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ORIGINAL ARTICLE

Combined intermittent and sustained hypoxia is a novel and deleterious cardio-metabolic phenotype

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Abstract

Study Objectives: Chronic obstructive pulmonary disease and obstructive sleep apnea overlap syndrome is associated with excess mortality, and outcomes are related to the degree of hypoxemia. People at high altitudes are susceptible to periodic breathing, and hypoxia at altitude is associated with cardio-metabolic dysfunction. Hypoxemia in these scenarios may be described as superimposed sustained hypoxia (SH) plus intermittent hypoxia (IH), or overlap hypoxia (OH), the effects of which have not been investigated. We aimed to characterize the cardio-metabolic consequences of OH in mice.

Methods: C57BL/6J mice were subjected to either SH (FiO_2 = 0.10), IH (FiO_2 = 0.21 for 12 h, and FiO_2 oscillating between 0.21 and 0.06, 60 times/hour, for 12 h), OH (FiO_2 = 0.13 for 12 h, and FiO_2 oscillating between 0.13 and 0.06, 60 times/hour, for 12 h), or room air (RA), n = 8/group. Blood pressure and intraperitoneal glucose tolerance test were measured serially, and right ventricular systolic pressure (RVSP) was assessed.

Results: Systolic blood pressure transiently increased in IH and OH relative to SH and RA. RVSP did not increase in IH, but increased in SH and OH by 52% ($p < .001$) and 20% ($p = .001$). Glucose disposal worsened in IH and improved in SH, with no change in OH. Serum low- and very-low-density lipoproteins increased in OH and SH, but not in IH. Hepatic oxidative stress increased in all hypoxic groups, with the highest increase in OH.

Conclusions: OH may represent a unique and deleterious cardio-metabolic stimulus, causing systemic and pulmonary hypertension, and without protective metabolic effects characteristic of SH.

Statement of Significance

The chronic obstructive pulmonary disease and obstructive sleep apnea (COPD/OSA) overlap syndrome is highly prevalent and is associated with poor outcomes, including higher mortality than in those patients with either disease alone. There have been no investigations to date regarding the mechanisms which might account for these outcomes. The hypoxia of COPD/OSA overlap may be modeled by combined sustained hypoxia (SH) and intermittent hypoxia (IH) ("overlap hypoxia"), and this regimen may also represent hypoxia in other conditions such as high altitude. We have found that overlap hypoxia is a unique and deleterious stimulus, relative to either SH or IH alone, causing both systemic and pulmonary hypertension, hepatic oxidative stress, altered glucose metabolism, and dyslipidemia. Combined SH and IH is thus in many ways the "worst of both worlds."

Key words: overlap syndrome; hypoxia modeling; glucose dysregulation; pulmonary hypertension; oxidative stress

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Introduction

The chronic obstructive pulmonary disease and obstructive sleep apnea (COPD/OSA) overlap syndrome is highly prevalent [1], and is associated with higher mortality than in either disease alone [2–4]. Recent data suggest that outcomes are associated with the severity of nocturnal hypoxemia [4]. The pattern of hypoxemia in patients with the overlap syndrome can vary [1] but may be described in many patients as superimposed sustained hypoxemia (due to chronic lung disease) and intermittent hypoxemia (due to OSA). A similar pattern of hypoxemia may be observed in other clinical states, such as at high altitudes, in which sustained hypoxemia results from low oxygen tension in a hypobaric environment relative to sea level. In such an environment, periodic breathing is highly prevalent [5], and the presence of more severe hypoxemia is associated with worsening cardiovascular and metabolic risk [6,7].

Rodent models of sustained hypoxia (SH) and intermittent hypoxia (IH) are useful to demonstrate particular cardiometabolic effects and to understand mechanisms downstream of hypoxic exposure which might account for these effects. SH causes pulmonary hypertension in rodents [8, 9] but does not elevate systemic blood pressure [10]. SH improves glucose disposal [11] and reduces fasting glucose [12]. By contrast, IH increases systemic blood pressure [10, 13], and only few studies have demonstrated an effect of IH on pulmonary pressures [14–17]. Moreover, IH worsens glucose disposal and increases fasting glucose [18, 19]. SH and IH also have different effects on adipose tissue composition, and on gene expression in the lungs [20, 21]. Our group has been struck by the disparate effects of SH and IH, and to our knowledge, combining features of SH and IH in rodents to recapitulate the hypoxemia of COPD/OSA overlap or high-altitude periodic breathing has never previously been tested rigorously. In this experiment, we aimed to determine how superimposed SH and IH (or overlap hypoxia, OH) might impact cardio-metabolic outcomes by exposing mice to either SH, IH, or OH, to understand the effect of hypoxemia in the overlap syndrome.

Methods

Mice

C57BL/6J mice, age 6 weeks, n = 8/group, half female, were used. Mice were fed a regular chow diet ad libitum, and were exposed to light 7 am to 7 pm daily. Food intake and mouse weight were recorded daily. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, and were performed in alignment with the Declaration of Helsinki.

Experimental protocol and hypoxia exposure

Each group of mice was exposed to a different hypoxia regimen for 40 days, using an OxyCycler A84XOV Multi-Chamber Dynamic Oxygen Controller (BioSpherix Ltd., Parish, NY). Group 1 was exposed to SH (constant FiO_2 of 0.10). Group 2 was exposed to IH (FiO_2 fluctuating between 0.21 and 0.06 once/min, for 12 h per day during the light phase, and FiO_2 0.21 during the dark phase). Group 3 was exposed to OH, a combination of sustained and IH (FiO_2 fluctuating between 0.13 and 0.06 once/min, for 12 h per day during the light phase, and FiO_2 0.13 during the dark phase). Group 4 was exposed to room air (RA). A FiO_2 of 0.13 was chosen as the baseline for OH because in preliminary experiments this stimulus caused peripheral saturations in mice to fall to 87%–92%, mimicking the resting saturations in patients with severe COPD. An FiO_2 of 0.13 also approximates the partial pressure of inspired oxygen at an altitude of approximately 3800 m [22], corresponding to data from well-characterized populations [6, 7]. Oxyhemoglobin saturations were recorded in a separate group of mice (n = 3) during each oxygen profile using a PhysioSuite pulse oximetry system (Kent Scientific Corp., Torrington, CT).

Measurement of blood pressure and right ventricular systolic pressure

Systemic blood pressure was measured at baseline and weekly thereafter, using a CODA high throughput mouse tail-cuff system (Kent Scientific Corp.). Acclimatization to blood pressure measurement was performed by placing the mice in the appropriate restrainer without inflation of the tail cuffs, on 2 days prior, and the day prior, to baseline measurement. Just prior to sacrifice, right ventricular systolic pressure (RVSP) was obtained using a Millar pressure volume catheter (PVR-1030) inserted into the right external jugular vein and advanced into the right ventricle, as previously described [23]. RVSP tracings were recorded for 2 min.

