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Chronic alcohol-induced hepatic insulin resistance and ER stress ameliorated by PPAR- δ agonist treatment

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Abstract

Background—Chronic alcoholic liver disease is associated with hepatic insulin resistance, dysregulated lipid metabolism with increased toxic lipid (ceramide) accumulation, lipid peroxidation, and oxidative and endoplasmic reticulum (ER) stress. Peroxisome-proliferator activated receptor (PPAR) agonists are insulin sensitizers that can restore hepatic insulin responsiveness in both alcohol and non-alcohol-related steatohepatitis. Herein, we demonstrate that treatment with a PPAR- δ agonist enhances insulin signaling and reduces the severities of ER stress and ceramide accumulation in an experimental model of ethanol-induced steatohepatitis.

Methods—Adult male Long Evans rats were pair fed with isocaloric liquid diets containing 0% or 37% ethanol (caloric) for 8 weeks. After 3 weeks on the diets, rats were treated with vehicle or PPAR- δ agonist twice weekly by i.p. injection.

Results—Ethanol-fed rats developed steatohepatitis with inhibition of signaling through the insulin and insulin-like growth factor-1 receptors, and Akt activated pathways. Despite continued ethanol exposure, PPAR- δ agonist co-treatments increased Akt activation, reduced multiple molecular indices of ER stress and steatohepatitis.

Conclusions—These results suggest that PPAR- δ agonist rescue of chronic alcoholic liver disease is mediated by enhancement of insulin signaling through Akt/metabolic pathways that reduce lipotoxicity and ER stress.

Keywords

alcoholic liver disease; experimental model; PPAR agonists; steatohepatitis; insulin resistance; ER stress; ceramides

Introduction

Alcohol abuse is a leading cause of morbidity and mortality from chronic liver disease.

Alcohol-induced liver disease (ALD) can progress from simple steatosis to steatohepatitis,

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fibrosis, and then cirrhosis, ultimately culminating in liver failure or hepatocellular carcinoma¹. Chronic ALD is mediated by combined effects of insulin resistance^{2–8} and toxic injury^{9, 10}. Hepatic insulin resistance is caused by defects in intracellular¹¹, including impairments in receptor binding and receptor tyrosine kinase activation^{2, 3}. Ethanol also inhibits tyrosine phosphorylation of insulin receptor substrate (IRS) proteins that are needed to transmit insulin and insulin-like growth factor (IGF) receptor signals^{6, 11}. Consequently, chronic ALD inhibits Erk mitogen-activated protein kinase (MAPK), which mediates DNA synthesis and liver regeneration, and phosphatidylinositol-3-kinase (PI3 Kinase)¹⁰, which promotes cell growth, survival, glucose utilization, and energy metabolism^{11, 12}. Hepatotoxic effects of alcohol are mediated by inflammation, oxidative stress, mitochondrial dysfunction, and acetaldehyde-induced adduct formation¹⁰. Therefore, combined effects of chronic insulin/IGF resistance and hepatotoxicity lead to sustained impairments in cell survival, energy metabolism, and DNA synthesis, and promote injury and cell loss through increased DNA damage, oxidative stress, and apoptosis^{5, 11}. Moreover, inflammation with activation of stellate cells promotes liver fibrosis, exacerbating chronicity and progression of ALD¹³.

Insulin resistance impairs lipid homeostasis, promotes lipolysis¹⁴, and increases toxic lipids, including ceramides, which further compromise insulin signaling¹⁵ via inhibition of PI3K-Akt^{16, 17}. Insulin resistance drives endoplasmic reticulum (ER) stress because vital ER functions, i.e. protein synthesis, modification, and folding, calcium signaling, and lipid biosynthesis¹⁸ utilize glucose for energy, and insulin resistance impairs glucose utilization. Ethanol promotes hepatocellular injury and death via all 3 major ER stress sensor cascades: PERK, IRE-1 α , and ATF6^{19–22}. In addition, ethanol increases ER resident sterol regulatory binding proteins (SREBP)-1c and 2, up-regulating fatty acid/triglyceride synthesis, beta oxidation (SREBP-1a), and cholesterol synthesis (SREBP2)²³. ER stress potentiates insulin resistance and lipolysis leading to increased ceramide production^{19, 20, 22, 24} and worsening of inflammation and insulin resistance^{23, 25}. Therefore, it is not surprising that ER stress in ALD is marked by lipid dyshomeostasis and activation of pro-ceramide and pro-inflammatory pathways that increase toxic lipid generation^{19, 20, 22, 25, 26}.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that regulate gene transcription^{27, 28}. PPARs mediate their effects by heterodimerizing with retinoid $\times$ receptor²⁷. Three isoforms of PPARs exist: PPAR- α , PPAR- δ (also PPAR- β), and PPAR- γ . PPAR- α is abundantly expressed in brown adipose tissue and liver, and activated by polyunsaturated fatty acids and fibrates. PPAR- α regulates adipocyte growth, differentiation, lipid metabolism, lipoprotein synthesis, and tissue inflammatory responses^{27–29}. PPAR- δ is most abundant in gut, kidney and heart. PPAR- δ mediates adaptive responses to the environment²⁸. PPAR- γ is mainly expressed in adipose tissue, colon, immune cells, and retina²⁷. PPAR- γ influences storage of fatty acids in adipose tissue by regulating lipogenic metabolic and transport pathways^{27–29}. PPAR-agonists have been used to treat insulin resistance in type 2 diabetes²⁹ and non-alcoholic fatty liver disease^{29, 30}, and prevent development of alcohol-induced steatohepatitis²⁷.

Previously, we found that PPAR- δ agonists could restore ethanol-impaired hepatic insulin/IGF resistance and liver regeneration in a rat model of chronic ALD^{4, 31}. These

effects were associated with enhanced signaling through PI3K, and reduced DNA damage, lipid peroxidation, and oxidative stress^{4, 31}. The present study extends our understanding of how PPAR- δ agonists mediate their therapeutic effects in chronic ALD. Moreover, given the roles lipotoxicity, ER stress, and mitochondrial dysfunction as mediators of ALD^{11, 12}, we examined therapeutic effects of PPAR- δ agonist treatments on hepatic insulin signaling through Akt, mechanisms of ceramide accumulation, and indices of ER stress.

