Peroxisome Proliferator–Activated Receptor γ Regulates Chronic Alcohol-Induced Alveolar Macrophage Dysfunction

Samantha M. Yeligar^{1,2}, Ashish J. Mehta², Frank L. Harris¹, Lou Ann S. Brown¹, and C. Michael Hart²

¹Emory University, Department of Pediatrics, Division of Neonatal-Perinatal Medicine, Emory and Children's Healthcare of Atlanta Center for Developmental Lung Biology, Atlanta, Georgia; ²Emory University, Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, Atlanta Veterans Affairs and Emory University Medical Centers, Decatur, Georgia

Abstract

Peroxisome proliferator–activated receptor (PPAR) γ is critical for alveolar macrophage (AM) function. Chronic alcohol abuse causes AM phagocytic dysfunction and susceptibility to respiratory infections by stimulating nicotinamide adenine dinucleotide oxidases (Nox), transforming growth factor- β_1 , and oxidative stress in the AM. Because PPAR γ inhibits Nox expression, we hypothesized that alcohol reduces PPAR γ , stimulating AM dysfunction. AMs were examined from: (1) patients with alcoholism or control patients; (2) a mouse model of chronic ethanol consumption; (3) PPAR γ knockout mice; or (4) MH-S cells exposed to ethanol *in vitro*. Alcohol reduced AM PPAR γ levels and increased Nox1, -2, and -4, transforming growth factor- β_1 , oxidative stress, and phagocytic dysfunction. Genetic loss of PPAR γ recapitulated, whereas stimulating PPAR γ activity attenuated

alcohol-mediated alterations in gene expression and phagocytic function, supporting the importance of PPAR γ in alcohol-induced AM derangements. Similarly, PPAR γ activation *in vivo* reduced alcohol-mediated impairments in lung bacterial clearance. Alcohol increased levels of microRNA-130a/-301a, which bind to the PPAR γ 3' untranslated region to reduce PPAR γ expression. MicroRNA-130a/-301a inhibition attenuated alcohol-mediated PPAR γ reductions and derangements in AM gene expression and function. Alcohol-induced Toll-like receptor 4 endocytosis was reversed by PPAR γ activation. These findings demonstrate that targeting PPAR γ provides a novel therapeutic approach for mitigating alcohol-induced AM derangements and susceptibility to lung infection.

Keywords: alcohol; alveolar macrophage; microRNA; phagocytosis; bacterial clearance

Alveolar macrophages (AMs) comprise a critical cellular component of innate and acquired immunity in the lung (1). Recent evidence demonstrated that the nuclear hormone receptor, peroxisome proliferator–activated receptor (PPAR) γ , mediated AM differentiation from fetal monocytes within the lung through up-regulation of

the cytokine, granulocyte/macrophage colony–stimulating factor (GM-CSF) (2). In contrast, the role of PPAR γ in the maintenance of AM identity and function in the adult has not been established.

Chronic alcohol abuse increases susceptibility to respiratory infections (3) and acute respiratory distress syndrome (4), and causes significant morbidity

and mortality. In the United States, approximately 80,000 annual deaths are attributable to excessive drinking, making alcohol abuse the third leading lifestyle-related cause of death (5). Compared with matched subjects without alcoholism, patients with a history of alcohol use disorders and pneumonia are twice as likely to develop sepsis (6), and those

(Received in original form March 9, 2015; accepted in final form December 15, 2015)

This work was supported in part by National Institute on Alcohol Abuse and Alcoholism (NIAAA) T32 training grant 5T32AA013528-08, by Emory Alcohol and Lung Biology Center grant 1P50AA135757, by Department of Veterans Affairs (Biomedical Laboratory Research and Development) Merit Review Award 1I01BX001910, by NIAAA Ruth L. Kirschstein Institutional National Research Service Award 1F32AA020724-01, by American Heart Association Scientist Development Grant 13SDG13930003, by NIAAA K99 grant 1K99AA021803-01A1, by NIAAA R01 grant 5R01-012197, and by Department of Veterans Affairs (Clinical Science Research and Development) Career Development Award 1K2CX000643.

The contents of this report do not represent the views of the U.S. Department of Veterans Affairs or the U.S. Government.

Author Contributions: S.M.Y. designed and analyzed experiments and prepared the manuscript; A.J.M. obtained samples from recruited subjects; F.L.H. obtained samples from animal experiments; L.A.S.B. and C.M.H. designed experiments and prepared the manuscript.

Correspondence and requests for reprints should be addressed to C. Michael Hart, M.D., Atlanta VAMC, Emory University, 1670 Clairmont Road (151-P), Decatur, GA 30033. E-mail: michael.hart3@va.gov

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 1, pp 35–46, Jul 2016

Copyright © 2016 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2015-0077OC on December 17, 2015

Internet address: www.atsjournals.org

Clinical Relevance

Increased susceptibility to lung infections in subjects with alcoholism is due in part to increased oxidative stress in the alveolar macrophage (AM) that impairs phagocytic function. The current study provides novel evidence that stimulating peroxisome proliferator-activated receptor (PPAR) γ with rosiglitazone attenuates alcohol-induced: (1) expression of microRNA-130a and -301a that down-regulate PPAR γ expression, (2) AM oxidative stress and immune dysfunction, and (3) impairments in bacterial clearance in cell line studies *in vitro*, in mouse studies *in vivo*, and in human AMs isolated from patients with alcoholism. The data presented here demonstrate that PPAR γ ligands represent a novel therapeutic approach for attenuating increased susceptibility to respiratory infections in subjects with a history of chronic alcohol abuse.

with sepsis are twice as likely to develop acute respiratory distress syndrome (7).

AMs phagocytose and clear infectious particles from the lower airways (8), and chronic ethanol consumption impairs AM bacterial phagocytosis (9), increasing the risk for lung infection and injury (9, 10). Although the mechanisms are incompletely defined, several studies have provided important mechanistic insights for the deleterious effects of alcohol on AM phagocytosis. Toll-like receptor (TLR) 4 is a cell surface receptor that recognizes bacteria (11, 12). In a mouse macrophage model using RAW264.7 cells, ethanol suppresses LPS-induced macrophage TLR4 clustering, inflammatory response, and actin cytoskeleton remodeling (13), which, in turn, impairs phagocytosis (14, 15). Collectively, these studies suggest that chronic alcohol ingestion may alter machinery that contributes to phagocytic dysfunction and impaired bacterial clearance.

In addition, chronic alcohol ingestion stimulates AM oxidative stress, and attenuating this alcohol-induced oxidative stress reduces the deleterious effects of alcohol on the AMs (10). Reduced nicotinamide adenine dinucleotide oxidase

(Nox) enzymes, including Nox1, Nox2, and Nox4, are critical sources of reactive oxygen species (ROS) in the AMs (16, 17), and Nox2 is essential for respiratory burst involved in killing microbes after phagocytosis (16). However, excessive ROS production suppresses AM phagocytosis (18). Chronic ethanol ingestion stimulates AM oxidative stress through up-regulation of Nox1, Nox2, Nox4 (19), and transforming growth factor (TGF)- β_1 (20). TGF- β_1 enhances Nox4 expression and activity in the vasculature and lungs (21–23), and alcohol-induced increases in TGF- β_1 suppress AM phagocytic function (20). Collectively, these reports suggest that increased AM Nox expression underlies the induction of oxidative stress and phagocytic dysfunction that characterize the alcoholic AM phenotype.

