HEPATOLOGY COMMUNICATIONS, VOL. 1, NO. 4, 2017

Intermittent Hypoxia Is a Proinflammatory Stimulus Resulting in IL-6 Expression and M1 Macrophage Polarization

Esperance Schaefer, Winona Wu, Christina Mark, Andrew Yang, Erik DiGiacomo, Charles Carlton-Smith, Shadi Salloum, Cynthia Brisac, Wenyu Lin, Kathleen E. Corey, and Raymond T. Chung

The biological factors that promote inflammation or nonalcoholic steatohepatitis (NASH) in the setting of nonalcoholic fatty liver disease remain incompletely understood. Clinical studies have demonstrated an association between obstructive sleep apnea (OSA) and both inflammation and fibrosis in NASH, but the mechanism has not been identified. In this study, we use *in vitro* modeling to examine the impact of intermittent hypoxia on the liver. Hepatocyte, stellate cell, and macrophage cell lines were exposed to intermittent or sustained hypoxia. Candidate genes associated with inflammation, fibrosis, and lipogenesis were analyzed. Circulating cytokines were assessed in human serum of patients with nonalcoholic fatty liver disease. Intermittent hypoxia results in significant induction of interleukin (IL)-6 expression in both hepatocytes and macrophages. The increase in IL-6 expression was independent of hypoxia inducible factor 1 induction but appeared to be in part related to antioxidant response element and nuclear factor kappa B activation. Mature microRNA 365 (miR-365) has been demonstrated to regulate IL-6 expression, and we found that miR-365 expression was decreased in the setting of intermittent hypoxia. Furthermore, macrophage cell lines showed polarization to an M1 but not M2 phenotype. Finally, we found a trend toward higher circulating levels of IL-6 in patients with OSA and NASH. *Conclusion:* Intermittent hypoxia acts as a potent proinflammatory stimulus, resulting in IL-6 induction and M1 macrophage polarization. Increased IL-6 expression may be due to both induction of antioxidant response element and nuclear factor kappa B as well as inhibition of miR-365 expression. Higher levels of IL-6 were observed in human samples of patients with OSA and NASH. These findings provide biological insight into mechanisms by which obstructive sleep apnea potentiates inflammation and fibrosis in patients with fatty liver disease. (*Hepatology Communications* 2017;1:326-337)

Introduction

Nonalcoholic fatty liver disease (NAFLD) has emerged as an epidemic liver disease, affecting up to 46% of all adult Americans.[1] However, only a subset of patients with NAFLD, those with active inflammation (nonalcoholic steatohepatitis [NASH]), are likely to progress to significant liver damage, cirrhosis, hepatocellular carcinoma (HCC), and death. Multiple factors, both genetic and environmental, have been associated with a higher risk of NASH.

Abbreviations: ARE, antioxidant response element; ARG1, arginase 1; cDNA, complementary DNA; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HIF1α, hypoxia inducible factor 1 alpha subunit; IFNγ, interferon gamma; IH, intermittent hypoxia; IL, interleukin; miR, mature microRNA; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NFκB, nuclear factor kappa-light-chain enhancer of activated B; OSA, obstructive sleep apnea; PBS, phosphate-buffered saline; PMA, phorpbol myristate acetate; qPCR, real-time polymerase chain reaction; RT-PCR, quantitative reverse-transcription polymerase chain reaction; SH, sustained hypoxia; STAT-3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor alpha.

Received December 8, 2016; accepted April 12, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1045/suppinfo.

Supported by a KL2/Catalyst Medical Research Investigator Training award (to E.S.) from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health (NIH) Award KL2 TR001100). The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic healthcare centers, or the NIH. R.T.C. was supported by NIH DK078772 and the Massachusetts General Hospital Research Scholars Program.

Obstructive sleep apnea (OSA) has been identified as one such risk factor.

OSA is a disease characterized by intermittent decreases in oxygenation during sleep and has long been clinically associated with cardiovascular disease, stroke, and mortality risk.[2,3] The underlying biology is thought to be multifactorial but in large part due to the proinflammatory stimulus of chronic intermittent hypoxia. OSA has also been significantly associated with both inflammation and fibrosis in NAFLD, and this finding has been observed in both adult and pediatric populations.[4-7] The liver has a unique dual blood supply from the portal vein and hepatic artery, and the oxygen tension within the hepatic parenchyma is lower than that of other tissues. The partial pressure ranges from 60-65 mmHg in the periportal areas to 30-35 mmHg in the perivenous blood within the hepatic lobule[8] compared to the physiologic oxygen tension of 74-100 mmHg in other tissues. Thus, the liver may have a unique susceptibility to hypoxic insult in the setting of OSA.

Given the clinical association between OSA and cardiovascular and stroke risk, intermittent hypoxia (IH) has been extensively studied in *in vitro* models of cardiovascular and metabolic diseases and has been shown to have an impact on inflammatory markers in adipocytes and endothelial cells.[9-12] However, there has been limited exploration into the biological impact of IH on the liver and risk of liver steatosis, inflammation, and fibrosis.

In this study, we used an *in vitro* model of short-term IH to examine the impact of IH on liver tissue, including both parenchymal and nonparenchymal cell types. We found that IH promoted the expression of interleukin (IL)-6 in both hepatocyte-derived and macrophage cell lines. We additionally demonstrate that even short-term IH promotes a proinflammatory M1 macrophage polarization.

Materials and Methods

CELL CULTURE AND HYPOXIA EXPERIMENTS

Huh 7 hepatoma cells, LX-2 stellate cells, and U937 or THP monocyte cell lines were maintained in Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech) and 1% penicillin/streptomycin (Lonza/Biowhittaker, Walkersville, MD) and maintained at 37°C humidified air with 5% CO_2. THP-1 monocyte cells lines were maintained in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. THP-1 cells were matured to macrophages using phorpbol myristate acetate (PMA) at a concentration of 50 ng/mL. After 24 hours, the PMA-containing medium was removed and replaced with fresh medium. The cells were then maintained as mature adherent macrophages for 5-7 days prior to

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep4.1045

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the Massachusetts General Hospital, Gastrointestinal Unit, Boston, MA.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Esperance Schaefer, M.D., M.P.H.
Liver Center and Gastrointestinal Division
Massachusetts General Hospital
55 Fruit Street
Boston, MA
E-mail: eschaefer@partners.org
Tel.: +1-617-724-6004
or

Raymond T. Chung, M.D.
Liver Center and Gastrointestinal Division
Massachusetts General Hospital
55 Fruit Street
Boston, MA
E-mail: rtchung@partners.org
Tel.: +1-617-724-7562

experimentation. Hypoxia experiments were performed using Modular Incubator Chambers (Billups Rothenberg Inc., San Diego, CA). For sustained hypoxia (SH), the chamber was flushed with 1% O_2, 5% CO_2, and 94% N_2 pressurized gas obtained from AirGas (Hingham, MA) until the partial pressure of O_2 in the chamber was 1% and maintained for 6 hours. Oxygen tension was monitored using the Draeger PAC 3500 0_2 gas monitor (Draeger, Lubeck, Germany). For IH, the chamber was flushed with 1% O_2, 5% CO_2, and 94% N_2 pressurized gas until the partial pressure of oxygen was 1% and the chamber was maintained at 1% O_2 for 20 minutes. The plates were then removed from the chamber and placed in a 21% O_2 and 5% CO_2 environment for 40 minutes. The 20-minute hypoxia then 40-minute normoxia cycles were repeated over the course of 6 hours, with lysates extracted immediately for the SH group and 10 minutes after completion of hypoxia for the IH experiments.

