Hyperlipidemia and lipid peroxidation are dependent on the severity of chronic intermittent hypoxia

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OBSTRUCTIVE SLEEP APNEA (OSA) is caused by recurrent closure of the upper airway during sleep, which leads to repetitive periods of intermittent hypoxia (IH). OSA is a common disorder affecting 2–9% women and 4–24% men in the United States (50). Obesity is a major risk factor for OSA, and prevalence of OSA exceeds 50% in obese men (36, 48, 50). OSA is associated with metabolic abnormalities, including insulin resistance (16, 36) and dyslipidemia (7, 31, 39), independent of underlying obesity. Multiple perturbations of lipid metabolism occur in patients with OSA, including increases in total serum cholesterol and triglyceride levels, a decrease in high-density lipoprotein (HDL), loss of antioxidant capacity of HDL, increases in peroxidation of serum lipids, and an increase in oxidized low-density lipoprotein (LDL) (7, 17, 21, 31, 39, 45). However, the comorbidity imposed by obesity has made it difficult to establish a direct causal link between the physiological disturbances of OSA and the development of dyslipidemia.

We have previously developed a murine model of IH, mimicking an oxygen profile in patients with OSA (34, 35, 44). We have shown that an acute, 5-day IH exposure to 5% nadir of inspired oxygen can produce hyperlipidemia in lean C57BL/6J mice (25). The hypoxia-induced changes in lipid metabolism were mediated via hepatic stearoyl coenzyme A desaturase 1 (SCD-1) (25), an enzyme of cholesterol ester and triglyceride biosynthesis that induces dyslipidemia by increasing lipoprotein secretion (9, 33). We have also shown that the acute 5-day IH exposure decreased levels of scavenger receptor B1 (SR-B1) in the liver, which can inhibit reverse cholesterol transport, leading to hypercholesterolemia (25). However, it is unknown whether hyperlipidemia will develop when more moderate levels of IH are administered over a prolonged time period, an experimental paradigm that more closely simulates the physiological disturbances that characterize the majority of OSA patients.

Therefore, the purpose of the present study was to explore the effects of a 28-day chronic IH (CIH) exposure at both 5% (severe) and 10% (moderate) levels of nadir of inspired oxygen on lipid metabolism and lipid peroxidation in lean C57BL/6J mice. We examined changes in serum and liver lipid levels, liver lipid peroxidation, hepatic SCD-1 levels, and lipoprotein secretion and hypothesized that CIH will lead to dyslipidemia and lipid peroxidation in proportion to the severity of the hypoxic stimulus.

METHODS

A total of 48 wild-type male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) were used in the study. All mice were 10–11 wk of age at the beginning of the study, which was approved by the Johns Hopkins University Animal Care and Use Committee and complied with the American Physiological Society guidelines for animal studies. For all blood samples, injections, and surgical procedures, anesthesia was induced and maintained with 1–2% isoflurane administered through a facemask.

A gas control delivery system was designed to regulate the flow of room air, nitrogen, and oxygen into customized cages housing the mice as previously described (34). During each period of IH, the fractional inspired O2 (FiO2) was reduced from 20.9 either to 4.9 ± 0.1% (severe CIH) or to 9.8 ± 0.2% (moderate CIH) over a 30-s period and then rapidly reoxygenated to room air levels in the subsequent 30-s period. CIH was administered for 28 consecutive days in a controlled environment (22–24°C with a 12:12-h light-dark cycle; lights on at 0900) while mice were fed ad libitum with standard

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Purina chow diet (4% fat, 0.15% cholesterol). Control mice were exposed to intermittent room air (IA) for 28 days in identical chambers at identical rates to the IH exposure with FIO₂ remaining at 20.9%. Since weight loss was anticipated in the CIH groups (25, 34), IA mice were weight matched to mice exposed to CIH by varying food intake from 2.5 to 4.5 g/day. The CIH and IA states were induced during the 12-h light phase alternating with 12 h of constant room air during the dark phase.

All serum and tissue samples were obtained during exposure to CIH or control conditions. Mice fasted for 5 h before bleeding by direct cardiac puncture. After blood withdrawal, the animals were euthanized with pentobarbital (60 mg ip). Livers were surgically removed and immediately frozen for future analysis.

