatocytes and hepatoma cells (28, 33). Furthermore, a study using CYP2E1⁺ HuH7 cells harboring HCV replicon clearly demonstrated that ethanol metabolism, but not ethanol itself, impaired IFN-α signaling (16). However, we do not know whether ethanol metabolism synergizes with HCV to reduce STAT-1 methylation. In fact, this issue has not been adequately addressed because most of the HCV-ethanol in vitro studies were performed on the cell lines, which either do not express the two main ethanol-metabolizing enzymes, alcohol dehydrogenase (ADH) and cytochrome P4502E1 (CYP2E1), or express only CYP2E1. However, to use human hepatocytes for HCV-ethanol studies as previously suggested (44) is not the best option because hepatocytes in primary cultures rapidly dedifferentiate and lose the expression of ethanol-metabolizing enzymes within 24 h, whereas in vitro infection of these cells with HCV requires about 5 days, during which they lose the expression of both ethanol-metabolizing enzymes (37). Thus...
hepatocytes cannot metabolize alcohol by the time they become considerably infected. Here, we sought to mimic the effects of ethanol metabolites, by exposing stably transfected CYP2E1+/Huh7.5 cells to physiologically relevant levels of ethanol and to acetaldehyde (Ach) that was continuously generated by an extra-cellular Ach-generating system (AGS).

We hypothesized that, during HCV infection, ethanol metabolites interfere with innate immunity by suppressing methylation of major IFN signal transduction factor, STAT-1, thereby promoting liver injury. This hypothesis is based on our previous findings that ethanol metabolism diminishes methyltransferase activities in the liver (18) and reduces IFN-α signaling, which is a key step in innate immunity induction in HCV-infected liver cells. Here, we describe a pathogenic insight into the role of both ethanol metabolism and HCV in IFN-regulated activation of antiviral genes that control HCV replication. We present evidence that the ethanol metabolite, Ach, suppresses ISG activation attributable to protein phosphatase 2A (PP2A)-dependent impairment of STAT-1 methylation, which provides a temporal increase in HCV RNA and sensitizes cells to Ach-induced apoptosis.

MATERIALS AND METHODS

Reagents and Media

High-glucose DMEM, Williams medium, and FBS were purchased from Invitrogen (Carlsbad, CA). Human recombinant IFN-α was from MACS-Milenyi Biotech (San Diego, CA). TransAM DNA-binding ELISA kit was from Active Motive (Carlsbad, CA). Antibody to phosphorylated STAT-1 (Tyr 701) was from Cell Signaling (Beverly, MA); antibodies to the STAT-1, protein inhibitor of activated STAT-1 (PIAS-1), and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mono-dimethyl arginine and methylated lysine antibodies were from Abcam (Cambridge, MA). PP2A, CYP2E1, and pPP2A were from EMD Millipore (Temecula, CA) and LifeSpan Biosciences (Seattle, WA), respectively. Anti-ADH was a gift from Dr. Michael Felder (University of South Carolina). Reagents used for RNA isolation, cDNA synthesis, and real-time PCR were from Life Technologies (Carlsbad, CA). Other reagents, all of analytical grade quality, were from Sigma (St. Louis, MO).

Animal Studies

C57Bl/6d mice transgenically expressing HCV structural proteins obtained from Dr. S. Weinman (Kansas University Medical Center) were characterized elsewhere (21, 30). Mice (6–8 wk old) were divided into four groups (n = 6 per group): control, ethanol, betaine, and betaine + ethanol. They were pair fed control and ethanol Lieber De Carli diets for 10 days, with or without 2% betaine (wt/vol); then were gavaged with PBS/maltose dextran or ethanol on day 11 and killed 9 h after gavaging, as described in detail for a chronic-acute ethanol study (2). Four hours before death, each mouse was injected intraperitoneally with mouse IFN-α (1,000 IU). In this study, we observed no differences in alcohol consumption between ethanol and ethanol + betaine groups. There was no difference in liver weight either between each group (control: 1.01 ± 0.05 g; ethanol: 1.18 ± 0.06 g; betaine: 1.08 ± 0.03 g; ethanol + betaine: 1.03 ± 0.06 g) or body weight among the four groups after feeding.