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Quality and concentration of the extracted RNA was determined using the Nanodrop 2000 spectrophotometer. Complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Target gene expression was determined with quantitative reverse-transcription polymerase chain reaction (RT-PCR) using the DyNAmo HS SYBR Green real-time PCR (qPCR) system (Thermo Scientific, Waltham, MA) and performed on the Quant Studio 3 Real-Time PCR system (Thermo Scientific). MicroRNA (miRNA) was isolated from total RNA using the miRNeasy Mini kit (QIAGEN). cDNA from miRNA was generated using the miScript RT II kit (QIAGEN), and quantitative qPCR was performed using the miSCRIPT SYBR Green qPCR system (QIAGEN). Messenger RNA (mRNA) and miRNA primers are listed in Supporting Table 1.

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA). RT-qPCR data are represented as the mean ± SEM. The two-tailed Student t test was used. A P value <0.05 was considered statistically significant.

WESTERN BLOT

Protein was obtained from cellular lysates using radio immunoprecipitation assay lysis and extraction buffer (Thermo Scientific) supplemented with protease inhibitor (Sigma Aldrich, St. Louis, MO). For cytokine assessment, samples were treated with BD GolgiPlug (BD Biosciences, San Jose, CA) according to the manufacturer's instruction prior to the initiation of the experiments. Proteins were separated on NuPage Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene fluoride membrane. Antibodies to hypoxia inducible factor 1 alpha subunit (HIF1α) were obtained from AbCam (Cambridge, MA) and diluted to a concentration of 1:100. Antibodies to IL-6 were obtained from Cell Signaling (Cell Signaling, Danvers, MA) and diluted to a concentration of 1:1,000. β-actin was used as a control. The membranes were incubated overnight at 4°C and developed the following day using Pierce ECL Plus and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

REPORTER CELL IMAGING AND FLOW CYTOMETRY

Antioxidant response element (ARE)- and nuclear factor kappa-light-chain enhancer of activated B cells (NFκB)-green fluorescent protein (GFP) Huh7 reporter cells were generated as described.[13] Appropriate response of the respective reporters was determined by stimulation for 48-72 hours with *tert*-butylquinone for ARE reporters and tumor necrosis factor alpha (TNF-α) for NFκB reporters. Following experimentation, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS immediately following SH and 10 minutes following the last hypoxic exposure in IH. The cells were washed again and then incubated with 4',6-diamidino-2-phenylindole diluted 1:10,000 in PBS. GFP fluorescence was then assessed using the EVOS FL digital inverted microscope (Life Technologies, Grand Island, NY). Quantitative assessment of GFP-positive cells was performed using flow cytometry with the BD FACSCalibur Flow Cytometer (BD Biosciences) calculated using the percentage of positive GFP cells with data analyzed using the Flowing software (version 2.5.1; Turku Centre for Biotechnology, Turku, Finland).

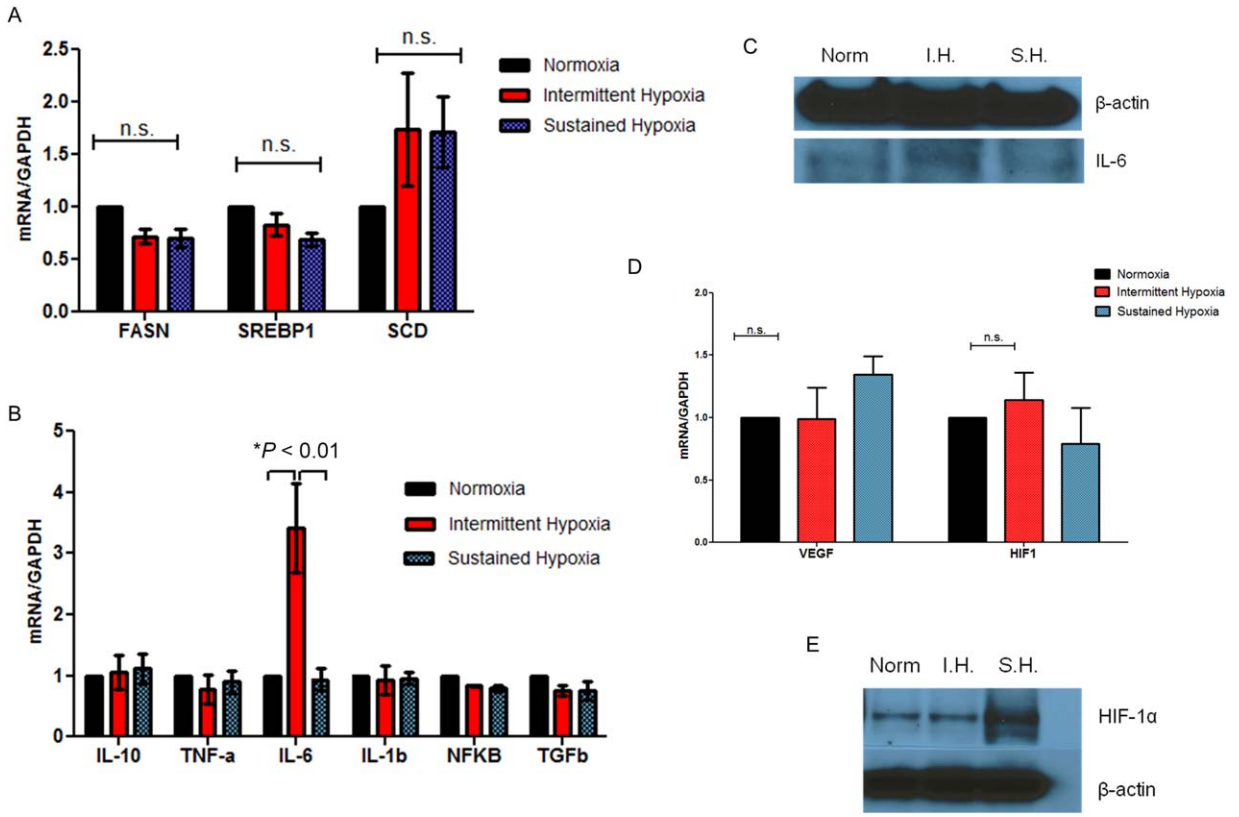

FIG. 1. IH results in increased IL-6 expression in Huh7 hepatoma cells. Using a candidate-gene approach, we examined the changes in expression levels of important drivers of lipogenesis and markers of inflammation. (A) We saw no significant change in FASN, SREBP1, and SCD, although a trend toward increased expression was observed in SCD. (B) There was a significant induction of IL-6 expression following the condition of IH compared to either normoxia or sustained hypoxia. No other examined gene demonstrated a change in expression level. (C) Increased intracellular protein level of IL-6 was confirmed by western blot. (D) No change in VEGF or HIF1a were observed at the mRNA level following the condition of either intermittent or sustained hypoxia, but (E) an increase in protein expression was seen in the sustained hypoxia group alone. qPCR data are presented as mean ± SEM, and significance was determined by a two-tailed Student *t* test. Abbreviations: FASN, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; n.s., not significant; SCD, stearoyl-co-A desaturase; SREBP1, sterol regulatory element binding protein 1; VEGF, vascular endothelial growth factor.