In addition to its role in AM differentiation, evolving evidence demonstrates that PPAR γ activation attenuates Nox expression. Synthetic thiazolidinedione (TZD) PPAR γ ligands, such as rosiglitazone (RSG), are employed clinically to enhance insulin sensitivity during type 2 diabetes treatment (24). However, TZDs attenuated Nox expression in several models, resulting in decreased oxidative stress-related dysfunction and pathology (25–27). Furthermore, loss of PPAR γ function in selected systems was sufficient to activate NF- κ B and stimulate Nox expression (28). Based on limited evidence that TZDs attenuated alcohol-induced Nox expression in lung homogenates (29), we sought to further examine the relationship between chronic alcohol ingestion, PPAR γ , and Nox expression, oxidative stress, and phagocytic dysfunction in the AMs.

To elucidate the molecular mechanisms that regulate PPAR γ during chronic alcohol exposure, we examined microRNAs (miRs) that post-transcriptionally target and suppress PPAR γ expression. In the current study, using the species conserved database, TargetScan (Whitehead Institute for Biomedical Research, Cambridge, MA), and *in silico* analysis, putative binding sites for miR-130a and -301a were identified in the 3' untranslated region (UTR) of PPAR γ , suggesting that increases in miR-130a and -301a could reduce PPAR γ expression. We hypothesized that chronic alcohol ingestion would decrease PPAR γ expression through increased levels of miR-130a and -301a to increase the activity

of Nox and TGF- β_1 expression, enhance AM oxidative stress, and impair phagocytic function.

The findings in the current study provide novel evidence that alcohol reduces PPAR γ by increasing AM miR-130a/301a levels. The importance of PPAR γ in these derangements was supported by studies demonstrating that genetic depletion of PPAR γ was sufficient to stimulate a profile of AM gene expression and phagocytic function observed in the alcoholic AM phenotype. Importantly, stimulating PPAR γ activity with exogenous pharmacological ligands reversed alcohol-induced derangements in AM function and improved bacterial clearance from the lung *in vivo*. Taken together, the current findings emphasize that an environmental stimulus, such as chronic alcohol ingestion, can reduce AM PPAR γ to stimulate phagocytic dysfunction, thereby contributing to pathobiologically relevant alterations in AM function. These results demonstrate that targeting the PPAR γ receptor provides a novel therapeutic approach for attenuating alcohol-induced AM derangements and reducing susceptibility to lung infection and injury.

Materials and Methods

Human AMs

The Emory University Institutional Review Board (Atlanta, GA) and the Atlanta Veterans Affairs Medical Center (Atlanta, GA) Research and Development Committee reviewed and approved this project. Subjects with alcoholism and control subjects without alcoholism were recruited (30). All human subjects provided written informed consent before study enrollment. Bronchoalveolar lavage (BAL) was performed (30) and human AMs (hAMs) were isolated (19, 29). Selected cells were treated with 10 μ M RSG (Cayman Chemicals, Ann Arbor, MI) or methylcellulose vehicle for 24 hours. See the online supplement for further details.

Mouse Models

All animal protocols were reviewed and approved by the Emory University and Atlanta Veterans Affairs Medical Center Institutional Animal Care and Use Committee. Studies were performed in accordance with National Institutes of Health guidelines. Male C57BL/6J mice

(Jackson Laboratory, Bar Harbor, ME), aged 8–10 weeks, were treated with or without ethanol (20% wt/vol) in their drinking water for 12 weeks (19, 29), inducing clinically relevant blood alcohol levels (0.12%) (29). During the final 7 days, control and ethanol mice were gavaged daily with RSG (10 mg/kg/day in 100 μ l methylcellulose vehicle) or vehicle alone (25, 26). In separate studies, mouse AMs (mAMs) were isolated from tissue-targeted Tek tyrosine kinase (Tie2)-Carbapenem-resistant enterobacteriaceae (Cre)-driven PPAR γ knockout (PPAR γ -KO) mice (31, 32) and cultured for 24 hours (19). See the online supplement for additional details.

MH-S Cells

The mAM cell line, MH-S (ATCC, Manassas, VA), was used to study the effects of ethanol *in vitro*. In selected studies, MH-S cells were transiently transfected with 50 nM of either antisense miR (anti-miR) or control anti-miR. MH-S cells were cultured for 24 hours and treated with or without 0.08% ethanol for 3 days with or without 10 μ M RSG during the last 24 hours of ethanol exposure (29). See the online supplement for additional details.

AM Studies

Using quantitative RT-PCR, PPAR γ , Noxs, and TGF- β ₁ mRNA levels (19, 20) and PPAR γ -related miR-130a and -301a levels (33) were measured. Protein levels of PPAR γ , Noxs, and TGF- β ₁ (fluorescent immunomicroscopy) and TLR4 (confocal immunomicroscopy) were assessed. Cellular ROS production, using 2',7'-dichlorofluorescein-diacetate fluorescence (Invitrogen, Carlsbad, CA), and H₂O₂ release, using Amplex Red analysis (Invitrogen), was determined (10, 19). *In vitro* phagocytic capacity was measured using pHrodo *Staphylococcus aureus* BioParticles conjugate (Invitrogen) (34). See the online supplement for additional details.

Lung *Klebsiella pneumoniae* Clearance *In Vivo*

Male C57BL/6J mice with or without ethanol (20% wt/vol, 12 wk) were anesthetized, and 100 μ l of 2×10^6 CFU/ml *K. pneumoniae* (online supplement) was delivered intratracheally (10). After 4 hours, selected mice were gavaged (25, 26) with 10 mg/kg RSG in 100 μ l methylcellulose vehicle or vehicle alone and then killed after

24 hours. BAL fluid and lysed mAMs were cultured to determine *K. pneumoniae* concentrations.

Statistical Analysis

Data are presented as means ($\pm$ SEM). In studies with only two groups, Student's *t* test was employed. In studies with more than two groups, statistical significance was calculated using one-way ANOVA followed by Tukey-Kramer *post hoc* tests to detect differences between individual groups (GraphPad Prism version 5, San Diego, CA). A *P* value less than 0.05 was considered statistically significant.