Mice were treated according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. Animal use and protocols of ethanol feeding were approved by the Institutional Animal Care and Use Committee of the VA Medical Center, Omaha, NE.

To characterize the changes in protein methylation, Sadenosylmethionine:S-adenosylhomocysteine (SAM:SAH) ratios were measured by high-performance liquid chromatography in crude liver homogenates (19).

Betaine was used as a promethylating agent to correct impaired protein methylation via the restoration of a normal SAM:SAH ratio. Also, 2'-5'-oligoadenylate synthase-like protein (OASL) and ISG15 mRNAs (ISGs) were quantified in the liver tissue using RT-PCR.

Cells

Huh7.5 cells. Huh7.5 cells were transfected with pLV-G2 (CYP2E1) plasmid as previously described for other cell lines (6, 31) using Lipo TAXI (Invitrogen). Recombinant cells, designated RLW cells, were selected in culture medium containing G418 at 400 μg/ml (Fig. 1A). Clones were expanded and screened for CYP2E1 expression and activity. Because we were unable to transfect Huh7.5 cells with the ADH plasmid, we used exogenously produced acetaldehyde generated by a special in vitro system (AGS, see description below).

Fig. 1. Effects of acetaldehyde-generating system (AGS) on RLW cells. A: phenotype of RLW cells. Immunoblot (IB) analysis showing that RLW cells lack alcohol dehydrogenase (ADH) but express CYP2E1. Lane 1, molecular weight markers; lane 2, lysate primary mouse hepatocytes as a positive control; lanes 3–4, lysates of RLW cells. B: kinetics of acetaldehyde (Ach) production by AGS after indicated times of exposure to RLW cells. C: lactate dehydrogenase (LDH) activity in AGS- or ethanol (EtOH)-treated RLW cells. Cells were exposed to AGS or 50 mM EtOH for 48 h (earlier time points showed no cytotoxicity in preliminary experiments). Cells lysed by sonication were used as a positive control (100% LDH leakage). Percent cytotoxicity was calculated as a percentage of positive control in treated samples, means ± SE. A–C show representative data from 3 independent experiments with similar results.
Human hepatocytes. Human hepatocytes were from Triangle Research Laboratories (Research Triangle Park, NC) (2 batches, triplicate readings).

Cell Treatments

RLW cells were infected by JFH1 (HCV genotype 2a) virus at a multiplicity of infection (MOI) of 0.1 as previously described (40) or were left uninfected. To investigate whether ethanol metabolites affected IFN-α signaling, on day 2 after infection, cells were exposed for up to 48 h to AGS added directly to the culture medium. The AGS included yeast ADH (0.02 U/ml), 2 mM nicotinamide adenine dinucleotide and 50 mM ethanol (EtOH). One unit of ADH catalyzes the reduction of μM NADH formed per minute at 37°C. In the presence of RLW cells, the levels of Ach measured by gas chromatography in the medium fluctuate between about 250 (at 1–4 h of exposure) and 50 μM (at 18–48 h of exposure) (Fig. 1B). These levels of Ach correspond to the amount of Ach produced by ADH-expressing liver cells (6, 33) and to the physiological concentrations observed in the liver of ethanol consumers. At the end of treatments, cells were stimulated with IFN-α (time and dose depended on the end points of the experiment, see RESULTS). Cytotoxicity was measured by lactate dehydrogenase release to cell medium as described (6) (Fig. 1C). To assess the involvement of impaired protein methylation in the regulation of IFN-α signaling, all treatments were done in the presence or absence of 2 mM betaine, a promethylating agent. The optimal concentration of betaine used for in vitro studies in liver cells was determined earlier (20).

Human hepatocytes were attached to collagen-coated six-well plates (4×10^5 cells/well) and then infected via serum from an HCV patient (IRB no. 520-14-EX; HCV RNA is > 3 level of detection of HCV RNA. Real-time PCR analysis was performed for the expression of ISGs (mRNAs).

