Acetaldehyde Accelerates HCV-induced Impairment of Innate Immunity By Suppressing Methylation Reactions in Liver Cells

Murali Ganesan\textsuperscript{1,2}, Jinjin Zhang\textsuperscript{3}, Tatiana Bronich\textsuperscript{3}, Larisa I. Poluektova\textsuperscript{2,4}, Terrence M. Donohue, Jr\textsuperscript{1,2}, Dean J. Tuma\textsuperscript{1,2}, Kusum K. Kharbanda\textsuperscript{1,2} and Natalia A. Osna\textsuperscript{1,2}

\textsuperscript{1}Research Service, Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, NE 68105, USA,
\textsuperscript{2}Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68105, USA,
\textsuperscript{3}School of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68105, USA
\textsuperscript{4}Department of Pharmacology and Experimental Neuroscience, Omaha, NE 68105, USA

Short running title: Acetaldehyde, HCV and innate immunity

Corresponding author:

Natalia Osna, MD, Ph.D.
Internal Medicine, UNMC,
Veterans Affairs Nebraska-Western Iowa Health Care System,
4101 Woolworth Ave, Omaha, NE 68105, USA,
Phone: 1-(402) 995-3735,
Fax: 1-(402) 449-0604,
E-mail: nosna@unmc.edu

Copyright © 2015 by the American Physiological Society.
Background and Aims: Alcohol exposure worsens the course and outcomes of hepatitis C virus (HCV) infection. Activation of protective anti-viral genes is induced by interferon (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 IFNα signaling modulation are not well-elucidated. Here, we explored a possibility that ethanol metabolism potentiates HCV-mediated dysregulation of IFNα signaling in liver cells via impairment of methylation reactions. Methods: HCV-infected Huh7.5 CYP2E1+ cells and human hepatocytes were exposed to acetaldehyde-generating system (AGS) and stimulated with IFNα to activate Interferon-Sensitive Genes (ISG) via the Jak-STAT1 pathway. Results: We observed significant suppression of signaling events by acetaldehyde (Ach). Ach exposure decreased STAT1 methylation via activation of protein phosphatase 2A (PP2A) and increased the protein inhibitor of activated STAT1 (PIAS1)-STAT1 complex formation in both HCV+ and HCV− cells, preventing ISG activation. Treatment with a pro-methylating 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+Tg mice. HCV and Ach-induced impairment of IFN signaling temporarily increased HCV RNA levels followed by apoptosis of heavily infected cells. Conclusions: Ach potentiates the suppressive effects of HCV on activation of ISGs due to methylation-dependent dysregulation of IFNα 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.
**Key words**: Ethanol metabolism; interferon alpha signaling; HCV; hepatocytes; betaine.

**Abbreviations**:

HCV, hepatitis C virus; AGS, acetaldehyde generating system; ISGs, interferon-sensitive genes; Ach, acetaldehyde; PP2A, protein phosphatase 2A; PIAS-1, protein inhibitor of activated STAT-1; STAT-1, signal transducers and activators of transcription; PP2A, ADH, alcohol dehydrogenase; CYP2E1, cytochrome P4502E1; SAM:SAH, S-adenosylmethionine to S-adenosylhomocysteine; JFH1, Japanese fulminant hepatitis virus-1; GC, gas chromatography; LDH, lactate dehydrogenase; OAS1, 2′-5′-oligoadenylate synthetase 1; OASL, 2′-5′-oligoadenylate synthase-like protein; PKR, protein kinase R; IP, immunoprecipitation; IB, immunoblotting; PRMT1, protein arginine methyl transferase 1; AMI, arginine N-methyltransferase inhibitor-1; BIX, 2-(Hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride; OA, okadaic acid
Introduction

With an estimated 170 million chronically infected persons, HCV infection is the most common blood-borne infection in the world. The prevalence of hepatitis C is 7 to 10-fold higher in alcohol abusers than in the general population (17), making the combination of HCV-infection and alcohol abuse a very common cause of chronic liver disease. Alcohol consumption in HCV-infected patients exacerbates liver injury, leading to rapid progression to fibrosis, cirrhosis and even hepatocellular carcinoma (34). Alcohol consumption significantly reduces responsiveness of HCV patients to anti-viral 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 since hepatocytes are primary sites for HCV replication and ethanol metabolism, both of which suppress innate immunity in liver cells.

