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PPAR Agonist Effects on Notch Signaling Mediators in Experimental Chronic Alcohol-Induced Steatohepatitis

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Abstract

Background: Aspartyl-Asparaginyl-B-Hydroxylase (ASPH) is a downstream target of insulin and IGF signaling and promotes cell motility for liver remodeling and repair. ASPH functions in part by activating Notch and HIF-1α. PPAR agonists can ameliorate steatohepatitis, hepatic insulin resistance, and reduced ASPH expression in experimental alcoholic liver disease. Herein, we examine the effects of PPAR-α, PPAR-δ, and PPAR-γ agonists on Notch and HIF-1α signaling.

Methods: Long Evans rats were chronically fed control or ethanol-containing diets and treated with vehicle, or a PPAR-α, PPAR-δ, or PPAR-γ agonist. ASPH, Notch, and HIF-1α-related genes and proteins were measured in liver.

Results: ASPH, Notch, and HIF-1α signaling genes and/or proteins were inhibited by chronic ethanol feeding. PPAR-δ and/or PPAR-γ agonists normalized ASPH, HIF-1α, and PCNA protein in ethanol-exposed livers. In contrast, Notch signaling through HES-1 was not restored.

Conclusion: Therapeutic effects of PPAR-δ and PPAR-γ agonists in alcoholic liver disease are mediated by post-translational mechanisms that bolster ASPH-HIF-1α signaling. Alternative strategies are needed to circumvent ethanol-mediated uncoupling of cross-talk among insulin/IGF-1/ASPH-Notch networks.

Keywords: Alcoholic liver disease; Aspartyl-Asparaginyl-B-Hydroxylase; Notch; PPAR agonists; HIF-1α

Introduction

Alcohol abuse is a leading cause of liver-related morbidity and mortality [1,2]. Excessive chronic or binge alcohol consumption is a major risk factor for progressive Alcoholic Liver Disease (ALD), which is mediated by combined effects of insulin and Insulin-Like Growth Factor (IGF) resistance [3-5], inflammation [6,7], mitochondrial dysfunction, and oxidative and Endoplasmic Reticulum (ER) stress [8,9]. In the most severe cases, ALD can advance to cirrhosis with attendant liver failure and/or hepatocellular carcinoma [10]. Ethanol disrupts insulin/IGF receptor binding and tyrosine kinase activation, tyrosine phosphorylation of Insulin Receptor Substrate-1 (IRS-1), and downstream signaling through mitogen-activated protein kinase, which promotes liver growth and repair, and Phosphoinositide-3 Kinase (PI3K), which drives metabolism, cell survival, and motility. Ethanol further impairs insulin/IGF signaling by increasing expression of negative regulators of PI3K-Akt [11].

Aspartyl-Asparaginyl-B-Hydroxylase (ASPH) is a downstream target of insulin/IGF signaling, and has functional roles in cell adhesion and motility [12-14]. ASPH is a ~86 kD Type 2 transmembrane protein located in the Endoplasmic Reticulum (ER) [15,16]. Intracellularly, ASPH is physiologically cleaved yielding N-terminal and C-terminal fragments. The N-terminal fragment is virtually identical to Humbug, a truncated isoform that supports cell adhesion through regulation of Ca2+ flux from intracellular stores [17,18]. The C-terminal fragment contains a catalytic domain that promotes cell adhesion and motility by hydroxylating Epidermal Growth Factor (EGF)-like domains of Notch signaling proteins [13,14,19-22].

Notch signaling proteins, Notch-1 and Jagged-1, have demonstrated roles in cell migration, differentiation, and adhesion [23]. ASPH activates their networks by hydroxylating Notch-1 and, its ligand, Jagged-1 at their EGF-like domains. Binding of Jagged (or Delta-like family of proteins) to Notch's extracellular domain triggers two sequential cleavage events. The ectodomain of Jagged is cleaved by an A Disintegrin and Metalloprotease (ADAM) 17-like activity [24,25]. The second cleavage event occurs within the transmembrane domain of Notch, and is triggered by a complex formed between γ-secretase and prenisilin proteases [24]. These events cause the Notch Intracellular Domain (NID) to translocate from the plasma membrane to the nucleus where the NID removes co-repressors and recruits transcriptional co-activators to specific DNA sequences. The end results include transcriptional activation of target genes such as hairy and enhancer of split-1 (HES-1) and HES-related proteins [26,27]. These networks are important for maintaining the integrity of insulin/IGF, ASPH, and Notch cross-talk needed to promote cell migration for liver remodeling and regeneration.