Glucose tolerance tests

On the day prior to hypoxia or RA exposure, and again at days 15 and 30, intraperitoneal glucose tolerance tests (GTT) were performed, as previously described [24]. Briefly, mice were fasted for 5 h, beginning at 7:30 am. A basal glucose level was obtained by using the tail scratch technique at time 0 with a handheld glucometer (ACCU-CHEK Guide, Roche), and the mice were injected intraperitoneally with 1 g/kg glucose. Blood glucose was checked at 15, 30, 45, 60, 90, and 120 min after glucose injection. GTT was analyzed by subtracting fasting glucose levels and calculating the area under the curve (AUC) for each mouse.

Biochemical assays

Fresh liver samples were collected and flash frozen in liquid nitrogen, then stored at –80°C. Whole liver tissue was homogenized, and liver malondialdehyde (MDA), a marker of lipid peroxidation, was assessed (MilliporeSigma, St. Louis, MO). Liver glycogen concentration was measured by colorimetric assay (MilliporeSigma), and pro-inflammatory cytokine levels (tumor necrosis factor-α [TNF-α], interleukin 6 [IL-6], and interleukin 1β [IL-1β]) were assessed by ELISA (Invitrogen, Carlsbad, CA). Corticosterone and insulin were measured in serum by ELISA (Enzo Life Sciences, Farmingdale, NY; and MilliporeSigma, respectively). Triglycerides and cholesterol components were measured in serum with biochemical assays (MilliporeSigma).

RNA isolation, RT-qPCR, and high throughput sequencing

Total RNA was isolated from whole liver tissue using a Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was

synthesized (n = 6/group) using an Advantage RT for PCR kit (Clontech, Palo Alto, CA). RT-qPCR was performed with Taqman assays (Life Technologies, Carlsbad, CA). Target mRNA level was normalized to 18 s, using the formula Target/18 s = $2^{Ct(18s)-Ct(target)}$. For RNA sequencing, samples with RNA integrity number >8.0 were used (n = 4/group; male mice were used) to generate RNA sequencing libraries. Libraries were multiplexed and sequenced with 50 bp single end reads to a depth of approximately 50 million reads/sample. Sequencing reads were aligned to the mouse genome (GRCm38) using Bowtie v1.3.0 (Johns Hopkins University, Baltimore, MD), and read quantification and gene count matrix was generated with RSEM v1.3.0 [25] and GENCODE annotation (Mus_musculus.GRCm38.68.gtf). The edgeR package [26] was used to profile differential gene expression (DGE). Genes with sufficient count (>100 counts per million mapped reads [cpm]) in fewer than three samples were excluded from the analysis.

DGE and biological pathway analysis

For all genes meeting the specified count threshold, log fold changes (logFC) and p values (P) comparing three hypoxia exposures (SH, IH, and OH) to RA were calculated based on the exact test [27]. Gene enrichment and biological pathway analysis were performed using Ingenuity Pathway Analysis (Qiagen). P values were corrected for multiple testing using the Benjamini-Hochberg procedure. Differentially expressed genes were ranked based on their π values [28] defined by $\pi = |\text{LogFC}| * (-\log_{10}\text{P})$, which considers both the biological and statistical significance of a gene.

Statistical analysis and data availability

Comparisons between groups of mice for data resulting in single-point measurements were performed using one-way analysis of variance, with post-hoc comparisons made with Tukey's multiple comparison test. Mixed-effects spline regression models were used to analyze systemic blood pressure data. For these models, we incorporated random intercepts to account for individual differences between mice in baseline blood pressure. We noted a drop in blood pressure in all groups between baseline and week 1, indicating that acclimatization to the experiment might impact blood pressure independent of hypoxic exposure. To account for these effects, we placed an inflection point after 2 weeks. We performed additional sensitivity analyses to determine whether delaying the inflection point by one or two additional weeks impacted our findings. For all statistical comparisons, a p-value < .050 was the threshold used for statistical significance. Data are reported as mean ± S.E.M. Prism 8 software (GraphPad, San Diego, CA) was used for all analyses except RNA sequencing (as above) and mixed-effects models of blood pressure (R). RNA sequencing data are publicly available on the Gene Expression Omnibus (GSE189958, available here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189958). Other data underlying the findings in this article may be shared upon request to the corresponding author.

Results

Oxyhemoglobin saturations in different hypoxia profiles

Effects of the various hypoxia profiles on oxyhemoglobin saturations are shown in Figure 1. Mice in SH had saturations of 61.7%–70.7%. In IH, nadir saturations ranged from 53.3 ± 5.2% to 56.0 ± 4.7%, and peak saturations ranged from 90.3 ± 3.9% to 97.7 ± 1.3%. In OH, nadir saturations ranged from 33.3 ± 3.5% to 43.3 ± 3.0% and peak saturations ranged from 85.3 ± 1.2% to 89.3 ± 0.7%.

Body weight, liver weight, epididymal/periovarian fat weight

Mice in RA gained weight during the experiment duration (+1.3 ± 0.2 g, Figure 2A). In SH and IH, mice lost weight (SH: −0.7 ± 0.6 g, p = .022 compared to RA group; IH: −1.1 ± 0.4 g, p = .002), but in OH, weight change was similar to RA (+0.9 ± 0.2 g, p = 1.000). Liver weight at sacrifice was reduced in IH relative to RA (Figure 2B), but in other hypoxic groups was similar to RA. There was no difference in epididymal/periovarian fat weight between groups (Figure 2C).

Cardiovascular outcomes

Effects of hypoxic exposures on systemic blood pressure are shown in Figure 3A. Mixed-effects spline-regression models (Supplemental Tables 1 and 2) demonstrated that systolic blood pressure initially fell in mice in RA, but increased slightly between weeks 2–5. Compared to RA, mice in SH had no significant differences in systolic blood pressure. Mice in IH and OH had markedly attenuated blood pressure reductions over the initial 2 weeks of exposure. Sensitivity analyses demonstrated comparable results when the inflection point was delayed to 3 weeks. There were no differences in diastolic blood pressure. These data indicate that mice exposed to IH and OH experienced higher systolic blood pressure over the first 3 weeks of exposure. RVSP was elevated in mice exposed to SH (52% increase relative to RA, p < .001, Figure 3B) and OH (20% increase, p = .001), but was unchanged in IH (p = .689). Serum corticosterone was not impacted by any hypoxic exposure (Figure 3C). Serum triglycerides were increased in SH relative to RA (p < .001, Figure 3D), reduced in IH (p = .014), and unchanged in OH (p = .225). Serum low- and very-low-density lipoproteins (LDL/VLDL) were increased in SH and OH relative to RA (p = .033 for both) but unchanged in IH (p = .756).

Glucose-related and liver-specific outcomes

Fasting glucose (time point 0 glucose during serial GTT) was unchanged between baseline and day 30 in the RA group

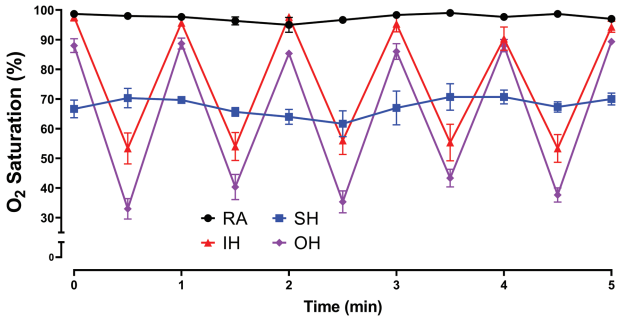

Figure 1. Oxyhemoglobin saturations in the three hypoxic conditions and RA. For each of IH and OH, nadir and peak saturations in each cycle of intermittent reduction in FiO_2 are shown.