Materials and Methods

Ethanol exposure model

Adult male Long Evans rats were pair-fed with isocaloric liquid diets containing 0% or 37% ethanol by caloric content (9.2% v/v) for 8 weeks^{3, 31}. After the first 3 weeks on the diets, rats were administered intra-peritoneal (i.p.) injections of saline or a PPAR- δ agonist (L-160,043; 2 μ g/Kg) on Mondays and Thursdays for the remaining 5 weeks^{4, 31}. Liver tissues harvested at the end of the experiment were snap-frozen and stored at -80°C , or fixed and processed for histology and electron microscopy (EM). Our protocol was approved by the Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital and conformed to guidelines set by the National Institutes of Health. Detailed methods are provided in supplementary (S) files.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays

RNA extracted from liver was reverse transcribed and the cDNAs were used in hydrolysis probe-based duplex qRT-PCR assays in which β -actin served as the internal control. β -actin primers were Y555-labeled, and the gene of interest probes were FAM-labeled. Gene-specific primer sequences and matched probes were determined with the ProbeFinder Software (STable 1). PCR amplifications were performed in a LightCycler 480 PCR machine. Fluorescence signals corresponding to the genes of interest were acquired in the FAM Channel (Em: 520–530 nm), and the β -actin signal was acquired in the Y555/HEX channel (Em: 550–568 nm). Results were analyzed using LightCycler® Software 4.0.

Enzyme-linked immunosorbent assay (ELISA)

ER stress pathway activation was assessed by measuring PERK, pPERK [T980], EIF2 α , pEIF2 α , GADD34/PP1, C/EBP homologous protein (CHOP)/GADD153, p53, and Bax immunoreactivity by direct binding duplex ELISAs. Immunoreactivity was normalized to large ribosomal protein³². Protein of interest immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and Amplex UltraRed soluble fluorophore (Ex 565 nm/Em 595 nm). Binding of biotin-conjugated anti-large ribosomal protein (RPLPO) was detected with streptavidin-conjugated alkaline phosphatase and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore (Ex360/Em450). Alternatively, β -actin was used as the normalizing control. Fluorescence was measured in a SpectraMax M5 microplate reader. The ratio of specific protein/RPLPO (or β -actin) immunoreactivity was calculated for inter-group comparisons. Ceramide immunoreactivity was quantified by ELISA³².

Bead-based multiplex ELISA

We assessed the integrity of signaling through insulin and IGF-1 receptors, IRS-1, and Akt pathways using the Akt Total and Phospho 7-Plex Panels. The Akt Total 7-Plex panel measured: insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3 β (GSK-3 β). The Akt Phospho 7-Plex panel measured: pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246-PRAS40, pTpS421/424-p70S6K, and pS9-GSK3 β . Immunoreactivity was measured in a BioPlex 200.

Statistical Analysis

Box plots reflect the median (horizontal bar), 95% confidence interval limits (upper and lower boundaries of boxes), and range (whiskers). Inter-group comparisons were made by two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests. Statistics were performed with GraphPad Prism 5 software.

Results

Effects of ethanol and PPAR- δ agonist treatments on steatohepatitis

Histological studies demonstrated that the chronic ethanol feeding caused steatohepatitis as previously reported², and that the PPAR- δ agonist treatments reduced hepatic steatosis, inflammation, and apoptosis, and nearly restored the normal chord-like architecture, as described³¹. EM studies demonstrated RER cisternae with stacked, parallel arrays of flattened and narrowed, elongated tubules in close juxtaposition to mitochondria in control livers (Figs 1A–1B). The main effects of PPAR- δ agonist treatments were to reduce the compactness of RER stacks and enmesh mitochondria amongst the RER (Figs 1C–1D). Ethanol increased the density and size of lipid droplets and glycogen granules, variability in mitochondrial size, disorganization of the RER. In addition, ER ribosomes were irregularly spaced with large gaps, and cisternae were focally dilated. Mitochondria were enmeshed with the RER rather than distributed at the perimeters of stacked cisternae (Figs 1E–1F). PPAR- δ agonist treatments reduced lipid droplet size and abundance, increased organization and compactness of RER, and rendered mitochondrial morphology more uniform (Figs 1G–1H).

Effects of PPAR- δ agonist treatments on hepatic insulin and IGF signaling

Multiplex ELISAs demonstrated reduced In-R (Figure 2A) and tyrosine phosphorylated In-R (Figure 2B), and increased IRS-1 (Figure 2G) in ethanol-exposed livers. In contrast, similar mean levels of IGF-1R (Figure 2D), tyrosine phosphorylated IGF-1R (Figure 2E), and serine phosphorylated IRS-1 (Figure 2H) were observed in control and ethanol-exposed vehicle-treated livers. PPAR- δ agonist treatments rendered the In-R and tyrosine phosphorylated In-R levels similar in control and ethanol-exposed livers, and IGF-1R (Figure 2D), IRS-1 (Figure 2G), tyrosine phosphorylated IGF-1R (Figure 2E), and serine phosphorylated IRS-1 (Figure 2H) significantly higher in the ethanol group. The higher mean ratio of pYpY1162/1163-In-R/Total In-R in control versus ethanol-fed, PPAR- δ agonist treated livers (Figure 2C) reflects enhanced In-R signaling in control but not ethanol-

exposed livers. The higher p^{S312} -IRS-1/Total IRS-1 vehicle-treated control versus ethanol-exposed livers also reflects more robust upstream insulin/IGF-1 signaling (Figure 2I). Otherwise, relative phosphorylation levels of In-R, IGF-1R and IRS-1 were similar in control and ethanol exposed livers. Compared with vehicle, PPAR- δ agonist treatments significantly reduced expression of IGF-1R ($P<0.01$), phosphorylated IGF-1R ($P<0.01$), IRS-1 ($P<0.01$), and phosphorylated IRS-1 ($P<0.001$), and increased the relative levels of phosphorylated In-R ($P<0.05$) in control livers, whereas in ethanol-fed rats, PPAR- δ agonist treatments only decreased IRS-1 expression ($P<0.01$). Therefore, the main group effects of PPAR- δ agonist treatments were mediated by reductions in protein expression and phosphorylation in control livers, rather than stimulation of ethanol-exposed livers.