Activation of an anti-viral innate immune response is based on IFN signaling. Type 1 Interferons per se, possess no anti-viral properties; however, IFNα signaling activates interferon-sensitive genes (ISGs) that encode the expression of anti-viral proteins to control HCV replication. IFNα signaling proceeds by endogenous IFNα binding to membrane-bound receptors, which activate Janus kinases and then phosphorylate the signal transduction and activators of transcription (STAT) 1 and 2. Activated STAT1 and STAT2 form a complex with interferon-regulated factor 9 (IRF9), which translocates from the cytosol to the nucleus. Following translocation, phosphorylated STAT-1 attaches to specific regions of DNA to activate anti-viral 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\(^+\) Huh 7 cells harboring HCV replicon, clearly demonstrated that ethanol metabolism, but not ethanol itself impaired IFN\(\alpha\) signaling (16). However, we do not know whether ethanol metabolism synergizes with HCV to reduce STAT1 methylation. In fact, this issue has not been adequately addressed since 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 hrs, while 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 extracellular acetaldehyde-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 IFNα 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 anti-viral genes that control HCV replication. We present evidence that the ethanol metabolite, Ach, suppresses ISG activation due to 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 Dulbecco’s Modified Eagle Medium (DMEM), Williams Medium, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Human recombinant interferon alpha (IFNα) was from Miltenyi Biotech Inc (MACS, 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, PIAS-1, β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mono-dimethyl Arginine and methylated lysine antibodies were from Abcam Inc (Cambridge, MA). PP2A, CYP2E1 and pPP2A were from EMD Millipore (Temecula, CA) and LifeSpan Biosciences, Inc (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/6J mice transgenically expressing HCV structural proteins obtained from Dr. S. Weinman (Kansas University Medical Center) were characterized elsewhere (21, 30). Mice (6-8 weeks 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 (w/v); then were gavaged with PBS/maltose dextran or ethanol on day 11 and sacrificed 9 hrs after gavaging as described in details for chronic-acute ethanol study (2). Four hrs before sacrifice, each mouse was injected IP with mouse IFNα, (1000 IU). In this study, we observed no differences in alcohol consumption between ethanol and ethanol+betaine groups. There was neither a difference in liver weight between each group (control: 1.01±0.05 g; ethanol: 1.18±0.06g; betaine: 1.08±0.03g; ethanol+betaine: 1.03 ±0.06g), nor in body weights 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 Biosafety Committees at VA Medical Center, Omaha, NE.

To characterize the changes in protein methylation, S-adenosylmethionine to S-adenosylhomocysteine (SAM:SAH) ratios were measured by HPLC in crude liver homogenates (19).
Betaine was used as a pro-methylating agent to correct impaired protein methylation via the restoration of normal SAM: SAH ratio. Also, OASL and ISG15 mRNAs (ISGs) were quantified in the liver tissue using RT-PCR.

Cells

Huh7.5 cells were transfected with pIV-G2 (CYP2E1) plasmid as previously described for other cell lines (6, 31) using Lipo TAXI (Invitrogen Corp., Carlsbad, CA). Recombinant cells, designated RLW cells, were selected in culture medium containing G418 at 400µg/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 special in vitro system (AGS, see description below).

Human hepatocytes were from Triangle Research Labs, LLC, Research Triangle Park, NC 27709 (two batches, triplicate readings).

Cell treatments

RLW cells were infected by JFH1 (HCV genotype 2a) virus at MOI=0.1 as previously described (40) or were left uninfected. To investigate whether ethanol metabolites affected IFNα signaling, on day 2 post infection, cells were exposed for up to 48 hrs to AGS added directly to the culture medium. The AGS included yeast ADH (0.02 U/ml), 2 mM Nicotinamide adenine dinucleotide (NAD) and 50 mM ethanol (EtOH). One unit of ADH catalyzes the reduction of µM of NADH formed per minute at 37C. In the presence of RLW cells, the levels of Ach measured by gas chromatography (GC) in the medium fluctuate between about 250 (at 1-4 hrs exposure) and 50 µM (at 18-48 hrs exposure) (Fig.1 B). 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 IFNα (time and dose depended on the end-points of the experiment, see Results).