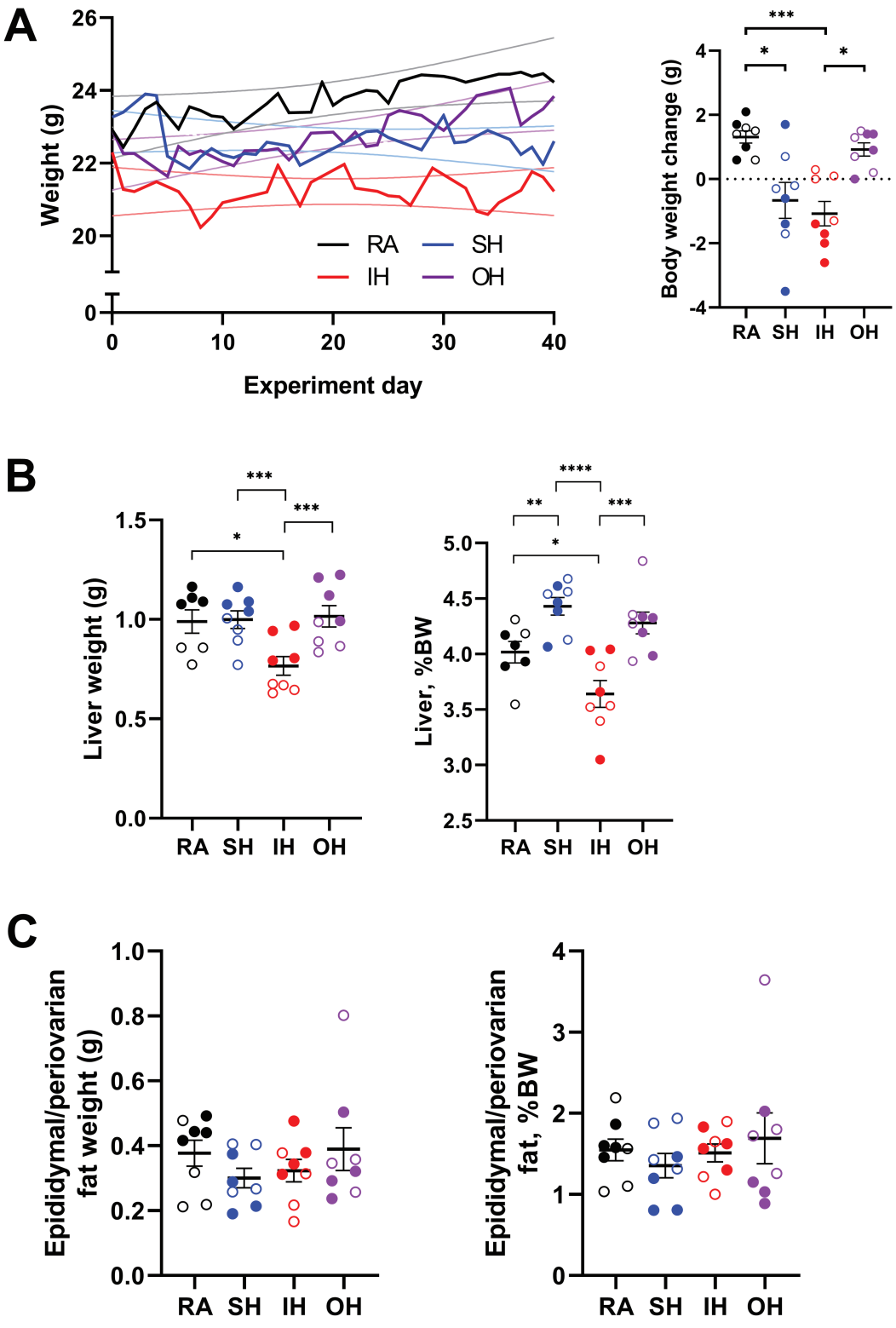

Figure 2. Changes in body composition over experiment duration. (A) Body weight over time (left), and change in body weight between baseline and the day of sacrifice, in each experimental condition (right). (B) Liver weight (left) and liver weight as a percentage of total body weight (right) at the time of sacrifice. Liver weight was reduced in IH mice relative to RA (0.766 ± 0.047 g vs. 0.990 ± 0.058 g, p = .020), but was no different in SH or OH (0.999 ± 0.044 g, p = 0.980; and 1.016 ± 0.054 g, p = 0.980, respectively). Liver weight expressed as a percentage of total body weight at the time of sacrifice was also reduced in IH (3.6% vs. 4.0%, p = .043), increased in SH (4.4%, p = .031), and not significantly changed in OH (4.2%, p = .150). (C) Epididymal/periovarian fat weight (left) and epididymal/periovarian fat as a percentage of total body weight (right) at the time of sacrifice. Data from male mice are in filled circles. *, p < .050; **, p < 0.010; ***, p < .005; ****, p < .001.

(132 ± 5 to 129 ± 12 mg/dL, p = .808, Figure 4A), but was reduced in SH (150 ± 11 to 93 ± 6 mg/dL, p = .001) and in OH (147 ± 7 to 106 ± 4 mg/dL, p < .001), and was increased in IH (127 ± 5 to 153 ± 2 mg/dL, p = .001). When comparing the change in fasting glucose between groups, the reductions in fasting glucose in SH and OH were both different from RA (SH vs. RA:

