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## Chronic intermittent hypoxia causes hepatitis in a mouse model of diet-induced fatty liver

Vladimir Savransky,<sup>1</sup> Shannon Bevans,<sup>1</sup> Ashika Nanayakkara,<sup>1</sup> Jianguo Li,<sup>1</sup> Philip L. Smith,<sup>1</sup> Michael S. Torbenson,<sup>2</sup> and Vsevolod Y. Polotsky<sup>1</sup>

<sup>1</sup>Division of Pulmonary and Critical Care Medicine, Department of Medicine, and <sup>2</sup>Department of Pathology, Johns Hopkins University, Baltimore, Maryland

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**Savransky V, Bevans S, Nanayakkara A, Li J, Smith PL, Torbenson MS, Polotsky VY.** Chronic intermittent hypoxia causes hepatitis in a mouse model of diet-induced fatty liver. *Am J Physiol Gastrointest Liver Physiol* 293: G871–G877, 2007. First published August 9, 2007; doi:10.1152/ajpgi.00145.2007.—Obstructive sleep apnea (OSA) causes chronic intermittent hypoxia (CIH) during sleep. OSA is associated with nonalcoholic steatohepatitis (NASH) in obese individuals and may contribute to progression of nonalcoholic fatty liver disease from steatosis to NASH. The purpose of this study was to examine whether CIH induces inflammatory changes in the liver in mice with diet-induced hepatic steatosis. C57BL/6J mice ( $n = 8$ ) on a high-fat, high-cholesterol diet were exposed to CIH for 6 mo and were compared with mice on the same diet exposed to intermittent air (control;  $n = 8$ ). CIH caused liver injury with an increase in serum ALT ( $461 \pm 58$  U/l vs.  $103 \pm 16$  U/l in the control group;  $P < 0.01$ ) and AST ( $637 \pm 37$  U/l vs.  $175 \pm 13$  U/l in the control group;  $P < 0.001$ ), whereas alkaline phosphatase and total bilirubin levels were unchanged. Histology revealed hepatic steatosis in both groups, with mild accentuation of fat staining in the zone 3 hepatocytes in mice exposed to CIH. Animals exposed to CIH exhibited lobular inflammation and fibrosis in the liver, which were not evident in control mice. CIH caused significant increases in lipid peroxidation in serum and liver tissue; significant increases in hepatic levels of myeloperoxidase and proinflammatory cytokines IL-1 $\beta$ , IL-6, and CXCL chemokine MIP-2; a trend toward an increase in TNF- $\alpha$ ; and an increase in  $\alpha_1(I)$ -collagen mRNA. We conclude that CIH induces lipid peroxidation and inflammation in the livers of mice on a high-fat, high-cholesterol diet.

nonalcoholic fatty liver disease; cytokine; inflammation; obstructive sleep apnea; lipid peroxidation

OBSTRUCTIVE SLEEP APNEA (OSA) is a recurrent upper-airway obstruction during sleep, leading to periods of chronic intermittent hypoxia (CIH) (16). OSA occurs in 4–24% of adult men and 2–9% of adult women in the U.S., but the prevalence exceeds 30–50% in the obese population (48, 68). OSA is associated with all manifestations of metabolic syndrome, including hypertension, insulin resistance, glucose intolerance, and dyslipidemia, independent of obesity (12, 39, 40, 44, 47, 48, 50). Recent evidence indicates that OSA is also associated with nonalcoholic steatohepatitis (NASH) (60, 61).

Nonalcoholic fatty liver disease (NAFLD) is a common condition with a prevalence among the general population between 14 and 24%, the major risk factors being obesity and insulin resistance (2, 6, 20, 41). NAFLD includes a spectrum of

the disease severity, ranging from steatosis without inflammation to NASH and liver cirrhosis (6, 10, 11). Day et al. (10) proposed a “two-hit” model to explain the progression of NAFLD. The “first hit” involves the accumulation of triglyceride in hepatocytes and has been specifically attributed to insulin resistance and obesity. Obesity and insulin resistance are characterized by increased adipocyte mass and increased hormone-sensitive lipase activity, which leads to upregulation of lipolysis and increased uptake of free fatty acids by the liver (6). In turn, increased free fatty acid uptake induces triglyceride biosynthesis and hepatic steatosis. When hepatic steatosis progresses to NASH, hepatic lobules become infiltrated with a mixed population of inflammatory cells. Inflammation is followed by or accompanied by hepatocyte ballooning and necrosis, appearance of Mallory bodies, and, finally, perisinusoidal fibrosis or cirrhosis (7, 35). The progression of hepatic steatosis to NASH was attributed to a “second hit” that leads to the development of liver inflammation and fibrosis (10). Progression of NAFLD to NASH was linked to oxidative stress and lipid peroxidation in the liver, leading to inflammation (3, 6, 7, 17, 29, 42, 49, 57). However, the causes of oxidative stress and hepatic inflammation in NASH are incompletely characterized.

