CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Hepatitis C and Alcohol Exacerbate Liver Injury by Suppression of FOXO3

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Hepatitis C virus (HCV) infection exacerbates alcoholic liver injury by mechanisms that include enhanced oxidative stress. The forkhead box transcription factor FOXO3 is an important component of the antioxidant stress response that can be altered by HCV. To test whether FOXO3 is protective for alcoholic liver injury, we fed alcohol to *FOXO3*^{-/-} mice. After 3 weeks, one third of these mice developed severe hepatic steatosis, neutrophilic infiltration, and >10-fold alanine aminotransferase (ALT) elevations. In cell culture, either alcohol or HCV infection alone increased FOXO3 transcriptional activity and expression of target genes, but the combination of HCV and alcohol together caused loss of nuclear FOXO3 and decreased its transcriptional activity. This was accompanied by increased phosphorylation of FOXO3. Mice expressing HCV structural proteins on a background of reduced expression of superoxide dismutase 2 (SOD2; *Sod2*^{+/-}) also had increased liver sensitivity to alcohol, with elevated ALT, steatosis, and lobular inflammation. Elevated ALT was associated with an alcohol-induced decrease in SOD2 and redistribution of FOXO3 to the cytosol. These results demonstrate that FOXO3 functions as a protective factor preventing alcoholic liver injury. The combination of HCV and alcohol, but not either condition alone, inactivates FOXO3, causing a decrease in expression of its target genes and an increase in liver injury. Modulation of the FOXO3 pathway is a potential therapeutic approach for HCV-alcohol-induced liver injury. (*Am J Pathol* 2013, 183: 1803–1814; <http://dx.doi.org/10.1016/j.ajpath.2013.08.013>)

Alcohol consumption has long been recognized as an important risk factor for more rapidly progressive liver disease in patients with chronic hepatitis C infection.¹ Chronic hepatitis C virus (HCV) infection also predisposes to more severe outcomes of drug-induced liver disease² and alcoholic hepatitis.³ Although no single mechanism has been shown to be responsible for this synergy, several potential mechanisms have been identified. These include effects on mitochondrial reactive oxygen species production and cell death pathways, endoplasmic reticulum stress, activation of mitogen-activated protein kinases, innate immune responses, dendritic cell function, and proteasome inhibition, and direct effects of alcohol on viral replication pathways.^{4–7}

Several mechanisms normally protect the liver from alcoholic injury, and these include antioxidant enzymes, such as

superoxide dismutase⁸ and thioredoxin.⁹ HCV can impair antioxidant defenses by decreasing activation of nuclear factor erythroid 2-related factor 2 (Nrf-2)¹⁰ and heme oxygenase-1,¹¹ two important components of the antioxidant response, and these have been postulated to play a role in ethanol-HCV synergy.¹¹ Understanding the mechanisms by which these occur could, therefore, lead to novel prophylactic and therapeutic approaches to alcoholic liver disease.

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There is emerging evidence that the FOXO transcription factor family plays a critical role in hepatic metabolic, antioxidant, and cell death responses.¹² The O branch of the forkhead box superfamily consists of four mammalian proteins, FOXO1, FOXO3, FOXO4, and FOXO6, and controls expression of genes regulating antioxidant response, cell cycle arrest, gluconeogenesis, apoptosis, and autophagy.^{13,14} FOXO3, in particular, is activated by oxidative stress and plays a role in longevity, cell cycle arrest, protection from oxidative stress, induction of apoptosis, and tumor suppression.^{13,15,16} It is a direct transcriptional activator of the antioxidant proteins superoxide dismutase 2 (SOD2), catalase, and peroxiredoxin III.¹⁷ FOXO3 transcriptional activity is regulated by a complex pattern of post-translational modifications that regulate both the nuclear-cytosolic distribution and affinity for specific transcriptional sites. The best understood of these is phosphorylation by Akt, considered to be an inactivating modification, which causes nuclear export of the protein, but other modifications, such as alternative phosphorylation sites, acetylation, ubiquitination, and methylation, also regulate protein function.¹⁸ The role of FOXO in injury processes is complex because FOXO transcriptional programs can be either cytoprotective or cytotoxic, and well-documented examples of both phenomena are numerous.¹⁹ There have been a limited number of reports of the effect of HCV infection on FOXO activity, and these have shown that activation of FOXO1 or FOXO3 by HCV may contribute to insulin resistance²⁰ and autophagy induction.²¹ FOXO3 has also recently been shown to be part of a proapoptotic pathway in human peripheral blood cells.²² Whether FOXOs play a role in protection against alcoholic liver injury and whether HCV effects on FOXO3 are part of the liver synergy have not been examined.

In this study, we used FOXO3 knockout mice to determine whether this transcription factor is required for hepatic protection from alcohol and further examined the effects of HCV and alcohol separately and in combination on FOXO3 activity. The results indicate that FOXO3 is a novel alcohol protection factor that is disrupted by the HCV-alcohol combination.

Materials and Methods

Cell Culture, Plasmids, and Viral Infection

Huh7.5 cells [obtained from Dr. Charles Rice (Rockefeller University, New York, NY)] were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. DNA coding for the Japanese fulminant hepatitis 1 (JFH1) sequence was obtained from Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan). Plasmid was propagated and reverse transcribed, and the resulting RNA was used to transfect (by electroporation) Huh7.5 cells for the production of intact viral particles, as described.²³ 3×10^5 Huh7.5 cells were

seeded onto T-25 flasks and infected the following day with HCV at a multiplicity of infection of 0.5-1.0. At 48 hours after infection, control and infected cells were seeded onto 96-well plates or 8-well chamber slides and treated as described. pECE-HA-FOXO3 expression plasmid was provided by Michael Greenberg (Harvard Medical School, Boston, MA) via Addgene (Cambridge, MA).

pMirTarget reporter construct with a 3'-untranslated region (UTR) of *SOD2* gene (transcript variant 1) downstream of firefly luciferase was from OriGene (Rockville, MD).

