Published in final edited form as:

Free Radic Biol Med. 2009 July 1; 47(1): 47–54. doi:10.1016/j.freeradbiomed.2009.03.021.

Acrolein, a ubiquitous pollutant and lipid hydroperoxide, inhibits antiviral activity of interferon-alpha: relevance to Hepatitis C

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Abstract

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease and can lead to hepatocellular carcinoma and end-stage liver disease. The current FDA-approved treatment for HCV (pegylated interferon-alpha (IFN α) with ribavirin) is effective only in about 50% of patients. Epidemiological evidence suggests that obesity, alcohol, smoking and environmental pollutants may contribute to resistance to IFN α therapy in HCV. Acrolein, a ubiquitous environmental pollutant and major component of cigarette smoke, is also generated endogenously by cellular metabolism and lipid peroxidation. This study examines the effects of acrolein on (i) IFN α -mediated signaling and antiviral gene expression in cultured and primary human hepatocytes, and (ii) HCV replication in an HCV-replicon system. Our data demonstrate that non-toxic concentrations of acrolein significantly inhibited IFN α -induced tyrosine phosphorylation of both cytoplasmic and nuclear STAT1 and STAT2, without altering the total levels. Also, acrolein down-regulated IFN α stimulated gene transcription, resulting in reduced expression of antiviral genes. Importantly, acrolein abolished the IFN α mediated downregulation of HCV viral expression in the HCV-replicon system. This study defines mechanisms involved in resistance to IFN α and identifies the pathogenic role of acrolein, and potentially other environmental pollutants, in suppressing IFN α antiviral activity, and establishes their adverse impact on HCV therapy.

Keywords

Environmental pollutant; acrolein; hepatitis C virus; interferon alpha; JAK-STAT signaling; lipid peroxidation products

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease (1). Chronic HCV infection can lead to cirrhosis, liver cancer and end-stage liver disease, making HCV

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an enormous burden on the health care system in the US and worldwide (2–5). The current FDA approved standard of care for chronic HCV is pegylated IFN α combined with ribavirin; however, treatment is ineffective in ~ 50% of patients (particularly with HCV genotype 1, prevalent in USA) (6–8).

IFN α is a ubiquitously expressed, immunomodulatory cytokine that has antiviral and anti-HCV activity (9, 10). IFN α action in cells is mediated by specific cell surface receptors IFNAR1 and IFNAR2, and involves activation of the JAK-STAT (receptor associated Janus family tyrosine kinases - signal transducer and activator of transcription family of proteins) pathway (11, 12). Upon stimulation by IFN α , upstream kinases Jak1 and Tyk2 are activated by phosphorylation; they, in turn, phosphorylate STAT1 and STAT2. Tyrosine phosphorylated STAT1 and STAT2 heterodimerize, translocate into the nucleus, and together with nuclear p48/IRF-9 proteins form the ISGF3 (interferon stimulated gene factor-3) transcription factor complex (9–12). ISGF-3 binds to the interferon-stimulated response element (ISRE) in the promoters of IFN α stimulated genes, and induces the expression of several antiviral proteins, such as dsRNA-activated PKR, oligoadenylate synthetases (OAS), Mx proteins, and numerous IFN α stimulated genes (ISGs) (11, 12).

IFN α mediated antiviral gene expression is subject to negative regulation by dephosphorylation via serine/tyrosine protein phosphatases (PP2A, SHP-1, SHP-2, PTP1B) and by association with inhibitory proteins such as SOCS (suppressor of cytokine signaling proteins) or PIAS (protein inhibitor of activated STAT) (reviewed in 9). Upon stimulation by IFN α , STATs are rapidly phosphorylated, enabling nuclear translocation and DNA binding; this is followed by dephosphorylation of STATs by protein phosphatases. Thus, protein phosphatases control STAT activity by maintaining STAT in a latent cytosolic unphosphorylated form and by terminating nuclear STAT activity by dephosphorylation.