Results

Treatment with PPAR γ Ligands *Ex Vivo* Reverses Chronic Alcohol Ingestion-Induced Oxidative Stress and Phagocytic Dysfunction in hAMs

Alcohol-induced AM oxidative stress and phagocytic dysfunction are consequences of increased expression and activity of Nox1, Nox2, Nox4 (19), and TGF- β ₁ (20). To determine the role of PPAR γ in these alcohol-induced derangements, hAMs were isolated from subjects with alcoholism or control subjects without alcoholism, then treated with or without RSG *ex vivo*. As shown in Figure 1A, hAMs isolated from subjects with alcoholism demonstrated decreased PPAR γ and increased Nox1, Nox2, Nox4, and TGF- β ₁ mRNA levels, as well as comparable alterations in the protein levels of these targets (Figure 1B; see also Figures E1A–E1E in the online supplement). Compared with hAMs from control subjects, chronic alcohol abuse also stimulated ROS production (Figure 1C) and H₂O₂ generation (Figure 1D), and reduced the phagocytic index (Figure 1E). Treating hAMs with RSG *ex vivo* for 24 hours significantly attenuated these chronic alcohol-induced AM derangements (Figures 1A–1E and Figures E1A–E1E). These findings demonstrate, for the first time, that patterns of chronic alcohol ingestion in patients are sufficient to cause significant reductions in AM PPAR γ expression. These results further indicate that reduced PPAR γ is associated with oxidative stress and AM dysfunction in hAMs, and that existing pharmacological PPAR γ ligands can rapidly attenuate these alcohol-induced derangements to improve AM function.

Treatment with PPAR γ Ligands *In Vivo* Reverses Chronic Alcohol Ingestion-Induced Oxidative Stress and Phagocytic Dysfunction in mAMs

To confirm the therapeutic potential of RSG as an intervention to attenuate alcohol-induced AM dysfunction, a mouse model of chronic ethanol consumption was used, and mAMs were isolated from control and ethanol-fed mice. Comparable to the findings in hAMs in Figure 1, chronic ethanol feeding in mice for 12 weeks decreased mAM PPAR γ and increased Nox1, Nox2, Nox4, and TGF- β ₁ mRNA levels (Figure 2A) and caused comparable alterations in the protein levels of these targets (Figure 2B). Treating mice with RSG *in vivo* by oral gavage during the last week of ethanol exposure attenuated both ethanol-induced reductions in PPAR γ and increases in Nox and TGF- β ₁ mRNA (Figure 2A) and protein (Figure 2B) levels. Chronic ethanol ingestion also stimulated mAM ROS production (Figure 2C) and H₂O₂ generation (Figure 2D), and caused a nearly 50% reduction in phagocytic index (Figure 2E). *In vivo* treatment with RSG attenuated ethanol-induced oxidative stress (Figures 2C and 2D) and completely restored phagocytic function (Figure 2E) in mAMs. Taken together, the findings in Figures 1 and 2 illustrate that chronic ethanol ingestion in the mouse model recapitulates the majority of derangements observed in hAMs isolated from patients with a history of chronic alcohol abuse. These results also provide novel evidence that short-term treatment with PPAR γ ligands attenuates alcohol-induced derangements in AM gene expression and phagocytic function.

Reductions in PPAR γ Are Sufficient to Stimulate AM Oxidative Stress and Phagocytic Dysfunction *In Vivo*

To determine whether reductions in PPAR γ play a causative or associative role in the effects of ethanol on AM function, tissue-targeted PPAR γ -KO mice were employed (31, 32). Compared with littermate controls, AMs from PPAR γ -KO mice exhibited approximately 90% reductions in PPAR γ mRNA levels and increased Nox and TGF- β ₁ mRNA levels (Figure 3A). Comparable reductions in mAM PPAR γ protein and increases in Nox and TGF- β ₁ protein levels (Figure 3B) were seen in PPAR γ -KO

Figure 1. Alcohol-induced human alveolar macrophage (hAM) oxidative stress and phagocytic dysfunction are reversed with the peroxisome proliferator-activated receptor (PPAR) γ ligand, rosiglitazone (RSG). hAMs were collected from control subjects (Con; $n = 5$) and from subjects with an alcohol use disorder (Alc; $n = 5$). hAMs were then treated with or without RSG *ex vivo* for 24 hours. (A) mRNA levels of PPAR γ , reduced nicotinamide adenine dinucleotide oxidase (Nox) 1, Nox2, Nox4, and transforming growth factor (TGF)- β 1 were measured by quantitative RT-PCR (qRT-PCR) ($n = 5$, in duplicate), normalized to 9 s mRNA, and expressed as mean $\pm$ SEM, relative to control. (B) Protein expression of PPAR γ , Nox1, Nox2, Nox4, and TGF- β 1 was determined in hAMs ($n = 5$, 10 fields per experimental condition) using confocal fluorescence microscopy. Quantification of fluorescence values were normalized to 4',6-diamidino-2-phenylindole (DAPI) nuclear stain and expressed as mean relative fluorescence units (RFU) per cell ($\pm$ SEM), relative to control. hAM reactive oxygen species (ROS) production was measured by 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence assay (C), and H₂O₂ generation was measured by Amplex Red assay (D) ($n = 5$, in duplicate). (E) hAM phagocytic ability was assessed by phagocytosis assay ($n = 5$, 10 fields per experimental condition). Phagocytic index was calculated from the percentage of cells positive for bacterial uptake multiplied by the RFU of *Staphylococcus aureus* per cell. All values are expressed as mean $\pm$ SEM relative to control. * $P < 0.05$ versus Con, $\phi P < 0.05$ versus Alc.

mice. Oxidative stress, as measured by 2',7'-dichlorofluorescein-diacetate fluorescence (Figure 3C) and Amplex Red (Figure 3D) assay, was also stimulated in AMs from PPAR γ -KO mice. Phagocytic capacity of PPAR γ -KO mAMs was impaired by 47 (± 3.5)% (Figure 3E). Ethanol feeding did not significantly exacerbate the detrimental effects of knocking down PPAR γ in mAMs, and RSG treatment did not significantly improve the detrimental effects of combined ethanol and PPAR γ -KO (data not shown). These data indicate that reductions in PPAR γ are sufficient to replicate the ethanol-induced AM phenotype.

Alcohol Increases AM PPAR γ -Related miR-130a and -301a: Attenuation with PPAR γ Ligands

To further examine the molecular mechanisms responsible for alcohol-mediated reductions in AM PPAR γ levels, PPAR γ -related miR-130a and -301a expression were examined. Compared with control subjects, levels of miR-130a and -301a were increased in hAMs (Figure 4A) and mAMs (Figure 4B) after exposure to alcohol *in vivo*, suggesting that alcohol suppresses PPAR γ expression by increasing PPAR γ -related miRs. Conversely, RSG treatment in hAMs (Figure 4A) *ex vivo* and mAMs *in vivo* (Figure 4B) partially attenuated

ethanol-induced miR-130a and -301a levels. These data indicate that preservation of a threshold level of PPAR γ signaling through treatment with exogenous ligands can attenuate proximal, upstream ethanol-induced signaling derangements in the AMs.