Further studies characterized effects of chronic ethanol feeding and PPAR- δ agonist treatments on Akt signaling. Ethanol fed rats had lower hepatic levels of GSK-3 β , PRAS40, and p70S6K, but similar levels of Akt relative to control (Figures 3A, 3D, 3G, 3J). The main effects of the PPAR- δ agonist were to significantly reduce expression of GSK-3 β in both control and ethanol-exposed livers ($P<0.0001$), and PRAS40 ($P<0.001$) and p70S6K ($P<0.05$) in control livers. In addition, Akt was modestly reduced by PPAR- δ agonist treatment of control livers ($P=0.016$). In contrast, the PPAR- δ agonist treatments did not modulate expression of Akt, PRAS40 or p70S6K in ethanol-exposed livers. Consequently, PPAR- δ agonist treatments resulted in significantly higher levels of Akt and similar levels of GSK-3 β and PRAS40 in control and ethanol-exposed livers, and no meaningful change in PRAS40 expression.

Ethanol significantly decreased p^{S473} -Akt and p^{T246} -PRAS40, but not p^{S9} -GSK-3 β or p^{T246} -PRAS40. The PPAR- δ agonist treatments reversed ethanol's inhibitory effects on Akt signaling, as demonstrated by the significantly higher levels of p^{S473} -Akt, p^{S9} -GSK-3 β , and p^{T246} -PRAS40 relative to control (Figures 3B, 3E, 3H). The calculated ratios of phosphorylated/total Akt, GSK-3 β , and p70S6K were similar in vehicle-treated, control and ethanol-exposed livers, whereas the mean level of p^{T246} -PRAS40 was significantly lower in the ethanol group (Figures 3C, 3F, 3I, and 3L). PPAR- δ agonist treatments significantly increased p^{S473} -Akt/Total Akt and p^{T246} -PRAS40/Total PRAS40 in ethanol-exposed relative to control livers (Figures 3C, 3I). These effects were due to increased levels of p^{S473} -Akt ($P<0.0001$) and p^{S473} -Akt/total Akt (both $P<0.0001$) in ethanol-exposed livers, increased p^{T246} -PRAS40/Total PRAS40 in ethanol-exposed livers ($P<0.05$), and decreased p^{T246} -PRAS40 and p^{T246} -PRAS40/Total PRAS40 in control livers (both $P<0.0001$). Another notable effect of the PPAR- δ agonist was to significantly increase the mean levels of p^{S9} -GSK-3 β /Total GSK-3 β in both control and ethanol-exposed livers relative to the corresponding vehicle-treated groups (both $P<0.0001$). The net effect was to inhibit GSK-3 β activity in both groups.

Effects of PPAR- δ agonist treatment on hepatic ceramides

Duplex qRT-PCR assays measured gene expression corresponding to de novo ceramide biosynthesis (Ceramide synthases 1, 2, 4, and the subunits 1 (regulatory) and 2 (catalytic) of serine palmitoyl transferase subunit (SPTLC1 and 2)), catabolic enzymes (sphingomyelin phosphodiesterases 1 and 3 (SMPD1 and SMPD3) and ceramidases (CERD)), and the

salvage pathway, which is used for biosynthesis of complex sphingolipids (ganglioside GM3 synthase and UDP-glucose ceramide glucosyltransferase (UGCG)) (Table 1). Chronic ethanol feeding resulted in higher levels of SPTLC1, SMPD3, GM3Syn, and UGCG. Bonferroni posttests demonstrated significant inter-group differences with respect to SMPD3 (Table 1). CERD3 expression was significantly lower in the ethanol+vehicle group. PPAR- δ agonist treatments increased expression of CERS2, CERS4, SMPD1, and CERD2 in control livers, but generally not in ethanol-exposed livers. The only exception was SMPD3, which was reduced by PPAR- δ agonist treatment, rendering the levels comparable to control (Table 1). Despite these shifts in ceramide gene expression, PPAR- δ agonist treatments did not significantly reduce hepatic lipid (Nile Red assay) or ceramide (ELISA) levels in either the control or ethanol group (Figure 4).

ER stress in ALD-effects of PPAR- δ agonist treatments

To examine the effects of PPAR- δ agonist treatments on ALD-induced ER stress, we measured mRNA and protein levels of ER stress mediators³³. Chronic ethanol feeding significantly up-regulated tryptophanyl-tRNA synthetase (WARS), ER degradation-enhancing α -mannosidase-like 1 (EDEM), the transcription factor ATF-4, the chaperone GRP78/BiP, and CHOP relative to control (Table 2). PPAR- δ agonist treatments significantly increased expression of BAX, P58IPK, the homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 (HERPUD), ATF-4, and GRP78/BiP in control samples, resulting in either similar (WARS, EDEM, CHOP), or significantly higher (BAX, P58IPK, HERPUD, ATF-4, GRP78) levels of ER stress gene expression relative to the PPAR- δ agonist, treated ethanol exposed livers (Table 2). Protein disulphur isomerase (PDI) was similarly expressed in control and ethanol-treated rats, irrespective of PPAR- δ agonist treatment. There were no examples in which PPAR- δ agonist treatments significantly increased ER stress gene expression, and only one example, i.e. CHOP whereby the PPAR- δ agonist significantly reduced hepatic ER stress gene expression in livers of chronic ethanol-fed rats.