Serum lipids were measured with kits from Wako Diagnostics (Richardson, VA). In addition, pooled serum from each experimental group was subjected to gel filtration HPLC on two tandemly connected TSK-Gel Lipopropak XL columns (300 × 7.8 mm) with simultaneous measurement of triglyceride and cholesterol using an on-line dual detection system, according to LipoSEARCH technology (Skylight Biotech, Tokyo). Serum leptin and insulin were detected with ELISA kits from Linco Research (St. Charles, MO). Lipids were extracted from the liver with chloroform-methanol, according to Bligh-Dyer procedure (1), and measured using kits from Wako Diagnostics. Lipid peroxidation in liver tissue was assessed by malondialdehyde (MDA) assay with a kit from Oxford Biomedical Research (Oxford, MI).

Total RNA was extracted from liver using Trizol (Life Technologies, Rockville, MD), and cDNA was synthesized using Advantage RT for PCR kit from Clontech (Palo Alto, CA). Real-time reverse-transcriptase PCR (RT-PCR) was performed with primers from Invitrogen (Carlsbad, CA) and Taqman probes from Applied Biosystems (Foster City, CA). The sequences of primers and probes were previously described (24, 25). The mRNA expression levels were normalized to 18S rRNA concentrations using the following formula: gene of interest/18S normalized to 18S rRNA concentrations using the following formula:

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\frac{[\text{gene of interest}]}{[\text{18S}]} = \frac{[\text{cycle of interest}] - [\text{cycle of interest}]_0}{[\text{cycle of interest}]_0}
\]

where [C₀] is cycle threshold.

An aliquot of the liver tissue from each mouse was homogenized in sucrose solution (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT) with complete protease inhibitor (Roche, Manheim, Germany). The microsomal fraction was isolated as previously described (27). Aliquots (70 μg of protein) were analyzed by 4–15% SDS-PAGE followed by immunoblot assays of SCD-1 (microsomal fraction), using goat polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), scavenger receptor B1 (SR-B1, total cell lysate), using rabbit polyclonal antibodies from Santa Cruz, LDL receptor (LDLR, total cell lysate), using rabbit antibodies kindly donated by Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX), and β-tubulin (total cell lysate and microsomal fraction), using mouse monoclonal antibodies from Sigma (St. Louis, MO). Goat anti-rabbit-horseradish peroxidase (HRP) conjugate was from Bio-Rad, bovine anti-goat-HRP conjugate was from Santa Cruz, and ECL system was from Amersham (Piscataway, NJ). Densitometry was performed using ChemiDoc XRS system from Bio-Rad (Hercules, CA) and UN-SCAN-IT Gel Automated Digitizing System, version 5.1 software (Silk Scientific, Orem, UT). The results were expressed as ratios of optical density of the bands representing proteins of interest (SCD-1, LDLR, SR-B1) to optical density of the band representing β-tubulin.

Lipoprotein secretion was determined as previously described (26, 37). Briefly, in a separate experiment, 16 lean male C57BL/6J mice (14 wk old, 24–27 g) were exposed to severe CIH with a FIO₂ nadir of 5% (n = 8) or IA control conditions (n = 8) for 28 days. At the end of the exposure, mice were fasted for 5 h, bled by a retro-orbital sinus puncture (50 μl), and then injected into the tail vein with Triton WR-1339 (Sigma), 12.5 mg in 100 μl of 0.9% NaCl. After the injection, mice were returned to the IH or control chamber. Mice were bled by a retro-orbital sinus puncture (50 μl) again 1 h and 2 h after the Triton injection and then euthanized. Serum triglyceride levels were measured using test kits from Wako Diagnostics.

All values are reported as means ± SE. Comparisons within and between the CIH and IA groups were performed using ANOVA, paired t-test (between day 0 and day 28 within a group) or unpaired t-test (between groups). A repeated-measures ANOVA was performed in the lipoprotein secretion experiment. A P value of <0.05 was considered significant.

## RESULTS

Mice exposed to severe CIH with a FIO₂ nadir of 5% lost 7% of body weight by day 7 and maintained stable weight from day 7 until the end of the exposure (Table 1). Weight loss occurred in settings of mildly decreased food intake and elevated serum leptin levels (Table 1). By day 28, mice exposed to severe CIH were significantly lighter than control animals (P < 0.05), despite food restriction in the control group. In contrast, mice exposed to moderate CIH with a FIO₂ nadir of 10% preserved their weight within the first week of the