The molecular mechanisms underlying resistance to IFN α therapy are not fully understood and depend upon both viral and host-specific factors including HCV genotype, viral load, age, and genetic background. Epidemiological data have identified potentially modifiable cofactors that may contribute to the failure of HCV therapy, including obesity, diabetes, and external factors such as alcohol, smoking and pollutants (10). A recent study suggests that cigarette smoking may adversely influence HCV treatment outcomes, since smokers suffering from chronic hepatitis C have a lower response rate to IFN α compared to non-smokers (13). Also, increasing evidence suggests that increased lipid peroxidation and oxidative stress may play a pathogenic role in chronic hepatitis C and may be an important factor influencing IFN α therapy outcomes (14–19). Indeed, recent data show that H₂O₂ can directly inhibit IFN α signaling in a cultured hepatocyte cell line (20).

Acrolein is the most reactive and toxic aldehyde produced endogenously as a byproduct of cellular lipid peroxidation (LPO); it is also formed by the metabolism of polyamines, hydroxyl-amino acids, and anti-cancer drugs such as cyclophosphamide (21). Acrolein is a pervasive environmental and industrial pollutant, and is identified by the U.S. EPA as one of the 188 most hazardous air pollutants (22). Humans are exposed to acrolein via automobile exhaust, wood, plastic and cigarette smoke, overheated cooking fats and drinking water (21, 23). Importantly, acrolein forms irreversible, covalent carbonyl-retaining Michael adducts with proteins, phospholipids and nucleic acids, with deleterious consequences to their function (24–28). Moreover, protein adducts of acrolein (particularly, acrolein-FDP-lysine) have been detected in several chronic diseases, including Alzheimer's disease (29), atherosclerosis and renal failure (30), and Type II diabetes (31).

This study tests the hypothesis that acrolein, a ubiquitous environmental pollutant and endogenous lipid peroxidation byproduct, contributes to poor response and resistance to

IFN α therapy in hepatocytes. Our data demonstrate that non-lethal concentrations of acrolein significantly inhibit IFN α mediated JAK/STAT signaling, leading to reduced expression of antiviral proteins in HepG2 cells, and enhanced HCV viral expression in the HCV replicon system. The study identifies the pathogenic role of LPO products and environmental pollutants (such as acrolein) in repressing IFN α antiviral activity, and establishes their adverse impact on anti-HCV therapy.

MATERIALS AND METHODS

Reagents

Human interferon alpha (IFN α) was purchased from PBL Biomedical Laboratories (Piscataway, NJ). General chemicals, acrolein and β -actin antibody were purchased from Sigma Aldrich (St. Louis, MO). Antibodies against phospho-STAT1 (Tyr701), STAT 1, phospho- STAT 2, and STAT 2 were purchased from Cell Signaling (Beverly, MA). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA).

Cell culture

HepG2, a human hepatoma cell line obtained from American Type Culture Collection (Rockville, MD) was used as described previously (32). The HCV replicon (Clone B) was obtained from Apath LLC (St. Louis, MO) and used in accordance with company instructions. All treatments were performed on sub-confluent monolayers of cells. Primary human hepatocytes were obtained from ZenBio (Research Triangle Park, NC) and used in accordance with company instructions.

RNA isolation, RT- PCR and Real Time PCR analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and subjected to real time PCR using SYBR green I dye reagents with an ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The specific primers were obtained from Superarray (Frederick, MD). The relative gene expression was analyzed using $2^{-\Delta\Delta C_t}$ method by normalizing with GAPDH gene expression in all the experiments.

Western Blot Analysis

Following treatment, cytosolic and nuclear protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis and visualized using the enhanced chemiluminescence light (ECL) reagents (Amersham, Arlington Heights, IL).

Cell Viability-MTT assay

Cell survival/cell death was measured in cells treated for 24h by the MTT (3, (4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay as described previously (32).