Peroxisome Proliferator Activated Receptors (PPARs) are transcription factors and nuclear receptors that heterodimerize with retinoid x receptors and bind to peroxisome proliferator response elements throughout the genome. Complex binding activates

transcription of genes involved in various biological functions, including lipid metabolism [28,29]. PPARs are expressed as three isoforms: PPAR-α, PPAR-δ, and PPAR-γ. PPAR-α plays an essential role in β-oxidation of fatty acids, and is expressed in liver as well as kidney, heart, skeletal muscle, and brown adipose tissue [30]. PPAR-α is naturally activated by polyunsaturated fatty acids and fibrates. PPAR-δ regulates lipid catabolism and oxidative phosphorylation, and is mainly expressed in muscle, kidney, heart, and liver [31]. Natural ligands include saturated and unsaturated fatty acids. PPAR-γ promotes adipocyte differentiation and has a role in lipogenesis [32]. PPAR-γ is primarily expressed in adipose tissue and activated by polyunsaturated fatty acids [33]. Thiazolidinediones, a class of synthetic PPAR-γ agonists with insulin-sensitizing properties, have been approved by the Food and Drug Administration for treatment of type 2 diabetes [34].

We previously showed PPAR-δ and PPAR-γ agonists can ameliorate ethanol-induced hepatic insulin resistance and correspondingly restore liver structure [35-37] while normalizing ASPH expression. Since ASPH promotes cell migration via activation of Notch [19,22,38,39], we extended our studies by evaluating the effects of different PPAR agonists on ASPH and Notch signaling genes and proteins to determine if restored hepatic architecture was linked to the re-activation of Notch networks. In addition, we examined the effects of PPAR agonist treatments on hypoxia inducible factor-1α (HIF-1α), a positive regulator of ASPH, and Proliferating Cell Nuclear Antigen (PCNA), which mediates hepatocellular proliferation.

Materials and Methods

Reagents

Long Evans rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA). Liquid diets were from Bio Serv (Frenchtown, NJ, USA). PPAR-α (GW7647, 10 nM), PPAR-δ (L-165,041, 10 nM), or PPAR-γ (Fmoc-Leu, 20 μM) agonists were from Cal Biochem (Carlsbad, CA, USA). Bicinchoninic Assay (BCA) reagent kit was purchased from Pierce (Rockford, IL, USA), and reagents for duplex Enzyme-Linked Immunosorbent (ELISA) assays, including Amplex ultrared reagent and 4-Methylumbelliferyl Phosphate (MUP) were purchased from Invitrogen (Carlsbad, CA, USA). 96-well Fluoro Nuncmaxisorp and optiplates were purchased from Thermo Scientific (Rochester, NY, USA). Rabbit Polyclonal Antibody to Large Acidic Ribosomal Protein (RPLPO) was from Proteintech (Chicago, IL, USA). Alkaline phosphatase-conjugated streptavidin was from Vector Laboratories (Burlingame, CA, USA). The Spectra Max M5 Microplate Reader was from Molecular Dynamics (Sunnyvale, CA, USA). Graph Pad Prism 6 software was purchased from Graph Pad Software (San Diego, CA, USA).

Chronic ethanol exposure model

Adult (~220-250 g) male rats were fed with isocaloric liquid diets containing 0% (control) or 37% ethanol (9.2% v/v) for 8 weeks [35,40,41]. From Week 2 through the remainder of the experiment, rats in each group were given twice weekly intraperitoneal (i.p) injections of vehicle (saline), a PPAR-α (GW7647; 2.5 μg/Kg), a PPAR-δ (L-160,043; 2 μg/Kg), or a PPAR-γ (Fmoc-Leu; 1.8 mg/Kg) agonist. The PPAR doses, route of administration, and frequency were based on an established protocol [35,40] [7,36]. Upon sacrifice, portions of fresh liver (N=8/group) were frozen, and stored at -80°C. Rats were monitored daily to ensure proper nutritional intake and maintenance of body weight. The rats lived under humane conditions with 12-hour light/dark cycles, and free access to food. All experiments were conducted according to guidelines established by the National Institutes of Health and approved by the institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital.