Cytotoxicity was measured by lactate dehydrogenase (LDH) release to cell medium as described (6), (Fig.1 C). To assess the involvement of impaired protein methylation in the regulation of IFNα signaling, all treatments were done in the presence or absence of 2 mM betaine, a pro-methylating 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 6-well plates (4x10^5 cells/well) and then infected via serum from an HCV+ patient (IRB# 520-14-EX, HCV RNA is 3x10^6 copies/ml), 50 µl/well. Infected cells were cultured in William’s Medium supplemented with antibiotics and 5% human serum of AB group for 3 days and then exposed to either medium or 50 mM ethanol, or to AGS for 48 hrs. After incubation, the cells were stimulated with human IFNα, 200 IU, for 4 hours, and then lysed and processed for real-time PCR as described below. HCV infection in hepatocytes was confirmed by detection of HCV RNA.

**RNA isolation and Real-time PCR:** ISGs with anti-viral activities, such as viperin, 2’-5’-oligoadenylate synthetase 1 (OAS1), protein kinase R (PKR), 2’-5’-oligoadenylate synthase-like protein (OASL) and ISG15 were quantified by real-time PCR. Total RNA was isolated from cells using Trizol Reagent. A 2-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 (RQ). 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 v2.0 (Applied Biosystems): 5'UTRF GACCGGGTCCTTTCTTGGAT; 5'UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCCGC-NFQ.

**Immunoblotting (IB) and Immunoprecipitation (IP):** Cell lysates were prepared in 0.5M EDTA, 2MTris, 20mM Na₃VO₄, 200mM Na₄P₂O₇, 100mM PMSF, 1M 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 AB, Sweden) for overnight in a rotating shaker at 4°C, followed by washing and incubation with SDS-PAGE sample solubilizing buffer at 95°C for 10 mins. Isotype –specific IgG were used as a negative control. Complexes were subsequently subjected to denaturing SDS-PAGE in polyacrylamide gels. Immunoblotting was performed as described previously, blots were developed using Odyssey® infrared imaging system and protein band was quantified using Li-Cor software (39). Beta 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 hrs. After 48 hrs of AGS treatment, cells were washed with PBS, fixed with 4% PFA 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 co-localization of HCV core protein with the lipid droplets. First, cells were incubated with antibody to HCV core (clone: C7-50, ThermoScientific, Cat# MA1-080, dilution 1:300) for 1 hr. Then the cells were 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 63X lens in a LSM 710 confocal microscope (Carl Zeiss, Peabody, MA).

Statistical analyses

Data from at least three independent experiments are expressed as mean values ± standard error. Comparisons among multiple groups were determined by one-way ANOVA, using a Tukey 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, treated with AGS (in the presence or absence of betaine) for 48 hrs, and then activation of anti-viral 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 IFNα-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 IFNα signaling. Subsequent betaine exposure either fully or partially restored ISG expression.

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

ISG activation by IFNα 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%, while the AGS suppressed binding by 42% compared with untreated IFNα controls (Fig.3A, B). The magnitude of this reduction was comparable in both HCV+ and HCV− RLW cells. Betaine treatment partially (up to 69-75% of IFNα control) restored Ach-impaired DNA binding of STAT-1.