Luciferase Reporter Assays

HepG2 cells were transiently transfected for 24h with an ISRE *cis*-reporting system (Stratagene, La Jolla, CA) using the transfection reagent FugeneTM6 according to the manufacturer's instructions (Roche, Indianapolis, IN). To control for transfection efficiency, cells were transfected in a single large batch and reseeded into 24-well plates for treatments. Total cell extracts were analyzed for luciferase activity using a commercial assay kit (Promega, Madison, WI). Results were normalized by protein concentration.

Protein phosphatase Assay

The Serine/Threonine or Tyrosine Phosphatase Assay System (Promega, Madison, Wisconsin) was used following the manufacturer's instructions. In this assay, serine/threonine phosphatase activity was selectively measured using the serine phosphopeptide substrate, RRA(pT)VA peptide. Tyrosine phosphatases activity was measured using DADE(pY)LIPQQG peptide, which serves as substrate for most cellular tyrosine phosphatases.

Statistical Analysis

All data are expressed as mean $\pm$ SD. The method of analysis used was unpaired analysis of variance with the Student's t-test, with data from three experiments. Differences were considered statistically significant for $P < 0.05$.

RESULTS

We studied the effects of acrolein, an LPO derivative and environmental pollutant, on IFN α signaling in human hepatoma cells (HepG2 cells), owing to the fact that they are a well-characterized, commonly used model system for human hepatocytes. However, we confirmed key findings in primary human hepatocytes. Additionally, we examined the effects of acrolein on HCV viral replication using an HCV replicon system.

Effect of acrolein on cell survival

Acrolein is known to be hepatotoxic and induces cell death in various cell types at high concentrations (22). To determine the appropriate non-lethal working concentrations of acrolein, we initially tested the cytotoxic effects of various concentrations of acrolein on HepG2, HCV replicon cells and primary human hepatocytes (Figure 1). We found that acrolein was relatively non-toxic at concentrations lower than 50 μ M (2.5 μ M - 50 μ M), and significant cell death (greater than 50%) was seen at doses higher than 75 μ M. As anticipated, primary hepatocytes were slightly more sensitive to acrolein compared to the established cell lines, and a statistically significant (greater than 40%) decrease in survival was seen at 50 μ M. Although we evaluated the effects of acrolein over a wide range of concentrations (both toxic and non-toxic), proteins and RNA were extracted only from the adherent (living) cells, and equal amounts of protein or RNA were used for further analysis.

Effect of ACR on IFN α -induced activation of the STAT proteins in HepG2 cells

A key event in the antiviral signaling initiated by IFN α is activation by tyrosine phosphorylation of STAT1 and STAT2, followed by nuclear translocation and binding to the IFN α response elements on target genes. We evaluated the effect of acrolein on STAT1 and STAT2 tyrosine phosphorylation, which is an essential event and serves as an excellent marker for IFN α signaling. As shown in Fig. 2, IFN α stimulation resulted in tyrosine phosphorylation of both STAT1 and STAT2 in cytoplasmic extracts, while untreated HepG2 cells show no activation of STATs. A dose-dependent decrease in phosphorylated STAT1 (Fig. 2A) and STAT2 (Fig. 2B) was observed in cytoplasmic extracts of cells exposed to acrolein starting at a concentration of 10 μ M, without alteration of the total levels of STAT1 and STAT2. Additionally, nuclear localization of phosphorylated STAT1 and STAT2 was greatly reduced demonstrating that acrolein effectively suppressed IFN α signaling and decreased activity of STAT1 and STAT2. An important mechanism for down-regulation of the JAK/STAT pathway is the induction of newly synthesized inhibitory proteins such as SOCS and PIAS. This possibility was ruled out since inhibition by acrolein was rapid and effective within 30min, and pretreatment with the protein synthesis inhibitor cycloheximide

did not abolish acrolein-mediated inhibition of STAT activation (data not shown). Additionally, protein levels of SOCS2 and PIAS1 were unchanged (data not shown).