Immunofluorescence and Image Analysis

Cells grown on coverslips were washed in PBS three times and fixed for 10 minutes in 4% paraformaldehyde in PBS at room temperature. For indirect immunofluorescence, fixed cells were permeabilized in 1% Triton X-100 in PBS for 5 minutes at room temperature. The coverslips were inverted and touched to 40- μ L droplets of blocking buffer [4% goat serum and 1% bovine serum albumin in PBS-Tween (0.05%)] on a clean parafilm sheet for 45 minutes at room temperature. Cells were then incubated in PBS with mouse anti-HCV core antibody (MA1-080; ThermoScientific, Pittsburgh, PA) and rabbit anti-FOXO3 antibody (75D8; Cell Signaling, Danvers, MA) for 1 hour at room temperature. After washing with PBS, coverslips were incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG (1:1000) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes, Carlsbad, CA) for 1 hour in the dark at room temperature. Coverslips were additionally incubated with DAPI for 20 minutes at room temperature to stain nuclear DNA.

Images were acquired using a 40 $\times$ oil objective on an Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY) equipped with a CoolSNAP HQ² charge-coupled device camera and MetaMorph version 7.1.1.0 software (Molecular Devices, Sunnyvale, CA). Image analysis was performed using CellProfiler version 2.0 release 11052 (Broad Institute of MIT and Harvard, Cambridge, MA, <http://www.cellprofiler.org>) run on Linux kernel 3.0.29 in Linuxmint version 13 operating system, using a quad core Intel i5 processor (Intel Corp., Santa Clara, CA) with 8 GB ram. Post-processing and statistical analysis were done with R version 3.01 (<http://www.r-project.org>) using packages Cairo, coin, ggplot2, grid, plyr, and psych. The CellProfiler pipeline and R scripts used for the analysis are available from author R.R. Briefly, DAPI channel images were used to segment nucleus objects and fluorescein isothiocyanate channel images were used to measure FOXO3 staining intensity for each pixel in each nucleus object. Fluorescein isothiocyanate channel images also were used to segment cell objects and cytoplasm objects. Texas Red channel objects were used to measure HCV core staining intensity in cytoplasm objects. Mean per-pixel FOXO3 staining per nucleus was calculated for each nucleus object to control for variation in nuclear area. The distributions of mean FOXO3 nuclear intensities were compared across treatment groups. For treatment groups HCV and

HCV-ethanol, nuclear objects from HCV core-negative cells were excluded from the analysis.

Real-Time PCR

RNA was extracted from cultured hepatoma cells using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). cDNA was generated with the random primer method using the RNA reverse transcription kit (catalogue no. 4368814; Applied Biosystems, Grand Island, NY). Quantitative real-time RT-PCR was performed in a CFX96 real-time system (Bio-Rad, Hercules, CA) using specific sense and antisense primers in a 25- μ L reaction volume containing 12.5 μ L of IQ SYBR Green supermix (Bio-Rad), 0.5 μ L of 10 μ mol/L primer stock, 8 μ L of cDNA (1:10 diluted), and 0.5 μ L double-distilled H₂O. The amplification consisted of an initial incubation of 95°C for 5 minutes (hot start), followed by 40 amplification cycles (15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C), and a final melting curve from 55°C to 95°C. Oligonucleotide primers were the following: glyceraldehyde-3-phosphate dehydrogenase, 5'-CAGCCTCAAGATCATCAGCA-3' (sense) and 5'-GTCTTCTGGGTGGCAGTGAT-3' (antisense); FOXO3, 5'-TCTACGAG TGGATGGTGC GTT-3' (sense) and 5'-CGACTATGCAGTGACAGGTTGTG-3' (antisense); Bcl-2-like protein 11 (Bim), 5'-CACATGAGCACATTTCCCTCT-3' (sense) and 5'-AAGGCACAAAACCTGCAGTAA-3' (antisense); SOD2, 5'-TTCAATAAGGAACGGGGACA-3' (sense) and 5'-GTAAGCGTGCTCCACACA-3' (antisense); growth arrest and DNA damage-inducible protein GADD45 alpha (GADD45a), 5'-CGCCTGTGAGTGAGTGC-3' (sense) and 5'-CTTATCCATCCTTTCCGGTCTT-3' (antisense); and SOD1, 5'-AAGATGACTTGGGCAAAGGT-3' (sense) and 5'-AATCCCAATCACACCACAAG-3' (antisense).

Luciferase Reporter Assays

Huh7.5 cells were cultured either without infection or for 4 days after JFH1 infection. Cells were then seeded onto 24-well plates. After 24 hours, cells were serum starved for 16 hours and then cotransfected using Lipofectamine-LTX (Invitrogen, Carlsbad, CA) with FHRE-luciferase reporter vector²⁴ and pRL-tk vector (renilla luciferase reporter) for normalization of transfection efficiency. The pFHRE-luciferase plasmid was provided by M. Greenberg via Addgene. Cells were subsequently incubated for 24 hours before lysis and luciferase determination. For ethanol treatment, experimental cells were incubated with 50 mmol/L ethanol for 48 hours before lysis. Lysates were used to measure luciferase activity using the Dual luciferase assay kit (Promega, Madison, WI) on a single-tube Glomax 20/20 luminometer (Promega). Results were expressed as firefly luciferase/renilla luciferase activity.