miRNA-365 EXPRESSION

miRNA-365 precursors, inhibitors, and scramble miRNA were obtained from Ambion (Life Technologies, Carlsbad, CA) and were transfected into Huh7, U937, and THP-1, along with hepatocyte-derived ARE-GFP and NFκB reporter cells. Hepatocyte cell lines were plated at a density of 150,000 cells per well, macrophage cell lines (U937 and THP-1) were plated at a density of 500,000 cells per well, and reverse transfection was performed with the HiPerfect Transfection Reagent (QIAGEN) per the manufacturer's instructions. Macrophage cell lines were treated with PMA at the time of reverse transfection. RNA was obtained 48 hours following transfection, and miRNA transcripts were analyzed with RT-PCR.

HUMAN CYTOKINE ARRAY

Human serum was obtained from two distinct clinical populations of patients with known NAFLD from the Massachusetts General Hospital Liver Center and Weight Center. These populations consisted of 12 patients who were diagnosed with NASH and OSA and 12 patients who were diagnosed with simple steatosis and without OSA through tissue pathology and polysomnography. Four healthy controls were also studied. These patients were all nondiabetic and matched for important clinical characteristics, including body mass index, sex, ethnicity, and tobacco use.

Concentrations of IL-6, IL-8, IL-10, interferon gamma (IFNγ), and TNF-α in these sera were assessed in duplicate using the Millipore Human High

Sensitivity T-Cell Panel (Millipore) and analyzed on a Luminex 200 imaging system (Luminex Corporation) with xPONENT software. Quantitative measurements were averaged within each distinct clinical cohort and in-house control group and compared by performing two sample, unpaired, two-tailed *t* tests using GraphPad Prism version 5.0 (GraphPad software).

ETHICS STATEMENT

Human samples used in this study were collected as part of a NAFLD repository. All patients provided written consent, and the study was approved by the Partners' Health Care Human Research Committee.

Results

IH PROMOTES IL-6 EXPRESSION IN HEPATOCYTE-DERIVED CELL LINES INDEPENDENT OF HIF1α INDUCTION

To determine the impact of IH on hepatocytes, we used Huh7 hepatoma cells and exposed the cells to conditions of normoxia, IH, and sustained hypoxia (SH). Hepatocytes comprise the vast majority of liver parenchymal cells and have numerous important physiologic functions, including synthesis of cholesterol and fatty acids. Given the association between OSA and severity of fatty liver, we hypothesized that IH would promote expression of important lipogenic genes. However, we found no expression differences between sterol regulatory element binding protein 1c and fatty acid synthase between conditions of normoxia, IH, and SH. Only stearoyl-co-A desaturase expression was found to have a nonsignificant increase in both IH and SH compared to normoxia controls (Fig. 1A).

Hepatocytes play a role in hepatic injury through the elaboration of proinflammatory or profibrotic cytokines. Using a candidate gene approach, we examined the impact of IH on the expression of several well-described mediators of hepatic inflammation. Interestingly, we found that only one of the examined cytokines had increased expression under conditions of IH. IL-6 was increased 3.4-fold compared to normoxia (SH expression was 0.93-fold of normoxia). Increased levels of intracellular IL-6 were confirmed at the protein level (Fig. 1C). Other inflammatory cytokines that were assessed, including IL-10, TNF-α, IL-1β, or transforming growth factor β (TGF-β), did not exhibit significant changes in expression under conditions of IH or SH. There was also no change in NFκB gene expression following these interventions (Fig. 1B). This significant induction of IL-6 expression following IH is supported by evidence from prior animal models[14] and clinical data in OSA.[15]

The HIF1α pathway is the canonical pathway associated with hypoxic insult, and prior research examining the effect of HIF1α on IL-6 expression has produced conflicting results, with some studies suggesting that HIF1α promotes IL-6 expression.[16] We thus sought to determine whether IH led to HIF1α or vascular endothelial growth factor induction. We found no increase in expression levels at the mRNA level of HIF1α or vascular endothelial growth factor (Fig. 1D). At the protein level, we saw a modest increase in HIF1α under the conditions of SH (6-hour stimulus) but no increase with IH (Fig. 1E). Thus, the observed IL-6 induction following short-term IH is likely caused by an HIF-independent mechanism.

INCREASED IL-6 IN HEPATOMA CELLS IS PARTLY DUE TO ANTIOXIDANT RESPONSE ELEMENT AND NFκB ACTIVATION

The antioxidant response element (ARE) exerts transcriptional control over a number of genes thought to play a cytoprotective role in the setting of oxidative stress and is known to promote the expression of IL-6.[17,18] Similarly, NFκB is known to promote expression of IL-6 alongside other proinflammatory cytokines and has been shown to be induced by IH in other cellular models.[11] To determine whether either of these important regulators played a role in the observed IL-6 induction following IH, we used two GFP reporter constructs in Huh7 hepatoma cells.

To confirm appropriate responsiveness of each cell line, positive controls were used and examined with immunofluorescence microscopy. ARE-GFP reporter cells were treated with *tert*-butylhydroquinone, and NFκB-GFP reporter cells were treated with TNF-α. We found appropriate GFP responses after a 24-48-hour duration of exposure quantified using flow cytometry for GFP+ cells (52.78% and 65.39% for ARE-positive and NFκB-positive cells, respectively). We then exposed the reporter cells to the conditions of normoxia or IH and assessed ARE and NFκB activation using both immunofluorescence and flow