Effect of acrolein on IFN α -mediated ISRE transcriptional activation in HepG2 cells

To evaluate the effect of acrolein on IFN α dependent gene transcription, we analyzed IFN α mediated transcriptional activation of an ISRE-dependent luciferase reporter construct in transiently transfected HepG2 cells treated with increasing concentrations of acrolein followed by IFN α stimulation. The IFN α -responsive reporter yielded a significant, almost twenty-fold induction of luciferase activity upon IFN α treatment (Fig. 3), while no induction was observed with acrolein alone at 25 μ M or 50 μ M. Correspondent with the observed down regulation of STAT1 activation in acrolein treated cells, IFN α -dependent ISRE reporter activity was significantly and dose-dependently reduced by pretreatment with acrolein, starting from a concentration of 10 μ M (Fig. 3).

Effect of acrolein on mRNA levels of IFN α -inducible antiviral genes in HepG2 cells

We examined the effect of acrolein on IFN α -induced gene expression in HepG2 cells. The antiviral genes PKR, OAS, ISG15 and ISG54 were used as indicators of IFN α -induced gene expression and messenger RNA levels of PKR (A), OAS (B), ISG15 (C), and ISG54 (D) were measured in HepG2 cells. Treatment of HepG2 cells with IFN α caused a large increase in mRNA levels of all the antiviral genes; fold induction of the different genes varied from approximately 3-fold for PKR to 30-fold for ISG54. Pretreatment with acrolein (10 μ M - 100 μ M) dose-dependently reduced IFN α -mediated expression of all the genes, and statistically significant down regulation was observed at or above 25 μ M acrolein in each case (Fig. 4). In all experiments, mRNA levels of GAPDH were used for normalization; these levels were not affected by acrolein at any concentrations, indicating that the effect of acrolein was not caused by nonspecific toxicity (not shown).

Effect of acrolein on protein levels of IFN α -inducible PKR and OAS in HepG2 cells

We examined the effect of acrolein on cellular levels of the antiviral proteins OAS and PKR. These proteins are induced by IFN α and are known to contribute to the antiviral effects of IFN α . Pretreatment with acrolein caused a significant down regulation of IFN α -mediated expression of both proteins (Fig 5A). Densitometric analysis revealed that IFN α induced a 1.3- and 1.2-fold upregulation in the protein levels of PKR and OAS, respectively, which was inhibited by acrolein (Fig 5B).

Effect of acrolein on IFN α -inducible antiviral genes in primary human hepatocytes

Although HepG2 cells are a well-established surrogate model system for human hepatocytes and have been extensively used for liver studies, they are immortalized, cultured hepatoma cells, which may not exhibit the same responses as primary hepatocytes. Hence, we used commercially available primary human hepatocytes to extend our HepG2 findings and validate the inhibitory effect of acrolein on IFN α -inducible antiviral genes (Fig. 6). We examined mRNA levels of the antiviral genes ISG15 (6A), ISG54 (6B) and OAS (6C) in primary human hepatocytes stimulated with IFN α without or with pretreatment with 25 μ M acrolein. Treatment of cells with IFN α led to a significant increase in mRNA levels of all antiviral genes. Although the magnitude of induction of each gene was different in the primary hepatocytes, the variability in magnitude was significantly less than that seen in HepG2 cells. There was an approximate 3–6 fold induction of all genes. Pretreatment with acrolein (25 μ M) significantly reduced IFN α -mediated expression of all antiviral genes. In all experiments, results were normalized with GAPDH mRNA levels, which were unaffected by acrolein.