RNA isolation and real-time PCR. ISGs with antiviral activities, such as viperin, 2′-5′-oligoadenylate synthetase 1 (OAS1), protein kinase R, OASL, and ISG15, were quantified by real-time PCR. Total RNA was isolated from cells using Trizol reagent. A two-step procedure was used, in which 200 ng RNA was reverse-transcribed to cDNA using the high-capacity reverse transcription kit. In the second step, the cDNA was amplified using TaqMan Universal Master Mix II with fluorescent-labeled primers (TaqMan gene expression systems). These were incubated in a Model 7500 qRT-PCR thermal cycler. The relative quantity of each RNA transcript was calculated by its threshold cycle (Ct) after subtracting that of the reference cDNA (GAPDH). RNA was also isolated from mouse liver tissue, and ISGs were quantified (using primer probes for mice) in the same way as specified for human cells. Data are expressed as the quantity of transcript. The relative HCV RNA expression level in infected cells was quantified using the following primers and probe for this consensus sequence designed with the help of PrimerExpress Software v. 2.0 (Applied Biosystems, Foster City, CA): 5′ UTRF GACCGGTCCTTTCTTGG-GAT; 5′ UTRR CCAACACTACTGCTAGCAGTCT; probe FAM-ATTGCGGGTGCCCGCC-NFQ.

Immunoblotting and immunoprecipitation. Cell lysates were prepared in 0.5 M EDTA, 2 M Tris, 20 mM Na3VO4, 200 mM Na4P2O7, 100 mM PMSF, 1 M NaF, 20% Triton X-100, and aprotinin, pH 7. Nuclear fractions were collected using an Active Motif kit (Carlsbad, CA). Immunoprecipitations were done by incubating each Ag-Ab complex with protein G Sepharose (GE Healthcare Biosciences, Uppsala, Sweden) overnight in a rotating shaker at 4°C, followed by washing and incubation with SDS-PAGE sample-solubilizing buffer at 95°C for 10 min. Isotype-specific IgG was used as a negative control. Complexes were subsequently subjected to denaturing SDS-PAGE in polyacrylamide gels. Immunoblotting was performed as described previously.

Fig. 2. IFN-sensitive gene (ISG) activation in RLW cells exposed to AGS. Hepatitis C virus (HCV)-infected RLW cells were exposed to 50 mM ethanol or AGS for 48 h in the presence or absence of betaine, and then cells were treated with 200 U of IFN-α for 4 h. Treatment with this IFN dose for 4 h did not affect HCV RNA levels in the cells (not shown). Real-time PCR analysis was performed for the expression of ISGs (mRNAs): A: 2′-5′-oligoadenylate synthetase 1 (OAS1). B: 2′-5′-oligoadenylate synthase-like protein (OASL). C: viperin. D: protein kinase R (PKR). GAPDH was used to normalize the gene of interest. Data are generated from 3 independent experiments and presented as means ± SE. Bars with different letters are significantly different at P = 0.05.
blots were developed using Odyssey infrared imaging system, and the protein band was quantified using Li-Cor software (39). β-Actin was used as the loading control to normalize the proteins.

**HCV core and BODIPY staining.** 50,000 cells/well were seeded onto coverslips inserted in each well of a 24-well plate. Cells were infected with JFH-1 virus (MOI = 0.1). After overnight incubation of cells with virus, virus-containing medium was removed and replenished with fresh media. Cells were cultured for another 48 h. After 48 h of AGS treatment, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at 37°C, permeabilized with 0.5% Triton X-100 for 5 min at room temperature, and blocked for 30 min with 5% goat serum in PBS. Cells were stained to study the colocalization of HCV core protein with the lipid droplets. First, cells were incubated with antibody to HCV core (clone: C7-50, dilution 1:300; catalog no. MA1-080; Thermo Scientific, Rockford, IL) for 1 h. The cells were then washed and incubated with the mixture of Alexa Fluor 594-labeled secondary antibody for HCV core and BODIPY (to stain lipid droplets, 1:100) for another hour. Nuclei were labeled with DAPI. The coverslips were transferred to microscope slides for imaging by using a ×63 lens in a LSM 710 confocal microscope (Carl Zeiss, Peabody, MA).

**Statistical Analyses**

Data from at least three independent experiments are expressed as means ± SE. Comparisons among multiple groups were determined by one-way ANOVA, using a Tukey’s post hoc test. For comparisons between the two groups, we used Student’s t-test. A probability value of 0.05 or less was considered significant.