In Vitro RSG Treatment and miR Inhibition Reverse Ethanol-Induced AM Gene Alterations

To confirm the role of miRs in alcohol-mediated alterations in PPAR γ that lead to AM derangements, an *in vitro* model using the mAM MH-S cell line was used. Similar to findings in AMs from ethanol-fed mice, *in vitro* treatment of MH-S cells

Figure 2. RSG reverses ethanol-induced mouse alveolar macrophage (mAM) oxidative stress and phagocytic dysfunction *in vivo*. mAMs were collected from Con and ethanol-fed (EtOH; 12 wk) mice with or without oral RSG for the last 7 days of ethanol feeding. (A) mRNA levels of PPAR γ , Nox1, Nox2, Nox4, and TGF- β_1 were measured in mAMs ($n = 5$, in duplicate). All mRNA values were measured by qRT-PCR, normalized to 9 s mRNA, and expressed as mean $\pm$ SEM relative to control. (B) Protein expression of PPAR γ , Nox1, Nox2, Nox4, and TGF- β_1 was determined in mAMs ($n = 5$, 10 fields per experimental condition), as measured by computer analysis of confocal fluorescence microscopic images. Quantification of all fluorescence values were normalized to DAPI nuclear stain and expressed as mean RFU per cell ($\pm$ SEM) relative to control or total RFU. mAM ROS production was measured by DCFH-DA fluorescence assay (C), and H₂O₂ generation was measured by Amplex Red assay (D) ($n = 5$, in duplicate). mAM phagocytic ability was assessed by phagocytosis assay ($n = 5$, 10 fields per experimental condition) (E). Phagocytic index was calculated from the percentage of cells positive for bacterial uptake multiplied by the RFU of *S. aureus* per cell. All values are expressed as mean $\pm$ SEM relative to control. * $P < 0.05$ versus Con, $\phi P < 0.05$ versus EtOH.

with ethanol decreased PPAR γ levels, and increased Nox1, Nox2, Nox4, and TGF- β_1 (Figures E2A and E2B), miR-130a (Figure 5A), and miR-301a levels (Figure E3A). RSG treatment attenuated ethanol-induced alterations in PPAR γ , Nox1, Nox2, Nox4, TGF- β_1 (Figures E2A and E2B), miR-130a (Figure 5A), and miR-301a levels (Figure E3A). Selected MH-S cells were also transfected with control anti-miR, anti-miR-130a (Figure 5), or anti-miR-301a (Figure E3). Compared with MH-S cells transfected with a control anti-miR, anti-miR-130a inhibited basal miR-130a levels by approximately 58% (Figure 5A) and anti-miR-301a inhibited basal miR-301a

levels by approximately 56% (Figure E3A). Anti-miR-130a did not affect basal miR-301a levels, and anti-miR-301a did not affect basal miR-130a levels, in these transfected MH-S cells (data not shown).

In MH-S cells treated with ethanol, increases in miR-130a levels were attenuated by treatments with RSG, anti-miR-130a, or combined RSG plus anti-miR-130a (Figure 5A). In control cells, RSG treatment had no effect on miR-130a (Figure 5A), PPAR γ (Figure 5B), or PPAR γ 3'UTR luciferase activity (Figure 5C). In contrast, treatment with anti-miR-130a increased basal PPAR γ mRNA levels and PPAR γ 3'UTR luciferase activity in control cells (Figures 5B and 5C). Ethanol-mediated reductions in

PPAR γ mRNA levels and PPAR γ 3'UTR luciferase activity were partially restored by treatments with RSG, fully restored by anti-miR-130a, and further increased by RSG plus anti-miR-130a (Figures 5B and 5C). RSG had no effect on basal expression of Nox1, Nox2, Nox4, or TGF- β_1 , whereas ethanol-induced expression of these targets was attenuated by treatments with RSG, anti-miR-130a, or combined RSG plus anti-miR-130a (Figures 5D–5G). Similar results were also observed in experiments using anti-miR-301a (Figures E3A–E3G). Collectively, these data indicate that RSG and anti-miR-130a or anti-miR-301a can reverse ethanol-induced reductions in PPAR γ and thereby normalize Nox1, Nox2, Nox4, and TGF- β_1 expression.

Figure 3. Reductions in mAM PPAR γ expression are sufficient to replicate the alcoholic AM phenotype. mAMs were collected from Tek tyrosine kinase–Carbapenem-resistant enterobacteriaceae–driven PPAR γ knockout (PPAR γ KO) and littermate control (Littermate Con) mice. (A) mRNA levels of PPAR γ , Nox1, Nox2, Nox4, and TGF- β ₁ were measured in mAMs ($n = 7$, in duplicate). All mRNA values were measured by qRT-PCR, normalized to 9 s mRNA, and expressed as mean $\pm$ SEM relative to Littermate Con. (B) Protein expression of PPAR γ , Nox1, Nox2, Nox4, and TGF- β ₁ was determined in mAMs ($n = 7$, 10 fields per experimental condition) using confocal fluorescence microscopy. Fluorescence intensities were normalized to DAPI nuclear stain and expressed as mean RFU per cell ($\pm$ SEM) relative to Littermate Con. mAM ROS production was measured by DCFH-DA fluorescence assay (C), and H₂O₂ generation was measured by Amplex Red assay (D) ($n = 7$, in duplicate). mAM phagocytic ability was assessed by phagocytosis assay ($n = 7$, 10 fields per experimental condition) (E). Phagocytic index was calculated from the percentage of phagocytic cells multiplied by the RFU of *S. aureus* per cell. All values are expressed as mean $\pm$ SEM relative to Littermate Con. * $P < 0.05$ versus Littermate Con.

The regulation of these targets by RSG in ethanol-treated cells, but not control cells, suggests that RSG regulates ethanol-stimulated pathways that alter miR-130a and -301a rather than directly modifying the transcriptional activity of the promoter elements of miR-130a and -301a, PPAR γ , Noxs, or TGF- β ₁.

RSG Treatment and miR Inhibition Reverse Ethanol-Induced AM Oxidative Stress and Dysfunction *In Vitro*

To evaluate the role of miR-130a and -301a in ethanol-induced AM oxidative stress and dysfunction *in vitro*, MH-S cells were transiently transfected with anti-miR or control anti-miR followed by treatment with ethanol with or without RSG.

Compared with MH-S cells transfected with a control anti-miR, anti-miR-130a attenuated basal ROS production (Figure 6A) and basal H₂O₂ generation (Figure 6B), consistent with anti-miR-130a–induced reductions in Nox expression (Figures 5D–5F). Ethanol-induced ROS production and H₂O₂ generation were decreased by treatments with RSG, anti-miR-130a, or combined RSG plus anti-miR-130a (Figures 6A and 6B). Furthermore, ethanol-induced phagocytic dysfunction was partially attenuated by RSG treatment, but more effectively restored by treatments with anti-miR-130a or combined RSG plus anti-miR-130a (Figure 6C). Similar results were also

observed in experiments using anti-miR-301a (Figures E4A–E4C). Taken together, these data demonstrate that ethanol-induced AM oxidative stress and dysfunction can be attenuated by targeting PPAR γ with RSG or with strategies that prevent increases in PPAR γ -related miRs and subsequent reductions in PPAR γ expression.