**Ethanol Metabolism and PIAS1-STAT1 Complex Formation**

Protein inhibitor of activated STAT-1 (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.3C, D, E). Only the combination of CYP2E1 and Ach increased PIAS-1-pSTAT-1 complex formation by about 2-fold. This effect was even more prominent in HCV+ than in non-infected cells (AGS+IFNα vs IFNα) 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 (Fig. 4 A, B, C and Fig. 5 A, B, C). The magnitude of Ach-induced reduction in STAT1 methylation was higher in HCV-infected than in non-infected 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. Next, the specific inhibitors of protein arginine methyltransferase (PRMT1), lysine methyltransferase and the pan-methylation inhibitor, tubercidin, were used to relate the suppression in STAT-1 methylation to the downstream step of IFNα signaling, an impaired attachment of STAT-1 to DNA (Fig. 5 D). As shown, 50 μM AMI (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- by 60% of IFNα-stimulated control, indicating that DNA attachment of activated STAT1 is regulated by more than one methyltransferase. In addition, we observed a dose-dependent decline by AMI on STAT-1 DNA binding (Fig. 5 E), 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 STAT1 Methylation by Alcohol Metabolism
It is known that STAT-1 arginine methylation is suppressed by HCV through the protein phosphatase 2A (PP2A)-dependent down-regulation of PRMT1 activity (7). To date, the involvement of PP2A in the regulation of methylation by Ach 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 hrs in the presence or absence of the PP2A inhibitor, okadaic acid (OA, 5nM). Cell lysates were immunoprecipitated with anti-methyl arginine and then probed for STAT-1 by WB. Tubercidin treatment (2.5 µM) for 24 hrs 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 that phosphorylation decreases PP2A activity (14). The pPP2A/total PP2A ratio after Ach treatment is presented in Fig. 6 C, D, E, F. pPP2/PP2A ratio was lower in HCV-infected than in non-infected cells and was further suppressed by Ach, indicating that PP2A activity is induced by Ach and is higher in HCV-infected than in non-infected 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. Fig. 7 A shows that in RLW cells, HCV RNA was transiently increased 3-fold
after 24 hrs of incubation with AGS, but after 48 hrs 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±2.5 in untreated cells to 0.448±0.448 in the cells exposed for 48 hrs to AGS, (p=0.01). This suggests 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 hrs 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 hrs (Fig. 7C, 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 anti-viral 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, B, C, 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 IFNα-treated samples (p<0.07), and this increase was significantly reversed by betaine (p<0.02) (Fig. 8 E).
To link the *in vitro* and *in vivo* effects of ethanol metabolism on ISG activation, we measured OASL and ISG15 mRNAs in HCV+Tg mice given control and ethanol liquid diets with or without betaine. Ethanol feeding significantly (p<0.01) reduced ISGs, while inclusion of betaine in the ethanol diet partially restored ISG expression (Fig. 9 A, 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 9 C), indicating suppression of methylation reactions in livers of HCV+ ethanol-fed mice, which was restored by betaine co-treatment.

**Discussion**

In this study, we addressed a clinically relevant mechanism of methylation-related impairment in IFNα signaling triggered by ethanol metabolism. By itself, IFNα, 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 anti-viral proteins. Thus, dysregulation of IFN signaling reduces protection from HCV, thereby allowing HCV-infection progression (24, 35). While 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 IFNα signaling via impairment of methylation reactions. We focused our investigation on downstream events in IFNα signaling that depend upon STAT-1 methylation (26) and directly activate ISGs. HCV infection reduces STAT-1
methylation via PP2A-induced down-regulation of PRMT1, which catalyzes methylation of STAT-1 on Arg 31(7-9). This mechanism seems to be quite universal for viral hepatitis since similar changes in protein methylation were also observed in HBV-infection(5). However, previously it was not clear whether ethanol metabolites, particularly Ach, potentiate HCV-induced suppression of IFNα signaling.

To examine the effects of ethanol metabolism on IFNα 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 Huh 7.5 cells during 48 hrs 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 pro-methylating agent, betaine. Importantly, Huh7.5 cells are responsive to betaine in restoration of methylation cycle, since they express betaine-homocysteine S-methyltransferase (BHMT). In contrast to the traditional pro-methylating 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 IFNα 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 IFNα signaling. These changes (usually, more profound in HCV-infected RLW cells) were also observed in HCV− cells, indicating that while Ach potentiates the suppression of IFNα signaling by HCV, it also decreases IFNα-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-methylation 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 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 ISGs activation. The mechanism, by which Ach interferes with IFNα-induced activation of anti-viral genes in HCV-infected cells can be summarized as follows: Suppression of STAT-1 methylation by HCV can be further exacerbated by Ach due to its ability to activate PP2Ac by down-regulating PP2A phosphorylation (Fig.10 A). 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 IFNα-induced ISG activation. The ethanol-elicited reduction in ISGs 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 anti-viral 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 amount were gradually increased up to 24 hrs of AGS treatment, but surprisingly, declined at 48 hrs almost back to pre-treatment levels. Moreover, after 48 hrs, 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-hr 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 intracellular amount of HCV core protein were not absolutely cleared up by Ach, but just decreased after 48 hrs, indicating low viral replication. Our explanation of these events is that long-term persistence of HCV in liver cells is possible when 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 RNA 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 pro-apoptotic effects, leading to induction of apoptotic cell death in heavily infected RLW cells clearly seen after 48 hrs of AGS treatment. Indeed, the levels of cleaved caspase 3 (terminal caspase) were highest after 48 hrs 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). Fig. 10B summarizes a proposed pathogenic mechanism of Ach-induced progression of HCV-induced hepatitis, which is now under investigation in our laboratory.