**RESULTS**

**Ethanol Metabolism and ISG Activation in HCV-Infected Liver Cells**

RLW cells were infected with HCV and treated with AGS (in the presence or absence of betaine) for 48 h, and then

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**Fig. 3.** Effects of EtOH or Ach on STAT-1 attachment to DNA and protein inhibitor of activated STAT-1 (PIAS-1)-STAT-1 complex. After exposure to medium, 50 mM ethanol, or AGS for 48 h in the presence or absence of betaine, cells were treated with 1,000 IU of IFN-α for 30 min to measure pSTAT-1 attachment to DNA in nuclear fractions by ELISA. Nontreated (with either AGS, ethanol, or IFN-α) cells were used as a control. A: HCV− cells. B: HCV+ cells. Values from cells treated with IFN-α only are expressed as 100%. Results are expressed as the percentage of DNA binding in IFN-α-stimulated cells and are means ± SE. Bars with different letters indicate significant differences at P ≤ 0.05. C: IP with anti-PIAS-1. Lane 1, nontreated cells; lane 2, IFN-α; lane 3, EtOH + IFN-α; lane 4, EtOH + IFN-α + betaine; lane 5, AGS + IFN-α; lane 6, AGS + IFN-α + betaine, representative IP data. D and E: quantification of pSTAT-1. PIAS-1 ratio obtained in HCV− and HCV+ cells, the results of 3 independent experiments, means ± SE. Bars with different letters are significantly different at P ≤ 0.05.
activation of antiviral ISGs was induced (for details, see figure legends). As shown in Fig. 2, A–D, in CYP2E1+ cells, only the AGS, not ethanol alone, suppressed IFN-α-induced activation of ISGs, indicating that Ach or the combination of Ach with CYP2E1-generated ethanol metabolites (but not CYP2E1-generated ethanol metabolites in the absence of Ach) regulate this downstream step of IFN-α signaling. Subsequent betaine exposure either fully or partially restored ISG expression.

**Ethanol Metabolism and STAT-1 Attachment to DNA**

ISG activation by IFN-α depends on the binding of activated (phosphorylated) STAT-1 to DNA. Here, STAT-1 attachment to DNA was measured in nuclear extracts using a TransAM DNA binding ELISA kit. In HCV+ RLW cells, ethanol exposure alone suppressed DNA binding by only 13%, whereas the AGS suppressed binding by 42% compared with untreated IFN-α controls (Fig. 3, A and B). The magnitude of this reduction was comparable in both HCV+ and HCV− RLW cells. Betaine treatment partially (up to 69–75% of IFN-α control) restored Ach-impaired DNA binding of STAT-1.

**Ethanol Metabolism and PIAS-1-STAT-1 Complex Formation**

PIAS-1 competes with DNA for the binding to STAT-1. Lysates from ethanol/AGS-treated HCV+ and HCV− RLW cells were immunoprecipitated with anti-PIAS-1 and then probed for pSTAT-1 (Fig. 3, C–E). Only the combination of CYP2E1 and Ach increased PIAS-1-pSTAT-1 complex formation by about twofold. This effect was even more prominent in HCV+ than in noninfected cells (AGS + IFN-α vs. IFN-α) and was prevented by betaine treatment.

**Ethanol Metabolism and STAT-1 Methylation**

Cell lysates from treated HCV+ and HCV− cells were immunoprecipitated either with antibody to methyl arginine or methyl lysine and then probed for STAT-1. Ach reduced methylation of STAT-1 on both residues (Figs. 4, A–C, and 5, A–C). The magnitude of Ach-induced reduction in STAT-1 methylation was higher in HCV-infected than in noninfected cells (for methyl arginine, 50% vs. 27% and for methyl lysine, 62% vs. 38%, respectively), indicating that HCV synergizes with Ach in suppressing protein methylation. The effect of ethanol metabolites on STAT-1 methylation was reversed by betaine treatment.