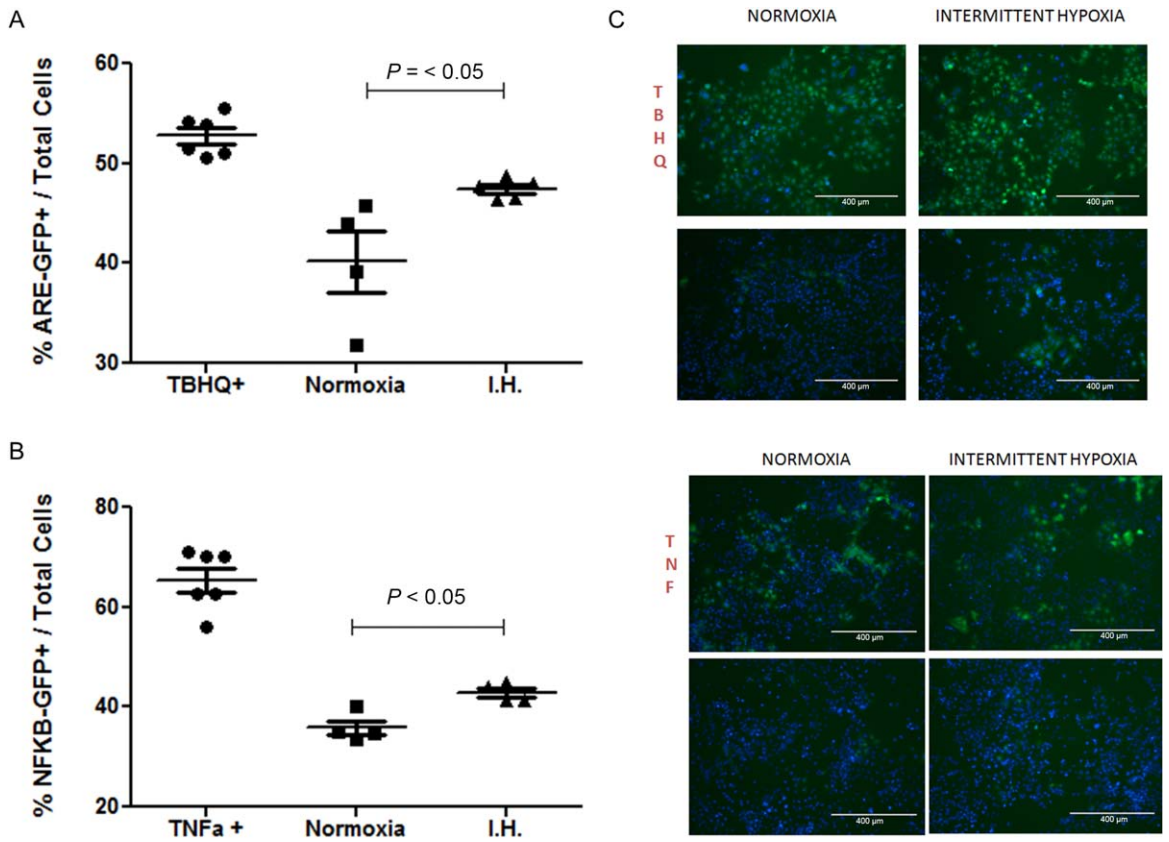

FIG. 2. IH induces both ARE and NFκB signaling. Using ARE-GFP and NFκB-GFP Huh7 reporter cells, we determined whether IH resulted in ARE or NFκB induction. (A,B) *t*BHQ treatment and TNF-α treatment for 48 hours were used as a positive control for ARE and NFκB response, respectively. Using flow cytometry, the expression of GFP+ cells was determined as % positive/total cells, comparing normoxia and IH. The percentage of ARE-GFP-positive cells was significantly increased following IH, and the percentage of NFκB-GFP-positive cells was also increased. (C) These findings were confirmed with immunofluorescence studies. Flow cytometry data are presented as mean ± SEM, and significance was determined by a two-tailed Student *t* test. Abbreviation: *t*BHQ, *tert*-butylquinone.

cytometry. We found that there was a significant increase in ARE-GFP expression following IH compared to normoxia (47.48% versus 38.32%, $P = 0.01$), and there was a smaller but significant increase in NFκB activation (42.24% versus 36.61%, $P = 0.046$) (Fig. 2A,B). These findings were confirmed with immunofluorescence studies, which demonstrated a clear increase in ARE expression (Fig. 2C).

miRNA-365a INHIBITION PROMOTES IL-6 EXPRESSION BUT DOES NOT ALTER ARE OR NFκB ACTIVATION

IH resulted in a 1.24-fold increase in ARE and a 1.15-fold increase in NFκB activation but induced IL-6 expression at a level of 3.4-fold higher than normoxia. These data suggest that these abrupt alterations in IL-6 expression are directed by pathways other than ARE- or NFκB-dependent transcriptional activation. Mature miRNA-365 (miR-365) has been described as a direct negative regulator of IL-6.[19] miRNAs are known to have differential expression and are repressed following SH.[20] However, the impact of IH on miRNA expression has yet to be clearly defined. One recent study in mice has implied that miRNA profiles can potentially be affected by IH.[21] We hypothesized that IH might additionally lead to differential miR-365 expression, which in turn contributes to the observed phenotype of increased IL-6 mRNA levels within our cell-based experiments.

We confirmed that miR-365 was expressed at lower levels in Huh-7 hepatoma cells exposed to IH (48.25%