RSG Improves Clearance of *K. pneumoniae* from the Lung *In Vivo*

TLR4 is a cell surface receptor that recognizes bacteria for uptake by phagocytic cells (11, 12). To determine if alcohol-induced AM phagocytic dysfunction is due to alterations in the ability of TLR4

Figure 4. RSG attenuates ethanol-induced increases in PPAR γ -related microRNAs (miRs) in hAMs and mAMs. miR levels of PPAR γ -related miR-130a and -301a were measured in hAMs collected from Con and Alc treated with or without RSG *ex vivo* for 24 hours (A), and mAMs collected from Con and EtOH (12 wk) mice with or without oral RSG for the last 7 days of ethanol feeding (B). Levels were measured using qRT-PCR ($n = 5$, in duplicate), normalized to 5 U ribosomal mRNA, and expressed as mean $\pm$ SEM relative to control. * $P < 0.05$ versus Con, $\phi P < 0.05$ versus EtOH or Alc.

to recognize gram-negative bacteria, mAMs were isolated from control and ethanol-fed mice treated with or without RSG *ex vivo*. Although preliminary data did not show that ethanol transcriptionally regulated TLR4 expression (data not shown), chronic ethanol ingestion impaired the expression of TLR4 on the MH-S cell surface (Figure 7A) and mAM cell surface (Figure 7B). These ethanol-induced derangements were attenuated by *ex vivo* treatment with the PPAR γ ligand, RSG (Figures 7A and 7B).

To determine if RSG therapy can improve bacterial clearance in a mouse model of chronic alcohol consumption, BAL fluid and mAMs were isolated from control and ethanol-fed mice that were challenged with *K. pneumoniae* and treated with or without RSG *in vivo* for 24 hours. BAL fluid CFUs represented extracellular killing of *K. pneumoniae*, and AM CFUs represented intracellular killing of the bacteria. Compared with control *K. pneumoniae* mice, *in vivo* ethanol exposure impaired clearance of *K. pneumoniae* bacteria from BAL fluid (Figure 7C) and mAMs (Figure 7D). RSG treatment in ethanol-fed mice restored bacterial clearance from BAL fluid and mAMs to levels observed in control mice (Figures 7C and 7D). Collectively, these data indicate that RSG treatment *in vivo* attenuates ethanol-induced AM dysfunction and improves *K. pneumoniae* clearance from the lung.

Discussion

Chronic ethanol consumption increases susceptibility to lung injury (9) by stimulating AM expression of Nox1, Nox2, Nox4 (19), and TGF- β_1 (20) to enhance oxidative stress and subsequent phagocytic dysfunction. PPAR γ activation with RSG *in vivo* attenuated chronic ethanol-induced Nox expression and activity in mouse lungs and reduced susceptibility to lung injury (29). Although Noxs and TGF- β_1 are principal mediators of ethanol-induced oxidative stress, the specific mechanisms by which ethanol increased levels of these mediators were not determined. The current study provides novel evidence that alcohol increases specific AM miR to reduce PPAR γ , and that reductions in PPAR γ stimulate increases in Nox and TGF- β_1 expression, causing AM oxidative stress and phagocytic dysfunction. Furthermore, these animal and clinical studies (summarized in Figure E5) demonstrate that stimulating PPAR γ not only attenuates alcohol-induced alterations in levels of miR, PPAR γ , Noxs, and TGF- β_1 in AMs, but also restores phagocytic function and bacterial clearance. These results thereby identify a novel and promising therapeutic strategy to mitigate the adverse effects of chronic alcohol ingestion in the lung.

The central role of PPAR γ in the alcoholic AM phenotype was established using a Tie2-Cre tissue-targeted approach

to knockdown mAM PPAR γ . Although Tie2 is also expressed by endothelial cells (32), its expression in monocytes and monocyte-derived macrophages (31) significantly depleted AM PPAR γ mRNA and protein levels, which then increased Nox and TGF- β_1 expression, enhanced oxidative stress, and impaired phagocytic function. Although oxidative stress reduces PPAR γ expression in selected tissues and pathological conditions, the results in PPAR γ -KO mice place PPAR γ upstream of Noxs and TGF- β_1 , supporting the rationale for investigating the molecular mechanisms by which ethanol represses PPAR γ , and justifying the examination of miRs that target and reduce PPAR γ expression. miRs have been implicated in the pathobiology of pulmonary diseases (35), and have been studied as master regulators of alcohol-induced disorders (36). miRs suppress or inhibit translation of mRNA to protein by binding to seed sequences in the 3'UTR of their target mRNAs (37). Using TargetScan and *in silico* analysis, putative binding sites for miR-130a and -301a were identified in the 3'UTR of PPAR γ . Using *in vivo* and *in vitro* models of chronic alcohol ingestion, we showed, for the first time, that alcohol increased the expression of PPAR γ -related miR-130a and -301a in hAMs, mAMs, and MH-S cells.

The molecular mechanisms involved in alcohol-induced miR-130a and -301a expression remain to be defined. miR promoters contain redox-sensitive

Figure 5. RSG treatment and miR-130a inhibition reverse ethanol-induced AM alterations in gene expression *in vitro*. Selected MH-S cells were transiently transfected with control anti-miR or anti-miR-130a and treated with either Con or EtOH (0.08%) conditions for 3 days. Selected MH-S cells were also treated with RSG for the last 24 hours of Con or EtOH exposure. (A) PPAR γ -related miR-130a levels were measured by qRT-PCR ($n=3$, in duplicate), normalized to 5 S ribosomal mRNA, and expressed as mean $\pm$ SEM relative to control. PPAR γ mRNA levels (B) were measured by qRT-PCR, and PPAR γ 3' untranslated region (UTR) activity (C) was measured by luciferase assay, normalized to protein concentrations, and expressed as mean $\pm$ SEM relative to control. mRNA levels of Nox1 (D), Nox2 (E), Nox4 (F), and TGF- β $_1$ (G) were measured by qRT-PCR ($n=3$, in duplicate), normalized to 9 S mRNA, and expressed as mean $\pm$ SEM relative to control. * $P < 0.05$ versus Con, $\Phi P < 0.05$ versus EtOH.