The decrease in protein methylation is attributed to suppression of appropriate methyltransferase activities. The specific inhibitors of protein arginine methyltransferase (PRMT1), lysine methyltransferase and the pan-methylation inhibitor, tubercidin, were next used to relate the suppression in STAT-1 methylation to the downstream step of IFN-α signaling, an impaired attachment of STAT-1 to DNA (Fig. 5D). As shown, 50 μM arginine methyltransferase inhibitor (AM1) (a PRMT1 inhibitor) reduced STAT-1 binding to DNA by 20%; 10 μg/ml BIX (an inhibitor of histone lysine methyltransferase) suppressed it by 30%, and tubercidin reduced it by 60% of IFN-α-stimulated control, indicating that DNA attachment of activated STAT-1 is regulated by more than one methyltransferase. In addition, we observed a dose-dependent decline by AM1 on STAT-1 DNA binding (Fig. 5E), underscoring the importance of arginine methylation for the regulation of STAT-1 attachment to DNA and the downstream ISG activation.
Mechanism of the Regulation of STAT-1 Methylation by Alcohol Metabolism

It is known that STAT-1 arginine methylation is suppressed by HCV through PP2A-dependent downregulation of PRMT1 activity (7). To date, the involvement of PP2A in the regulation of methylation by Acetaminophen additive to HCV effect has not been explored. To study whether PP2A regulates the impairment of STAT-1 arginine methylation, RLW cells were treated with AGS-generated Ach for 48 h in the presence or absence of the PP2A inhibitor, okadaic acid (OA, 5 nM). Cell lysates were immunoprecipitated with anti-methyl arginine and then probed for STAT-1 by Western blot. Tubercidin treatment (2.5 μM) for 24 h served as a positive control for the reduction of STAT-1 methylation. OA by itself did not affect STAT-1 methylation, but PP2A suppression was protected from the reduction of STAT-1 methylation by Ach (Fig. 6A). Furthermore, OA treatment reversed an Ach-induced enhancement in PIAS-1-STAT-1 complex formation (Fig. 6B), indicating that PP2A has an impact on Ach-impaired STAT-1 methylation and PIAS-1-STAT-1 complexion. To investigate whether Ach activates PP2A, we measured the effect of Ach on total and phosphorylated PP2Ac (pPP2Ac) subunit because phosphorylation decreases PP2A activity (14). The pPP2A/total PP2A ratio after Ach treatment is presented in Fig. 6C–F. pPP2A/PP2A ratio was lower in HCV-infected than in noninfected cells and was further suppressed by Ach, indicating that PP2A activity is induced by Ach and is higher in HCV-infected than in noninfected cells. Activation of PP2A by Ach was prevented by betaine treatment.

Effects of Ethanol/Ethanol Metabolism on HCV RNA

To study the impact of HCV- and ethanol metabolism-induced changes in IFN signaling on HCV replication, we measured the levels of HCV RNA in infected cells treated with...
AGS. Figure 7A shows that, in RLW cells, HCV RNA was transiently increased threefold after 24 h of incubation with AGS, but, after 48 h of AGS exposure, it declined to near control levels. Similar kinetics of HCV core protein localized to lipid droplets was observed in cells after AGS treatment (Fig. 7B); the intensity of HCV core fluorescence per cell fell from 3.8 ± 0.11006 to 2.5 in untreated cells to 0.448 ± 0.448 in the cells exposed for 48 h to AGS (P = 0.01). This suggests that a temporary rise followed by depletion of HCV RNA in HCV-infected cells occurs in response to Ach. The decline in expression of HCV core-positive cells and HCV RNA is likely related to Ach-elicited induction of apoptosis in heavily infected cells after 24 h of AGS exposure. Hence, we measured cleaved caspase 3 as the downstream parameters for apoptosis and found that the amount of cleaved caspase 3 increased during exposure to Ach, reaching a maximum after 48 h (Fig. 7, C and D).

As a proof of concept that suppression of ISG and persistence of HCV RNA observed in hepatocyte-like hepatoma cells resembles the effects of ethanol metabolism in primary liver cells, we repeated the same AGS treatments in human hepatocytes. We found a similar reduction in expression of antiviral ISGs after cell treatment with AGS as observed in Huh7.5 cells; this reduction was apparently methylation dependent because it was attenuated by betaine (Fig. 8, A–D). As expected, ethanol exposure alone did not affect ISG levels because expression of ethanol-metabolizing enzymes in the hepatocytes was extinguished. This was confirmed by the lack of Ach present in the media of the ethanol-treated cells. Thus, in human hepatocytes, AGS induced a numeric increase in HCV RNA compared with IFN-α-treated samples (P = 0.07), and this increase was significantly reversed by betaine (P < 0.02) (Fig. 8E).