Western Blots and Immunoprecipitation

Proteins were extracted from liver tissue by homogenization using a glass tissue grinder (Kontes Duall 20; Kontes Glass

Co, Vineland, NJ) in tissue lysis buffer containing 50 mmol/L Tris, pH 7.4, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 1% SDS, 1% NP-40, and protease and phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis, MO) on ice. Whole cell lysates were prepared from cells that had been washed and harvested by centrifugation in PBS, pH 7.5. Cell pellets were resuspended in radioimmunoprecipitation assay buffer that contained 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1 mmol/L EDTA, and 1% protease and phosphatase inhibitors (Sigma-Aldrich). Lysates were centrifuged at 15,000 $\times g$ for 15 minutes; supernatants were collected, and protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Cytosolic and nuclear proteins were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions.

Protein extracts (15 μ g) were subjected to 10% SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and blocked in 3% bovine serum albumin and Tris-buffered saline (TBS)/0.1% Tween-20 (TBS-T) at room temperature for 1 hour. Primary antibodies used were anti-FOXO3 (75D8), anti-phospho-FOXO3 (Ser253), anti-pan Akt (11E7), anti-phospho-Akt (Ser473), anti-Bim, and rabbit anti-caspase 3 (D175) from Cell Signaling, anti-SOD2 (sc-30080), goat anti-intercellular adhesion molecule (ICAM) 1, and β -actin from Santa Cruz Biotechnology (Santa Cruz, CA), anti-HCV core from Affinity Bioreagents (Golden, CO), and rabbit anti-SOD2 and mouse anti- β -actin from Sigma-Aldrich. Secondary antibodies were from Southern Biotechnology Associates (Birmingham, AL). Immunoblots were detected using the Electrochemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ). Expression levels were evaluated by quantification of relative density of each band normalized to that of the corresponding β -actin band density using a digital imager, AlphaEase FC (α Innotech, San Leandro, CA), with NIH ImageJ software version 1.48c (NIH, Bethesda, MD). For protein half-life measurement, cells were treated for indicated times with 100 μ g/mL cycloheximide before harvesting.

Mouse Strains and Alcohol Feeding

FOXO3^{-/-} mice were provided by Dr. Kana Miyamoto (Keio University, Tokyo, Japan) and were generated as described.²⁵ Heterozygotes were bred together, obtaining both knockout and wild-type (WT) littermates. They were used for alcohol feeding experiments at 3 to 6 months of age. HCV transgenic mice (SL-139 line) were generated on a C57BL/6J background, as previously described.²⁶ These mice are a hemizygous model and express HCV structural proteins (core, E1, E2, and p7) in hepatocytes under control of the murine albumin enhancer/promoter. *Sod2*^{+/-} mice on a C57BL/6J background have been previously described.²⁷ They were crossed with the HCV transgenic mice to

produce the *HCV/Sod2*^{+/-} genotype. All mice were housed in a temperature-controlled, specific, pathogen-free environment with 12-hour light-dark cycles and fed regular mouse chow and water ad libitum. *HCV/Sod2*^{+/-} mice were used for alcohol feeding at 6 to 9 months old. All animal handling procedures were approved by the Institutional Animal Care and Use Committees at the University of Texas Medical Branch (Galveston, TX) and the University of Kansas Medical Center (Kansas City, KS).

Mice were fed either a high-fat liquid diet with 35% of the calories supplied by ethanol (product F1258; Bio-Serv Inc., Frenchtown, NJ) or a control diet with maltodextrin isocalorically substituted for ethanol (product F1259; Bio-Serv Inc.). No other food was provided, but they had free access to water. Mice were initially acclimated to the control liquid diet for 3 days, and then ethanol concentration was increased stepwise to 1.6%, 3.2%, 4.8%, and, finally, 6.4% (v/v) at 3-day intervals. This ethanol concentration was maintained to the end of a 3-week period. The duration of alcohol exposure was calculated from the first day of the 1.6% alcohol feeding solution. Control mice were pair fed the amount consumed the previous day by the alcohol-fed mice to prevent differences in caloric intake. At the end of the feeding period, mice were sacrificed and livers and venous blood were obtained. Serum was stored at -80°C. Liver samples were fixed in formalin for histological examination or snap frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry

Liver tissue sections (5 µm thick) were prepared from paraffin-embedded samples. For analysis of histological features, H&E sections were evaluated by an experienced liver pathologist (M.O.) in a blinded manner and scored for percentage steatosis, severity of liver inflammation (0, none; 1, mild; 2, moderate; 3, severe), and percentage necrosis. Immunostaining on formalin-fixed sections was performed by deparaffinization and rehydration, followed by antigen retrieval by heating in a pressure cooker (121°C) for 5 minutes in 10 mmol/L sodium citrate, pH 6.0. Peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 minutes. Sections were rinsed three times in TBS/TBS-T and incubated in 5% normal goat serum in TBS-T at room temperature for 1 hour. After removal of blocking solution, slides were placed into a humidified chamber and incubated overnight with rabbit anti-FOXO3 (EP1949Y) purchased from Millipore, diluted 1:500 in TBS-T containing 1% normal goat serum at 4°C. Antigen was detected using the SignalStain Boost IHC detection reagent (no. 8114; Cell Signaling Technology, Beverly, MA), developed with diaminobenzidine (Dako, Carpinteria, CA), counterstained with hematoxylin (Sigma-Aldrich), and mounted.