Figure 6. RSG and anti-miR-130a reverse ethanol-induced AM oxidative stress and dysfunction *in vitro*. Selected MH-S cells were transiently transfected with control anti-miR or anti-miR-130a (A–C) and treated with either Con or EtOH (0.08%) conditions for 3 days. Selected MH-S cells were also treated with RSG for the last 24 hours of Con or EtOH exposure. ROS production was measured by DCFH-DA fluorescence assay (A) and H₂O₂ generation was measured by Amplex Red assay (B) in MH-S cells ($n = 3$, in duplicate). MH-S cell phagocytic function was assessed by phagocytosis assay ($n = 3$, 10 fields per experimental condition), and phagocytic index (C) was calculated from the percentage of phagocytic cells multiplied by the RFU of *S. aureus* per cell. All values are expressed as mean $\pm$ SEM relative to control. * $P < 0.05$ versus Con, ^φ $P < 0.05$ versus EtOH.

transcription factor binding sites (38). *In silico* analysis of PPAR γ -related miR promoters revealed putative binding sites for CCAAT/enhancer binding protein α , which can activate miR expression (39), and activator protein-1, which is increased in chronic alcohol consumption (40). Similarly, NF- κ B activation increased miR-301 levels in pancreatic cancer cells (41). Furthermore, the hypoxia-inducible factor family of transcription factors, which are increased during alcohol abuse (33), were up-regulated in AMs lacking PPAR γ (2), and are known to stimulate the miR-130/-301 family (42). The examination of these transcriptional pathways in AMs during chronic alcohol exposure should provide fertile areas for future investigations of the mechanisms involved in ethanol-induced PPAR γ -related miR expression. The importance of increases in miR-130a/-301a is supported by evidence that either anti-miR-130a (Figure 5) or -301a (Figure E3) attenuated ethanol-induced reductions in PPAR γ expression and increases in Nox1, Nox2, Nox4, TGF- β 1, and oxidative stress (Figure 6 and Figure E4). Species-conserved putative binding sites for miR-130a and -301a were identified in the PPAR γ 3'UTR. However, no putative binding sites were identified for these miRs on the 3'UTRs of Nox1, Nox2, Nox4, or TGF- β 1. These data illustrate that miR-130a and -301a directly regulate PPAR γ expression, but that downstream targets of chronic ethanol ingestion, such as Nox and TGF- β 1, are regulated through

PPAR γ -induced suppression of their promoter elements (29, 43).

The miR-130a and -301a family has been implicated in oxidative stress-related pathologies, such as atherosclerosis, inflammation, and angiogenesis (44, 45). Consistent with this concept, previous studies in an experimental model of hypoxia-induced pulmonary hypertension reported increased lung levels of miR-130a and -301a (46) and reduced PPAR γ levels (28). Recently, miR130a/-301a was shown to play a critical role in regulating PPAR γ and downstream proliferative signaling pathways involved in pulmonary vascular remodeling and pulmonary hypertension pathogenesis (42). Interestingly, PPAR γ ligands modulate the expression of redox signaling and oxidative stress targets, including Nox4 and TGF- β 1 (47). miR-130a and -301a are members of the same family of miRs, and bind to the same seed sequence in the PPAR γ 3'UTR. Our studies show that miR-130a and -301a responses to alcohol are nearly identical in AMs, similar to alterations in miR-130a and -301a levels in mouse lungs and pulmonary artery smooth muscle cells (42). Our data demonstrate the critical role of either miR-130a (Figure 5) or -301a (Figure E3) as destabilizers of PPAR γ expression, further supporting the importance of PPAR γ signaling in maintaining a balanced redox environment crucial for normal AM phagocytic function.

Although our data provide evidence that alcohol increases miR-130a/-301a levels to reduce PPAR γ expression, studies

employing the PPAR γ ligand, RSG, suggest that PPAR γ , in turn, regulates alcohol-induced miR-130a/-301a levels (Figure 4). RSG had no effect on basal expression levels of miR-130a, miR-301a, PPAR γ , Nox, or TGF- β 1 in control AMs. However, activation of PPAR γ with RSG attenuated ethanol-mediated increases in miR-130a/-301a levels. RSG, as a synthetic PPAR γ ligand, mediates suppression of the activity of proinflammatory transcription factors, such as NF- κ B, through transrepression mechanisms (27). Because PPAR γ promotes antiinflammatory signaling cascades (48), we postulate that RSG inhibits alcohol-induced proinflammatory transcriptional pathways by maintaining a threshold of PPAR γ activity through down-regulation of both miR-130a and miR-301a and up-regulation of factors that likely regulate the expression of Nox proteins and TGF- β 1. Redox-sensitive and inflammatory-mediated downstream targets of miR-130a/-301a, such as the hypoxia-inducible factor family of transcription factors and signal transducer and activator of transcription 3 (42) or NF- κ B-repressing factor (41), are of particular interest for future studies.

Studies examining hAMs from subjects with alcoholism and control subjects without alcoholism emphasize the clinical relevance of our findings. Similar to ethanol-exposed mAMs and MH-S cells, hAMs from subjects with alcoholism showed reductions in PPAR γ expression and increases in the expression of miR-130a

Figure 7. RSG promotes clearance of *Klebsiella pneumoniae* from the lung in alcohol-fed mice. Selected microscopy images and quantification of Toll-like receptor (TLR) 4 subcellular localization at 60 $\times$ magnification and 50% of the Z-plane depth are provided for (A). MH-S cells treated with either Con or EtOH (0.08%) with or without RSG (10 μ M, 24 h) and (B) mAMs collected from Con or EtOH (12 wk) mice with or without RSG *ex vivo* (10 μ M, 24 h). Cell plasma membranes were stained with peanut agglutinin (PNA). Scale bars: 20.0 μ m. Quantification of all fluorescence values were normalized to DAPI nuclear stain ($n=5$, 10 fields per experimental condition) and expressed as mean RFU per cell ($\pm$ SEM) relative to control or total RFU. Mice fed EtOH or Con diets for 12 weeks received intratracheal *K. pneumoniae* (Kleb; 200 CFU) and then 4 hours later were treated with or without 10 mg/kg RSG ($n=4-5$). After 24 hours, clearance of *K. pneumoniae* was assessed in bronchoalveolar lavage (BAL) fluid (C) and lysed mAMs (D) that were cultured on MacConkey agar, where concentration of *K. pneumoniae* bacteria was quantified as CFU. CFU in BAL fluid was normalized to number of isolated macrophages. All values are expressed as mean $\pm$ SEM relative to untreated controls with bacterial challenge. * $P < 0.05$ versus Con + Kleb, $^{\phi}P < 0.05$, versus EtOH + Kleb.

and -301a, Noxs, TGF- β_1 , as well as oxidative stress and phagocytic dysfunction (Figure 1). Therapeutic intervention with RSG *ex vivo* (24 h) partially reversed these alcohol-induced derangements. The difference in RSG treatment methods and duration between mice (10 mg/kg/d by oral gavage for 7 d *in vivo*) and human subjects (10 μ M for 24 h *ex vivo*) may account for the perceived variations in RSG effectiveness in our studies. However, to our knowledge, this is the first study using clinical samples to implicate PPAR γ -related miRs in mediating alcohol-induced AM derangements. These results illustrate that strategies to activate PPAR γ can reverse alcohol-induced impairments in AM phagocytic function. Combined with our *in vivo* *K. pneumoniae* challenge data, where acute RSG treatment restored lung bacterial clearance and AM phagocytic function during chronic ethanol consumption (Figure 7), our studies set the stage for future clinical trials using RSG as a novel therapeutic strategy for attenuating respiratory infections seen in subjects with alcoholism. Although our studies describe RSG treatment of hAMs for 24 hours *ex vivo* to attenuate alcohol-induced derangements, optimization of dose and duration of PPAR γ ligand treatment in the management of patients with alcoholism will require additional investigation.