| Table 1. Baseline characteristics of lean C57BL/6J exposed to chronic intermittent hypoxia and intermittent air control conditions for 4 wk |
|---|---|---|---|
| | Severe CIH (FIO₂ Nadir 5%) | Moderate CIH (FIO₂ Nadir 10%) |
| n | 8 | 8 | 8 | 8 |
| Body weight, g | | | | |
| Day 0 | 24.2 ± 0.4 | 24.2 ± 0.5 | 23.3 ± 0.5 | 23.4 ± 0.6 |
| Day 7 | 22.8 ± 0.3 | 22.4 ± 0.3 | 23.8 ± 0.3 | 23.9 ± 0.3 |
| Day 28 | 24.7 ± 0.5 | 22.5 ± 0.4** | 26.8 ± 0.6 | 26.9 ± 0.7** |
| Food intake, g/day | | | | |
| Day 0 | 3.6 ± 0.1 | 3.9 ± 0.1 | 3.8 ± 0.1 | 3.9 ± 0.1 |
| Day 7 | 2.7 ± 0.1 | 3.3 ± 0.1** | 3.7 ± 0.2 | 3.7 ± 0.1 |
| Day 28 | 2.5 ± 0.6 | 2.5 ± 0.6 | 1.5 ± 0.2 | 1.9 ± 0.2 |
| Fasting serum insulin, ng/ml | 1.0 ± 0.2 | 2.4 ± 0.2 | 1.0 ± 0.2 | 2.4 ± 0.2 |
| Liver cholesterol content, mg/g of tissue | 0.89 ± 0.05 | 0.79 ± 0.07 | 0.83 ± 0.11 | 0.81 ± 0.07 |
| Liver triglyceride content, mg/g of tissue | 6.1 ± 0.3 | 6.3 ± 0.4 | 8.2 ± 0.4 | 7.1 ± 0.3 |
| Liver phospholipid content, mg/g of tissue | 8.2 ± 0.2 | 7.2 ± 0.8 | 7.6 ± 0.8 | 8.0 ± 0.3 |

Values are means ± SE. FIO₂, fractional inspired O₂. *P < 0.05 for differences between chronic intermittent hypoxia (CIH) and intermittent air control condition (IA) within an exposure; †P < 0.05 for difference between severe and moderate CIH; ‡P < 0.05 for difference between day 0 and day 7 or between day 0 and day 28; §P < 0.05 for difference between day 7 and day 28.

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exposure, subsequently gaining 15% of body weight. Serum leptin level during moderate CIH did not differ from the control group (Table 1). By the end of the experiment, food intake approached normal 3.5–4 g/day, and pair feeding was no longer required at both levels of hypoxia. CIH for 4 wk did not affect fasting serum insulin levels. Severe CIH induced significant increases in serum fasting total cholesterol levels with a nearly 50% rise in LDL cholesterol (LDL-C) and more modest increases in high-density lipoprotein cholesterol (HDL-C) and serum phospholipids (Fig. 1). Compared with weight-matched IA controls, severe CIH increased serum triglyceride levels, despite a decrease in body weight (Table 1). In contrast to severe CIH, moderate CIH did not affect serum lipid levels. Mice exposed to moderate CIH exhibited lower fasting levels of total cholesterol, LDL-C, and HDL-C, and higher levels of serum triglycerides, compared with mice exposed to severe CIH (Fig. 1). The higher levels of serum triglycerides in the IA controls for the moderate CIH exposure could be attributed to greater body weight compared with the IA controls for the severe CIH exposure (Table 1). Neither severe nor moderate CIH altered liver lipid content (Table 1).

Lipid measurements by enzymatic assays were verified in pooled serum by HPLC using LipoSEARCH technology, which revealed that severe CIH increased total cholesterol levels to 114 mg/dl compared with 98.9 mg/dl in IA controls, with corresponding increases in HDL-C levels (94.03 and 77.7 mg/dl, respectively). In contrast to the enzymatic assay, HPLC did not show an increase in LDL-C levels in pooled serum during severe CIH. This phenomenon is likely related to increased lipid peroxidation (see Fig. 2), which leads to LDL oxidation (43), changing the eluting profile and masking detection of LDL (47). Similarly to the enzymatic assays, HPLC did not reveal any changes in serum lipids after exposure to moderate CIH.

Analysis of lipid peroxidation in the liver tissue showed that severe CIH led to a 65% increase in lipid peroxidation levels, while moderate CIH caused only a 25% increase (Fig. 2). Our data suggest that CIH induces lipid peroxidation in a dose-dependent manner.

Real-time PCR with total RNA from the liver revealed that severe CIH caused marked upregulation of SCD-1 and modest, but statistically significant, downregulation of LDLR, whereas mRNA levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) and SR-B1 receptor were unchanged (Fig. 3A). Moderate CIH had no effect on analyzed genes of lipid metabolism (Fig. 3B). Immunoblot in liver tissue showed that severe CIH caused a threefold increase in SCD-1 protein levels and no change in LDLR and that moderate CIH had no effect on any of the studied proteins (Fig. 4).