Cell death was detected *in situ* in mouse liver paraffin-embedded sections by enzymatic labeling of DNA strand breaks with a TUNEL assay (In Situ Cell Death Detection Kit, catalogue no. 11684809910; Roche Diagnostics, Indianapolis,

IN), according to the manufacturer's instructions, and followed by counterstaining with *N*-ethyl-*N*-[4-([4-(ethyl[(3-sulfophenyl)methyl]amino)phenyl](4-hydroxy-2-sulfophenyl)methylene)-2,5-cyclohexadien-1-ylidene]-3-sulfo-benzene-methanaminium, disodium salt (fast green FCF). Intensity of TUNEL staining was quantitated on a scale of 1 to 5 by determining the percentage of positive nuclei per high-power field.

Statistical Analysis

Results are expressed as means ± SD. A Student's *t*-test, rank-sum test, χ^2 test, or one-way analysis of variance with Tukey's post hoc test was performed as indicated. *P* < 0.05 was considered significant.

Results

Effects of Alcohol Feeding on *FOXO*^{-/-} Mice

To determine whether FOXO3 is a modifier of alcoholic liver injury, we assessed the effects of ethanol on *FOXO3*^{-/-} mice. We placed constitutive *FOXO3*^{-/-} mice on a Lieber-DeCarli alcohol diet for 3 weeks and compared them with WT littermates. Figure 1A demonstrates that ethanol feeding of WT mice caused a modest alanine aminotransferase (ALT) elevation (103 ± 91, *n* = 17) compared with control diet (59 ± 51, *n* = 10) (*P* = 0.17), and histological characteristics showed only moderate to mild zone 2 steatosis with minimal inflammation or necrosis (Figure 1B). These results are consistent with the widely reported observation that Lieber-DeCarli alcohol feeding produces only mild liver injury in mice.²⁸⁻³⁰ In contrast, after 3 weeks, alcohol-fed *FOXO3*^{-/-} mice had a wide variation in ALT, with a mean of 302 ± 396 (*n* = 23). Seven of 23 *FOXO3*^{-/-} mice, but no WT mice, had ALT elevations >10-fold (*P* = 0.014), and each of these livers showed severe macrovesicular steatosis with pan-lobular inflammation, prominent presence of neutrophils, and frank necrosis (Figure 1B). Examples of TUNEL staining of WT and *FOXO3*^{-/-} mice on ethanol are shown in Figure 1C, and higher-magnification examples of necrosis and neutrophilic infiltration are shown in Figure 1D. Steatosis, inflammation, and TUNEL positivity were significantly greater in the ethanol-fed *FOXO3*^{-/-} mice compared with ethanol-fed WT mice (Figure 1E). As shown in Figure 1A, not all *FOXO*^{-/-} mice developed severe liver injury. The lower half of the ALT distribution was identical for ethanol-treated WT and *FOXO*^{-/-} mice [45 ± 16 (*n* = 8) versus 49 ± 15 (*n* = 11)], but the half of the mice with the greatest ALT elevations were dramatically different [155 ± 99 (*n* = 9) for WT versus 534 ± 438 (*n* = 12) for *FOXO3*^{-/-} (*P* = 0.03)].

Effects of HCV and Ethanol on FOXO3

The previously described results suggested that FOXO3 could be an alcohol hepatoprotection factor. We then used Huh7.5 cells as a model system to directly examine the effects of HCV and alcohol on FOXO3. These cells were

Figure 1 Effects of alcohol feeding on *FOXO3*^{-/-} mice. *FOXO3*^{-/-} mice or WT littermates were fed either a Lieber-DeCarli liquid alcohol diet (E) or a control isocaloric alcohol-free liquid diet (C) for 3 weeks, as described in [Materials and Methods](#). **A:** Serum ALT values from individual mice are shown. $P = 0.022$. **B:** Representative H&E-stained liver histopathological characteristics from WT and *FOXO3*^{-/-} mice on either a control or an ethanol (EtOH) diet. **C:** Examples of TUNEL staining performed on alcohol-fed livers from WT and *FOXO3*^{-/-} mice. **D:** Higher-magnification images showing presence of neutrophils and areas of necrosis in ethanol-fed *FOXO3*^{-/-} mouse livers with elevated ALT. **E:** Analysis of steatosis, inflammation, and nuclear TUNEL staining in the four groups, as previously described. * $P < 0.05$, ** $P < 0.01$ by *t*-test comparing alcohol-treated *FOXO3*^{-/-} mice with alcohol-treated WT mice. The distribution of inflammation grade scores was significantly different for the two populations of alcohol-treated mice by χ^2 test. Original magnification, $\times 10$ (B and C).

chosen because they support the full life cycle of HCV infection³¹ and metabolize alcohol via alcohol dehydrogenase (ADH). [Figure 2A](#) demonstrates that Huh7.5 cells express comparable levels of ADH protein, as found in human or mouse liver. Infection with the JFH1 strain of HCV increased endogenous FOXO3 transcriptional activity, as evidenced by increased FHRE-luciferase reporter activity ([Figure 2B](#)). HCV infection also increased nuclear translocation of FOXO3, as evidenced by immunofluorescence imaging of FOXO3 ([Figure 2C](#)). Images obtained as in [Figure 2C](#) were quantitatively analyzed by an image analysis algorithm that used a DAPI-stained image to identify nuclei, and separately assessed nuclear and cytoplasmic average intensity for 400 to 600 cells for each condition. The results, shown in [Figure 2D](#), confirm that HCV significantly increased nuclear FOXO3 intensity, shifting the distribution to the right. Western blot analysis of FOXO3 in cytosolic and nuclear fractions also showed a more than twofold increase in nuclear FOXO3 after HCV infection ([Figure 2E](#)).