OSA is associated with systemic oxidative stress and serum lipid peroxidation, which are proportional to the severity of CIH (30, 56). Given that OSA is also associated with NASH and chronic liver injury (51, 60, 61), it is possible that OSA leads to NASH. We have recently used a mouse model of CIH and have shown that CIH leads to liver lipid peroxidation in direct proportion to the severity of the hypoxic stimulus (32). We have also demonstrated that, in mice on a regular diet, CIH causes liver injury, although significant inflammation was not present (53). We hypothesized that an interaction of CIH with a second insult may be necessary for the development of NASH. Indeed, OSA is prevalent in individuals with underlying obesity and insulin resistance (48, 68), suggesting the presence of hepatosteatosis (2, 6, 20, 41). It is conceivable that CIH interacts with coexisting hepatosteatosis, leading to NASH.

The purpose of the present study was to examine the effects of CIH on the liver in the presence of diet-induced hepatosteatosis. We exposed C57BL/6J mice on a high-fat, high-cholesterol diet to CIH or control conditions for 6 mo and examined 1) serum indices of hepatocellular injury, 2) liver histopathology, and 3) lipid peroxidation and levels of proinflammatory cytokines in liver tissue.

Address for reprint requests and other correspondence: V. Y. Polotsky, Division of Pulmonary and Critical Care Medicine, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (e-mail: vpolots1@jhmi.edu).

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## METHODS

**Animals.** A total of 16 wild-type, 6- to 8-wk-old, male, lean C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) were used in the study. The study 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 face mask.

**Experimental design.** 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 (45). A maximum of three mice were housed continuously in a single customized cage (27 × 17 × 17 cm) with constant access to food and water. A series of programmable solenoids and flow regulators altered the inspired oxygen fraction over a defined and repeatable profile that simulated the timing and magnitude of arterial oxygen desaturation changes seen in OSA patients (59). During each period of intermittent hypoxia (IH), inspired oxygen fraction was reduced from 20.9 to  $4.9 \pm 0.1\%$  over a 30-s period and then rapidly reoxygenated to room air levels in the subsequent 30-s period. We have recently reported that such a regimen of IH resulted in repetitive arousals, similar to human OSA (46). We have now performed additional validation of our model measuring an arterial blood gas (ABG) in a separate subset of mice ( $n = 4$ ). A femoral arterial line was placed under 1–2% isoflurane anesthesia, and an ABG was measured in awake, nonanesthetized mice both during IH and normoxia 48 h after the femoral line insertion. Blood draw was performed over a 60-s interval and reflected average values over a hypoxic cycle or a corresponding interval during normoxia.

Eight mice were fed a high-fat, high-cholesterol diet (TD.88051; Harlan Teklad, Madison, WI; 4 kcal/g, 15.8% fat, 1.25% cholesterol) and were placed in the IH chamber for six consecutive months. Eight control mice on a high-fat, high-cholesterol diet were exposed to intermittent room air (IA; control group) for the same period of time in identical chambers. All animals were kept in a controlled environment (22–24°C with a 12:12-h light:dark cycle; lights on at 0900) with free access to food and water. The IH and IA states were induced during the light phase, alternating with 12 h of constant room air during the dark phase.

**Sample collection.** Mice fasted for 5 h before bleeding and death. Arterial blood (1 ml) was obtained by direct cardiac puncture under 1–2% isoflurane anesthesia. Serum was separated and frozen at  $-80^{\circ}\text{C}$ . After blood withdrawal, the animals were euthanized with pentobarbital (60 mg ip). Liver was surgically removed and weighed. Liver tissue was either frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for further studies, fixed in buffered 10% formalin for histological examination, or immediately frozen in Sakura Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA).