Duplex ELISAs measured PERK (Figure 5A), pPERK (Figure 5B), eIF2a (Figure 5C), p-eIF2a (Figure 5D), PDI (Figure 5E), IRE-1 (Figure 5F), GRP78/BiP (Figure 5G), CHOP (Figure 5H), ERO1 (Figure 5I), and Calnexin (Figure 5J). Results were normalized to β -actin. Chronic ethanol feeding significantly increased expression of PERK ($P<0.0001$), PDI ($P<0.0001$), CHOP ($P<0.01$), and Calnexin ($P<0.05$), and decreased expression of eIF2A ($P<0.0001$) and p-eIF2a ($P<0.0001$) relative to vehicle-treated controls. Relative to the corresponding vehicle-treated groups, PPAR- δ agonist treatments reduced expression of pPERK ($P<0.05$) in ethanol-fed rats, and eIF2a ($P<0.0001$) and p-eIF2a ($P<0.0001$) in control rats; the latter resulted in similar levels of eIF2a and p-eIF2a in the control and ethanol-exposed livers. In addition, PPAR- δ agonist treatments reduced hepatic expression of PDI ($P<0.0001$), BIP ($P<0.05$), ERO1 ($P<0.05$), IRE-1 ($P<0.05$), CHOP ($P<0.0001$), and Calnexin ($P<0.05$) in ethanol-fed rats (Figures 5E–5J). Consequently, the mean levels of each were either similar to, or below control.

Discussion

This study examined the effects of PPAR- δ agonist treatment of experimental chronic ALD, focusing on the: 1) ultrastructural pathology; 2) integrity of insulin/IGF-1 signaling through Akt pathways; 3) activation of pro-ceramide mechanisms and ceramide accumulation; and 4) ER stress mechanisms. An important objective was to determine the degree to which structural, biochemical, and molecular abnormalities in chronic ALD could be resolved by insulin sensitizer treatments vis-à-vis continued high-level ethanol exposures. EM studies showed that PPAR- δ agonist-mediated reductions in hepatic lipid content were associated with reduced size and density of lipid droplets. However, hepatic steatosis was not resolved as lipid content remained significantly elevated.

Chronic ALD-associated impairment in hepatic insulin/IGF signaling was manifested by the reduced levels of insulin receptor expression, and reduced tyrosine phosphorylation of insulin and IGF-1 receptors. The main effects of PPAR- δ agonist treatments were to increase the relative levels of insulin receptor, IGF-1 receptor, and IRS-1, and either increase or normalize the levels of phosphorylated insulin and IGF-1 receptors and IRS-1 in ethanol-exposed livers. Therefore, insulin sensitizer treatments can support insulin and IGF-1 signaling at proximal points within the cascade.

Chronic ethanol-exposed livers had profoundly impaired signaling through Akt, as was manifested by the significantly reduced levels of total and phosphorylated Akt, increased levels of total GSK-3 β , and decreased levels pS⁹-GSK-3 β and pS⁹-GSK-3 β /GSK-3 β ⁴. Similarly, activation of mTOR networks via p70S6K and PRAS40 were impaired. Inhibition of PI3K-Akt promotes apoptosis, DNA damage, mitochondrial dysfunction, and oxidative stress^{2, 4, 5}. Constitutive inhibition of Akt and activation of GSK-3 β could have contributed to the metabolic impairments mediating lipid dyshomeostasis and ultimately ER stress in chronic ethanol-fed rats. The finding that the PPAR- δ agonist treatments restored Akt and PRAS40 signaling and reduced GSK-3 β activation in chronic ethanol-exposed livers, indicates that PPAR agonists can rescue chronic ALD by enhancing insulin/IGF signaling through key metabolic pathways and inhibiting GSK-3 β . As an additional mechanism, the anti-inflammatory effect of PPAR agonists could have helped to restore insulin signaling and reduce oxidative-stress²⁸. For example, PPAR- γ agonist prevention of experimental ALD can be mediated by inhibition of lipopolysaccharide-induced Kupffer cell production of pro-inflammatory cytokines³⁴. Moreover, PPAR- δ seems to have an important role in suppressing Kupffer cell-mediated hepatic dysfunction, liver injury, and inflammation³⁵. Therefore, the mechanisms by which PPAR- δ agonists restore hepatic insulin signaling in experimental ALD could be indirect and mediated by inhibition of Kupffer cell function.

Progression of chronic ALD is likely mediated by aberrant shifts in membrane lipid composition with attendant disruption of intracellular signaling²⁴, accumulation of toxic lipids that promote oxidative stress, ROS generation, adduct formation, and ER stress¹¹. We focused our studies on ceramides because they have lipotoxic effects on hepatocytes²⁰, contribute to the pathogenesis of steatohepatitis,^{7, 36} promote insulin resistance, inflammation, and oxidative stress³⁷, and impair Akt/PKB signaling³⁸. Chronic ethanol feeding up-regulated expression of pro-ceramide genes in the biosynthetic, catabolic, and

salvage pathways, and significantly increased hepatic ceramide levels. Mechanistically, insulin resistance promotes lipolysis, including hydrolysis of complex lipids, resulting in increased ceramide production. Increased expression of ceramide synthase genes could be explained by increased fatty acid load that occurs with steatohepatitis^{7, 36, 39}. Although gangliosides such as GM3 synthase can mediate insulin resistance³⁷, we did not detect increased GM3 synthase expression in our model. PPAR- δ agonist treatments reduced hepatic SMPD3 expression and ceramide levels. SMPD3 generates ceramide via a catabolic pathway. However, the PPAR- δ agonist treatments had minimal effect on the levels of most pro-ceramide genes in the chronic ALD model, and correspondingly, hepatic ceramide levels remained significantly elevated in the ethanol group.

ER stress is promoted by insulin resistance, inflammation, and ceramide accumulation, and ER stress exacerbates insulin resistance, inflammation, oxidative stress, and ceramide accumulation^{26, 40, 41}. In our model of chronic ALD, ER stress signaling was up-regulated at multiple levels in the pathway, including chaperones, ubiquitin proteasome mechanisms, transcription factors and pro-apoptosis signaling. EM studies revealed that chronic ethanol feeding resulted in pronounced disorganization and disruption of RER tubules, as initially reported in relation to human ALD^{42, 43}. Therefore, in addition to corroborating recent studies showing that ethanol increases ER stress in liver⁴⁴, this study correlates molecular indices of ER stress with ultra-structural abnormalities in the ER. Although the PPAR- δ agonist significantly reduced CHOP expression and restored the ER architecture (EM), the overall effect was quite limited as many components of the ER stress pathway were unaffected. Most of the inter-group differences observed were due to responses among the controls, similar to the findings with respect to pro-ceramide mechanisms.