We next examined the effects of alcohol on FOXO3. When uninfected cells were exposed to 50 mmol/L ethanol for 48 hours, there was a similar increase of FHRE-luciferase activity ([Figure 2B](#)). However, the ethanol-induced increase in FOXO3 activity was not associated with a change in the nuclear FOXO3 intensity, as assessed

by immunofluorescence ([Figure 2, C and D](#)) or Western blot analysis ([Figure 2E](#)).

Paradoxically, although both HCV and ethanol increased FOXO3 reporter activity ([Figure 2B](#)), treatment of HCV-infected cells with ethanol resulted in a decrease in FHRE-reporter activity ([Figure 2B](#)) and a significant decrease of nuclear FOXO3 detected either by immunofluorescence ([Figure 2, C and D](#)) or Western blot analysis ([Figure 2E](#)). Although HCV is known to produce cytotoxicity after prolonged infection in cell culture,³² there was no increase in cytotoxicity as measured by lactate dehydrogenase release at the 48-hour time point of this experiment in any of the conditions. There were, thus, two separate effects of ethanol on FOXO3: transcriptional activation without an increase in the nuclear content in the absence of HCV and suppression of nuclear translocation and transcriptional activation in the presence of HCV. To determine the role of oxidative stress and ethanol metabolism on these effects, we performed luciferase reporter experiments in the presence of the antioxidant, N-acetylcysteine, and the ethanol metabolism inhibitor, 4-methylpyrazole. N-acetylcysteine completely blocked the ability of ethanol to directly increase FOXO3 activity, but it had no effect on direct HCV activation or on the ability of ethanol to suppress HCV activation. Both effects, however, required

Figure 2 HCV infection and alcohol alter FOXO3 in Huh7.5 cells. **A:** Western blots showing relative abundance of alcohol dehydrogenase in Huh7.5 cells, Huh7 cells, Huh7.5 cells with an RFP-tagged reporter protein to identify HCV infection, human liver (Hu), and mouse liver (M). **B:** Luciferase reporter assays using a synthetic FHRE reporter were performed in uninfected and HCV-infected Huh7.5 cells, as described in [Materials and Methods](#). * $P < 0.01$. $n = 9$ to 10 independent experiments. **C:** Immunofluorescence images of Huh7.5 cells 48 hours after HCV infection and/or treatment with 50 mmol/L ethanol. FOXO3 (green), anti-HCV core protein (red), and DAPI staining for nuclei (blue). The **arrow** in the HCV/ethanol (EtOH) condition indicates one cell in the field that is not HCV infected. **D:** Distribution of nuclear FOXO3 intensity quantitated by automated image analysis. Data were collected from four separate experiments. P values were calculated by analysis of variance. **E:** Western blot analysis of FOXO3 in nuclear (N) and cytosolic (C) fractions isolated from Huh7.5 cells treated as in **B**. Numbers represent intensity of the nuclear FOXO3 band and are representative of three independent experiments. **F:** Luciferase reporter assays using a synthetic FHRE reporter in control and HCV-infected Huh7.5 cells in the presence of N-acetylcysteine or 4-methylpyrazole. Data are presented as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. $n = 3$. 4-MP, 4-methylpyrazole; NAC, N-acetylcysteine.

ethanol metabolism because they were prevented by 4-methylpyrazole (Figure 2F).

Effects of HCV and Ethanol on FOXO3-Dependent mRNA and Protein Expression

We next examined the effects of HCV and ethanol on mRNA levels of FOXO3 target genes. Consistent with its effect on FOXO3 nuclear distribution and reporter activity, HCV infection resulted in an increase in steady-state mRNA levels for four FOXO3 target genes (*FOXO3* itself, *SOD2*, *BIM*, and *GADD45*), but had no effect on the non-FOXO-dependent antioxidant gene, *SOD1* (Figure 3A). An examination of the effects of ethanol on the time course of changes in FOXO3-dependent mRNA levels (Figure 3B) also confirmed the previous results. Uninfected cells had an increase in *FOXO3* target gene mRNA with time of exposure to alcohol, but HCV-infected cells experienced a decrease in target

gene expression after 48 to 72 hours of ethanol exposure (Figure 3B).

Effects of the transcriptional changes on protein expression were examined by using Western blot analyses of whole cell lysates (Figure 3C). Uninfected cells had a progressive increase in the protein levels of *SOD2*, *BIM*, and *FOXO3* during the course of exposure to 50 mmol/L ethanol for 48 hours, consistent with the transcriptional increases observed. However, although HCV infection increased FOXO3 transcriptional activity and target gene mRNA levels, it did not increase the steady-state protein concentrations of *SOD2*, *Bim*, and *FOXO3* (Figure 3C). Nonetheless, after 48 hours of ethanol exposure, HCV-infected cells had decreased levels of all three proteins consistent with the suppression of FOXO3 activity at that time point (Figure 3C). The decrease in *SOD2* protein in HCV-infected cells was approximately 50% at 48 hours of ethanol exposure (Figure 3D).