Gene expression studies

Total RNA was isolated from liver with the EZ1 RNA Universal Tissue Kit and the BIO Robot EZ1 (Qiagen Inc., Valencia, CA, USA). RNA was reverse transcribed with random oligonucleotide primers and the AMV First Strand cDNA synthesis kit. The cDNAs were used to measure gene expression by qPCR analysis with gene-specific primers [39]. Primers were designed using Mac Vector 10 software (Mac Vector, Inc., Cary, NC, USA) and target specificity was verified using NCBI-BLAST (Basic Local Alignment Search Tool). The Master ep-Real plex instrument and software (Eppendorf AG, Hamburg, Germany) were used to detect amplified signals from triplicate reactions. Using the average CT values, the ng levels of mRNA or 18S rRNA were calculated from standard curves. Relative mRNA abundance was calculated from the ng ratios of mRNA to 18S rRNA in the same samples.

Duplex Enzyme-Linked Immunosorbent Assay (ELISA)

Liver tissue was homogenized in buffer (50 mM Tris pH 7.5, 150 Mm NaCl, 5 mM ethylene diaminetetra acetic acid pH 8.0, 50 mMNaF, 0.1% triton x-100) containing protease and phosphatase inhibitors [35]. Homogenates were centrifuged and clarified supernatants were used to measure immunoreactivity by ELISA. Protein concentrations were measured with the BCA assay. Immunoreactivity was measured by a duplex ELISA as previously described [42].

In brief, sample aliquots containing 100 ng proteins in 50 μl were adsorbed to the bottoms of maxisorp 96-well plates by overnight incubation, and then coated with 1% bovine serum albumin in TRIS buffered saline (TBS-BSA) to minimize non-specific binding. Primary antibodies (0.5-1 μg/ml) were applied overnight at 4°C, and immunoreactivity was detected with horseradish peroxidase-conjugated antibodies and the Amplex Ultra Red soluble fluorophore (Ex 530 nm/Em 590 nm). Subsequently, the samples were incubated with biotinylated RPLPO and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase and 4-MUP fluorophore (Ex 360 nm/Em 460 nm). Fluorescence was detected with the SpectraMax M5 microplate reader. Non-specific binding was assessed with parallel incubations in which the primary or secondary antibody was omitted. The calculated ratios of target protein/RPLPO fluorescence were used for inter-group statistical comparisons. All assays included least 6 biological replicates, in 4 technical replicates.

Statistical analysis

Inter-group statistical comparisons were made by two-way Analyses Of Variance (ANOVA) and the Fisher posttest using Graph Pad Prism 6 software.

Results

Effects of Ethanol and PPAR Agonist Treatments on ASPH-Notch Pathway Genes: Two-way ANOVA tests demonstrated significant effects of ethanol on ASPH, Jagged-1, and HES-1 expression,

significant effects of PPAR agonist treatments on ASPH, HES-1 and HIF-1α expression, and ethanol x PPAR interactive effects on ASPH, HES-1, and FIH expression (Table 1). In contrast, no significant ethanol or PPAR agonist effects were observed with respect to Notch-1.

	Ethanol Effect		PPAR Agonist Effect		Ethanol x PPAR Effect	
mRNA	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
ASPH	27.86	<0.0001	6.507	0.001	3.902	0.015
Notch-1	0.641	N.S.	1.061	N.S.	0.101	N.S.
Jagged-1	26.55	<0.0001	1.703	N.S.	0.649	N.S.
HES-1	20.01	<0.0001	3.993	0.014	11.92	<0.0001
FIH	1.9	N.S.	1.94	N.S.	9.288	<0.0001
HIF-1α	0.408	N.S.	3.224	0.032	1.869	N.S.