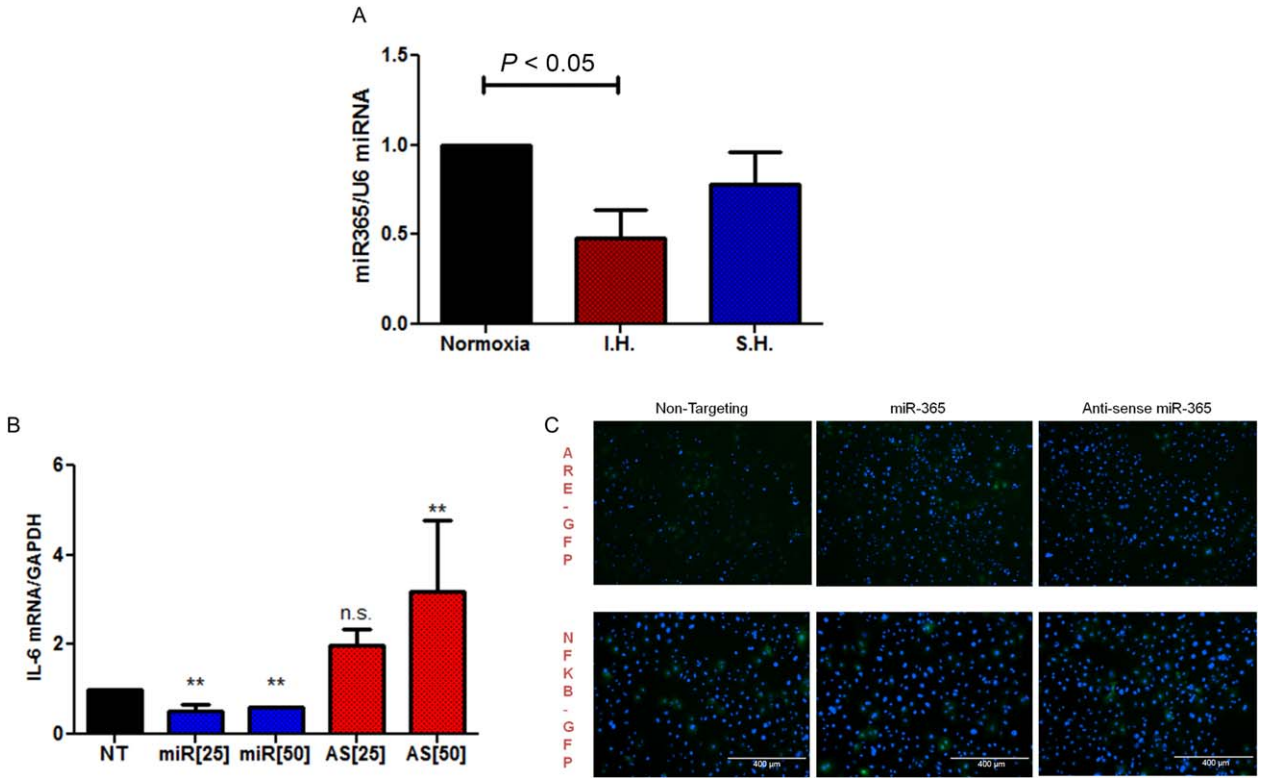

FIG. 3. IH results in lower expression of miR-365, a known regulator of IL-6. (A) Using RT-qPCR, levels of miR-365 were assessed in Huh7 hepatoma cells following exposure to IH and sustained hypoxia. There was significant reduction in miR-365 following intermittent but not sustained hypoxia. (B) Huh7 cells were transfected with an miR-365 mimic (miR) and an miR-365 inhibitor (AS) at two different concentrations (25 nM, 50 nM), along with a nontargeting scramble miRNA (NT). We observed decreased IL-6 expression in cells transfected with the mimic and increased expression with the inhibitor compared to the NT. (** denotes *P* value <0.05 compared to NT) (C) ARE-GFP and NFκB-GFP reporter cells were used to determine a change in ARE or NFκB activity following the miR-365 mimic or inhibitor. No change in activity was observed with the mimic or the inhibitor. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

$\pm$ 15.5%, $P < 0.05$) compared to normoxia. There was also a smaller and nonsignificant decrease in miR-365 expression observed in SH (77.9% $\pm$ 18.1%, $P = 0.31$) (Fig. 3A).

To confirm the effect of miR-365 on IL-6 expression in hepatoma cells, we transfected the Huh7 cells with an miR-365 mimic and antagomir at different concentrations (25 nM, 50 nM) and determined the effect on IL-6 expression. IL-6 mRNA expression was decreased by miR-365 overexpression (44.3% $\pm$ 15.6% and 59.3% $\pm$ 1.8%, respectively, for 25 nM and 50 nM). In contrast, miR-365 blockade by an antagomir resulted in a 1.9-fold and 3.1-fold increase in expression for 25 nM and 50 nM, respectively (Fig. 3B). Using the ARE-GFP and NFκB-GFP reporter systems, we additionally confirmed that miR-365 overexpression and antagonism did not affect ARE or NFκB activation (Fig. 3C). Together, these data suggest that the increased elaboration of IL-6 observed in hepatoma cells following IH likely occurs through multiple mechanisms, which include both the independent effects of ARE and NFκB activation as well as decreased expression of miR-365.

IH PROMOTES M1 MACROPHAGE POLARIZATION BUT DOES NOT AFFECT STELLATE CELL ACTIVATION

While hepatocytes comprise the vast majority of cells within the liver, hepatic resident macrophages (Kupffer cells) and hepatic stellate cells are critical mediators of the clinical manifestations of inflammation and fibrosis. We used similar *in vitro* perturbations to determine the effect of IH on these

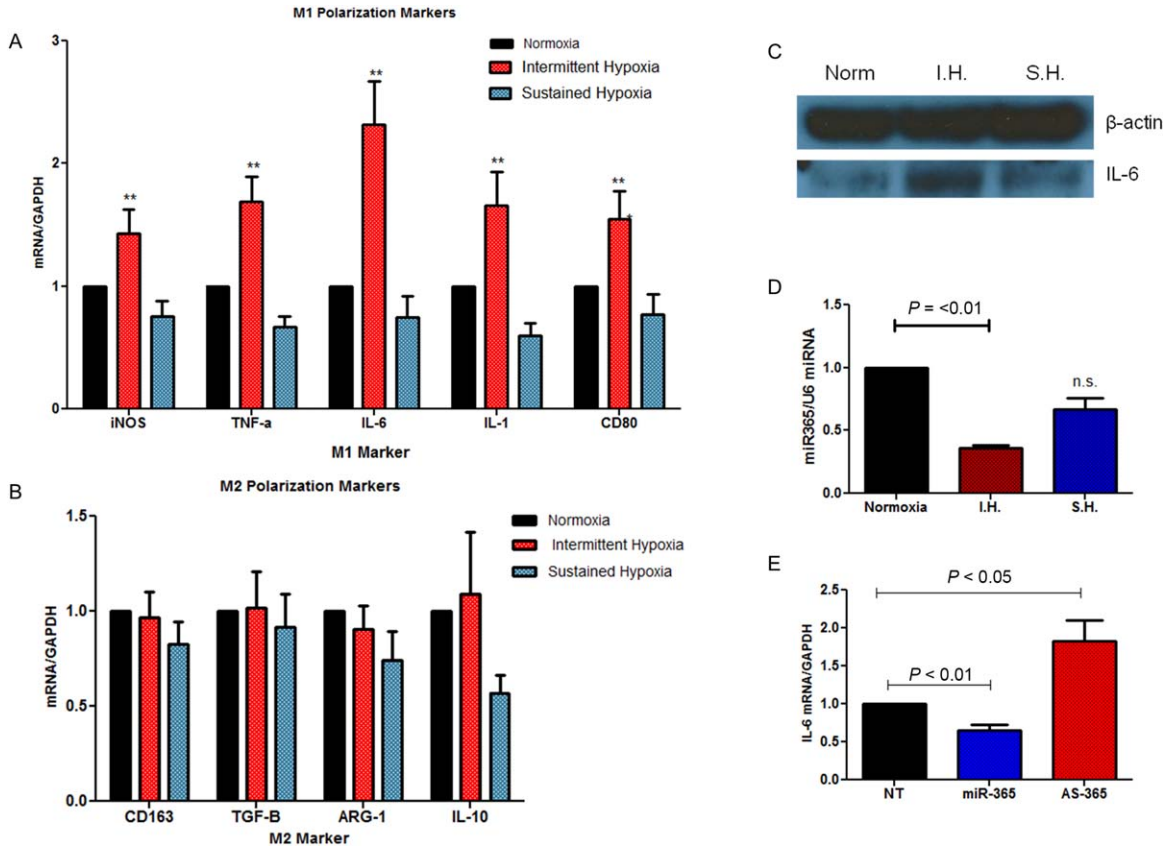

FIG. 4. IH polarizes macrophages to an M1 phenotype. THP-1 macrophages were matured with PMA 5-7 days prior to experimentation and allowed to mature, then were exposed to intermittent or sustained hypoxia. Markers of M1 and M2 macrophage polarization were determined using RT-qPCR. (A) mRNA expression levels of M1 expression markers, including iNOS, TNF-α, IL-6, IL-1, and CD80 were all significantly increased. (** denotes *P* value <0.05 compared to normoxia.) (B) There was no change in expression of M2 expression markers, including CD163, TGF-*β*, ARG-1, and IL-10. (C) Increased intracellular protein level of IL-6 was confirmed by western blot in U937 macrophages. (D) Using RT-qPCR, intracellular levels of miR-365 were assessed in THP-1 macrophages following exposure to IH and SH. We demonstrated that miR-365 expression is significantly decreased following IH. (E) U937 and THP-1 macrophages were transfected with an miR-365 mimic (miR-365) and an miR-365 inhibitor (AS-365) at a concentration of 50 nM compared to a nontargeting miRNA scramble (NT) also at 50 nM. We found IL-6 expression to be decreased following transfection with the mimic and increased in the setting of transfection with the antagomir. qPCR data are presented as mean ± SEM, and significance was determined by a two-tailed Student *t* test. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase.

nonparenchymal liver cells. We found that IH exerted no effect on the expression of the profibrotic gene expression in LX2 stellate cells, with no increase observed in mRNA levels of collagen1a, α-smooth muscle actin, or connective TGF (Supporting Fig. S1). Thus, using cell line models of hepatic stellate cells, there did not appear to be a significant impact of short-term IH on hepatic stellate cell activation.