Fig. 6. Effects of AGS on protein phosphatase 2A (PP2A). Cells were treated with AGS in the presence or absence of the PP2A inhibitor, okadaic acid (OA), and exposed to IFN-α, 1,000 IU for 30 min. A: PP2A-dependent regulation of STAT-1 methylation by Ach. IP, anti-methyl arginine; IB, STAT-1. Lane 1, IFN-α; lane 2, IFN-α + OA; lane 3, AGS + IFN-α; lane 4, AGS + IFN-α + OA; lane 5, tubericidin + IFN-α. B: PP2A-dependent regulation of PIAS-1-pSTAT-1 complex formation. IP, PIAS-1; IB, pSTAT-1. Lanes are designated as lane 1, nontreated (control) cells; lane 2, IFN-α; lane 3, IFN-α + OA; lane 4, AGS + IFN-α; lane 5, AGS + IFN-α + OA; lane 6, tubericidin + IFN-α. Both A and B are representative data from 3 independent experiments with similar results. C–F: effects of Ach on PP2A activation in HCV− (C and D) and HCV+ (E and F) cells. IB, phosphorylated PP2A and total PP2A; β-actin was used as the loading control. Lane 1, control; lane 2, IFN-α; lane 3, IFN-α + betaine; lane 4, AGS + IFN-α; lane 5, AGS + IFN-α + betaine. C and E: representative IB data on PP2A phosphorylation. D and F: quantification of phospho-PP2A/PP2A ratios from 3 independent experiments presented as means ± SE. Bars with different letters are significantly different at P ≤ 0.05.
To link the in vitro and in vivo effects of ethanol metabolism on ISG activation, we measured OASL and ISG15 mRNAs in HCV transgenic (Tg) mice given control and ethanol liquid diets with or without betaine. Ethanol feeding significantly \((P < 0.01)\) reduced ISGs, whereas inclusion of betaine in the ethanol diet partially restored ISG expression (Fig. 9, A and B). In addition, the SAM:SAH ratio (a hallmark of methylation reactions) in livers of these animals was lower in ethanol-fed than in control mice (Fig. 9C), indicating suppression of methylation reactions in livers of HCV ethanol-fed mice, which was restored by betaine cotreatment.

**DISCUSSION**

In this study, we addressed a clinically relevant mechanism of methylation-related impairment in IFN-\(\alpha\) signaling triggered by ethanol metabolism. By itself, IFN-\(\alpha\), which is endogenously produced by liver and immune cells, does not possess antiviral properties. However, it activates signaling events in the cells by inducing ISGs that encode synthesis of antiviral proteins. Thus dysregulation of IFN signaling reduces protection from HCV, thereby allowing HCV infection progression (24, 35). Although HCV-induced hijacking of innate immunity has been extensively investigated (22, 36), the role of alcohol and, especially, alcohol metabolism in potentiation of HCV-induced defects of IFN signaling remains unclear.

Here, we explored the possibility that alcohol exposure to liver cells promotes HCV-elicited dysregulation of IFN-\(\alpha\) signaling via impairment of methylation reactions. We focused our investigation on downstream events in IFN-\(\alpha\) signaling that depend on STAT-1 methylation (26) and directly activate ISGs. HCV infection reduces STAT-1 methylation via PP2A-induced downregulation of PRMT1, which catalyzes methylation of STAT-1 on Arg 31 (7–9). This mechanism seems to be quite universal for viral hepatitis because similar changes in protein methylation were also observed in HBV infection (5). However, previously it was not clear whether ethanol metabolites, particularly Acetaldehyde, potentiate HCV-induced suppression of IFN-\(\alpha\) signaling.

To examine the effects of ethanol metabolism on IFN-\(\alpha\) signaling, we created hepatocyte-like conditions in HCV-infected Huh7.5 cells by stable transfection with CYP2E1 plasmid and in vitro exposure to AGS that contains ADH to...
generate physiological levels of Ach. AGS induced little to no toxic effects in Huh7.5 cells during 48-h treatment.