Figure 3 Effects of HCV and alcohol on FOXO3 transcriptional activity. **A:** Real-time RT-PCR for FOXO3-dependent genes, *FOXO3*, *SOD2*, *Bim*, and *GADD45*, and a control, non-FOXO target gene, *SOD1*, in RNA isolated from control (black bars) and 5-day HCV-infected (gray bars) Huh7.5 cells. $n = 6$ to 18 individual RNA preparations. P values by Wilcoxon signed-rank test for the comparison of infected and noninfected conditions were as follows: *FOXO3*, $P < 0.001$; *SOD2*, $P < 0.001$; *Bim*, $P < 0.005$; *GADD45*, $P < 0.05$; *SOD1*, $P = 0.86$. **B:** Time course of mRNA expression changes determined by real-time PCR for FOXO3 and its target genes, *Bim* and *SOD2*, in control (black bars) and HCV-infected (gray bars) cells treated with 50 mmol/L ethanol for the indicated times. $N = 3$ to 6 individual preparations. **C:** HCV-infected and noninfected Huh7.5 cells were incubated with 50 mmol/L ethanol for 48 hours. Western blots for FOXO3, SOD2, Bim, and β -actin (loading control) are presented for whole cell lysates prepared at different times of alcohol exposure. Similar results were obtained in four independent experiments. **D:** SOD2 protein levels were measured by densitometry analysis of immunoblots performed as in **A**. $n = 4$. **E:** Kinetics of SOD2 degradation after cycloheximide treatment, determined as in **D** ($n = 3$ experiments). **F:** pMiR-Target-Luciferase-SOD2 3'-UTR reporter expression in HCV-infected and noninfected Huh7.5 cells incubated where indicated with 50 mmol/L ethanol for 48 hours. $n = 3$. * $P < 0.05$, ** $P < 0.01$.

The observation that HCV increased SOD2 mRNA more than fourfold, but did not change steady-state protein levels, suggested that HCV regulated SOD2 by another mechanism as well, either a change in protein stability or suppression of mRNA translation. Figure 3E shows that there was no effect of HCV on SOD2 half-life after inhibition of protein synthesis with cycloheximide. Figure 3F shows the results obtained using the pMiR-Target-Luciferase-SOD2 3'-UTR reporter system (OriGene) to assess the presence of miRNAs able to suppress SOD2 mRNA translation. This result shows that HCV, but not alcohol, causes a suppression of SOD2 translation. This result shows that HCV, but not alcohol, caused an miRNA-dependent suppression of SOD2 translation. This mechanism, along with an HCV-alcohol-induced decrease in SOD2 mRNA, likely contributes to the decrease in SOD2 protein level after prolonged HCV-alcohol exposure.

Effects of HCV and Alcohol on FOXO3 Phosphorylation and Stability

Because FOXO3 activity and nuclear localization are negatively regulated by Akt-dependent phosphorylation,²⁴ we assessed whether this phosphorylation modification was affected by the ethanol-HCV combination. As previously reported,³³ HCV infection, but not ethanol, caused Akt phosphorylation and, thus, activation (Figure 4A). Figure 4B demonstrates that ethanol itself did not increase the accumulation of the S-253 phosphorylated form of FOXO3 in noninfected cells, but in HCV-infected cells, ethanol initially increased P-S253-FOXO3 in the first 6 hours after ethanol exposure. At longer times, both P-FOXO3 and total FOXO3 decreased, consistent with the phosphorylated form being targeted for degradation, and accounting for the loss of

Figure 4 Alcohol- and HCV-induced phosphorylation of FOXO3. **A:** Western blots of Huh7.5 whole cell lysates were performed for total and phospho-Akt. Results are representative of three independent experiments. **B:** Whole cell lysates were prepared at indicated times (in hours) after alcohol exposure and Western blots performed for S-253 phosphorylated and total FOXO3. **C:** Rate of degradation of HA-FOXO3 determined by serial immunoblots after cycloheximide treatment. Numbers indicate hours after cycloheximide addition. **D:** Densitometry analysis of three experiments performed as in **C**. Data are presented as means $\pm$ SD. * P < 0.05, ** P < 0.01. EtOH, ethanol.

FOXO3-dependent protein expression at the later time points. To determine whether an increase in FOXO3 degradation could account for the HCV-ethanol effects, we measured FOXO3 stability after cycloheximide treatment. Figure 4, C and D, shows that the HCV-ethanol combination produced a 40% decrease in the half-life of FOXO3 protein after cycloheximide inhibition of protein synthesis.

Effects of Alcohol on HCV Transgenic Mice

The previous results demonstrate that FOXO3 can act as an alcohol hepatoprotective factor and that the combination of HCV and alcohol causes phosphorylation and loss of FOXO3 activity in cell culture. To determine whether the HCV-alcohol effects on FOXO3 can contribute to liver injury, we next evaluated alcoholic liver injury in an HCV animal model. We used mice that have liver-specific expression of the HCV protein core, E1, E2, and p7 and display increased mitochondrial oxidative stress.²⁶ To maximize the chance to observe an oxidative liver injury, we also crossed the HCV mice with Sod2 heterozygous knockout mice (*Sod2*^{+/-}). The latter mice have a mild, subclinical oxidative stress phenotype and have previously shown utility in toxicological studies.³⁴ The *Sod2*^{+/-} mice had no obvious phenotype, and SOD2 expression was approximately 60% of control.²⁷ In the absence of alcohol, SOD2 level was not influenced by the presence of HCV protein expression.

Each of the four genotypes (WT, HCV, *Sod2*^{+/-}, and HCV/*Sod2*^{+/-}) was fed an ethanol-containing Lieber-DeCarli diet for 3 weeks. Serum ALT values are shown in Figure 5A, and typical histological results are shown in Figure 5B. Liver injury, as evidenced by elevated ALT level, primarily occurred in the HCV/*Sod2*^{+/-} group (P < 0.001). A further examination of the characteristics of liver injury in the HCV/*Sod2*^{+/-} group demonstrated increased expression of

ICAM-1, caspase 3 cleavage (Figure 5C), and histological findings of severe steatosis, with lobular inflammation, and ballooning degeneration of hepatocytes (Figure 5, B and D).