Macrophage activation has been described to play an important role in the development of hepatic inflammation and fibrosis in the setting of fatty liver disease, and macrophage polarization may differ in different stages of disease progression. M1 macrophages are considered to be proinflammatory macrophages with increased expression of TNF-α, IL-1*β*, IL-6, clusters of differentiation (CD)80, and inducible nitric oxide synthase. An M1 bias has been observed in human liver biopsy samples with NASH, but no fibrosis and has been associated with inflammation in mouse models.[22,23] M2 macrophages are thought to be the dominant cell type in the later stages of the injury response and associated with healing and fibrosis, producing IL-10, TGF-*β*, and arginase 1 (ARG1), among others.[24] To determine the effect of IH on

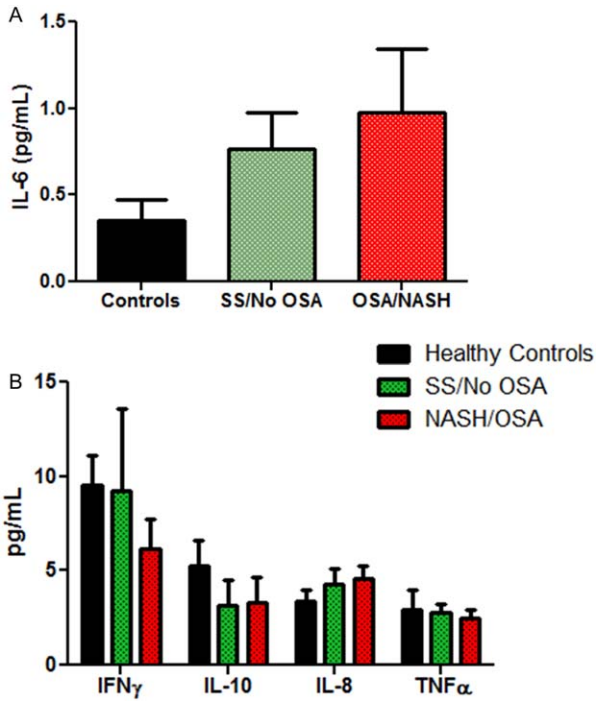

FIG. 5. Individuals with OSA and NASH have a trend toward higher circulating IL-6 levels and no significant difference in other circulating cytokines. Using a Luminex array, circulating levels of select cytokines were examined in patients with simple steatosis and absence of OSA (SS/no OSA, n = 12) or biopsy-proven NASH and polysomnography-proven OSA (OSA/NASH, n = 12) or healthy controls (n = 4). (A) There was a trend toward higher levels of circulating IL-6 in patients with OSA/NASH. (B) No significant difference in the levels of other circulating cytokines were observed.

macrophage polarization, we determined the expression of classic M1 and M2 genes in THP macrophages following the 6-hour exposure to IH.

We found substantial and significant induction of markers of M1 activation following exposure to conditions of IH. IL-6 mRNA expression was increased 2.3-fold following IH ($P = 0.005$) and was unchanged in the setting of SH ($P = 0.20$). Intracellular protein levels of IL-6, likewise, were elevated in the setting of IH (Fig. 4A,C). There were also significant increases in inducible nitric oxide synthase, TNF-α, IL-1β, and CD80 expression (Fig. 4A). We did not, however, observe any changes in expression markers associated with the M2 macrophage phenotype: IL-10, TGF-β, CD163, or Arg1. (Fig. 4B). These data suggest that IH, even of short duration, provides an important stimulus for macrophage polarization toward the proinflammatory M1 phenotype.

We next sought to determine whether, similar to our findings in hepatocyte-derived cell lines, the IL-6 induction and M1 macrophage polarization was driven by alterations in expression in miR-365. We found miR-365 to have significantly lower expression levels in macrophages following IH (36.0% $\pm$ 2.6%, $P = 0.001$) but was not significantly decreased following SH (67.9%, $\pm$ 9.0%, $P = 0.17$) (Fig. 4D). We then transfected macrophage cell lines with a mimic and antagomir of miR-365 to assess the effect on IL-6 expression and M1 macrophage polarization. We again observed that the mimic significantly reduced IL-6 expression (34.6% $\pm$ 6.9% reduction, $P = 0.007$) and the antagomir significantly increased IL-6 expression (1.82-fold $\pm$ 0.28 increase, $P = 0.03$) (Fig. 4E). Thus, we confirmed that a decrease in miR-365 may contribute to the observed IL-6 expression. However, we did not see a significant change in other markers of M1 polarization, such as TNF-α and IL-1β (Supporting Fig. S2). Therefore, the mechanisms by which IH promotes M1 polarization are independent of alterations in miR-365 expression and merit further investigation.

PATIENTS WITH OSA AND NAFLD HAVE HIGHER LEVELS OF CIRCULATING IL-6

Based on the data from cellular models, IL-6 may play an important role in the development of inflammation and fibrosis in the setting of liver disease. We thus sought to determine whether there were higher levels of circulating IL-6 observed in patients with OSA and NASH compared to those patients without OSA and only simple steatosis.

We identified 24 samples from a cohort of patients with NAFLD who had biopsy-proven NAFLD and polysomnography-diagnosed presence or absence of OSA for the determination of circulating cytokines. Selected subjects either had NASH *and* OSA (NASH/OSA) or simple steatosis *and* no diagnosis of OSA (SS/no OSA) and were matched on body mass index, age, and ethnicity. No subjects had diabetes, and no subjects were current smokers. The clinical characteristics of the patients are shown in Supporting Table 2. These samples were analyzed using the Luminex array for analysis of circulating cytokines.

We found a trend toward higher levels of circulating IL-6 in patients with OSA and NASH, but the differences between the groups did not reach statistical significance (Controls, 0.35 versus OSA/NASH 0.98 pg/mL, $P = 0.33$; OSA/NASH versus SS/no OSA, 0.77

pg/mL, $P = 0.31$) (Fig. 5A). There was no difference in circulating IFNγ, IL-10, IL-8, or TNF-α levels among the groups (Fig. 5B). These findings are limited by the small sample size but are consistent with prior data examining OSA alone in both children and adults[25-27] and suggest that there is likely important human relevance to our *in vitro* findings.

Discussion

While several clinical studies have linked OSA, which is a condition marked by chronic IH, to higher degrees of inflammation and fibrosis in NAFLD, the mechanism underlying this association remains incompletely understood. Hepatic injury in NAFLD occurs by pathways of sterile inflammation, and the best described triggers for inflammation include activation of the inflammasome by Toll-like receptor signaling and mitochondrial dysfunction due to oxidative stress and lipotoxicity.[28,29] IH as clinically manifested by OSA would represent a unique trigger for sterile hepatic inflammation.