The present study revealed that only the combination of CYP2E1-generated alcohol metabolites and AGS-generated Ach efficiently suppressed activation of multiple antiviral ISGs in HCV-infected RLW cells. We confirmed these findings by similar experiments with human hepatocytes infected with HCV, as well as by results of in vivo studies. To attribute Ach-mediated ISG suppression to impaired protein methylation, AGS-exposed cells were treated with or without the promethylating agent, betaine. Importantly, Huh7.5 cells are responsive to betaine in restoration of methylation cycle because they express betaine-homocysteine S-methyltransferase. In contrast to the traditional promethylating agent SAM, betaine does not lower hepatoma cell viability and thus is better suited for in vitro studies on hepatoma cells.

Analyzing the events in IFN-α signaling, which are upstream from ISG activation, we found that, in HCV + RLW cells, AGS suppressed the binding of phosphorylated STAT-1 to DNA. Ach likely plays a pivotal role in these effects because, in CYP2E1-expressing cells, exposure to ethanol alone did not suppress IFN-α signaling. These changes (usually, more profound in HCV-infected RLW cells) were also observed in HCV− cells, indicating that, although Ach potentiates the suppression of IFN-α signaling by HCV, it also decreases IFN-α-induced signal transduction in the absence of HCV. In our hands, Ach-mediated reduction in STAT-1 attachment to DNA was attenuated by betaine. Furthermore, a decline of STAT-1 attachment to DNA by specific PRMT1 or lysine methyltransferase inhibitors, as well as by the pan-methyltransferase inhibitor, tubercidin, supports the notion that there is important methylation-dependent regulation of this signaling step.

PIAS-1 is a negative regulator of IFN signaling that complexes with activated STAT-1 and competes for its attachment to DNA (38). Here, Ach enhanced complex formation between

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Fig. 8. Effects of AGS on ISG activation and HCV RNA levels in primary human hepatocytes. HCV-infected human hepatocytes were exposed to 50 mM ethanol or AGS for 48 h in the presence or absence of betaine, and then cells were treated with 200 U IFN-α for 4 h. Real-time PCR analysis was performed for the expression of ISGs (mRNAs). A: OAS1. B: OASL. C: viperin. D: PKR. GAPDH was used for normalization. E: HCV RNA in hepatocytes treated with AGS for 48 h. GAPDH was used to normalize the gene of interest. All data are generated from 3 independent experiments and presented as means ± SE. Bars with different letters are significantly different at \( P \leq 0.05 \).
PIAS-1 and activated STAT-1. This latter event was blocked by betaine treatment and thus is methylation dependent. Furthermore, blocking of PP2A activity by the PP2A inhibitor, OA, reversed the activating effects of Ach on PIAS-1-STAT-1, indicating that the increase in PIAS-1-STAT-1 complex formation by Ach is controlled by PP2A. As demonstrated earlier, HCV uses the PP2A mechanism to deactivate PRMT1, which methylates STAT-1 on Arg 31 (7, 15). Our results indicated that Ach potentiated HCV-induced impairment in STAT-1 methylation via PP2A by enhancing PP2A activity (by blocking PP2Ac phosphorylation to inactivate the enzyme). Furthermore, we found that Ach, in addition to suppressing STAT-1 arginine methylation (as HCV does), also blocks lysine methylation. Even if we observed a modest suppression of STAT-1 methylation by Ach, these changes seemed to be enough to increase PIAS-1-STAT-1 complex formation, which, in turn, prevented STAT-1 attachment to DNA and subsequent ISG activation. The mechanism, by which Ach interferes with IFN-α-induced activation of antiviral genes in HCV-infected cells can be summarized as follows: suppression of STAT-1 methylation by HCV can be further exacerbated by Ach because of its ability to activate PP2Ac by downregulating PP2A phosphorylation (Fig. 10A). The results of in vivo studies on HCV+ Tg mice confirmed that chronic ethanol feeding, not only suppressed protein methylation in the liver by lowering the SAM:SAH ratio, but also inhibited IFN-α-induced ISG activation. The ethanol-elicited reduction in ISG expression was prevented (fully or partially) by inclusion of betaine in the liquid diet.