Table 1: PPAR-Agonist Treatment Effects on Notch Pathway Gene Expression in Experimental Chronic Alcohol-Induced Liver Injury: Two-Way ANOVA Summary. Adult Long Evans rats were chronically fed isocaloric control or ethanol-containing liquid diets. Rats in each group were also treated with vehicle (saline) or a PPAR agonist. Gene expression data were analyzed by two-way ANOVA and the Fisher multiple comparison posttest. See text for abbreviations.

ASPH expression was significantly reduced in vehicle-treated, ethanol exposed relative to control livers (P<0.0001). Among controls, PPAR-δ or PPAR-γ agonist treatments significantly reduced hepatic ASPH expression relative to vehicle (Figure 1A), whereas in the ethanol groups, PPAR agonist treatments had modest (PPAR-α) or no appreciable effects on hepatic ASPH expression. Consequently, the PPAR agonist treatments reduced (PPAR-α, PPAR-δ) or abolished (PPAR-γ) the magnitude of inter-group differences in hepatic ASPH mRNA due to inhibition of ASPH expression in controls (Figure 1A).

Notch-1 expression was similar for control and ethanol exposed livers, and no significant differences occurred with respect to PPAR agonist treatments (Figure 1B).

Jagged-1 mRNA expression was significantly reduced in livers of ethanol-fed, vehicle-, PPAR-α, or PPAR-γ agonist treated rats relative to corresponding controls (Figure 1C). In contrast, the PPAR-δ agonist increased hepatic expression of Jagged-1 in the ethanol group, rendering the levels similar to control. Therefore, the PPAR-δ agonist treatments rescued ethanol-mediated inhibition of Jagged-1 expression.

HES-1 expression was similar in vehicle-treated control and ethanol-exposed livers. However, treatment with the PPAR-α or PPAR-δ agonist selectively increased HES-1 in control but not ethanol-exposed livers, rendering the inter-group differences highly statistically significant. In contrast, hepatic HES-1 mRNA levels were unaffected by the PPAR-γ agonist treatments (Figure 1D).

Hepatic FIH expression was significantly higher in vehicle-treated, ethanol-fed relative to control rats (Figure 1E). In controls, the PPAR-α, PPAR-δ, and PPAR-γ agonists similarly increased hepatic FIH expression, whereas in ethanol-fed rats, the PPAR-α and PPAR-δ agonists had no significant effect on hepatic FIH, and the PPAR-γ agonist significantly reduced FIH expression (P<0.01).

Although hepatic HIF-1α expression varied within group, between-group differences were not statistically significant, i.e. control and ethanol-exposed livers within each treatment group had similar mean levels of HIF-1α mRNA. However, among controls, the PPAR-α agonist increased hepatic HIF-1α relative to the effects of PPAR-δ (P<0.01) and PPAR-γ (P<0.05) agonists (Figure 1F).