In this study, we use an *in vitro* model of IH to characterize the impact of cycling oxygen tension on resident liver cells involved in disease progression. Through the use of this *in vitro* model and cell lines, this study for the first time carefully examines each of the major cell types involved in NAFLD. We demonstrate that hepatocyte-derived cells subjected to short-term IH do not exhibit marked induction of genes associated with steatosis but rather there is an early and dramatic induction of IL-6. This finding is not unexpected given a prior animal model showing increased IL-6[14] and observed clinical associations, particularly the demonstration of elevated IL-6 in an obese pediatric population with OSA.[30] However, we further demonstrate here that hepatocytes may be an important source of both intrahepatic and circulating IL-6.

This rise in IL-6 associated with IH potentially plays an important role in the development of hepatic inflammation. IL-6 has pleiotropic effects and promotes the inflammatory response by multiple mechanisms, including T-cell proliferation, B-cell differentiation, and monocyte maturation. Our findings suggest that IH promotes IL-6 production in hepatocytes, which may be an important trigger in driving the inflammatory cascade that eventually promotes the development of NASH. The increased production of IL-6, however, may have other important clinical sequelae, including HCC.

An additional important downstream effect of IL-6 signaling is phosphorylation of signal transducer and activator of transcription 3 (STAT-3) with induction of STAT-3 target genes, including induction of the suppressor of cytokine signaling 3, which serves to dampen the inflammatory response. Thus, the increased expression of IL-6 may also play a regulatory role in decreasing the inflammatory response by negative feedback mechanisms.[31] However, suppressor of cytokine signaling 3 activation has also been shown to play a role in insulin resistance[32] and development of hepatic steatosis.[33]

The demonstration of higher levels of circulating IL-6 in patients with OSA and NASH supports the clinical implications of our *in vitro* findings, although the strength of our observations is limited by the small sample size of subjects. Both disease processes have been described to be associated with elevated serum IL-6 levels, but the interplay between liver disease and OSA with circulating IL-6 has yet to be investigated.[26,34] A more comprehensive characterization of IL-6 levels in a larger cohort of patients with NASH and without OSA and with OSA and without NASH will be critical to better characterize the relative contributions of each disease process.

Cirrhosis is a clear risk factor for the development of HCC, but an emerging body of literature suggests that HCC may develop in NASH even in the absence of advanced fibrosis.[35] IL-6 is considered to play an important role in HCC development and metastases, and its deletion in macrophages using a murine model protected against the development of HCC.[36,37] The significant and abrupt increase in IL-6 expression from our *in vitro* models combined with the higher levels in human samples raises the important possibility that OSA may play an important role not just in hepatic inflammation but also in tumorigenesis. OSA may be an important risk factor for HCC development, and this pathogenetic association merits further investigation.

The macrophage response to this *in vitro* model of IH was not, however, restricted to increased IL-6. Expression levels of markers associated with the proinflammatory M1 were also observed, suggesting that IH is a sterile driver of M1 macrophage polarization. M1 macrophages have been implicated in the early pathogenesis of fatty liver disease[22] and have also been observed in mouse models following cytochrome P450 2E1-induced oxidative stress.[23] These findings provide evidence that OSA may promote inflammation via the rapid polarization of monocytes to activated M1 macrophages.

Finally, in our inquiry into the mechanisms of IL-6 induction, we found that canonical pathways of IL-6 production, including ARE and NFκB activation, appear to be involved but do not alone account for the increased observed levels of IL-6. miRNAs have been shown to be differentially regulated in the setting of SH,[20] but differential regulation in the setting of IH has yet to be investigated. In this study, we demonstrate that at least one miRNA, miR-365, is differentially expressed in the setting of IH and affects IL-6 expression in both hepatocytes and macrophages. miR-365 has been previously shown to be a regulator of IL-6[19] and has recently been described to be increased in other tissues in the setting of SH.[38] Our findings confirm that inhibition of miR-365 results in increased IL-6 expression but does not affect ARE or NFκB activation, highlighting an important role for miRNA in the genesis of sterile inflammation. Inhibition of miR-365, however, did not lead to changes in other markers of M1 macrophage polarization. These data suggest that while miR-365 may play a role in an IL-6 increase in both hepatocytes and macrophages, the mechanisms by which IH promotes M1 macrophage polarization do not rely on changes in expression of this miRNA and certainly merit additional investigation.

Limited prior research has evaluated the role of IH in the development of hepatic inflammation, with one prior study in mice demonstrating enhanced steatosis and elevated inflammatory markers in blood and whole liver tissue.[14] Our *in vitro* studies examining hepatocytes, stellate cells, and macrophages individually suggest that alteration in oxygenation specifically plays an important role in hepatic inflammation by way of IL-6 induction and macrophage activation. Additional inquiry into these mechanisms *in vivo* would be illustrative, and examination of alterations of intrahepatic miRNA expression may provide important clues to biological pathways involved in disease pathogenesis in NASH.

In summary, our study uses cell-culture modeling to provide biological insight into the observation linking OSA and NASH. We demonstrate that short-term IH acts as an important trigger for the production of proinflammatory cytokines and activation of macrophages. Elucidation of the pathways of sterile inflammation involved in this response may yield important insight into novel therapeutic targets.

Acknowledgment: We thank Dr. Manish Gala for assistance with design of hypoxia experiments and Dr. Russell Goodman, Dr. Annie Kruger, Dr. Nadia Alatrakchi, and Dr. Robert Schaefer for additional assistance with manuscript preparation.