The current studies, although demonstrating that chronic ethanol ingestion and PPAR γ knockdown produce comparable increases in AM oxidative stress and phagocytic impairment (Figures 2D–2F and 3C–3E), do not define the molecular mechanisms by which alcohol impairs AM phagocytosis. Other studies suggest that impaired clearance of *Streptococcus pneumoniae* in mice with macrophage-specific knockdown of PPAR γ is caused by

altered macrophage maturation and dysregulation of innate immune receptors, including TLRs (49). Therefore, depletion of PPAR γ could impair AM phagocytic function by multiple potential mechanisms. Alcohol-mediated decreases in PPAR γ may contribute to diminished AM differentiation and maturity (2), including down-regulation of the macrophage maturation marker, GM-CSF receptor β . Chronic alcohol ingestion perturbed AM differentiation, as evidenced by decreases in the expression of GM-CSF receptor β (30), which leads to AM phagocytic dysfunction. Furthermore, PPAR γ cross-talk with NF- κ B is mediated by the bacterial receptor, TLR4, in macrophages (50). Alcohol-induced perturbations in TLR4, combined with PPAR γ depletion up-regulating Nox4 via NF- κ B activation (27), suggest that phagocytic dysfunction is mediated by derangements in AM bacterial recognition and innate immune response. In our studies, *in vitro* phagocytosis assays were performed with *S. aureus*, a gram-positive bacterium, whereas *in vivo* phagocytosis and bacterial clearance assays were performed with *K. pneumoniae*, a gram-negative bacterium. We focused on *in vivo* studies using *K. pneumoniae*, rather than *S. aureus*, because it is a more clinically relevant pathogen in subjects with alcoholism (6). Studies by Jong and colleagues (6) demonstrate a high mortality rate in subjects with alcoholism with bacteremic *K. pneumoniae* pneumonia. Because TLR4 is known to have a role in the recognition of gram-negative bacteria, we investigated the response of TLR4 to alcohol as one possible mechanism by which phagocytosis of *K. pneumoniae* may be impaired. To our knowledge, the data presented herein are not only the first to demonstrate that alcohol-induced AM

endocytosis of the bacterial recognition receptor, TLR4 (Figures 7A and 7B), can be reversed with RSG treatment, but they are also the first to demonstrate that targeting PPAR γ with acute RSG treatment can improve lung and AM clearance of *K. pneumoniae* during chronic alcohol ingestion (Figures 7C and 7D). Although further studies are warranted to elucidate the associations between alcohol-induced decreases in PPAR γ expression, AM maturity, and TLR4-mediated bacterial recognition, these findings confirm that PPAR γ plays a critical role in lung immunity.

Collectively, our data demonstrate that alcohol-induced expression of PPAR γ -related miR-130a and -301a reduce PPAR γ levels, and that reduced PPAR γ increases expression and activity of Noxs and TGF- β_1 , leading to enhanced oxidative stress and impaired phagocytic function. The data presented herein extend our previous findings that ethanol increases AM oxidative stress and phagocytic dysfunction (10, 19) by demonstrating that PPAR γ plays an important role in both the pathogenesis and potential treatment of alcohol-induced derangements in both hAMs and mAMs. These findings indicate that targeting PPAR γ represents a novel therapeutic strategy for attenuating chronic alcohol-induced AM derangements. These results provide the necessary mechanistic data and proof of principle to further investigate PPAR γ ligands in pilot clinical studies in human subjects with a history of chronic alcohol ingestion. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The insightful comments of Dr. Ruxana Sadikot during the preparation of the manuscript are gratefully acknowledged.

References

1. Trapnell BC, Whitsett JA. GM-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. *Annu Rev Physiol* 2002;64:775–802.
2. Schneider C, Nobs SP, Kurrer M, Rehrauer H, Thiele C, Kopf M. Induction of the nuclear receptor PPAR- γ by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. *Nat Immunol* 2014;15:1026–1037.
3. Mehta AJ, Guidot DM. Alcohol abuse, the alveolar macrophage and pneumonia. *Am J Med Sci* 2012;343:244–247.
4. Moss M, Parsons PE, Steinberg KP, Hudson LD, Guidot DM, Burnham EL, Eaton S, Cotsonis GA. Chronic alcohol abuse is associated with an increased incidence of acute respiratory distress syndrome and severity of multiple organ dysfunction in patients with septic shock. *Crit Care Med* 2003;31:869–877.
5. Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. *JAMA* 2004;291:1238–1245.
6. Jong GM, Hsue TR, Chen CR, Chang HY, Chen CW. Rapidly fatal outcome of bacteremic *Klebsiella pneumoniae* pneumonia in alcoholics. *Chest* 1995;107:214–217.
7. Joshi PC, Guidot DM. The alcoholic lung: epidemiology, pathophysiology, and potential therapies. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L813–L823.
8. Lundahl J, Halldén G, Sköld CM. Human blood monocytes, but not alveolar macrophages, reveal increased CD11b/CD18 expression and adhesion properties upon receptor-dependent activation. *Eur Respir J* 1996;9:1188–1194.
9. Brown LA, Harris FL, Ping XD, Gauthier TW. Chronic ethanol ingestion and the risk of acute lung injury: a role for glutathione availability? *Alcohol* 2004;33:191–197.