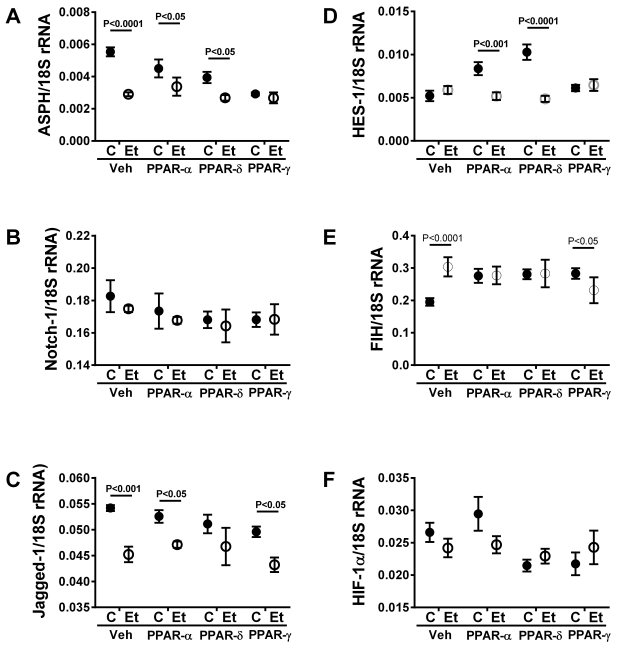

Figure 1: Effects of PPAR agonist treatments on hepatic expression of ASPH and Notch-pathway genes in experimental alcohol-related liver disease. Adult Long Evans rats were fed with isocaloric control or ethanol-containing liquid diets for 6 weeks, and treated with vehicle, or a PPAR-α, PPAR-δ, or PPAR-γ agonist by i.p. injection, twice weekly for the last 4 weeks of the feeding regimen. Liver RNA was reverse transcribed, and the resulting cDNAs were used in QPCR reactions to measure: A) ASPH, B) Notch-1, C) Jagged-1, D) HES-1, E) FIH, and F) HIF-1α. Gene expression was normalized to 18S rRNA measured in the same samples. Box plots depict the means (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (stems). Inter-group comparisons were made by two-way ANOVA (Table 1) with the Fisher posttest.

Differential PPAR agonist and ethanol effects on hepatic ASPH-Notch signaling proteins

Duplex ELISAs using the A85G6 and A85E6 monoclonal antibodies were respectively used to detect epitopes within the C-terminus and N-terminus of ASPH. Because the N-terminus of ASPH is nearly

identical to full-length Humbug, A85E6 also detects Humbug, whereas A85G6 is specific for ASPH. In addition, we used duplex ELISAs to measure Notch-1, Jagged-1, HIF-1α, and PCNA (Table 2 and Figure 2).

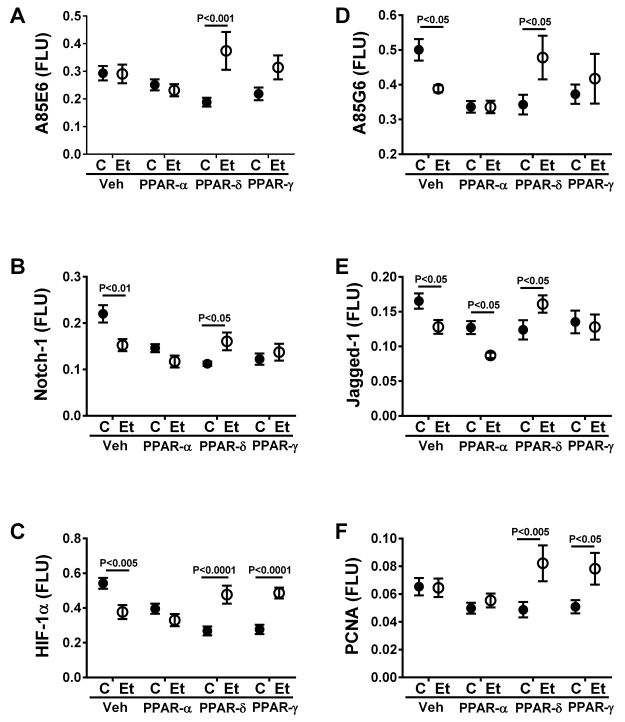

Figure 2: Effects of chronic ethanol exposure and PPAR agonist treatments on ASPH and Notch signaling molecules in liver. Rats chronically maintained on control or ethanol-containing (37%) liquid diets were treated with vehicle, or a PPAR-α, PPAR-δ, or PPAR-γ agonist. Livers homogenates were used to measure immunoreactivity to: ASPH using the A) A85E6 or B) A85G6 monoclonal antibodies, C) Notch-1, D) Jagged-1, E) HIF-1α), and F) proliferating cell nuclear antigen (PCNA) by duplex ELISAs. Results were normalized to levels of large ribosomal protein (RPLPO) measured in the same wells. Box plots depict the means (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (stems). Inter-group comparisons were made by two-way ANOVA (Table 2) with the Fisher post test.