There is ample evidence that PPAR agonists have therapeutic effects in experimental ALD^{4, 31, 45}. Despite the observed positive effects of the PPAR- δ agonist treatments, concerns exist about their therapeutic application in the context of ALD, since previous studies showed that PPAR activation or PPAR agonist treatments can inhibit expression of aldehyde dehydrogenases^{46–48} and cytochrome P450 genes and proteins⁴⁹. On the other hand, other evidence suggests that PPAR- γ agonists can induce CYP3A⁵⁰ and prevent endotoxin-induced inhibition of hepatic cytochrome P450- 3A2 and 2C11 proteins⁵¹.

In summary, we demonstrated that in an experimental model of chronic ALD, PPAR- δ agonists can significantly reduce hepatic insulin resistance and steatosis. However, the therapeutic effects with respect to pro-ceramide mechanisms, ceramide load, and ER stress are somewhat circumscribed. Therefore, in addition to PPAR agonist treatments, measures to reduce lipotoxicity and ER stress will likely be needed to improve outcomes in chronic ALD.

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Abbreviations

4-MUP	4-methylumbelliferyl phosphate
ALD	alcohol-induced liver disease
ANOVA	analysis of variance
BSA	bovine serum albumin
CERD	ceramidase
CERS	Ceramide synthase
CHOP	CEB{ jp,p;pgpis [rpteom
EDEM	ER degradation-enhancing α -mannosidase-like 1
EIF-2a	eukaryotic translation initiation factor 2A
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
Em	emission
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
ERO1	ER oxidoreductin 1
Ex	excitation
GADD	growth arrest and DNA damage in
GM3Syn	ganglioside GM3 synthase enzyme
GRP78/BiP	glucose regulated protein 78 kD/ immunoglobulin binding protein
GSK-3β	glycogen synthase kinase 3b
H&E-	hematoxylin and eosin
HERPUD	homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1
HRP	horseradish peroxidase
i.p.	intraperitoneal
IGF	insulin like growth factor
In-R	insulin receptor
IRE-1	inositol-requiring protein 1
IRS	insulin receptor substrate
MAPK	mitogen activated protein kinase
mTOR	mammalian target of rapamycin
P79S6K	ribosomal protein S6 kinase

PDI	protein disulfur isomerase
PERK	PKR-like ER kinase
PKC	protein kinase C
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator activated receptor
pPERK	phosphorylated PKR-like ER kinase
PPI	peptidyl-propyl isomerase
PRAS40	proline-rich Akt substrate 40 kDa
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
SMPD	sphingomyelin phosphodiesterase
SPTLC	serine palmitoyl transferase
SREBP	sterol regulatory binding proteins
TBS	Tris buffered saline
UGCG	UDP-glucose ceramide glucosyltransferase
UPR	unfolded protein response
WARS	tryptophanyl-tRNA synthetase

References

1. O'Shea RS, Dasarthy S, McCullough AJ. Alcoholic liver disease. *Hepatology*. 2010; 51:307–328. [PubMed: 20034030]
2. de la Monte SM, Yeon JE, Tong M, et al. Insulin resistance in experimental alcohol-induced liver disease. *J Gastroenterol Hepatol*. 2008; 23:e477–e486. [PubMed: 18505416]
3. Denucci SM, Tong M, Longato L, et al. Rat strain differences in susceptibility to alcohol-induced chronic liver injury and hepatic insulin resistance. *Gastroenterol Res Pract*. 2010; 2010
4. Pang M, de la Monte SM, Longato L, et al. PPARdelta agonist attenuates alcohol-induced hepatic insulin resistance and improves liver injury and repair. *J Hepatol*. 2009; 50:1192–1201. [PubMed: 19398227]
5. Ronis MJ, Wands JR, Badger TM, de la Monte SM, Lang CH, Calissendorff J. Alcohol-induced disruption of endocrine signaling. *Alcohol Clin Exp Res*. 2007; 31:1269–1285. [PubMed: 17559547]
6. Sasaki Y, Wands JR. Ethanol impairs insulin receptor substrate-1 mediated signal transduction during rat liver regeneration. *Biochem Biophys Res Commun*. 1994; 199:403–409. [PubMed: 7510107]
7. Setshedi M, Longato L, Petersen DR, et al. Limited therapeutic effect of N-acetylcysteine on hepatic insulin resistance in an experimental model of alcohol-induced steatohepatitis. *Alcoholism, clinical and experimental research*. 2011; 35:2139–2151.
8. Longato L, Ripp K, Setshedi M, et al. Insulin resistance, ceramide accumulation, and endoplasmic reticulum stress in human chronic alcohol-related liver disease. *Oxid Med Cell Longev*. 2012; 2012:479348. [PubMed: 22577490]

9. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology*. 2011; 141:1572–1585. [PubMed: 21920463]
10. Setshedi M, Wands JR, Monte SM. Acetaldehyde adducts in alcoholic liver disease. *Oxid Med Cell Longev*. 2010; 3:178–185. [PubMed: 20716942]
11. de la Monte, SM. Alcohol-induced liver and brain degeneration: roles of insulin resistance, toxic ceramides, and endoplasmic reticulum stress. In: Sova, M., editor. *Alcohol, Nutrition, and Health Consequences*. New York: Humana Press; 2012.
12. de la Monte SM, Longato L, Tong M, DeNucci S, Wands JR. The liver-brain axis of alcohol-mediated neurodegeneration: role of toxic lipids. *Int J Environ Res Public Health*. 2009; 6:2055–2075. [PubMed: 19742171]
13. Friedman SL, Nieto N. Cannabinoids provoke alcoholic steatosis through a conspiracy of neighbors. *Cell Metab*. 2008; 7:187–178. [PubMed: 18316020]
14. Kao Y, Youson JH, Holmes JA, Al-Mahrouki A, Sheridan MA. Effects of insulin on lipid metabolism of larvae and metamorphosing landlocked sea lamprey, *Petromyzon marinus*. *Gen Comp Endocrinol*. 1999; 114:405–414. [PubMed: 10336828]
15. Holland WL, Summers SA. Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. *Endocr Rev*. 2008; 29:381–402. [PubMed: 18451260]
16. Bourbon NA, Sandirasegarane L, Kester M. Ceramide-induced inhibition of Akt is mediated through protein kinase C ζ : implications for growth arrest. *J Biol Chem*. 2002; 277:3286–3292. [PubMed: 11723139]
17. Nogueira TC, Anhe GF, Carvalho CR, Curi R, Bordin S, Carpinelli AR. Involvement of phosphatidylinositol-3 kinase/AKT/PKC ζ /lambda pathway in the effect of palmitate on glucose-induced insulin secretion. *Pancreas*. 2008; 37:309–315. [PubMed: 18815554]
18. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. 2010; 140:900–917. [PubMed: 20303879]
19. Kaplowitz N, Than TA, Shinohara M, Ji C. Endoplasmic reticulum stress and liver injury. *Semin Liver Dis*. 2007; 27:367–377. [PubMed: 17979073]
20. Malhi H, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis*. 2008; 28:360–369. [PubMed: 18956292]
21. Sharma NK, Das SK, Mondal AK, et al. Endoplasmic reticulum stress markers are associated with obesity in nondiabetic subjects. *The Journal of clinical endocrinology and metabolism*. 2008; 93:4532–4541. [PubMed: 18728164]
22. Sundar-Rajan S, Srinivasan V, Balasubramanyam M, Tatu U. Endoplasmic reticulum (ER) stress & diabetes. *Indian J Med Res*. 2007; 125:411–424. [PubMed: 17496365]
23. Kaplowitz N, Ji C. Unfolding new mechanisms of alcoholic liver disease in the endoplasmic reticulum. *J Gastroenterol Hepatol*. 2006; 21(Suppl 3):S7–S9. [PubMed: 16958678]
24. Anderson N, Borlak J. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol Rev*. 2008; 60:311–357. [PubMed: 18922966]
25. Ronis MJ, Butura A, Korourian S, et al. Cytokine and chemokine expression associated with steatohepatitis and hepatocyte proliferation in rats fed ethanol via total enteral nutrition. *Exp Biol Med* (Maywood). 2008; 233:344–355. [PubMed: 18296740]
26. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 2004; 306:457–461. [PubMed: 15486293]
27. Gyamfi MA, Wan YJ. Pathogenesis of alcoholic liver disease: the role of nuclear receptors. *Exp Biol Med* (Maywood). 2010; 235:547–560. [PubMed: 20463294]
28. Yessoufou A, Wahli W. Multifaceted roles of peroxisome proliferator-activated receptors (PPARs) at the cellular and whole organism levels. *Swiss Med Wkly*. 2010; 140:w13071. [PubMed: 20842602]
29. Heald M, Cawthorne MA. Dual acting and pan-PPAR activators as potential anti-diabetic therapies. *Handb Exp Pharmacol*. 2011:35–51. [PubMed: 21484566]
30. Promrat K, Lutchman G, Uwaifo GI, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology*. 2004; 39:188–196. [PubMed: 14752837]

31. de la Monte SM, Pang M, Chaudhry R, et al. Peroxisome proliferator-activated receptor agonist treatment of alcohol-induced hepatic insulin resistance. *Hepatol Res.* 2011; 41:386–398. [PubMed: 21426453]
32. Longato L, Tong M, Wands JR, de la Monte SM. High fat diet induced hepatic steatosis and insulin resistance: Role of dysregulated ceramide metabolism. *Hepatol Res.* 2012; 42:412–427. [PubMed: 22176347]
33. Jager R, Bertrand MJ, Gorman AM, Vandenabeele P, Samali A. The unfolded protein response at the crossroads of cellular life and death during ER stress. *Biol Cell.* 2012
34. Enomoto N, Takei Y, Hirose M, et al. Prevention of ethanol-induced liver injury in rats by an agonist of peroxisome proliferator-activated receptor- γ , pioglitazone. *J Pharmacol Exp Ther.* 2003; 306:846–854. [PubMed: 12805475]
35. Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, et al. Alternative M2 activation of Kupffer cells by PPAR δ ameliorates obesity-induced insulin resistance. *Cell Metab.* 2008; 7:496–507. [PubMed: 18522831]
36. Lyn-Cook LE Jr, Lawton M, Tong M, et al. Hepatic Ceramide May Mediate Brain Insulin Resistance and Neurodegeneration in Type 2 Diabetes and Non-alcoholic Steatohepatitis. *J Alzheimers Dis.* 2009; 16:715–729. [PubMed: 19387108]
37. Holland WL, Knotts TA, Chavez JA, Wang LP, Hoehn KL, Summers SA. Lipid mediators of insulin resistance. *Nutr Rev.* 2007; 65:S39–S46. [PubMed: 17605313]
38. Ruvolo PP. Intracellular signal transduction pathways activated by ceramide and its metabolites. *Pharmacological research : the official journal of the Italian Pharmacological Society.* 2003; 47:383–392. [PubMed: 12676512]
39. de la Monte SM, Tong M, Lawton M, Longato L. Nitrosamine exposure exacerbates high fat diet-mediated type 2 diabetes mellitus, non-alcoholic steatohepatitis, and neurodegeneration with cognitive impairment. *Mol Neurodegener.* 2009; 4:54. [PubMed: 20034403]
40. Senkal CE, Ponnusamy S, Bielawski J, Hannun YA, Ogretmen B. Antiapoptotic roles of ceramide-synthase-6-generated C16-ceramide via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2010; 24:296–308. [PubMed: 19723703]
41. Boslem E, MacIntosh G, Preston AM, et al. A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking. *Biochem J.* 2011; 435:267–276. [PubMed: 21265737]
42. Iseri OA, Lieber CS, Gottlieb LS. The ultrastructure of fatty liver induced by prolonged ethanol ingestion. *Am J Pathol.* 1966; 48:535–555. [PubMed: 5936783]
43. Romert P, Matthiessen ME. Fine structure of hepatocytes from mini-pigs and mini-pig fetuses exposed to alcohol (ethanol) in vivo. *Acta Anat (Basel).* 1984; 120:190–195. [PubMed: 6516779]
44. Dara L, Ji C, Kaplowitz N. The contribution of endoplasmic reticulum stress to liver diseases. *Hepatology.* 2011; 53:1752–1763. [PubMed: 21384408]
45. Kong L, Ren W, Li W, et al. Activation of peroxisome proliferator activated receptor α ameliorates ethanol induced steatohepatitis in mice. *Lipids Health Dis.* 2011; 10:246. [PubMed: 22208561]
46. Alnouti Y, Klaassen CD. Tissue distribution, ontogeny, and regulation of aldehyde dehydrogenase (Aldh) enzymes mRNA by prototypical microsomal enzyme inducers in mice. *Toxicological sciences : an official journal of the Society of Toxicology.* 2008; 101:51–64. [PubMed: 17998271]
47. Canuto RA, Maggiora M, Trombetta A, Martinasso G, Muzio G. Aldehyde dehydrogenase 3 expression is decreased by clofibrate via PPAR γ induction in JM2 rat hepatoma cell line. *Chem Biol Interact.* 2003; 143–144. 29–35.
48. Crabb DW, Pinaire J, Chou WY, et al. Peroxisome proliferator-activated receptors (PPAR) and the mitochondrial aldehyde dehydrogenase (ALDH2) promoter in vitro and in vivo. *Alcoholism, clinical and experimental research.* 2001; 25:945–952.
49. Shaban Z, El-Shazly S, Ishizuka M, Kimura K, Kazusaka A, Fujita S. PPAR α -dependent modulation of hepatic CYP1A by clofibric acid in rats. *Arch Toxicol.* 2004; 78:496–507. [PubMed: 15127182]