Effect of acrolein on anti-HCV activity of IFN α in an HCV replicon system

Although the above experiments determined the effect of acrolein on hepatic anti-viral gene expression, they did not reveal any information about HCV viral replication. To determine the effect of acrolein on HCV viral replication and HCV-specific antiviral activity of IFN α , we used an HCV replicon expressing Huh7 cell line obtained from Apath LLC. This system has been well characterized and allows the direct assessment of effects on HCV viral replication. IFN α dose-dependently blocked HCV viral replication at concentrations that were much lower than those used in HepG2 cells and approximately 80% inhibition was seen at 100U/ml of IFN α (not shown). Based on this observation, we treated cells for 24h with 100U/ml IFN α , with a 30 min pretreatment with non-toxic concentrations of acrolein (5 μ M and 10 μ M), as determined by MTT-based cell survival data (Fig. 1). We observed that acrolein dose dependently reversed the antiviral effect of IFN α , allowing HCV replication to occur as efficiently as seen in UT (Fig. 7). In fact, 10 μ M acrolein pretreatment led to a small but reproducible increase in HCV RNA over the level seen in untreated cells. Thus, acrolein not only interfered with IFN α antiviral responses but slightly promoted HCV viral replication.

Effect of acrolein on protein phosphatases in HepG2 cells

One of the ways that acrolein exposure may cause reduced IFN α signaling and gene expression is by up regulation of protein phosphatases leading to dephosphorylation of critical signaling components, such as STATs. We investigated the effects of acrolein on the enzymatic activity of serine/threonine and tyrosine-specific protein phosphatases in HepG2 cells (Fig. 8A). Cells were treated with a non-toxic concentration of acrolein (25 μ M) for 30min or 1h. Phosphatase activity was measured using serine- or tyrosine-specific phosphopeptide substrates. A significant increase in both serine and tyrosine phosphatase activity was seen in cells treated with acrolein for 30min and 1h. At 30min, a 1.5-fold increase was seen in serine/threonine-phosphatase activity, with an even greater rise (~2.5-fold) in tyrosine-phosphatase activity. Parallel phosphatase assays were conducted in the presence of inhibitors to evaluate specificity using sodium orthovanadate (Na₃VO₄), a tyrosine phosphatase inhibitor and sodium fluoride (NaF), a serine/threonine phosphatase inhibitor. Additionally, the inhibitors were used to pretreat HepG2 cells to examine whether they would reverse the acrolein-induced inhibition of IFN α antiviral gene expression, using IFN α mediated up regulation of OAS mRNA as an indicator. As seen in Fig. 8B, IFN α induced a 13-fold increase in OAS message, which was decreased to less than 4-fold with acrolein exposure. Pretreatment of cells with NaF partially restored OAS mRNA levels to approximately 7-fold, while vanadate pretreatment raised OAS mRNA to about 9-fold. Although neither inhibitor completely restored OAS expression, partial protection was observed, suggesting that phosphatase activity contributes to the inhibitory effects of acrolein on IFN α mediated antiviral signaling. Further studies are needed to determine the exact contribution of phosphatases in the effect of acrolein.

DISCUSSION

Despite substantial research, the molecular mechanisms underlying resistance to IFN α therapy are not fully understood. Clinical studies have identified several modifiable cofactors that may contribute to the failure of anti-HCV therapy, including obesity, diabetes, alcohol abuse, smoking and environmental pollutants (34). Our study demonstrates that acrolein, which is both an environmental pollutant and a product of endogenous LPO, inhibits IFN α antiviral activity at non-lethal concentrations, and may have a significant impact on response to HCV therapy. Because acrolein is a reactive byproduct of LPO and can itself induce lipid peroxidation/oxidative stress, it may play a dual role in HCV disease

progression by mediating oxidative injury of hepatocytes, and by actively inhibiting IFN α antiviral function.

The predominant route of environmental exposure to acrolein is inhalation of smoke or automotive exhaust. However, since acrolein is also a constituent of several food substances and has been detected in drinking water, high acrolein exposure via ingestion is also possible. Individuals likely to receive the highest exposures include smokers and those inhaling second-hand smoke, persons in close proximity to sources of wood and plastic smoke, including those in the forest products and firefighting communities, and populations living or working in areas of dense automotive traffic (22). Although acrolein exposure is expected to primarily affect respiratory organs, in vivo hepatic effects have also been reported (22). Moreover, in vitro hepatotoxicity of acrolein has been clearly documented (21). Elevated acrolein levels can also occur endogenously in the liver due to the metabolism of (i) allyl alcohol, which is widely used in food flavoring and fire retardant industries and is a well-known hepatotoxicant; (ii) anti-cancer drugs such as cyclophosphamide; and (iii) carbohydrates, lipids, polyamines, and hydroxyl-amino acids (reviewed in 21). In this study, we find that non-cytotoxic concentrations of acrolein can dramatically inhibit IFN α antiviral activity. Hence, although endogenous hepatic acrolein may not reach high cytotoxic levels, the effects on certain signaling pathways may be profound, and may lead to major defects with important disease consequences.