Two- way ANOVA tests demonstrated significant ethanol effects on A85E6, Notch-1, and PCNA, and ethanol-driven statistical trends for HIF-1α expression (Table 2). Significant PPAR agonist effects occurred with respect to Jagged-1 and HIF-1α and a trend was observed with respect to A85G6 immunoreactivity. Significant ethanol x PPAR agonist interactive effects were observed with respect to A85G6, A85E6, Jagged-1, and HIF-1α, while trend effects occurred in relation to Notch-1 and PCNA expression (Table 2).

A85E6: Hepatic A85E6 immunoreactivity was somewhat reduced by PPAR agonist treatments, particularly PPAR-δ. Ethanol had no effect on hepatic A85E6 immunoreactivity, but treatment with the PPAR-δ agonist significantly increased and A85E6 immunoreactivity relative to control (Figure 2A).In contrast, PPAR-δ agonist treatments did not enhance A85E6 immunoreactivity relative to vehicle in ethanol-exposed livers.

A85G6: Among controls, A85G6 immunoreactivity was highest in the vehicle-treated group, and significantly suppressed (P<0.05-P<0.01) by the PPAR agonist treatments (Figure 2D). Ethanol+vehicle significantly reduced A85G6 immunoreactivity relative to control. PPAR-α and PPAR-δ agonist treatments had no significant effect on A85G6/ASPH expression, and because the levels were suppressed in corresponding controls, those inter-group differences were not statistically significant. In contrast, the PPAR-δ agonist significantly increased A85G6 immunoreactivity in the ethanol group, rendering the difference statistically significant from the paired control group.

Notch-1: Notch-1 immunoreactivity was highest in the vehicle-treated control livers. All 3 PPAR agonist treatments significantly reduced Notch-1 expression among controls (P<0.05) (Figure 2B). Chronic ethanol feeding significantly inhibited Notch-1 expression, and that effect was not rescued by any of the PPAR agonist treatments. However, due to greater degrees of PPAR-δ agonist suppression of Notch-1 expression in controls, the inter-group differences were statistically significant.

	Ethanol Effect		PPAR Agonist		Ethanol x Agonist	
Protein	**F-Ratio**	**P-Value**	**F-Ratio**	**P-Value**	**F-Ratio**	**P-Value**
ASPH/A85G6	0.37	N.S.	2.67	0.06	3.45	0.025
ASPH+Humbug/A85E6	6.65	0.013	0.768	N.S.	3.6	0.021
Notch-1	9.07	0.004	1.29	N.S.	2.33	0.089
Jagged-1	1.78	N.S.	4	0.014	4.05	0.013
HIF-1α	3.61	0.065	3.24	0.032	15.21	<0.0001
PCNA	9.07	0.004	1.29	N.S.	2.33	0.089

Table 2: PPAR-Agonist Treatment Effects on Notch Pathway Protein Expression in Experimental Chronic Alcohol-Induced Liver Injury: Two-Way ANOVA Summary. Adult Long Evans rats were chronically fed isocaloric control or ethanol-containing liquid diets. Rats in each group were also treated with vehicle (saline) or a PPAR agonist. Protein expression data were analyzed by two-way ANOVA and the Fisher multiple comparison posttest. See text for abbreviations.

Jagged-1: In control rats, Jagged-1 expression was highest in vehicle-treated rats as its expression was broadly inhibited by the PPAR agonists (all P<0.05) (Figure 2E). Jagged-1 expression was significantly reduced by ethanol, and further lowered by treatment with the PPAR-α agonist. In contrast, the PPAR-δ agonist slightly increased Jagged-1 immunoreactivity, but the suppressive effects in controls rendered the inter-group difference statistically significant. The PPAR-γ agonist had no effect on Jagged-1 immunoreactivity in ethanol-exposed livers, but the suppressive responses in controls caused the levels to be similar.

HIF-1α: Among controls, HIF-1α protein expression was highest with vehicle treatment, and relatively suppressed by the PPAR agonists (P<0.005) (Figure 2C). In the ethanol groups, vehicle or PPAR-α agonist treatment resulted in similarly low levels of HIF-1α immunoreactivity, whereas treatment with the PPAR-δ or PPAR-γ

agonist nearly normalized hepatic HIF-1α expression. Due to their suppressive effects in controls, the levels of HIF-1α were significantly higher in the PPAR-δ and PPAR-γ agonist treated, ethanol exposed livers relative to corresponding controls.