50. Kliewer SA, Lehmann JM, Milburn MV, Willson TM. The PPARs and PXR: nuclear xenobiotic receptors that define novel hormone signaling pathways. *Recent Prog Horm Res.* 1999; 54:345–367. discussion 67–68. [PubMed: 10548883]
51. Ueyama J, Kitaichi K, Nadai M, et al. Effect of pioglitazone on endotoxin-induced decreases in hepatic drug-metabolizing enzyme activity and expression of CYP3A2 and CYP2C11. *European journal of pharmacology.* 2004; 498:257–265. [PubMed: 15364003]

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Figure 1.

Effects of chronic ethanol feeding and peroxisomeproliferator activated receptor (PPAR)-d agonist treatments on hepatic ultrastructural pathology: Transmission electron microscopy studies of liver from (a, b) Control + Vehicle, (c, d) Control + PPAR-d agonist, (e, f) Ethanol + Vehicle, and (g, h) Ethanol + PPAR-d agonist treatment groups. (a, b) Control + vehicle treated livers had abundant mitochondria (m) distributed adjacent to or at the periphery of endoplasmic reticulum (ER) and coarse central and peripheral nuclear chromatin. (b and inset) ER (er) was arranged in parallel stacks and richly coated with

ribosomes. (c, d) PPAR-d agonist treatments reduced the abundance of mitochondria and density of nuclear chromatin, slightly increased small lipid droplets (*), but maintained the normal ER morphology. (e, f) Chronic ethanol feeding increased steatosis (lipid droplets, mitochondrial abundance and size with increased mitochondrial interspersation among RER tubules, but reduced the density of ER ribosomes and caused irregular dilatation of the ER tubules. (g, h) PPAR-d agonist treatment of ethanol-exposed livers partly restored the ultrastructure of hepatocytes, reducing mitochondrial and lipid droplet size and abundance, and rendering the ER tubules more regular, parallel and better populated with ribosomes. (Original magnifications: a, c, e, g: $\times 7100$; b, d, f, h: $\times 44\,000$)

Figure 2.

Effects of chronic ethanol feeding and peroxisome-proliferator activated receptor (PPAR)- δ agonist treatments on hepatic insulin signaling upstream pathway mediators. Liver protein homogenates were used in bead-based multiplex enzyme linked immunosorbent assays (ELISAs) to measure immunoreactivity to the (a) insulin receptor (InR), (d) insulin like growth factor-1 (IGF-1) receptor (IGF 1R), (g) insulin receptor substrate-1 (IRS-1), (b) pYpY^{1162/1163}-InR, (e) pYpY^{1135/1136}-IGF-1R, and (h) pS³¹²-IRS-1. Calculated phospho/total ratios of (c) InR, (f) IGF-1R, and (i) IRS-1 reflect relative levels of phosphorylation. Comparisons were made using 2-way analyses of variance (ANOVAs) with Bonferroni post-tests.

Figure 3.

Effects of chronic ethanol feeding and peroxisome-proliferator activated receptor (PPAR)- δ agonist treatments on hepatic insulin signaling downstream through Akt. Liver protein homogenates were used in bead-based multiplex enzyme linked immunosorbent assays (ELISAs) to measure immunoreactivity to (a) Akt, (d) glycogen synthase kinase 3b (GSK-3b), (g) proline-rich Akt substrate 40 kDa (PRAS40), (j) ribosomal protein S6 kinase (p70S6K), (b) pS⁴⁷³-Akt, and (e) pS⁹-GSK-3b, (h) pT²⁴⁶-PRAS40, and (k) pTpS^{421/424}-p70S6K. Calculated phospho/total ratios of (c) Akt, (F) GSK-3b, (i) PRAS40, and (l)

p70S6K reflect relative levels of phosphorylation. Comparisons were made using 2-way analyses of variance (ANOVAs) with Bonferroni post-tests.

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Figure 4.

Peroxisome-proliferator activated receptor (PPAR)- δ agonist treatments do not significantly reduce hepatic levels of lipid or ceramides in the context of chronic ethanol feeding. (a) Nile red fluorescence and (b) ceramide immunoreactivity were measured in liver homogenates with results normalized to protein content. Comparisons were made using 2-way analyses of variance (ANOVAs) with Bonferroni post-tests.

Figure 5.

Effects of peroxisome-proliferator activated receptor (PPAR)- δ agonist treatments on chronic ethanol-induced endoplasmic reticulum (ER) stress pathway activation in liver. Liver tissue homogenates were used in direct binding duplex enzyme linked immunosorbent assays (ELISAs) to measure (a) PKRlike ER kinase (PERK), (b) p-PERK (Thr980), (c) eIF2-a, and (d) p-eIF2-a (Ser51), (e) protein disulfur isomerase (PDI), (f) inositol-requiring protein 1 (IRE-1), (g) glucose regulated protein 78 kD/immunoglobulin binding protein (GRP-78/BiP), (h) CHOP, (i) ER oxidoreductin 1(ERO1), and (j) calnexin

immunoreactivity. Fluorescence light units (FLU) were measured (Ex 530 nm/Em 590 nm) in a Spectramax M5 microplate reader. Results were normalized to large ribosomal protein measured in the same samples. Comparisons were made using 2-way analyses of variance (ANOVAs) with Bonferroni post-tests.