Acrolein has been linked to various disease conditions that exhibit enhanced lipid peroxidation and oxidative stress, including renal failure (30), Alzheimer's disease (29, 35), diabetes (31), atherosclerosis (30, 36, 37). Our study suggests that HCV is another disease wherein acrolein may play a critical role in determining disease progression and therapy outcomes. Recent studies correlate elevated oxidative stress and lipid peroxidation in the pathogenesis of HCV (18, 19, 20). A role for oxidative stress in HCV is also supported by the fact that antioxidant therapy improves liver injury in some HCV patients (38). Indeed, the reported correlation between elevated oxidative stress/lipid peroxidation and HCV pathogenesis may be attributable, at least in part, to increases in acrolein concentrations. Importantly, acrolein adducts (specifically, acrolein-FDP-lysine adducts) have been used as a disease correlate in Alzheimer's disease (29, 35), and type 2 diabetes (31). We are currently evaluating whether or not elevated serum levels of acrolein adducts are prognostic for poor IFN α antiviral function; these acrolein-adduct levels may serve as a potential biomarker for non-response IFN α therapy in HCV.

Acrolein is a major component of cigarette smoke (25–140 μ g/cigarette). The adverse effects of smoking on the liver have recently been reviewed by El-Zayadi, and these include inflammation, apoptosis, excess iron deposition and oxidative stress and increased LPO (39). There is a growing association between smoking and severity of HCV, and associated HCC (40–44). Moreover, El-Zayadi et al. recently reported that smokers suffering from HCV tend to have a lower response rate to IFN α therapy (13). The concentrations of the acrolein metabolite S-(3-hydroxypropyl)mercaptopuric acid (HPMA) in the urine of smokers is shown to be 4-fold higher than in nonsmokers, where it is about 422 μ g/L (45), corresponding to a concentration of approximately 7.5 μ M of acrolein. Also, endogenous generation of acrolein in humans due to LPO is $\sim$ 10-fold higher than that of HNE. Undoubtedly, localized tissue-specific concentrations of acrolein at sites of generation or exposure are likely to be higher, particularly in disease conditions. Hence, the concentrations of acrolein used in this study are within the range of human exposure and levels of acrolein that are associated with pathological states. Our data demonstrate the direct inhibitory effects of acrolein on IFN α antiviral signaling in hepatocytes. The effects were seen at low, non-toxic levels of acrolein, suggesting that they are independent of apoptosis and the hepatotoxicity associated with acrolein. Thus, in addition to significantly contributing to

cigarette smoke-induced inflammation and hepatic injury, acrolein may directly lower response to IFN α therapy, thereby promoting HCV disease progression.