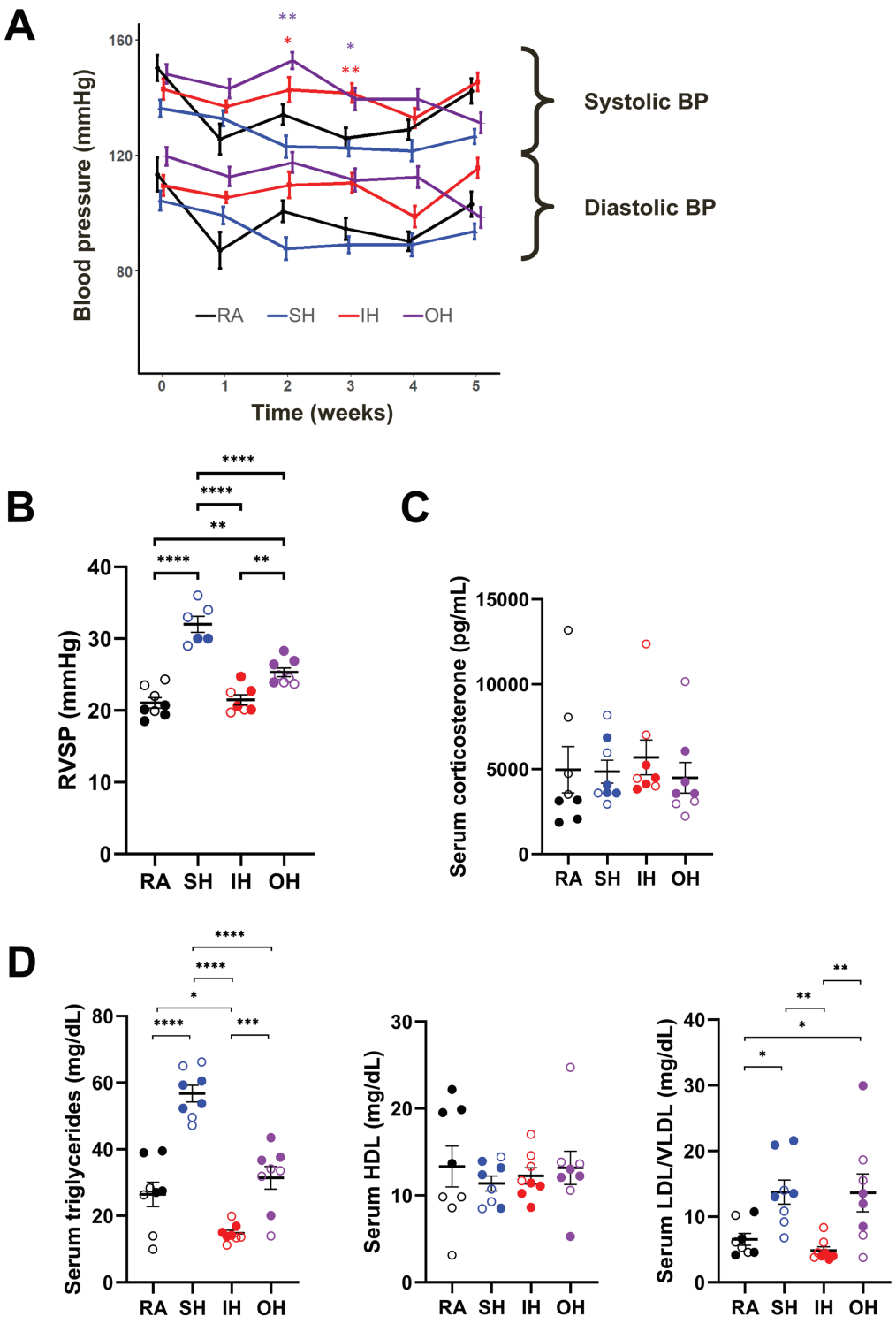

Figure 3. Cardiovascular changes over experiment duration. (A) Weekly blood pressure over experiment duration. Asterisks indicate a statistically significant change in systolic blood pressure over 2 or 3 weeks when compared to RA rather than direct group comparisons per week. (B) Right ventricular systolic pressure measured at the time of sacrifice. RVSP was elevated in OH relative to RA (25.3 vs. 21.0 mm Hg, 20% absolute increase, $p = .001$) and in SH relative to RA (32.0 mm Hg, 52% absolute increase, $p < .001$). IH did not increase RVSP (21.5 mm Hg, $p = .689$). (C) Serum corticosterone at the time of sacrifice. No differences were seen in any hypoxic group relative to RA (RA: 4970 ± 1362 pg/mL; SH: 4857 ± 672 pg/mL, $p = 1.000$; IH: 5694 ± 1020 pg/mL, $p = .958$; OH: 4491 ± 903 pg/mL, $p = .987$). (D) Serum lipids at sacrifice. Triglycerides (*left*) were increased in SH (RA: 26.4 ± 3.7 mg/dL; SH: 56.7 ± 2.5 mg/dL, $p < .001$) and were reduced in IH (14.8 ± 0.9 mg/dL, $p = .014$) but no change was observed in OH (31.4 ± 3.4 mg/dL, $p = .225$). HDL (*middle*) was unchanged in all hypoxic groups (RA: 13.3 ± 2.4 mg/dL; SH: 11.4 ± 0.9 mg/dL, $p = .956$; IH: 12.3 ± 0.9 mg/dL, $p = .984$; OH: 13.2 ± 1.9 mg/dL, $p = .984$). LDL and VLDL cholesterol fractions (*right*) were increased in SH and OH relative to RA (RA: 6.6 ± 0.9 mg/dL; SH: 13.7 ± 1.8 mg/dL, $p = .033$; OH: 13.6 ± 2.9 mg/dL, $p = .033$) but were unchanged in IH (4.8 ± 0.6 mg/dL, $p = .756$). Data from male mice are in filled circles. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein. *, $p < .050$; **, $p < .010$; ***, $p < .005$; ****, $p < .001$.