Table 1Effects of ethanol and PPAR- δ agonist treatments on pro-ceramide gene expression

Gene	C $\pm$ Veh	Et $\pm$ Veh	C $\pm$ PPAR δ	Et $\pm$ PPAR- δ	Ethanol	PPAR	Interaction	Bonferroni
CERS1	94.2 $\pm$ 39.0	97.2 $\pm$ 5.0	90.2 $\pm$ 22.0	92.7 $\pm$ 38.0	NS	NS	NS	
CERS2	37.0 $\pm$ 27.0	37.9 $\pm$ 24.0	46.9 $\pm$ 17.0	37.4 $\pm$ 29.0	NS	NS	0.0425	PPAR- δ *
CERS4	0.3 $\pm$ 0.03	0.3 $\pm$ 0.03	0.5 $\pm$ 0.07	0.3 $\pm$ 0.03	NS	NS	0.0116	PPAR- δ **
SPTLC1	97.2 $\pm$ 3.2	103.1 $\pm$ 3.9	98.6 $\pm$ 3.5	104.6 $\pm$ 3.3	NS	NS	NS	
SPTLC2	0.4 $\pm$ 0.03	0.4 $\pm$ 0.05	0.4 $\pm$ 0.1	0.3 $\pm$ 0.04	NS	NS	NS	
SMPD1	25.8 $\pm$ 2.9	21.8 $\pm$ 1.0	31.6 $\pm$ 1.3	20.9 $\pm$ 1.6	0.0005	NS	NS	PPAR- δ ***
SMPD3	29.0 $\pm$ 4.0	54.6 $\pm$ 4.4	42.8 $\pm$ 4.1	31.2 $\pm$ 5.6	NS	NS	0.0003	Veh ***
CERD2	0.3 $\pm$ 0.0006	0.2 $\pm$ 0.02	0.5 $\pm$ 0.08	0.2 $\pm$ 0.02	0.0022	NS	0.0481	PPAR- δ **
CERD3	73.3 $\pm$ 2.5	52.2 $\pm$ 4.1	71.9 $\pm$ 2.1	54.2 $\pm$ 4.6	<0.0001	NS	NS	Veh ***; PPAR- δ ***
GM3SYN	0.6 $\pm$ 0.06	1.0 $\pm$ 0.08	1.2 $\pm$ 0.21	0.9 $\pm$ 0.07	NS	0.0295	0.006	
UGCG	0.3 $\pm$ 0.04	0.4 $\pm$ 0.04	0.5 $\pm$ 0.1	0.3 $\pm$ 0.06	NS	NS	0.0079	

Gene expression was measured by a duplex probe-based qRT-PCR analysis. Values shown are $\times 10^{-2}$, N=8/group. Data were analyzed by 2-way ANOVA. Significant effects of ethanol, PPAR-d agonist, or interactions of the treatments are shown in columns 5–7.

Bonferroni post-test results:

* P<0.05,

** P<0.01;

*** P<0.001;

**** P<0.0001.

C=Control; Veh=vehicle; Et=Ethanol.

Table 2Effects of ethanol and PPAR- δ agonist treatments on ER stress genes

Gene	C $\pm$ Veh	Et $\pm$ Veh	C $\pm$ PPAR- δ	Et $\pm$ PPAR- δ	Ethanol	PPAR- δ	Interaction	Bonferroni
WARS	2.63 $\pm$ 0.2	3.9 $\pm$ 0.2	3.6 $\pm$ 0.2	3.5 $\pm$ 0.2	0.007	NS	0.0025	Veh ****
BAX	37.8 $\pm$ 1.4	34.7 $\pm$ 2.3	43.5 $\pm$ 0.9	32.1 $\pm$ 1.7	<0.0001	NS	0.0104	PPAR- δ ****
P58IPK	57.2 $\pm$ 4.1	56.9 $\pm$ 2.9	75.7 $\pm$ 3.2	58.4 $\pm$ 3.2	0.016	0.0069	0.0192	PPAR- δ **
PDI	73.2 $\pm$ 4.1	71.5 $\pm$ 4.1	78.7 $\pm$ 2.1	77.7 $\pm$ 2.3	NS	0.0079	NS	NS
HERPUD	44.9 $\pm$ 2.1	45.9 $\pm$ 1.9	55.1 $\pm$ 1.5	47.2 $\pm$ 1.7	0.0036	NS	0.0197	PPAR- δ **
EDEM	38.6 $\pm$ 1.4	48.4 $\pm$ 1.9	53.0 $\pm$ 3.9	45.1 $\pm$ 3.7	NS	0.002	0.0325	Veh *
ATF4	52.1 $\pm$ 1.5	61.4 $\pm$ 1.6	65.2 $\pm$ 2.1	59.7 $\pm$ 0.9	NS	0.0011	<0.0001	Veh ****; PPAR- δ *
GRP78	25.6 $\pm$ 0.7	36.6 $\pm$ 4.2	35.6 $\pm$ 1.7	33.4 $\pm$ 3.1	NS	0.0485	<0.0001	Veh ****; PPAR- δ *
CHOP	0.3 $\pm$ 0.1	1.2 $\pm$ 0.2	0.8 $\pm$ 0.2	0.6 $\pm$ 0.1	0.0329	NS	0.0002	Veh ****

Gene expression was measured by a duplex probe-based qRT-PCR analysis. Values shown are $\times 10^{-2}$, N=8/group. Data were analyzed by 2-way ANOVA. Significant effects of ethanol, PPAR-d agonist, or interactions of the treatments are shown in columns 5–7.

Bonferroni post-test results:

* P<0.05,

** P<0.01;

*** P<0.001;

**** P<0.0001.

C=Control; Veh=vehicle; Et=Ethanol.