In our study, we observed that acrolein up regulated the catalytic activity of both tyrosine and, to a lesser degree, serine/threonine phosphatases, suggesting one possible mechanism of inhibition of IFN α signaling by acrolein. Interestingly, another LPO aldehyde, 4-hydroxynonenal (HNE) is known to activate the serine/threonine phosphatase PP2A, albeit in a different cell type (46). We saw a 1.5-fold increase of ser/thr phosphatase activity and an increase in tyr-phosphatase of almost 2.5-fold. Since both sodium orthovanadate and sodium fluoride only partially reversed the effects of acrolein in this study, the exact mechanism of acrolein inhibition of IFN α antiviral activity may be multifactorial. Interestingly, oxidative stress induced by hydrogen peroxide inhibits IFN α mediated JAK–STAT signaling without activation of phosphatases (14). In seeming contrast to our observations, a recent report has demonstrated that acrolein, in fact, inactivates a tyrosine phosphatase (47). The authors observed that in vitro exposure to acrolein of the purified catalytic subunit of human protein tyrosine phosphatase 1B resulted in its potent time-dependent inactivation. However, the concentration of acrolein required to achieve a half-maximal rate of inactivation, K_i , was determined to be $2.3 \pm 0.6 \times 10^{-4}$ M, which is almost 10-fold higher than the effective acrolein concentration used here (25 μ M); this may explain the divergence of the results. Further studies using specific siRNA inhibition will be needed to unequivocally establish the contribution of phosphatases in the inhibition of IFN α antiviral function by acrolein.

Overall, our findings suggest that inhibition of the IFN α mediated JAK/STAT pathway due to increased acrolein concentrations in the liver could be one of the mechanisms explaining the resistance to antiviral action of IFN α in HCV infected patients with complicating conditions associated with high level of oxidative stress and LPO in the liver, such as advanced age, alcohol, iron overload, steatosis, fibrosis, as well as exposure to environmental and other hepatotoxins. Further studies are necessary to elucidate the exact mechanisms involved in the inhibition of IFN α signaling by acrolein in hepatocytes, and to determine whether or not acrolein adducts can serve as a biomarker for poor response to HCV therapy.

Acknowledgments

This research was supported by NIH grants (McClain and S Barve), VA (McClain) and AASLD-Sheila Sherlock Award (Cave).

List of Abbreviations

HCV	Hepatitis C virus
IFNα	interferon-alpha
JAK	Janus kinase
STAT	signal transducer and activator of transcription
ISGF	interferon-stimulated gene factor
ISG	interferon-stimulated genes
ISRE	interferon- α -stimulated response elements
IRF	interferon regulatory factor
OAS	2'5'-oligoadenylate synthetase 1

PKR	dsRNA-dependent protein kinase
GSH	glutathione
ELISA	Enzyme linked immunosorbant assay
Mrna	messenger ribonucleic acid
RT-PCR	real time polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
LPO	lipid peroxidation, IFNAR1, Interferon-alpha receptor 1 and IFNAR2, Interferon-alpha receptor 2

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Fig 1. Effect of Acrolein on Cell Survival

HepG2, HCV replicon cells or primary human hepatocytes were untreated (UT) or exposed to acrolein (2.5 μ M - 100 μ M) for 30min in serum free media, after which serum was added back with further incubation for 24h. Cell viability was measured by MTT assay. Data are normalized to UT values and expressed as % UT. Data represent mean $\pm$ S.D. (n=3). * = p<0.05, compared with UT.

Fig 2A **Fig 2B**

Fig. 2. Acrolein dose dependently downregulates tyrosine phosphorylation of STAT1 (A) and STAT2 (B) in HepG2 cells
 HepG2 cells were untreated (UT), pre-treated with 0, 5, 10, 25 or 50 μ M acrolein in serum-free media for 30 minutes and then stimulated with 1000 U/mL IFN α for 30 minutes. Equal amounts of cytoplasmic (CE) or nuclear lysates (NE) were analyzed by SDS-PAGE followed by immunoblotting with specific antibodies against tyrosine-phosphorylated STAT1 (A) or STAT2 (B), with total STAT1 and STAT2 and β -actin as controls.

Fig. 3. Acrolein dose dependently inhibits IFN α inducible ISRE-luciferase activity in HepG2 cells
HepG2 cells transiently transfected with an ISRE-luciferase reporter construct were untreated (UT), stimulated with 1000 U/mL IFN α for 6h (IFN), or pre-treated with 5, 10, 25, 50 μ M acrolein in serum-free media for 30 minutes and then stimulated with 1000 U/mL IFN α for 6h (A-5+I thru A-50+I). Total cell extracts were analyzed for luciferase activity. Data are presented as mean $\pm$ SD, n=3. * = p<0.05, compared with IFN alone.