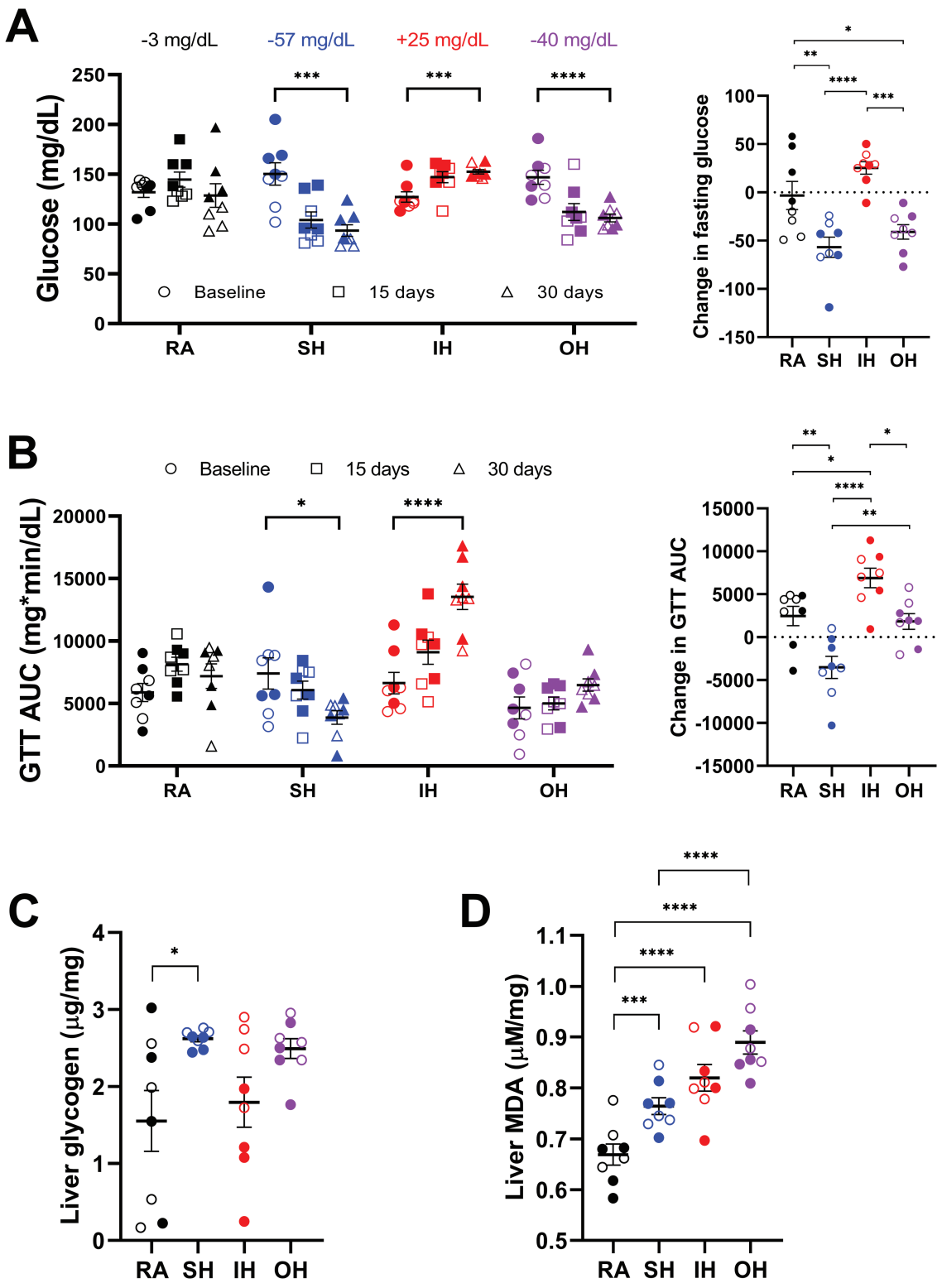

Figure 4. Changes in glucose metabolism and hepatic oxidative stress. (A, *left*) Fasting blood glucose measured at baseline (circles), day 15 GTT (squares), and day 30 GTT (triangles), demonstrating within-group effects over time. Fasting glucose decreased in SH and OH, and increased in IH. (*right*) Change in fasting glucose showing significant between-group effects. (B, *left*) Area under the GTT curve (GTT AUC). Glucose tolerance improved in SH and worsened in IH. (*right*) Change in GTT AUC showing significant between-group effects. (C) Hepatic glycogen content at sacrifice. Glycogen was elevated in SH relative to RA (RA: 1.55 ± 0.40 µg/mg liver tissue; SH: 2.62 ± 0.04 µg/mg, $p = .038$), but there was no difference in IH (1.80 ± 0.32 µg/mg, $p = .915$) or OH (2.49 ± 0.13 µg/mg, $p = .080$). (D) Liver malondialdehyde (MDA), a product of lipid peroxidation and a hepatic marker of oxidative stress, was increased in each hypoxic group relative to RA, and the magnitude of increase was highest in OH (RA: 0.67 ± 0.02 µM/mg liver tissue; SH: 0.76 ± 0.02 µM/mg, $p = .022$; IH: 0.82 ± 0.03 µM/mg, $p < .001$; OH: 0.89 ± 0.02 µM/mg, $p < .001$). Data from male mice are in filled points. *, $p < .050$; **, $p < .010$; ***, $p < .005$; ****, $p < .001$.

p = .004, OH vs. RA: p = .044), but unchanged in IH (IH vs. RA: p = .113).

We analyzed the GTT AUC as a measure of the dynamic response to glucose load (Figure 4B). GTT AUC from baseline to day 30 did not change in RA (5889 ± 723 to 7190 ± 979 mg/dL-min, p = .305), but decreased in SH (7403 ± 1252 to 3878 ± 539 mg/dL-min, p = .028), increased in IH (6637 ± 855 to 13536 ± 1015 mg/dL-min, p < .001), and was unchanged in OH (4653 ± 872 to 6480 ± 495 mg/dL-min, p = .096). In examining the change in GTT AUC over time, it was decreased in SH (SH vs. RA: p = .004), increased in IH (IH vs. RA: p = .019), and remained unchanged in OH (OH vs. RA: p = .690).

Liver glycogen was elevated in SH relative to RA, with no change in IH or OH (Figure 4C). Liver MDA, an oxidative stress marker, was increased in all hypoxic groups relative to RA (Figure 4D). Higher MDA concentrations were observed in OH relative to SH, but there were no differences between IH and SH, or OH and IH. Hepatic levels of pro-inflammatory cytokines were assessed, but we observed no differences between groups (Table 1).

Hepatic gene expression analysis

Because of the marked differences in glycemia and lipid metabolism among groups, hepatic gene expression patterns were analyzed by RNA sequencing, demonstrating highly variable gene expression profiles (Figure 5). Using a p-value of < .050 adjusted for multiple comparisons, there were 515 differentially expressed genes in IH versus RA, 86 differentially expressed genes in OH versus RA, and 25 in SH versus RA (Figure 5A). When using π values as a marker of significance as described above, this approach showed a more even distribution of genes with differential expression. The numbers of intersecting differentially expressed genes are shown in Figure 5B, and all intersecting genes are listed in Supplemental Table 3, ranked in order of descending π values for each overlapping group. Differentially expressed genes among all hypoxic conditions relative to RA included those significant for lipid metabolism (*Fasn*, *Hacl1*, *Insig2*, *Plin2*), glucose metabolism (*Gys2*), and circadian biology (*Dbp*). Among the most highly differentially expressed pathways were those impacting lipid metabolism and oxidative stress (Figure 5C). Supplemental Tables 4–6 show pathways which are significantly changed in SH versus RA, IH versus RA, and OH versus RA, respectively. RT-qPCR was used to examine expression changes in genes derived from RNA sequencing results, in a larger group of mice. We found differences in expression of several enzymes of glucose and triglyceride metabolism, such as *Gck*, *G6pc3*, *Lpin1*, *Srebf1*, *Cpt1a*, and *Dgat2* (Figure 5D).

DISCUSSION

The COPD/OSA overlap syndrome is associated with excess mortality [3, 4]. COPD has myriad pathophysiological effects, including expiratory airflow limitation, hypoxemia, hypercapnia, and respiratory muscle dysfunction. OSA causes IH, intrathoracic pressure swings, and repetitive arousals from sleep. There are few, if any, mechanistic data exploring the relative contribution of any of these effects to outcomes in the COPD/OSA overlap syndrome. Moreover, high altitude may have detrimental cardio-metabolic effects [6, 7]. Because outcomes in COPD/OSA overlap and at high altitude appear to be related to the severity of nocturnal hypoxemia, we investigated whether OH, as a model of either of these conditions, could worsen cardio-metabolic health in mice. We found that OH induces pulmonary hypertension and transient systemic hypertension, causes hepatic oxidative stress, elevates serum LDL/VLDL cholesterol (itself associated with atherosclerotic heart disease [29]), and results in a lack of glycemic "protection" that is seen in SH exposure alone (Figure 6). Moreover, RNA sequencing and RT-qPCR provide some insight about gene expression changes associated with these phenomena. In all, these data suggest that hypoxemia such as that experienced by many patients with COPD/OSA overlap or those living at high altitude, may be uniquely deleterious due to a combination of adverse cardio-metabolic effects.