PCNA: Hepatic PCNA expression was similar for control and ethanol exposed vehicle-treated rats (Figure 2F). Among controls, PPAR agonist treatments caused modest (non-significant) reductions in PCNA expression. Similarly, the PPAR-α agonist modestly reduced PCNA expression in ethanol-exposed livers. In contrast, the PPAR-δ and PPAR-γ agonist treatments significantly increased PCNA expression in the ethanol-exposed relative to corresponding controls, but not relative to ethanol+vehicle. Again, those inter-group differences were effectuated by the combined stimulatory responses in the ethanol groups and suppressive responses among controls.

Discussion

Progressive alcoholic liver disease is partly mediated by sustained insulin resistance [43-45] because ethanol disrupts insulin/IGF signaling at various steps in the cascade. Moreover, ethanol-mediated perturbations in membrane lipid composition impair insulin receptor binding and receptor tyrosine kinase activation [11], which are the most proximal steps in the pathway. Ethanol's inhibition of growth, survival, and metabolic functions is linked to reduced IRS-1 phosphorylation and attendant activation of PI3K-Akt [4,5,41,46,47], together with increased expression and activation of negative regulators of PI3K-Akt, e.g. PTEN [48,49]. Previous studies tested the hypothesis that hepatic insulin and IGF-1 resistance are pivotal in the pathogenesis of ALD by using insulin sensitizers to abrogate the adverse effects of ethanol. Those studies showed that PPAR-δ more than PPAR-γ agonists can restore ethanol-impaired insulin/IGF signaling and resolve many aspects of ALD histo- and ultrastructural pathology [35,36,40,50]. Importantly, these therapeutic responses were associated with increased expression of the insulin/IGF responsive ASPH, which is inhibited by ethanol [35]. We postulated that the PPAR agonist improvements in hepatic function and structure were likely mediated by ASPH's positive effects on cell motility and adhesion, which are needed for hepatocellular remodeling after injury [21,22,39].

ASPH's functions are likely due, in part, to the activation of Notch signaling networks since: 1) ASPH physically interacts with Notch-1 and its natural ligand, Jagged-1 [38], which have consensus sequences for ASPH hydroxylation [51]; 2) Notch-1 and Jagged-1 have established roles in cell adhesion and migration [52]; and 3) high levels of ASPH increase Notch-1, Jagged-1, and HES-1 expression, while reduced levels of ASPH have opposite effects [19,22]. Furthermore, Insulin-IGF-1/ASPH/Notch expression and signaling have been linked to HIF-1α expression and function [19,22,39]. Of further note is that FIH negatively regulates HIF-1α via hydroxylation [53], raising the possibility that hydroxylating networks are important regulators of liver structure and function. The present work extends our investigations into the uncoupling of cross-talk mechanisms among insulin/IGF-ASPH, Notch, and HIF-1α in chronic ALD, and the potential utility of PPAR agonists for restoring the integrity of these signaling networks.

The qRT-PCR analyses demonstrated ethanol-inhibition of ASPH and Jagged-1 expression, and an ethanol-mediated increase in FIH. In contrast, the mRNA levels of Notch-1, HES-1, and HIF-1α were similar in control and ethanol-exposed livers. In control livers, the main effects of PPAR-δ and PPAR-α agonist treatments were to down-regulate ASPH and stimulate HES-1 and FIH, whereas in the ethanol group, PPAR agonist treatments had almost no effect on mRNA levels corresponding to ASPH, the Notch pathway, or HIF-1α/FIH. Therefore, nearly all of the "normalizing" effects of PPAR agonist treatments on mRNA expression were due to responses in control livers or broadening of statistical variance, causing the means to overlap. More important, the agonist stimulation of HES-1 and FIH, and inhibition of ASPH reflect coupling of insulin responsive genes and mechanisms to Notch and HIF-1α networks in control livers, but general uncoupling of such responses in chronic ethanol-exposed livers. Therefore, any rescue effects of PPAR agonist insulin sensitizing agents with respect to ALD are not likely to be effectuated by changes in gene expression within these pathways. Instead, alternative mechanisms must be investigated.