Based on the results of this study, suppression of IFNα 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 anti-viral agents (DAA). Indeed, a promising protective effect of the pro-methylating agent, SAM, has been demonstrated in HCV-patients treated with recombinant IFNα (11) in the absence of alcohol. However,
while in non-alcoholic HCV patients 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 anti-viral effects of DAA to avoid a risk of re-infection 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 anti-viral 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 acetaldehyde generated from ethanol metabolism enhances HCV-
induced suppression of STAT-1 methylation, thereby reducing attachment of activated
STAT-1 to DNA due 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 pro-apoptotic 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.

Acknowledgment

We thank Dr. D. Clemens for providing CYP2E1 plasmid, Dr. C. Rice for Huh7.5 cells,
Dr. T. Wakita for JFH1 virus and Dr. S. Weinman for Tg HCV+ mice. We also thank Lee
Jaramillo and Joseph Hindman for excellent technical assistance.

Author Contributions: MG: Performed experiments, analyzed data, drafted
manuscript; JZ: performed experiments, prepared figures; TB,LP,TD,DT,KK: critical
revision of the manuscript for important intellectual content; NO: Conception and design of research, interpreted results of experiments, approved final version of manuscript.

**Financial Support:** This work was supported by Merit Review BX001673 from the Department of Veterans Affairs, Office of Research and Development (Biomedical Laboratory Research and Development), USA

**Disclosures:** All authors disclose no conflict of interests

**Figure Legends**

**Fig. 1.** Effects of AGS on RLW cells. (A) Phenotype of RLW cells: Immunoblot (IB) analysis showing RLW cells lack ADH, but express CYP2E. Lane 1: MW markers; Lane 2: Lysate primary mouse hepatocytes as a positive control; Lane 3-4 lysates of: RLW cells. (B) Kinetics of Ach production by AGS after indicated times of exposure to RLW cells. (C) LDH activity in AGS- or EtOH-treated RLW cells. Cells were exposed to AGS or 50 mM EtOH for 48 hrs (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 % of positive control in treated samples, Mean ± SEM. Panels A, B, C show representative data from 3 independent experiments with similar results.

**Fig. 2.** ISG activation in RLW cells exposed to AGS. HCV-infected RLW cells were exposed to 50mM ethanol or AGS for 48hrs in the presence or absence of betaine, and then cells were treated with 200 units of IFNα for 4hrs. Treatment with this IFN dose for 4 hrs did not affect HCV RNA levels in the cells (not shown). Real-time PCR analysis
was performed for the expression of ISGs (mRNAs): (A) OAS1, (B) OASL, (C) viperin, (D) PKR. GAPDH was used to normalize the gene of interest. Data are generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

**Fig. 3.** Effects of EtOH or Ach on STAT-1 attachment to DNA and PIAS-1-STAT-1 complex. After exposure to medium, 50 mM ethanol or AGS for 48hrs in the presence or absence of betaine, cells were treated with 1000 IU of IFNα for 30 min to measure pSTAT-1 attachment to DNA in nuclear fractions by ELISA. Non-treated (with either AGS or ethanol, or IFNα) cells were used as a control. A. HCV- cells; B. HCV+ cells. Values from cells treated with IFNα only are expressed as 100%. Results are expressed as the percent of DNA binding in IFNα-stimulated cells, and are mean values ± SEM. Bars with different letters indicate significant differences at p ≤ 0.05. Results are expressed as % of DNA binding in IFNα-stimulated cells, Mean ± SEM. (C) PIAS-1-STAT-1 complex formation in HCV- and HCV+ cells. IP: PIAS-1, IB: pSTAT-1. Lane 1: non-treated cells; Lane 2: IFNα; Lane 3: EtOH+IFNα; Lane 4: EtOH+IFNα+Betaine; Lane 5: AGS+IFNα; Lane 6: AGS+IFNα+Betaine, representative IP data; (D and E) Quantification of pSTAT1: PIAS1 ratio obtained in HCV- and HCV+ cells, the results of 3 independent experiments, mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