Table 1. Select pro-inflammatory cytokine levels in homogenized liver tissue, normalized to liver tissue weight, at the time of sacrifice (n = 8/group)

	RA	SH	IH	OH
TNF-α, pg/mg	7.5 ± 0.3	8.0 ± 0.5	8.2 ± 0.5	8.4 ± 0.5
IL-1β, pg/mg	40.0 ± 2.3	48.1 ± 4.7	48.9 ± 2.5	46.1 ± 4.1
IL-6, pg/mg	7.1 ± 0.4	6.9 ± 0.4	6.8 ± 0.3	6.9 ± 0.3

Data are reported as mean ± S.E.M. No between-group comparisons are significant to p < .050.

We note that our data are mostly in agreement with existing literature regarding the effects of SH and IH. SH at a FiO_2 of 0.10 improves glucose tolerance [11] and increases hepatic glycogen [12] and MDA levels, [11] and is widely used as a model of hypoxic pulmonary hypertension in mice [8, 9]. IH as in our model causes systemic hypertension in rodents [10, 13], worsens glucose tolerance [18, 19], increases MDA [30], and results in poor weight gain or weight loss [15, 31]. We observed all of these effects in our experiments. By contrast, IH has been shown to cause pulmonary hypertension [14–17], an effect we did not observe. However, there have been only limited investigations into this finding, and these groups either did not measure RVSP directly [15], exposed to IH for longer duration than in our protocol [16] or performed their work at some degree of altitude (Denver, CO^{14} or Albuquerque, NM^{17}), where the altitude of 1600–1700 m could reasonably have caused an effect of mild OH. Our investigation of the cardio-metabolic effects of OH are novel and thus there is no strong basis for comparison with existing literature.

Another important point regarding pulmonary hypertension as an outcome: Although we did not observe RVSP elevation in OH as severe as SH, the oxyhemoglobin saturations induced by SH in our experiment and models of hypoxic pulmonary hypertension are not realistic reflections of any common disease state. By contrast, our OH regimen may accurately reflect severe COPD/OSA overlap syndrome [1]. Thus, our finding of higher RVSP in OH compared to IH suggests that patients with COPD/OSA overlap may be at higher clinical risk for pulmonary hypertension than patients with OSA alone and that this increased risk is driven primarily by overall hypoxemia severity, in the context of pulmonary parenchymal destruction.

The effects of the hypoxic regimens on fasting glucose and glucose tolerance also merit consideration. Although both are associated with cardiovascular disease, there is a strong link between response to glucose load and cardiovascular outcomes [32]. Our findings broadly suggest that IH worsens and SH improves glucose metabolism and that these effects are perhaps counterbalanced in OH. These results may be broadly in line

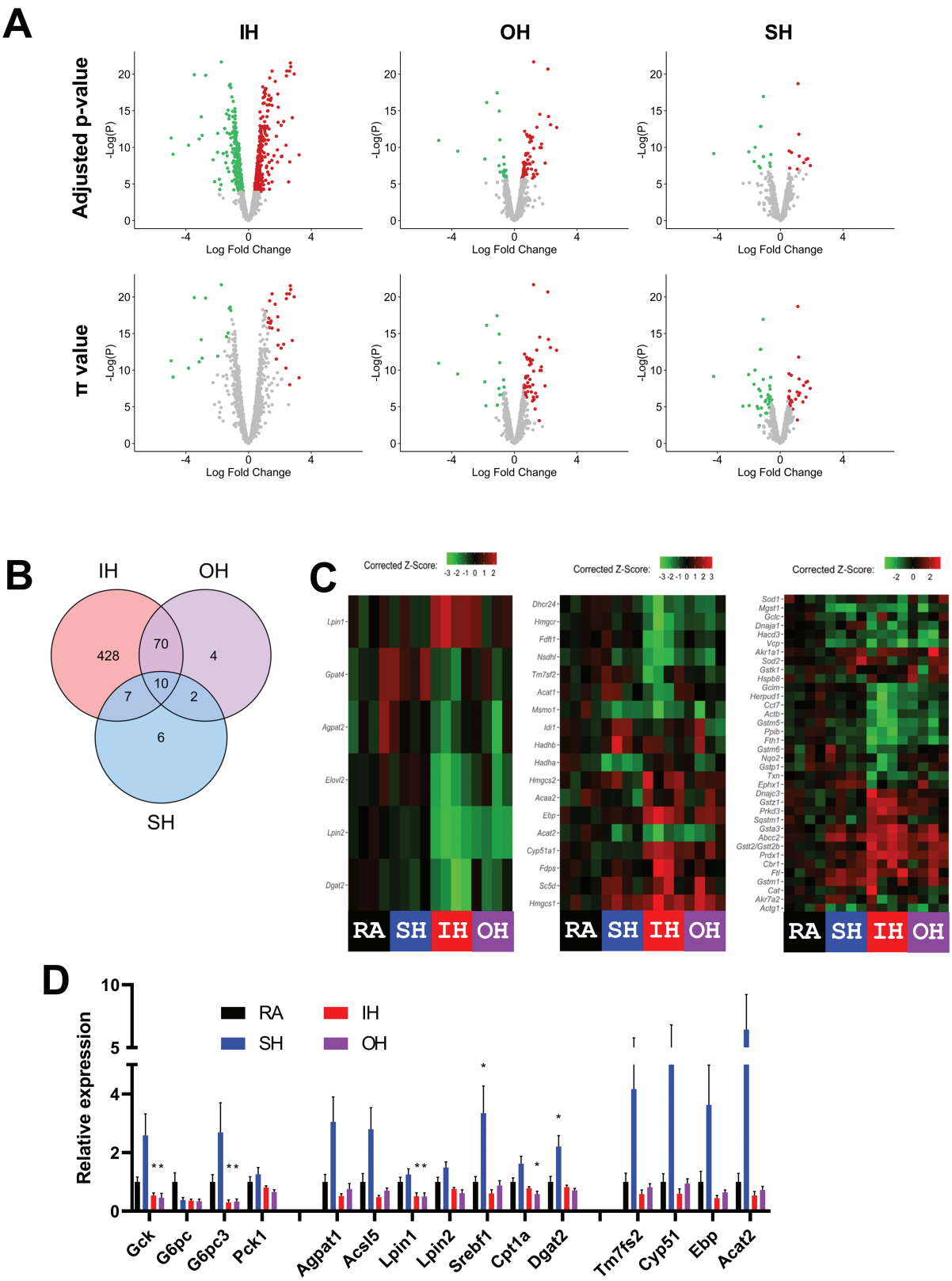

Figure 5. RNA sequencing and RT-qPCR from whole liver. (A) Volcano plots showing effects on hepatic gene expression in SH, IH, and OH, each relative to RA. The top panels show differentially expressed genes when assessed by adjusted p-value alone (green dots signifying underexpressed genes and red dots signifying overexpressed genes, each to $p < .050$), and the bottom panels show differentially expressed genes when assessed by π value, taking into account fold-change expression difference as a way of considering both the biological and statistical significance of gene expression changes (green and red dots signifying underexpressed and overexpressed genes, respectively, and the top 5% of π values in each hypoxic group are highlighted). Overall gene expression patterns varied considerably by hypoxic exposure. (B) Venn diagram of differentially expressed genes based on adjusted *p*-value. Genes in intersecting regions are listed in Supplemental Table 3. (C) Heat maps