10. Yeligar SM, Harris FL, Hart CM, Brown LA. Glutathione attenuates ethanol-induced alveolar macrophage oxidative stress and dysfunction by downregulating NADPH oxidases. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L429–L441.
11. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, Florquin S, van der Poll T. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect Immun* 2004;72:788–794.
12. van der Aar AM, Sylva-Steenland RM, Bos JD, Kapsenberg ML, de Jong EC, Teunissen MB. Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition. *J Immunol* 2007;178:1986–1990.
13. Dai Q, Pruett SB. Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton, and associated TNF- α production. *Alcohol Clin Exp Res* 2006;30:1436–1444.
14. Bird MD, Zahs A, Deburghgraeve C, Ramirez L, Choudhry MA, Kovacs EJ. Decreased pulmonary inflammation following ethanol and burn injury in mice deficient in TLR4 but not TLR2 signaling. *Alcohol Clin Exp Res* 2010;34:1733–1741.
15. Coppolino MG, Dierckman R, Loijens J, Collins RF, Pouladi M, Jongstra-Bilen J, Schreiber AD, Trimble WS, Anderson R, Grinstein S. Inhibition of phosphatidylinositol-4-phosphate 5-kinase α impairs localized actin remodeling and suppresses phagocytosis. *J Biol Chem* 2002;277:43849–43857.
16. Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med* 2002;166:S4–S8.
17. Piotrowski WJ, Marczak J. Cellular sources of oxidants in the lung. *Int J Occup Med Environ Health* 2000;13:369–385.
18. Liang Y, Harris FL, Brown LA. Alcohol induced mitochondrial oxidative stress and alveolar macrophage dysfunction. *Biomed Res Int* 2014;2014:371593.
19. Yeligar SM, Harris FL, Hart CM, Brown LA. Ethanol induces oxidative stress in alveolar macrophages via upregulation of NADPH oxidases. *J Immunol* 2012;188:3648–3657.
20. Brown SD, Brown LA. Ethanol (EtOH)-induced TGF- β 1 and reactive oxygen species production are necessary for EtOH-induced alveolar macrophage dysfunction and induction of alternative activation. *Alcohol Clin Exp Res* 2012;36:1952–1962.
21. Lassègue B, Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 2010;30:653–661.
22. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, et al. Transforming growth factor- β 1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L661–L673.
23. Brown DI, Griendling KK. Nox proteins in signal transduction. *Free Radic Biol Med* 2009;47:1239–1253.
24. Yki-Järvinen H. Thiazolidinediones. *N Engl J Med* 2004;351:1106–1118.
25. Hwang J, Kleinhenz DJ, Rupnow HL, Campbell AG, Thulé PM, Sutliff RL, Hart CM. The PPAR γ ligand, rosiglitazone, reduces vascular oxidative stress and NADPH oxidase expression in diabetic mice. *Vascul Pharmacol* 2007;46:456–462.
26. Nisbet RE, Bland JM, Kleinhenz DJ, Mitchell PO, Walp ER, Sutliff RL, Hart CM. Rosiglitazone attenuates chronic hypoxia-induced pulmonary hypertension in a mouse model. *Am J Respir Cell Mol Biol* 2010;42:482–490.
27. Lu X, Murphy TC, Nanes MS, Hart CM. PPAR γ regulates hypoxia-induced Nox4 expression in human pulmonary artery smooth muscle cells through NF- κ B. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L559–L566.
28. Bijli KM, Kleinhenz JM, Murphy TC, Kang BY, Adesina SE, Sutliff RL, Hart CM. Peroxisome proliferator-activated receptor γ depletion stimulates Nox4 expression and human pulmonary artery smooth muscle cell proliferation. *Free Radic Biol Med* 2015;80:111–120.
29. Wagner MC, Yeligar SM, Brown LA, Michael Hart C. PPAR γ ligands regulate NADPH oxidase, eNOS, and barrier function in the lung following chronic alcohol ingestion. *Alcohol Clin Exp Res* 2012;36:197–206.
30. Mehta AJ, Yeligar SM, Elon L, Brown LA, Guidot DM. Alcoholism causes alveolar macrophage zinc deficiency and immune dysfunction. *Am J Respir Crit Care Med* 2013;188:716–723.
31. Guignabert C, Alvira CM, Alastalo TP, Sawada H, Hansmann G, Zhao M, Wang L, El-Bizri N, Rabinovitch M. Tie2-mediated loss of peroxisome proliferator-activated receptor- γ in mice causes PDGF receptor- β -dependent pulmonary arterial muscularization. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L1082–L1090.
32. Kleinhenz JM, Kleinhenz DJ, You S, Ritzenthaler JD, Hansen JM, Archer DR, Sutliff RL, Hart CM. Disruption of endothelial peroxisome proliferator-activated receptor- γ reduces vascular nitric oxide production. *Am J Physiol Heart Circ Physiol* 2009;297:H1647–H1654.
33. Yeligar S, Tsukamoto H, Kalra VK. Ethanol-induced expression of ET-1 and ET-BR in liver sinusoidal endothelial cells and human endothelial cells involves hypoxia-inducible factor-1 α and microRNA-199. *J Immunol* 2009;183:5232–5243.
34. Brown SD, Gauthier TW, Brown LA. Impaired terminal differentiation of pulmonary macrophages in a Guinea pig model of chronic ethanol ingestion. *Alcohol Clin Exp Res* 2009;33:1782–1793.
35. Nana-Sinkam SP, Hunter MG, Nuovo GJ, Schmittgen TD, Gelinas R, Galas D, Marsh CB. Integrating the MicroRNome into the study of lung disease. *Am J Respir Crit Care Med* 2009;179:4–10.
36. Miranda RC, Pietrzykowski AZ, Tang Y, Sathyan P, Mayfield D, Keshavarzian A, Sampson W, Herold D. MicroRNAs: master regulators of ethanol abuse and toxicity? *Alcohol Clin Exp Res* 2010;34:575–587.
37. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol* 2007;23:175–205.
38. Piriyaopongsa J, Jordan IK, Conley AB, Ronan T, Smalheiser NR. Transcription factor binding sites are highly enriched within microRNA precursor sequences. *Biol Direct* 2011;6:61.
39. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* 2008;9:839–845.
40. Yeligar SM, Machida K, Tsukamoto H, Kalra VK. Ethanol augments RANTES/CCL5 expression in rat liver sinusoidal endothelial cells and human endothelial cells via activation of NF- κ B, HIF-1 α , and AP-1. *J Immunol* 2009;183:5964–5976.
41. Lu Z, Li Y, Takmi A, Li B, Zhang J, Conklin DJ, Young KH, Martin R, Li Y. miR-301a as an NF- κ B activator in pancreatic cancer cells. *EMBO J* 2011;30:57–67.
42. Bertero T, Lu Y, Annis S, Hale A, Bhat B, Saggat R, Saggat R, Wallace WD, Ross DJ, Vargas SO, et al. Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. *J Clin Invest* 2014;124:3514–3528.
43. Lu X, Bijli KM, Ramirez A, Murphy TC, Kleinhenz J, Hart CM. Hypoxia downregulates PPAR γ via an ERK1/2–NF- κ B–Nox4-dependent mechanism in human pulmonary artery smooth muscle cells. *Free Radic Biol Med* 2013;63:151–160.
44. Li T, Cao H, Zhuang J, Wan J, Guan M, Yu B, Li X, Zhang W. Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans. *Clin Chim Acta* 2011;412:66–70.
45. Urbich C, Kuehnbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008;79:581–588.
46. Caruso P, MacLean MR, Khanin R, McClure J, Soon E, Southgate M, MacDonald RA, Greig JA, Robertson KE, Masson R, et al. Dynamic changes in lung microRNA profiles during the development of pulmonary hypertension due to chronic hypoxia and monocrotaline. *Arterioscler Thromb Vasc Biol* 2010;30:716–723.
47. Green DE, Sutliff RL, Hart CM. Is peroxisome proliferator-activated receptor γ (PPAR γ) a therapeutic target for the treatment of pulmonary hypertension? *Pulm Circ* 2011;1:33–47.
48. Clark RB. The role of PPARs in inflammation and immunity. *J Leukoc Biol* 2002;71:388–400.
49. Gautier EL, Chow A, Spanbroek R, Marcelin G, Greter M, Jakubzick C, Bogunovic M, Leboeuf M, van Rooijen N, Habenicht AJ, et al. Systemic analysis of PPAR γ in mouse macrophage populations reveals marked diversity in expression with critical roles in resolution of inflammation and airway immunity. *J Immunol* 2012;189:2614–2624.
50. Necela BM, Su W, Thompson EA. Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor γ and nuclear factor- κ B in macrophages. *Immunology* 2008;125:344–358.