Fig. 1. Effect of chronic intermittent hypoxia (CIH) for 28 days with a fractional inspired O₂ (FIO₂) nadir of 5% and 10% on fasting serum total cholesterol (TC; A), low-density lipoprotein cholesterol (LDL-C; B), high-density lipoprotein cholesterol (HDL-C; C), phospholipid (PL) (D), and triglyceride (TG) (E) levels in C57BL/6J mice. *P < 0.05 for the difference between CIH and intermittent room air (IA) control.
Lipoprotein secretion was assessed in mice exposed to severe CIH by measuring serum triglyceride following administration of Triton WR-1339 (Fig. 5). Triton WR-1339 blocks very low-density lipoprotein (VLDL) catabolism, and triglyceride accumulation in the serum after administration of Triton allows one to estimate VLDL secretion in vivo (26). CIH led to a 1.5–2 fold increase in lipoprotein secretion compared with IA control.

DISCUSSION

The purpose of the study was to determine the impact of increasing severities of CIH on dyslipidemia and lipid peroxidation in lean mice. The data demonstrate that exposure to severe CIH with a 5% nadir of inspired oxygen levels caused hypercholesterolemia, a marked increase in hepatic SCD-1, an important enzyme of lipid metabolism and lipoprotein secretion, induced lipoprotein secretion in vivo, and caused lipid peroxidation in the liver. In contrast, moderate CIH with a 10% nadir of inspired oxygen levels did not produce hyperlipidemia or alter hepatic SCD-1 but did cause lipid peroxidation in the liver at a reduced level relative to severe CIH. These data confirm and extend our previous findings that acute 5-day exposure to severe IH can produce hyperlipidemia and alter hepatic SCD-1 activity. We now show that CIH leads to prolonged and sustained dyslipidemia and liver lipid peroxidation and that the degree of lipid peroxidation is dependent on the nadir of inspired oxygen level. In the discussion below, we will explore mechanisms affecting lipid metabolism during CIH and discuss the clinical implications of our work.

Severe CIH induced increases in serum cholesterol despite weight loss, which was particularly striking. Weight loss during severe CIH was likely related to increased levels of circulating leptin, an adipocyte-secreted hormone inducing satiety and metabolic rate (2, 10, 12, 28, 52), which was consistent with our previous reports in short-term IH (25, 34). In contrast, moderate CIH did not result in weight loss or an increase in serum leptin (Table 1).

CIH may increase serum lipid levels via three major mechanisms: 1) upregulation of cholesterol biosynthesis de novo, 2) upregulation of lipoprotein secretion, or 3) downregulation of reverse cholesterol transport (8, 22, 26, 32, 38). Given that CIH did not affect liver lipid content (Table 1) and did not change levels of HMG-CoAR, the rate-limiting enzyme of cholesterol biosynthesis (4), upregulation of cholesterol biosynthesis is unlikely. Our data demonstrate that hypercholesterolemia occurs due to induction of lipoprotein secretion by severe CIH (Fig. 5). Lipoproteins are secreted in the bloodstream as VLDL particles composed of cholesterol, cholesteryl esters, and triglyceride bound to apolipoprotein B (ApoB) (26, 42). SCD-1 catalyzes biosynthesis of monounsaturated fatty acids, which augments triglyceride and cholesteryl ester biosynthesis, leading to an increase in lipoprotein secretion (9, 33). We have now shown that severe CIH increases mRNA and protein levels of SCD-1, which is consistent with our previous observations in acute 5-day IH (25). Thus severe CIH may lead to dyslipidemia by upregulating lipoprotein secretion via the SCD-1 mechanism.
Another mechanism of hypercholesterolemia in mice exposed to severe CIH is downregulation of reverse cholesterol transport. Cholesterol is taken up by the liver through two major pathways: 1) direct uptake of HDL cholesterol by SR-B1, and 2) the transfer of cholesterol esters from HDL via cholesteryl ester transfer protein to VLDL and LDL with subsequent uptake by LDLR (3, 22, 53). Unlike acute 5-day IH (25), CIH had no impact on SR-B1 levels in the liver (Figs. 3 and 4). Severe CIH suppressed hepatic LDLR expression, which was not, however, translated in a significant decrease in LDLR protein levels. Thus there is no evidence that hyperlipidemia of severe CIH occurs via reverse cholesterol transport mechanisms.