Ethanol metabolism-induced changes in IFN signaling and subsequent activation of antiviral ISGs suggest that HCV RNA levels should go up in the cells with reduced innate immunity. However, more detailed in vitro studies on HCV-infected ethanol-metabolizing cells have shown that HCV RNA amounts were gradually increased up to 24 h of AGS treatment but, surprisingly, declined at 48 h almost back to pretreatment levels. Moreover, after 48 h, we observed no infectivity in cell supernatants (not shown), and the amount of HCV core protein associated with lipid droplets was also decreased compared with that after 24 h of incubation, indicating that infectious HCV particles did not leak from infected cells, nor were they harbored inside the cells. More importantly, HCV RNA and the intracellular amount of HCV core protein were not absolutely cleared up by Ach but just decreased after 48 h, indicating low viral replication. Our explanation of these events is that long-term persistence of HCV in liver cells is possible when a low level of HCV persistence in hepatocytes is controlled by lipid peroxidation, without interfering with cell viability (43). Furthermore, in our earlier study on HCV-infected scid Alb-uPA mice with humanized livers, in vivo ethanol feeding prolonged the persistence of HCV core protein compared with the same mice on control diet, without significant elevation of HCV RNA levels (32), mimicking a scenario of an ethanol-induced switch from acute to chronic HCV infection.

We believe that Ach, on one hand, induced temporal accumulation of HCV in the cells and, on the other hand, triggered proapoptotic effects, leading to induction of apoptotic cell death in heavily infected RLW cells clearly seen after 48 h of AGS treatment. Indeed, the levels of cleaved caspase 3 (terminal caspase) were highest after 48 h of Ach exposure to HCV+ HuH7-CYP (RLW) cells.

It is known that cell-to-cell communication in HCV-infected liver can be established via exosomes (3, 23) or apoptotic bodies (13). Apoptotic bodies may be captured by Kupffer and stellate cells, thereby promoting inflammation and fibrosis development (25, 27, 42). In addition, HCV proteins, including core, NS3 and NS5 induce fibrogenic effects on hepatic stellate cells (1, 41). Figure 10B summarizes a proposed pathogenic mechanism of Ach-induced progression of HCV-induced hepatitis, which is now under investigation in our laboratory.

On the basis of the results of this study, suppression of IFN-α signaling by HCV and Ach reduces protection of cells from the virus and enhances cell death by apoptosis. All of
these events are at least partially dependent on impaired protein methylation. Thus betaine should be included in the therapy regimen for HCV-infected alcoholic patients undergoing treatment with IFN type 1 and/or direct antiviral agents (DAA). Indeed, a promising protective effect of the promethylating agent, SAM, has been demonstrated in patients with HCV treated with recombinant IFN-α (11) in the absence of alcohol. However, whereas, in patients with nonalcoholic HCV, the combination of betaine with DAA is optional, we firmly believe that it becomes “a must” in HCV+ alcohol abusers and will substantially increase the effectiveness of DAA therapy in this category of patients. Activation of the immune system should follow antiviral effects of DAA to avoid a risk of reinfection, as this possibility exists after DAA treatment (4). In addition, betaine has been shown to reverse apoptosis of hepatocytes in alcohol-fed rodents (19, 29). Therefore, it, not only restores antiviral protection by ISG activation, but also prevents apoptotic body formation, which appears to be a pathogenic mechanism for ethanol metabolism-induced liver injury progression in HCV infection.

We conclude that Ach generated from ethanol metabolism enhances HCV-induced suppression of STAT-1 methylation, thereby reducing attachment of activated STAT-1 to DNA attributable to the PP2A-regulated increase in PIAS-1-STAT-1 complex formation. This ultimately results in blocked activation of ISGs. Enhanced replication of HCV sensitizes liver cells to the proapoptotic effects of ethanol metabolism. All the aforementioned negative events are based on impaired IFN signaling, are methylation dependent, and can be fully or partially reversed by betaine treatment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.G. and J.Z. performed experiments; M.G., T.B., L.I.P., K.K., and N.A.O. analyzed data; M.G., D.J.T., and N.A.O. interpreted results of experiments; M.G. and J.Z. prepared figures; M.G. and N.A.O. drafted manuscript; T.B., L.I.P., T.M.D., D.J.T., and K.K. edited and revised manuscript; N.A.O. conception and design of research; N.A.O. approved final version of manuscript.

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