REFERENCES

1) Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology 2011;140:124-131.
2) Redline S, Yenokyan G, Gottlieb DJ, Shahar E, O'Connor GT, Resnick HE, et al. Obstructive sleep apnea-hypopnea and incident stroke: the sleep heart health study. Am J Respir Crit Care Med 2010;182:269-277.
3) Gottlieb DJ, Yenokyan G, Newman AB, O'Connor GT, Punjabi NM, Quan SF, et al. Prospective study of obstructive sleep apnea and incident coronary heart disease and heart failure: the sleep heart health study. Circulation 2010;122:352-360.
4) Corey KE, Misdraji J, Gelrud L, King LY, Zheng H, Malhotra A, et al. Obstructive sleep apnea is associated with nonalcoholic steatohepatitis and advanced liver histology. Dig Dis Sci 2015; 60:2523-2528.
5) Sundaram SS, Halbower A, Pan Z, Robbins K, Capocelli KE, Klawitter J, et al. Nocturnal hypoxia-induced oxidative stress promotes progression of pediatric non-alcoholic fatty liver disease. J Hepatol 2016;65:560-569.
6) Sundaram SS, Sokol RJ, Capocelli KE, Pan Z, Sullivan JS, Robbins K, et al. Obstructive sleep apnea and hypoxemia are associated with advanced liver histology in pediatric nonalcoholic fatty liver disease. J Pediatr 2014;164:699-706.e691.
7) Trzepizur W, Boursier J, Mansour Y, Le Vaillant M, Chollet S, Pigeanne T, et al. Association between severity of obstructive sleep apnea and blood markers of liver injury. Clin Gastroenterol Hepatol 2016;14:1657-1661.
8) Jungermann K, Kietzmann T. Oxygen: modulator of metabolic zonation and disease of the liver. Hepatology 2000;31:255-260.
9) **Emin M, Wang G,** Castagna F, Rodriguez-Lopez J, Wahab R, Wang J, et al. Increased internalization of complement inhibitor CD59 may contribute to endothelial inflammation in obstructive sleep apnea. Sci Transl Med 2016;8:320ra321.
10) Taylor CT, Kent BD, Crinion SJ, McNicholas WT, Ryan S. Human adipocytes are highly sensitive to intermittent hypoxia induced NF-kappaB activity and subsequent inflammatory gene expression. Biochem Biophys Res Commun 2014;447:660-665.
11) Lee MY, Wang Y, Mak JC, Ip MS. Intermittent hypoxia induces NF-kappaB-dependent endothelial activation via adipocyte-derived mediators. Am J Physiol Cell Physiol 2016;310:C446-455.
12) Toffoli S, Roegiers A, Feron O, Van Steenbrugge M, Ninane N, Raes M, et al. Intermittent hypoxia is an angiogenic inducer for endothelial cells: role of HIF-1. Angiogenesis 2009;12:47-67.
13) Salloum S, Holmes JA, Jindal R, Bale SS, Brisac C, Alatrakchi N, et al. Exposure to human immunodeficiency virus/hepatitis C virus in hepatic and stellate cell lines reveals cooperative profibrotic transcriptional activation between viruses and cell types. Hepatology 2016;64:1951-1968.
14) Savransky V, Bevans S, Nanayakkara A, Li J, Smith PL, Torbenson MS, et al. Chronic intermittent hypoxia causes hepatitis in a mouse model of diet-induced fatty liver. Am J Physiol Gastrointest Liver Physiol 2007;293:G871-877.

15) Maeder MT, Strobel W, Christ M, Todd J, Estis J, Wildi K, et al. Comprehensive biomarker profiling in patients with obstructive sleep apnea. Clin Biochem 2015;48:340-346.
16) Zhu G, Tang Y, Geng N, Zheng M, Jiang J, Li L, et al. HIF-alpha/MIF and NF-kappaB/IL-6 axes contribute to the recruitment of CD11b+Gr-1+ myeloid cells in hypoxic microenvironment of HNSCC. Neoplasia 2014;16:168-179.
17) Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 2009;284:13291-13295.
18) Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 2014;6: a016295.
19) Xu Z, Xiao SB, Xu P, Xie Q, Cao L, Wang D, et al. miR-365, a novel negative regulator of interleukin-6 gene expression, is cooperatively regulated by Sp1 and NF-kappaB. J Biol Chem 2011;286:21401-21412.
20) Shen J, Xia W, Khotskaya YB, Huo L, Nakanishi K, Lim SO, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature 2013;497: 383-387.
21) Liu KX, Chen GP, Lin PL, Huang JC, Lin X, Qi JC, et al. Detection and analysis of apoptosis- and autophagy-related miRNAs of vascular endothelial cells in a mouse chronic intermittent hypoxia model. Life Sci 2016; doi: 10.1016/j.lfs.2016.08.005. [Epub ahead of print]
22) Bertola A, Bonnafous S, Anty R, Patouraux S, Saint-Paul MC, Iannelli A, et al. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. PLoS One 2010;5:e13577.
23) Seth RK, Das S, Pourhoseini S, Dattaroy D, Igwe S, Ray JB, et al. M1 polarization bias and subsequent nonalcoholic steatohepatitis progression is attenuated by nitric oxide donor DETA NONOate via inhibition of CYP2E1-induced oxidative stress in obese mice. J Pharmacol Exp Ther 2015;352:77-89.
24) Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. Cell Mol Life Sci 2015;72:4111-4126.
25) Huang YS, Guilleminault C, Hwang FM, Cheng C, Lin CH, Li HY, et al. Inflammatory cytokines in pediatric obstructive sleep apnea. Medicine (Baltimore) 2016;95:e4944.
26) Martinez-Ceron E, Barquiel B, Bezos AM, Casitas R, Galera R, Garcia-Benito C, et al. Effect of continuous positive airway pressure on glycemic control in patients with obstructive sleep apnea and type 2 diabetes. A randomized clinical trial. Am J Respir Crit Care Med 2016;194:476-485.
27) de Lima FF, Mazzotti DR, Tufik S, Bittencourt L. The role inflammatory response genes in obstructive sleep apnea syndrome: a review. Sleep Breath 2016;20:331-338.
28) Hoque R, Farooq A, Mehal WZ. Sterile inflammation in the liver and pancreas. J Gastroenterol Hepatol 2013;28 Suppl 1:61-67.
29) Mehta R, Jeiran K, Koenig AB, Otgonsuren M, Goodman Z, Baranova A, et al. The role of mitochondrial genomics in patients with non-alcoholic steatohepatitis (NASH). BMC Med Genet 2016;17:63.
30) Gileles-Hillel A, Alonso-Alvarez ML, Kheirandish-Gozal L, Peris E, Cordero-Guevara JA, Teran-Santos J, et al. Inflammatory markers and obstructive sleep apnea in obese children: the NANOS study. Mediators Inflamm 2014;2014:605280.
31) Wunderlich CM, Hovelmeyer N, Wunderlich FT. Mechanisms of chronic JAK-STAT3-SOCS3 signaling in obesity. JAKSTAT 2013;2:e23878.
32) Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, et al. Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. J Biol Chem 2003;278:13740-13746.
33) Ueki K, Kondo T, Tseng YH, Kahn CR. Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. Proc Natl Acad Sci U S A 2004;101:10422-10427.
34) Polyzos SA, Kountouras J, Polymerou V, Papadimitriou KG, Zavos C, Katsinelos P. Vaspin, resistin, retinol-binding protein-4, interleukin-1alpha and interleukin-6 in patients with nonalcoholic fatty liver disease. Ann Hepatol 2016;15:705-714.
35) Mohamad B, Shah V, Onyshchenko M, Elshamy M, Aucejo F, Lopez R, et al. Characterization of hepatocellular carcinoma (HCC) in non-alcoholic fatty liver disease (NAFLD) patients without cirrhosis. Hepatol Int 2016;10:632-639.
36) Zhuang PY, Wang JD, Tang ZH, Zhou XP, Quan ZW, Liu YB, et al. Higher proliferation of peritumoral endothelial cells to IL-6/sIL-6R than tumoral endothelial cells in hepatocellular carcinoma. BMC Cancer 2015;15:830.
37) Kong L, Zhou Y, Bu H, Lv T, Shi Y, Yang J. Deletion of interleukin-6 in monocytes/macrophages suppresses the initiation of hepatocellular carcinoma in mice. J Exp Clin Cancer Res 2016;35:131.
38) Mori A, Nishi H, Sasaki T, Nagamitsu Y, Kawaguchi R, Okamoto A, et al. HLA-G expression is regulated by miR-365 in trophoblasts under hypoxic conditions. Placenta 2016;45:37-41.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1045/suppinfo.