**Fig. 4.** Effects of Ach on arginine STAT-1 methylation. HCV+ and HCV- RLW cells were treated as described above. IP: Methyl arginine, IB: STAT-1. (A) HCV- and HCV+ cells:
Lane 1: Control; Lane 2: IFNα; Lane 3: IFNα+Betaine; Lane 4: AGS+IFNα; Lane 5: AGS+IFNα+Betaine, shown as a representative immunoprecipitation. (B, C) Quantification of IP data from 3 independent experiments, presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

Fig. 5. Effects of AGS on lysine STAT-1 methylation. RLW cells were treated as described above. IP: anti-methyl lysine, IB: STAT-1. (A) HCV− and HCV+ cells: Lane 1: Control; Lane 2: IFNα; Lane 3: IFNα + Betaine; Lane 4: AGS + IFNα; Lane 5: AGS + IFNα + Betaine, shown as a representative immunoprecipitation. (B, C) Quantification of IP data is done based on the results from 3 independent experiments. (D). Inhibition of STAT-1 attachment to DNA with specific methylation inhibitors. Cells were incubated with indicated methylation inhibitors (AMI-arginine methylation inhibitor, BIX- lysine methylation inhibitor, tubercidin-pan-methylation inhibitor) for 24 hrs and then exposed to IFNα for 30 min. Attachment of STAT1 to DNA was determined in nuclear extracts by ELISA. (E) Dose-dependent effects of AMI on the attachment of STAT-1 to DNA. Cells were incubated with various doses of AMI for 24 hrs and then processed as described in D. Data are from 3 independent experiments, presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

Fig. 6. Effects of AGS on PP2A. Cells were treated with AGS in the presence or absence of the PP2A inhibitor, okadaic acid (OA) and exposed to IFNα, 1000 IU for 30 min. (A) PP2A-dependent regulation of STAT-1 methylation by Ach. IP: anti-methyl arginine, IB: STAT-1. Lane 1: IFNα; Lane 2: IFNα + OA; Lane 3: AGS + IFNα; Lane 4:
AGS+IFNα+OA; Lane 5: Tubericidin + IFNα, (B) PP2A-dependent regulation of PIAS-1-pSTAT-1 complex formation. IP: PIAS-1, IB: pSTAT-1. Lines are designated as Lane 1: Non-treated (control) cells; Lane 2: IFNα; Lane 3: IFNα + OA; Lane 4: AGS + IFNα; Lane 5: AGS+IFNα+OA; Lane 6: Tubericidin + IFNα,. 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, D) and HCV⁺ (E, F) cells. IB: phosphorylated PP2A and total PP2A, beta actin was used as the loading control. Lane 1: control; Lane 2: IFNα; Lane 3: IFNα + Betaine; Lane 4: AGS + IFNα; Lane 5-AGS + IFNα + Betaine. (C,E): Representative IB data on PP2A phosphorylation. (D,F)- Quantification of phosphoPP2A/PP2A ratios from 3 independent experiments presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

**Fig. 7.** Effects of AGS on HCV infection and apoptosis. HCV-infected RLW cells were treated with AGS for the indicated time. (A): HCV RNA in RLW cells. GAPDH mRNA was used to normalize the gene of interest. (B) Immunostaining of RLW cells treated with AGS for 24 and 48 hrs: red - HCV core protein, green - Lipid droplets (LDs), yellow - co-localization of core protein and LDs; blue - nuclear staining; (C, D): IB: cleaved caspase-3 in both HCV- and HCV+ cells treated with 50 mM ethanol and AGS for 48 hrs. Beta actin was used as loading control. Lane 1: control (untreated cells); Lane 2: EtOH; Lane 3: AGS. All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.
**Fig. 8.** Effects of AGS on ISG activation and HCV RNA levels in primary human hepatocytes. HCV-infected human hepatocytes were exposed to 50mM ethanol or AGS for 48hrs in the presence or absence of betaine, and then cells were treated with 200 units of IFNα for 4hrs. 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 hrs. GAPDH was used to normalize the gene of interest. All data are generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

**Fig. 9.** Effects of ethanol and betaine on activation anti-viral genes in HCV+ mice (fed ethanol with or without betaine supplementation). Real-time PCR analysis was performed for the expression of ISGs (A) OASL and (B) ISG15. GAPDH was used to normalize the gene of interest. (C) SAM: SAH ratios in liver tissue from HCV+ EtOH fed mice. Data generated from 6 mice/group (4 groups, see Materials and Methods) are presented as Mean ± SEM. Bars with different letters are significantly different at p ≤ 0.05.