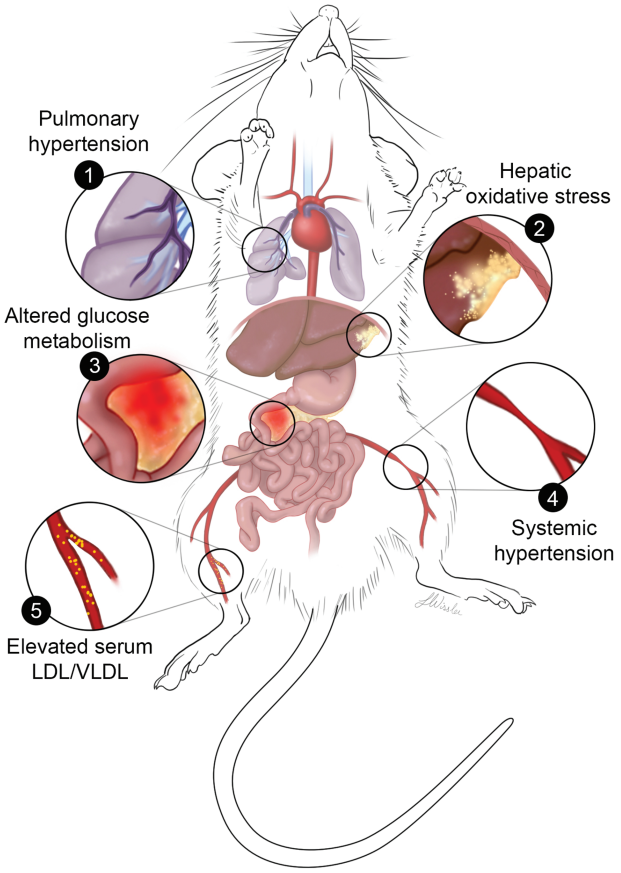

Figure 6. Summary of the deleterious effects of OH. OH causes (1) pulmonary hypertension (elevated RVSP), more so than IH; (2) Hepatic oxidative stress, to a larger degree than in other hypoxic groups, which is associated with dysglycemia [38] and may lead to further liver injury; [39] (3) Altered glucose metabolism, with reduced fasting glucose but worsened glucose disposal relative to SH; (4) Systemic hypertension, at least transiently, in a pattern similar to IH; and (5) Changes to serum lipids, including elevated serum LDL/VLDL cholesterol. Superimposed sustained and intermittent hypoxia thus induces a variety of deleterious changes which may account for poorer outcomes in patients with this pattern of hypoxemia.

with clinical experience; there is a higher prevalence of diabetes mellitus among COPD/OSA overlap patients than among matched patients with COPD alone [33], and higher hemoglobin A1c as a function of apnea-hypopnea index (AHI) in people living at high altitude [7].

An important limitation in the interpretation of our data rests in the choice of a FiO_2 of 0.10 for the SH group, rather than the baseline used in OH (0.13). Our main goal in this study was to determine broadly the impact of superimposed sustained and IH on various physiologic outcomes. In particular, we wished to know what additional cardio-metabolic dysfunction arises in overlap syndromes (COPD/OSA, or high altitude) relative to OSA alone. A FiO_2 of 0.10 was selected for SH because this stimulus is well described in the literature to induce particular effects on glucose metabolism and pulmonary pressures; we, therefore, have a valid comparator group that is well described. However, the effects of more modest SH, which might better recapitulate the hypoxemia of COPD or high altitude without concurrent sleep-disordered breathing, should also be investigated. In addition, future inquiry regarding the precise contribution of "swings" in oxyhemoglobin saturation, versus a reduction in mean saturation, would be helpful in explaining the changes we observed.

What our study does not provide is a full understanding of two major questions: First, is there a unique combination of outcomes that leads to worsened health in COPD/OSA? For instance, to what degree do dysglycemia, systemic hypertension, and pulmonary hypertension each contribute to mortality in COPD/OSA overlap? It is known that cardiovascular disease, hypertension, diabetes, and dyslipidemia are more common in the overlap syndrome than in OSA [33, 34]. The degree to which each co-morbidity contributes to adverse outcomes is unclear. Second, how do the different pathophysiologic features of COPD or OSA contribute to excess mortality in these patients? Aside from hypoxemia, what role is played by, for example, hypercapnia, or excess sympathetic tone from sleep-related arousals? [35, 36]

Our study has several strengths and some important limitations. First, we took care to model SH, IH, and OH in relevant ways, and OH in particular in a way that faithfully recapitulates oxyhemoglobin saturations in human disease. Second, mice were extensively characterized with respect to clinically meaningful outcomes. Third, mice were minimally disturbed from their respective hypoxic exposures during the entirety of the study period. Fourth, both male and female mice were used; we note that physiologic effects in female mice have historically been underexplored [37]. Although this study was not powered specifically to determine sex-related differences in any outcome of interest, there was nothing about our results that suggested any particular necessity to exclude female mice from experimentation. Despite these strengths, we lack data regarding the potential effects of acclimatization to hypoxic exposures—might this explain the transient effect of OH and IH on systemic blood pressure?—and, as described above, we lack insight about the effect of more modest SH. We also note that our study might have been underpowered to examine effects on some of the outcomes of interest. For instance, we note a trend of worsening glucose tolerance in OH over time; however, we would suggest that the impact of OH on glucose tolerance, if any, is of smaller effect size than IH or SH, which is an intriguing outcome in its own right. Finally, although the RNA sequencing, RT-qPCR, and measures of hepatic oxidative stress and pro-inflammatory cytokines offer insight into mechanisms that might explain the unique characteristics of OH, this topic merits further investigation. RNA sequencing in particular is limited in that gene expression changes at the time of sacrifice may not reflect earlier gene expression patterns which might result in some of the physiologic manifestations we report. In addition, we surmise that some of the observed effects may occur through alterations in hypoxia inducible factor (HIF) signaling in various tissues, and studies are ongoing to test this.

In conclusion, we have shown that the hypoxemia of COPD/OSA overlap syndrome, or high-altitude exposure, may

showing pathway changes in each experimental group, in representative canonical pathways of lipid metabolism and oxidative stress. *L–R:* Triacylglycerol Biosynthesis, Superpathway of Cholesterol Biosynthesis, and NRF2-mediated Oxidative Stress Response. Gene expression is normalized to the mean expression value of the RA group for each gene. (D) RT-qPCR showing gene expression changes in important genes of glucose metabolism (*left*), triglyceride synthesis and metabolism (*middle*), and cholesterol synthesis and metabolism (*right*). *, $p < .050$ when compared to gene expression in RA.

successfully be modeled in rodents and that this overlap hypoxemia leads to systemic and pulmonary hypertension, hepatic oxidative stress, elevations in serum LDL/VLDL, and alterations in glucose metabolism relative to SH and IH. We hope for further insight about the mechanisms by which hypoxemia in these clinical states may worsen outcomes, and advocate for careful attention to hypoxemia in patients with COPD/OSA overlap. The utility of only the most common metrics of disease severity, such as the AHI or FEV_1, may be insufficient to guide meaningful outcomes in the overlap syndrome.