Fig 4A

Fig 4B **Fig 4C** **Fig 4D**

Fig. 4. Acrolein dose-dependently inhibits IFN α inducible gene expression of antiviral genes in HepG2 cells

Messenger RNA levels of PKR (A), OAS (B), ISG15 (C), and ISG54 (D) were determined by real time PCR using total RNA from HepG2 cells that were untreated (UT), stimulated with 1000 U/mL IFN α for 2h (IFN), or pre-treated with acrolein (5uM-100uM) for 30 minutes followed by 1000U/mL IFN α for 2h (A10+IFN – A100+IFN). Data are presented as mean $\pm$ SD, n=3. * = p<0.05, compared with IFN alone.

Fig 5A **Fig 5B**

Fig 5. Effect of acrolein on IFN α -inducible antiviral proteins PKR and OAS in HepG2 cells
HepG2 cells were untreated (UT), pre-treated with 25 μ M acrolein in serum-free media for 30 minutes and then stimulated with 1000 U/mL IFN α for 24h. Equal amounts of total cellular extracts were analyzed by SDS-PAGE followed by immunoblotting with specific antibodies against OAS or PKR, and with β -actin as control.

Fig 6A **Fig 6B**

Fig 6C

Fig. 6. Acrolein dose-dependently inhibits IFN α inducible gene expression of antiviral genes in primary human hepatocytes

Messenger RNA levels of ISG15 (A), ISG54 (B) and OAS (C) were determined by real time PCR using total RNA from primary human hepatocytes that were untreated (UT), stimulated with 1000 U/mL IFN α for 2h (IFN), or pre-treated with acrolein (25uM) for 30 minutes followed by 1000U/mL IFN α for 2h (A25+IFN). Data are presented as mean $\pm$ SD, n=3. * = $p < 0.05$, compared with IFN.

Fig 7

Fig. 7. Non-toxic concentrations of acrolein dose-dependently reverse antiviral effects of IFN α on HCV Replicon

Huh7 cells expressing the HCV replicon were untreated (UT), stimulated with 100U/mL IFN α for 2h (IFN), or pre-treated with acrolein (5uM or 10uM) for 30 minutes followed by 100U/mL IFN α for 2h (A5+IFN and A10+IFN). Total cell RNA was isolated and subjected to real time PCR using HCV specific primers, with GAPDH for normalization. Data are presented as mean $\pm$ SD, n=3. * = p<0.05, compared to IFN.

Fig 8A

Fig 8B

Figure 8A. Acrolein increases protein phosphatase activity in HepG2 cells

HepG2 cells were incubated with 25 μ M acrolein for 0.5h or 1h. Serine/threonine- (white bars) and tyrosine- (black bars) phosphatase activity was measured from total cell extracts as described in Materials and Methods. Parallel assays were conducted in the presence of inhibitors 100 μ M sodium orthovanadate (Van), a tyrosine phosphatase inhibitor and 100 μ M sodium fluoride (NaF), a serine/threonine phosphatase inhibitor. Each column represents the mean $\pm$ S.D. of triplicate assays. (*= p<0.05 compared to No inhibitor-0h; # = p<0.05, compared to No inhibitor-30min; ** = p<0.05 compared to 1h-No inhibitor).

Protein phosphatase inhibitors partially reverse the effect of acrolein.

Messenger RNA level of OAS was determined by real time PCR using total RNA from HepG2 cells that were untreated (UT), stimulated with 1000 U/mL IFN α for 2h, without (IFN) or with pre-treated for 30 minutes with 25 μ M acrolein (A25+IFN). Phosphatase inhibitors sodium fluoride and sodium orthovanadate were added 30min prior to 25 μ M

acrolein, followed by 1000 U/mL IFN α for 2h (NaF+A+I and Van+A+I, respectively). Data are presented as mean $\pm$ SD, n=3. * = p<0.05, compared to A25+IFN.