**Fig.10.** (A): A proposed mechanism by which Ach synergizes with HCV to impair IFNα-induced ISG activation in liver cells: Ethanol metabolites (mainly, Ach) and HCV activate PP2A to decrease STAT-1 methylation. It causes PIAS-1-STAT-1 complex formation, which leads to the reduction of pSTAT-1 attachment to DNA and prevents ISG activation, thereby suppressing anti-viral protection in hepatocytes. (B): A proposed
mechanism of liver injury promotion in HCV-infected cells exposed to Ach: Ach induces apoptosis in HCV-sensitized hepatocytes; HCV-containing hepatocyte apoptotic bodies activate Kupffer cells to promote liver inflammation and stellate cells to promote liver fibrosis.

References


40. **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ.**


Figure 1

A

1  2  3  4

50 kDa  37 kDa

CYP2E1  ADH

B

μM Acetaldehyde

1 hr  4 hr  18 hr  24 hr  48 hr

C

Percent Leakage of LDH

Positive control  Untreated cells  48hrs Etoh  48hrs AGS

Figure 1
Figure 2
Figure 3

A

% of IFNa

HCV-

0
20
40
60
80
100
120

Control IFNa IFNo+Betaine EtOH+IFNa EtOH+IFNo+Betaine AGS+IFNa AGS+IFNo+Betaine

b
b,c
c
c
d
e

b
b,b,c
c
c
d

c

B

% of IFNa

HCV+

0
20
40
60
80
100
120

Control IFNa IFNo+Betaine EtOH+IFNa EtOH+IFNo+Betaine AGS+IFNa AGS+IFNo+Betaine

b
b
b,c
c
c
d
e

b
b,b,b,c
c
c
d

c

C

HCV-

IP with anti- PIAS-1

91 kDa

1 2 3 4 5 6

IB-pSTAT-1

91 kDa

1 2 3 4 5 6

IB-PIAS-1

78 kDa

D

E

pSTAT-1 / PIAS-1

1,Non-HCV Control 2,IFNa 3,EtOH+IFNa 4,EtOH+IFNo+Betaine 5,AGS+IFNa 6,AGS+IFNo+Betaine

1,HCV Control 2,IFNa 3,EtOH+IFNa 4,EtOH+IFNo+Betaine 5,AGS+IFNa 6,AGS+IFNo+Betaine

a

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b

b
Figure 4
Figure 5
Figure 6

A. IP with anti-methyl arginine

IB - STAT-1

Input STAT-1

91 kDa

B. IP with anti-PIAS-1

IB - pSTAT-1

IB - PIAS-1

91 kDa

78 kDa

C. HCV-

Phospho PP2A

Total PP2A

Beta actin

HCV-

1 2 3 4 5

36 kDa

36 kDa

42 kDa

D. Phospho / Total PP2A

HCV-

Control IFNα IFNα + Betaine AGS+ IFNα AGS+ IFNα + Betaine

36 kDa

36 kDa

42 kDa

E. HCV+

Phospho PP2A

Total PP2A

Beta actin

HCV+

1 2 3 4 5

36 kDa

36 kDa

42 kDa

F. Phospho / Total PP2A

HCV+

Control IFNα IFNα + Betaine AGS+ IFNα AGS+ IFNα + Betaine

36 kDa

36 kDa

42 kDa

Figure 6
Figure 7

A

![Bar graph showing HCV Quantity with groups labeled: Control, AGS 1hr, AGS 4hrs, AGS 18hrs, AGS 24hrs, AGS 48hrs.](image)

B

![Images of control and AGS treated cells.](image)

C

![Images showing Cleaved Caspase-3 and Beta actin with molecular weights.](image)

D

![Graph showing Cleaved Caspase-3/actin ratio with groups labeled: Control, Eth, AGS.](image)
Figure 8