We next assessed possible factors that correlate with alcohol-induced ALT increases in HCV/*Sod2*^{+/-} mice. ALT did not correlate with core protein expression or thioredoxin 2 oxidation (data not shown). In contrast, there was a correlation between ALT and SOD2 protein expression (Figure 6A), with the highest ALT elevations seen in mice whose livers had the lowest expression of SOD2. Although alcohol feeding had little effect on hepatic SOD2 protein levels in WT, *Sod2*^{+/-}, or HCV mice, it decreased SOD2 level in the HCV/*Sod2*^{+/-} group (Figure 6B).

To determine whether altered FOXO3 might account for decreases in SOD2 expression, we examined the subcellular distribution of FOXO3 in these livers by immunohistochemistry (Figure 6, C and D). In liver specimens from mice fed the control diet, FOXO3 staining was primarily in the nuclei of hepatocytes and bile duct epithelial cells. There were no differences between the genotypes. After alcohol treatment, WT and *Sod2*^{+/-} livers still had nearly exclusively nuclear localization of FOXO3, but the HCV and HCV/*Sod2*^{+/-} mice demonstrated hepatocytes with cytosolic FOXO3 staining as well (Figure 6C). Quantitative analysis demonstrated that >20% of the hepatocytes in ethanol-treated HCV/*Sod2*^{+/-} mice had cytosolic FOXO3 (P = 0.006 compared with ethanol-treated *Sod2*^{+/-} mice).

Discussion

This study evaluated the role of FOXO3 in HCV/alcohol liver injury. In mice, FOXO3 behaved as a hepatic protection factor to alcohol. *FOXO3*^{-/-} mice developed liver injury after a 3-week alcohol diet that had several characteristics of

Figure 5 Effects of alcohol feeding on mouse liver. Mice from the four genotypes were fed either a Lieber-DeCarli liquid alcohol diet or a control, alcohol-free liquid diet for 3 weeks, as described in *Materials and Methods*. **A:** Serum ALT values expressed for each mouse were expressed as fold elevation compared with WT mouse on the control diet. ALT in the ethanol-fed HCV/Sod2^{+/-} group was significantly different from the other groups by analysis of variance ($P < 0.001$). White squares, pair fed; red circles, alcohol fed. **B:** Representative H&E-stained liver histopathological characteristics for the different groups. **C:** Western blot analysis for inflammation and apoptosis markers in 3-week alcohol-fed and control-fed mice. **D:** Higher magnification of features of the HCV/Sod2^{+/-} alcohol-treated livers demonstrating ballooning degeneration (top panel) and lobular inflammation (bottom panel). Features are indicated by arrows. Casp, caspase.

alcoholic hepatitis, including severe steatosis, necrosis, and neutrophilic infiltration. In cell culture, either HCV or alcohol alone produced an increase in FOXO3 transcriptional activity, but the combination of HCV and alcohol together suppressed FOXO3 nuclear translocation, transcriptional activity, and expression of target genes such as *SOD2*. The HCV-alcohol combination caused an increase in FOXO3 phosphorylation at an Akt site, resulting in a shortened half-life of the transcription factor. Loss of SOD2 expression and FOXO3 cytosolic translocation were seen in alcohol-fed HCV transgenic mice and correlated with liver injury.

FOXO transcription factors play a major role in longevity, cell cycle arrest, gluconeogenesis, and antioxidant cytoprotection¹⁶; in some contexts, they can initiate apoptotic programs.¹⁹ FOXO transcriptional activity is regulated at several levels. Nuclear FOXO3 can be phosphorylated by Akt and related kinases, generating a binding site for 14-3-3 and causing cytosolic translocation, polyubiquitination, and degradation.³⁵ Relatively little is known about FOXOs in liver disease.¹² FOXO1 has been shown to promote gluconeogenesis,³⁶ and its activity is decreased by insulin via Akt activation. FOXOs are also required for lipid homeostasis.³⁷ HCV infection was shown to contribute to insulin resistance by increased FOXO1 activity,^{20,38} and a similar observation has been made for nonalcoholic steatohepatitis.³⁹ It has more recently been shown that FOXO3 activity is increased by starvation/malnutrition in HCV infection, where it modulates innate immunity signaling.²¹ These findings underline the importance of FOXO3 in the hepatic response to various stress stimuli. Our novel and unexpected observation that the alcohol-HCV combination inhibits FOXO3 activity thus suggests the possibility of alterations of several important

adaptive processes, including reactive oxygen species cytoprotection and cell proliferation.

The cellular and animal model systems used in these experiments each have important characteristics that make them relevant to human disease. Huh7.5 cells can be infected with HCV and support the complete viral life cycle.²³ These cells also metabolize alcohol via ADH, although they do not express appreciable levels of CYP2E1 (data not shown).