The protein-based studies confirmed previous findings with respect to ethanol-inhibition of ASPH and Humbug, and further demonstrated ethanol inhibition of Notch-1, Jagged-1, and HIF-1α. It is noteworthy that in control livers, the PPAR agonist treatment either had modest or suppressive effects on proteins studied, whereas in the ethanol-exposed livers, stimulatory/supportive effects occurred mainly with respect to the PPAR-δ and PPAR-γ agonists, whereas the PPAR-α agonist appeared to exacerbate the trends of ethanol+vehicle. Since these responses were not always associated with similar changes (magnitude or direction) in gene expression, post-transcriptional mechanisms were likely involved. Enhanced insulin/IGF-1 signaling through PI3K-Akt, together with the anti-oxidant effects of PPAR agonists, serves to reduce GSK-3β activity, and GSK-3β phosphorylation decreases ASPH protein expression [54]. Therefore, the PPAR agonist-mediated increases in ASPH/Humbug immunoreactivity could have been mediated by reductions in the levels of GSK-3β activity and ASPH/Humbug phosphorylation.

PPAR agonist effects on Notch-1 and Jagged-1 mRNAs were either neutral or slightly inhibitory in control and ethanol-exposed livers. However, at the protein level, PPAR agonists had decisively inhibitory effects on Notch-1 and Jagged-1 in controls, and varied effects in ethanol-exposed livers. With ethanol exposure, Notch-1 and Jagged-1 proteins were further suppressed by the PPAR-α agonist, and supported or slightly stimulated by the PPAR-δ and PPAR-γ agonists. Overall, it seems unlikely that these relatively modest changes in Notch-1 and Jagged-1 expression would account for the considerable therapeutic responses observed in previous studies. Instead, we postulate that the normalization of ASPH expression is critical in ethanol-exposed livers.

In control livers, the PPAR-α and PPAR-δ agonists enhanced expression of HES-1, which is consistent with the concept that insulin/IGF-1 signaling pathways cross-talk and activate Notch networks. However, chronic ethanol exposure abrogated this response. One interpretation of these findings is that ethanol-induced hepatic insulin resistance uncouples insulin/IGF-1/ASPH/Notch-1 cross-talk mechanisms needed to stimulate expression of target genes.

In control livers, FIH mRNA and HIF-1α protein were reciprocally modulated by PPAR agonist treatments, such that HIF-1α expression and activity were inhibited relative to vehicle treatment. In addition, HIF-1α mRNA was inhibited by PPAR-δ and PPAR-γ treatments. The significance of these responses is not entirely clear; nonetheless they do support the concept that HIF-1α expression and signaling cross-talk through insulin regulated networks. Ethanol reciprocally inhibited HIF-1α protein and stimulated FIH mRNA. Although FIH and

HIF-1α mRNAs were unaffected by the PPAR agonist treatments, at the protein level, HIF-1α and PCNA protein were both stimulated by PPAR-δ and PPAR-γ agonists. HIF-1α's role in cell motility/migration, its regulation by insulin/IGF, and its cross-talk with ASPH have been well described [39,55,56]. Therefore, ethanol-mediated suppression of HIF-1α protein corresponds with its inhibitory effects on insulin/IGF-1 stimulated ASPH and cell motility [14,19,54,57]. The therapeutic rescue effectuated by PPAR-δ and PPAR-γ agonists in ALD [35,36,40] correlate with the increases in ASPH, HIF-1α, and PCNA protein expression rather than Notch pathway activation. This suggests that insulin sensitizer treatments do not reverse ethanol-mediated uncoupling of the insulin/ASPH/Notch pathway, but instead utilize alternative mechanisms via HIF-1α to restore liver function. These finding offer new approaches for treating chronic ALD. Future studies will extend this work by identifying additional pathways that cross-talk with insulin/IGF-1/IRS-1 to modulate cell motility and remodeling, e.g. Wnt/β-catenin [58-60], and also are responsive to PPAR agonists.

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