Supplementary Material

Supplementary material is available at *SLEEP* online.

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Disclosure Statement

None declared.

References

1. McNicholas WT. COPD-OSA overlap syndrome: evolving evidence regarding epidemiology, clinical consequences, and management. *Chest*. 2017;**152**(6):1318–1326.
2. Du W, *et al*. Obstructive sleep apnea, COPD, the overlap syndrome, and mortality: results from the 2005–2008 National Health and Nutrition Examination Survey. *Int J Chron Obstruct Pulmon Dis*. 2018;**13**:665–674.
3. Marin JM, *et al*. Outcomes in patients with chronic obstructive pulmonary disease and obstructive sleep apnea: the overlap syndrome. *Am J Respir Crit Care Med*. 2010;**182**(3):325–331.
4. Kendzerska T, *et al*. Cardiovascular outcomes and all-cause mortality in patients with obstructive sleep apnea and chronic obstructive pulmonary disease (overlap syndrome). *Ann Am Thorac Soc*. 2019;**16**(1):71–81.
5. Pham LV, *et al*. Cross-sectional comparison of sleep-disordered breathing in native Peruvian highlanders and lowlanders. *High Alt Med Biol*. 2017;**18**(1):11–19.
6. Miele CH, *et al*. Increased cardiometabolic risk and worsening hypoxemia at high altitude. *High Alt Med Biol*. 2016;**17**(2):93–100.
7. Pham LV, *et al*. Cardiometabolic correlates of sleep disordered breathing in Andean highlanders. *Eur Respir J*. 2017;**49**(6):1601705.
8. Hislop A, *et al*. New findings in pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *Br J Exp Pathol*. 1976;**57**(5):542–554.
9. Stenmark KR, *et al*. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol*. 2009;**297**(6):L1013–L1032.
10. Hui AS, *et al*. Regulation of catecholamines by sustained and intermittent hypoxia in neuroendocrine cells and sympathetic neurons. *Hypertension*. 2003;**42**(6):1130–1136.
11. Ioja S, *et al*. Nocturnal hypoxia improves glucose disposal, decreases mitochondrial efficiency, and increases reactive oxygen species in the muscle and liver of C57BL/6J mice independent of weight change. *Oxid Med Cell Longev*. 2018;**2018**:9649608.
12. Chen XQ, *et al*. Effects of hypoxia on glucose, insulin, glucagon, and modulation by corticotropin-releasing factor receptor type 1 in the rat. *Endocrinology*. 2007;**148**(7):3271–3278.
13. Fletcher EC, *et al*. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. *Hypertension*. 1992;**19**(6 Pt 1):555–561.
14. Fagan KA. Selected contribution: pulmonary hypertension in mice following intermittent hypoxia. *J Appl Physiol (1985)*. 2001;**90**(6):2502–2507.
15. Campen MJ, *et al*. Acute and chronic cardiovascular effects of intermittent hypoxia in C57BL/6J mice. *J Appl Physiol (1985)*. 2005;**99**(5):2028–2035.
16. Nisbet RE, *et al*. The role of NADPH oxidase in chronic intermittent hypoxia-induced pulmonary hypertension in mice. *Am J Respir Cell Mol Biol*. 2009;**40**(5):601–609.
17. Snow JB, *et al*. Intermittent hypoxia augments pulmonary vasoconstrictor reactivity through PKCβ/mitochondrial oxidant signaling. *Am J Respir Cell Mol Biol*. 2020;**62**(6):732–746.
18. Polak J, *et al*. Intermittent hypoxia impairs glucose homeostasis in C57BL6/J mice: partial improvement with cessation of the exposure. *Sleep*. 2013;**36**(10):1483–90; 1490A. doi:10.5665/sleep.3040.
19. Drager LF, *et al*. Intermittent hypoxia exacerbates metabolic effects of diet-induced obesity. *Obesity (Silver Spring)*. 2011;**19**(11):2167–2174.
20. Gozal D, *et al*. Visceral white adipose tissue after chronic intermittent and sustained hypoxia in mice. *Am J Respir Cell Mol Biol*. 2017;**56**(4):477–487.
21. Wu W, *et al*. Network analysis of temporal effects of intermittent and sustained hypoxia on rat lungs. *Physiol Genomics*. 2008;**36**(1):24–34.
22. Peacock AJ. ABC of oxygen: oxygen at high altitude. *BMJ*. 1998;**317**(7165):1063–1066.
23. Jain PP, *et al*. Halofuginone, a promising drug for treatment of pulmonary hypertension. *Br J Pharmacol*. 2021;**178**(17):3373–3394.
24. Mesarwi OA, *et al*. Hepatocyte HIF-1 and intermittent hypoxia independently impact liver fibrosis in murine nonalcoholic fatty liver disease. *Am J Respir Cell Mol Biol*. 2021;**65**(4):390–402.
25. Li B, *et al*. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;**12**:323.
26. Robinson MD, *et al*. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;**26**(1):139–140.

27. Robinson MD, *et al*. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics*. 2008;**9**(2):321–332.
28. Xiao Y, *et al*. A novel significance score for gene selection and ranking. *Bioinformatics*. 2014;**30**(6):801–807.
29. Ference BA, *et al*. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur Heart J*. 2017;**38**(32):2459–2472.
30. Jun J, *et al*. Intermittent hypoxia has organ-specific effects on oxidative stress. *Am J Physiol Regul Integr Comp Physiol*. 2008;**295**(4):R1274–R1281.
31. Naghshin J, *et al*. Chronic intermittent hypoxia increases left ventricular contractility in C57BL/6J mice. *J Appl Physiol (1985)*. 2009;**107**(3):787–793.
32. Coutinho M, *et al*. The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. *Diabetes Care*. 1999;**22**(2):233–240.
33. Papachatzakis I, *et al*. Comorbidities in coexisting chronic obstructive pulmonary disease and obstructive sleep apnea—overlap syndrome. *Eur Rev Med Pharmacol Sci*. 2018;**22**(13):4325–4331.
34. Uzokov J, *et al*. Prevalence of metabolic syndrome in patients with chronic obstructive pulmonary disease. *Eur Respir J*. 2016;**48**(suppl 60):PA3776.
35. Loredo JS, *et al*. Relationship of arousals from sleep to sympathetic nervous system activity and BP in obstructive sleep apnea. *Chest*. 1999;**116**(3):655–659.
36. Kim JB, *et al*. Effect of arousal on sympathetic overactivity in patients with obstructive sleep apnea. *Sleep Med*. 2019;**62**:86–91.
37. Wald C, *et al*. Biomedical research. Of mice and women: the bias in animal models. *Science*. 2010;**327**(5973):1571–1572.
38. Ling PR, *et al*. Hyperglycemia induced by glucose infusion causes hepatic oxidative stress and systemic inflammation, but not STAT3 or MAP kinase activation in liver in rats. *Metabolism*. 2003;**52**(7):868–874.
39. Ha HL, *et al*. Oxidative stress and antioxidants in hepatic pathogenesis. *World J Gastroenterol*. 2010;**16**(48):6035–6043.