Although the long-term goal of these studies is to understand the mechanism of human liver disease, there are no available animal models that faithfully reproduce the phenotype of human alcoholic liver disease. However, several different alcohol administration models have been used with mice.³⁰ The addition of alcohol to the drinking water⁴⁰ does not generally produce liver effects, but liver injury in mice does occur with a voluntary liquid-feeding diet, first developed by Lieber and DeCarli,⁴¹ an intragastric infusion model, developed by Tsukamoto et al,⁴² and, more recently, an acute or chronic alcohol administration model, developed by Gao and colleagues,⁴³ in which a modified Lieber-DeCarli diet is supplemented with alcohol gavage. The degree of liver injury produced by these models depends on other dietary components and seems to require the unsaturated fatty acid composition of the administered liquid-feeding solutions.⁴⁴ We chose to use the Lieber-DeCarli liquid diet for these studies primarily because it is well documented to produce only mild liver injury in mice with mild steatosis, negligible inflammation, and only mild ALT elevations.^{28–30}

However, unlike the mild liver effects in WT C57BL/6 mice, we found that, in two mouse models in which FOXO3 was disrupted, Lieber-DeCarli alcohol feeding caused a true

Figure 6 SOD2 and FOXO3 expression and distribution in alcohol-fed mouse livers. **A:** Correlation of ALT with postalcohol SOD2 level in liver. SOD2 expression relative to β -actin was determined by densitometry of Western blots and plotted versus serum ALT. Blue diamonds, control-fed; red circles, alcohol fed. **B:** Comparison of the effects of alcohol feeding on SOD2 protein abundance in liver for the four genotypes. Liver homogenates were analyzed by using Western blot analysis after 3 weeks of alcohol or control diet feeding. Each lane represents homogenate from a separate mouse. **C:** FOXO3 immunohistological characteristics are presented. For each genotype, paraffin sections of liver were stained for FOXO3; and for each diet, images are shown at low power (10 $\times$ objective, left panels) and at higher magnification (right panels), as indicated. The arrow indicates a cell with cytosolic immunoreactivity for FOXO3. **D:** Quantitative analysis of proportion of hepatocytes displaying cytosolic FOXO3 staining. Black bars, control diet; gray bars, ethanol diet. $N = 3$ different mouse livers for each condition. $^{**}P < 0.01$ compared with *Sod2*^{+/-}.

liver injury phenotype. The severe steatosis, presence of inflammation, and elevated ICAM-1 expression are similar to that seen in acute alcoholic hepatitis in humans. In both *FOXO3*^{-/-} and *HCV/SOD2*^{+/-} mice, severe liver injury occurred in only approximately one third of the animals, with the other animals being indistinguishable from WT mice. The factors responsible for this heterogeneity are not known. In *HCV/SOD2*^{+/-} mice, there was a correlation of liver injury with SOD2 level, but this was not the case in the *FOXO3*^{-/-} mice, in which SOD2, FOXO1, and FOXO4 mRNA levels were not predictive of liver injury. Although strong TUNEL staining of nuclei occurred in the *FOXO3*^{-/-} mice developing liver injury, there was no correlation of

severity with caspase 3 cleavage, suggesting that apoptosis was not a primary mode of cell death in this model. This suggests that some other target of FOXO3 may be critical for pathogenesis and, in the HCV/SOD2 mice, low SOD2 levels could be a marker of low FOXO3 activity.

The cell culture studies clearly show that HCV and alcohol are each activators of FOXO3. HCV activation is associated with translocation of FOXO3 from cytosol to nucleus, but alcohol produces transcriptional activation without a prominent change in the nuclear/cytosolic ratio. In the case of alcohol, the changes in FOXO3 target proteins, such as SOD2, can be explained entirely by the changes in mRNA. HCV infection alone also produced a large increase in SOD2 mRNA, but without a corresponding change in SOD2 protein level (Figure 3C). This appears to reflect an miRNA-dependent HCV-induced suppression of SOD2 mRNA translation (Figure 3F). miR-222 inhibits translation of SOD2 mRNA,⁴⁵ and it is up-regulated in HCV-associated fibrosis⁴⁶ and is, thus, a good candidate for this effect. This decreased efficiency of SOD2 mRNA translation makes HCV-infected cells dependent on high SOD2 mRNA levels. When HCV-infected cells are treated with ethanol, FOXO3 activity decreases, SOD2 mRNA decreases, and there is a profound reduction in steady-state SOD2 protein levels (Figure 3C).

The loss of FOXO3 activity in the combined HCV/alcohol condition results from nuclear export and a decreased protein half-life that does not occur in the other conditions. It is associated with an initial increase in FOXO3 phosphorylation at the S253 Akt site, followed by a subsequent decrease in both total and P-253 FOXO3, as would be expected for a modification that targets the protein for degradation. Akt is activated by HCV in either the absence or the presence of ethanol, but it only results in robust FOXO3 phosphorylation when ethanol is present. It is likely that an ethanol-induced FOXO3 post-translational modification change makes it a better substrate for Akt phosphorylation. Recent studies from our laboratory have shown that the combination of HCV and alcohol specifically suppresses arginine methylation of FOXO3,⁴⁷ and this is expected to make it a better substrate for Akt phosphorylation, causing nuclear export and degradation.⁴⁸ Because the effects can be seen in our transgenic mice and infected cells, the HCV structural proteins, particularly core, may play a critical role in FOXO3 modulation. This would not be unexpected because the core protein itself has been implicated as a major cause of HCV-induced oxidative stress.^{26,49,50}

In conclusion, this study has identified FOXO3 as a novel protective factor for alcoholic liver injury. Loss of FOXO3 expression in mice results in a phenotype in which alcohol consumption causes severe liver injury that is histologically similar to human alcoholic hepatitis. Although HCV and alcohol each activate FOXO3, the HCV-alcohol combination suppresses this transcription factor, decreases expression of cytoprotective genes, and leads to liver injury. Further understanding the consequences of the HCV effects

will require a detailed molecular understanding of the viral and alcohol-induced FOXO3 modifications, and a more comprehensive understanding of the specific target genes affected. Because FOXO3 serves multiple functions, including insulin response and tumor-suppressor activity, understanding the mechanisms by which HCV alters its properties may have implications for several hepatic consequences of chronic HCV infection.

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