Unlike severe CIH, moderate CIH had no effect on serum lipid levels. We have previously shown that hypoxia affects lipid metabolic pathways in the liver via hypoxia inducible factor 1 (HIF-1) (23), a transcription factor composed of an O2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (49). Partial deficiency in HIF-1α in Hif1a−/− mice blunted hypoxia-induced increases in triglycerides in the serum and SCD-1 in the liver, suggesting that hypoxia-mediated upregulation of SCD-1 occurs via HIF-1 (23). HIF-1α is regulated posttranslationally by prolyl hydroxylase, which ubiquinates HIF-1α with resolution of hypoxia (18, 19). In our present study, we report that severe CIH increased hepatic SCD-1 levels, whereas moderate CIH had no effect (Figs. 3 and 4). It is conceivable that CIH with a Fo2 nadir of 10% does not prevent ubiquitination of HIF-1α, and induction of SCD-1 does not occur.

Another possible mechanism of differences between severe and moderate CIH is oxidative stress. Previous reports revealed that exposure of rodents to CIH of different severity causes lipid peroxidation in different organs (6, 40, 51). CIH with an Fo2 nadir of 10% increased MDA and isoprostane levels in the brain, as well as activity of NADPH oxidase, an enzyme producing superoxide (40, 51). Severe CIH with a Fo2 nadir of 4–6% increased MDA levels in the myocardium and decreased activity of superoxide dismutase, an important endogenous antioxidant (6). However, levels of lipid peroxidation during exposure to different levels of CIH were never compared. We have now shown that both severe CIH with an inspired oxygen nadir of 5% and moderate CIH with an inspired oxygen nadir of 10% lead to lipid peroxidation in the liver, and the levels of lipid peroxidation are related to the severity of hypoxia. Reactive oxygen species stabilize HIF-1α and HIF-1 regulates lipid metabolism (14, 29). Therefore, differences in oxidative stress between moderate and severe CIH could be an additional mechanism leading to HIF-1 activation and ensuing hyperlipidemia during severe, but not moderate, CIH.

Our present data on lipid peroxidation imply that not only severe but also moderate CIH may cause adverse metabolic outcomes. Indeed, lipid peroxidation in the liver observed in moderate CIH could induce inflammatory response and fibrosis, ultimately leading to nonalcoholic steatohepatitis (NASH) (5). Lipid peroxidation could occur not only in the liver but also in serum and arterial wall inducing oxidation of LDL.

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which would accelerate atherosclerosis (43). In agreement with our hypothesis, increased prevalence of atherosclerosis (11, 30) and NASH (46) as well as high levels of serum lipid peroxidation (21) were observed in patients with mild-moderate OSA, and severity of atherosclerosis and NASH directly correlated with the severity of nighttime hypoxia (11, 30, 46).

Our study had several limitations. First, measurements of sleep were not performed. It is conceivable that CIH with a \( \text{FIO}_2 \) nadir of 5% caused more significant sleep fragmentation than sleep were not performed. It is conceivable that CIH with a \( \text{FIO}_2 \) nadir of 10% and that the severity of metabolic impairment correlated with a degree of sleep fragmentation rather than with hypoxia per se. Second, control mice for severe CIH exposure exhibited higher levels of serum triglyceride and hepatic SCD-1 mRNA than control mice for moderate CIH exposure. This variability was probably related to significant weight gain (15), which was present in the latter, but not the former group, subjected to more rigorous food restriction. It is noteworthy that other SCD-1 inducers, i.e., insulin and cholesterol (33), did not differ between two control groups.

Third, hypoxic animals in the severe CIH exposure had a lower body weight than control animals, which, however, only accentuates the significance of our finding that severe CIH leads to hyperlipidemia despite weight loss.

Conclusion and clinical implications. In the present study we show that CIH leads to hypercholesterolemia and lipid peroxidation, in the absence of obesity, and that the degree of metabolic impairment is dependent on the severity of the hypoxic stimulus. It is difficult to translate the experimental regimen of CIH used in the present study to the clinical arena. However, we do know that naturally sleeping mice can tolerate acutely increasing hypoxia to levels below a 5% nadir of inspired oxygen before spontaneously arouses from sleep (41), indicating that the level of severe CIH used in the current study represents a physiological stimulus. Although we cannot define a comparable stimulus in the clinical setting, patients with severe OSA can exhibit intermittent hypoxemia with profound periods of arterial hemoglobin desaturation (13, 20). Our data would suggest that OSA patients with the most severe respiratory disturbances are at the greatest risk for the development of hyperlipidemia, but even patients with mild-moderate OSA may exhibit increased lipid peroxidation, accelerating the progression of NASH and atherosclerosis.

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