**Biochemical assays.** Serum ALT, AST, and alkaline phosphatase activity, serum total bilirubin, and albumin levels were measured by the Clinical Chemistry Laboratory of the Johns Hopkins Bayview Medical Center. Fasting serum cholesterol and triglycerides were measured by using test kits from Wako Diagnostics (Richmond, VA). Serum insulin levels were detected with ELISA kits from Linco Research (St. Charles, MO). Fasting blood glucose was measured with an Accu-Chek Comfort Curve kit from Roche Diagnostics (Indianapolis, IN). Liver was homogenized by using an Omni EZ Connect homogenizer (Omni International, Warrington, VA). Lipids were extracted from the liver with chloroform-methanol, according to the Bligh-Dyer procedure (4), and total cholesterol and triglyceride contents were measured with kits from Wako Diagnostics. Malondialdehyde was measured in serum and total liver lysate with a kit from Oxford Biomedical Research (Oxford, MI). IL-1 $\beta$ , TNF-1 $\alpha$ , and macrophage inflammatory protein-2 (MIP-2) levels in total liver lysate were determined with ELISA kits from R&D Systems (Minneapolis, MN). IL-6 levels in the liver were measured with an ELISA

kit from RayBiotech (Norcross, GA), and myeloperoxidase (MPO) levels in the liver were measured with an ELISA kit from HyCult Biotechnology (Uden, the Netherlands).

**Real time PCR in liver tissue.** Total RNA was extracted from liver by using Trizol (Life Technologies, Rockville, MD), and cDNA was synthesized by using Advantage RT-for-PCR kit from Clontech (Palo Alto, CA). Real-time RT-PCR was performed with  $\alpha_1(\text{I})$ -collagen primers and a probe from Invitrogen Applied Biosystems (Foster City, CA; catalog no. Mm 00801666-g1) and with 18s primers and a probe described previously (31, 33). The  $\alpha_1(\text{I})$ -collagen expression level was normalized to 18s rRNA by using the following formula:  $\alpha_1(\text{I})\text{-collagen}/18\text{s} = 2^{\text{Ct}(18\text{s}) - \text{Ct}[\alpha_1(\text{I})\text{-collagen}]}$ .

**Histology.** Paraffin-embedded tissue was sectioned at 3–5  $\mu\text{m}$  thickness and was stained with hematoxylin and eosin, periodic acid-Schiff, or Masson's trichrome. OCT frozen tissue was sectioned at 7–10  $\mu\text{m}$  thickness and was stained with Oil red O. Histological assessment of tissue morphology was performed by using an Olympus light microscope (Olympus, Tokyo, Japan) and was evaluated in a blinded fashion (by M. S. Torbenson).

**Statistical analyses.** All values are reported as means  $\pm$  SE. Comparisons within and between the groups of mice were performed by using unpaired *t*-test (between IH and IA groups). A *P* value of  $<0.05$  was considered significant.

## RESULTS

**Baseline characteristics and serum biochemistry in C57BL/6J mice on a high-fat diet exposed to CIH for 6 mo.** ABG during IH showed pH of  $7.43 \pm 0.01$ ,  $\text{Paco}_2$  of  $29.1 \pm 2.6$  mmHg, and  $\text{Pao}_2$  of  $51.7 \pm 4.2$  mmHg, which corresponds to oxyhemoglobin saturation ( $\text{Sao}_2$ )  $\sim 80\%$ , commonly seen in patients with severe OSA (1, 25). ABG during normoxia showed pH of  $7.43 \pm 0.01$ ,  $\text{Paco}_2$  of  $27.1 \pm 2.0$  mmHg, and  $\text{Pao}_2$  of  $99.7 \pm 2.1$  mmHg, indicating that IH-induced hypoxemia resolves completely on cessation of the exposure and that IH does not significantly affect  $\text{Paco}_2$  level, which is lower than in human subjects at baseline (59). CIH for 6 mo completely abolished weight gain, which was observed in control animals (Table 1). It resulted in nearly a 20% difference in body weight between the CIH and control groups by the end of the exposure. Hypoxic mice exhibited a small decrease in the liver weight, which was related to differences in body weight between the groups. In contrast, a decrease in epididymal fat accumulation persisted after adjustment for body weight (Table 1). Prolonged exposure to CIH resulted in a decrease in fasting blood glucose without change in serum insulin, which is probably related to higher insulin sensitivity in mice with lower body weight and lower levels of adiposity. CIH did not affect fasting serum cholesterol and triglyceride levels (Table 1).

Our most striking biochemical finding was that CIH caused a greater than threefold increase in serum AST levels and a greater than fourfold increase in serum ALT levels (Fig. 1), suggesting that CIH led to hepatocellular injury. There were no significant changes in serum alkaline phosphatase levels (Fig. 1) or serum total bilirubin levels, and serum albumin was slightly increased (Table 1), indicating that CIH did not induce overt cholestasis and did not affect hepatic synthetic function.

**Liver histology.** Histopathology of the liver showed mild hepatic macrovesicular steatosis in both CIH and control groups. There was no difference in the amount of either macrovesicular or microvesicular steatosis on hematoxylin and eosin stains (Fig. 2, A and B), but Oil red O staining showed mild accentuation of fat staining, especially in the zone 3

Table 1. Baseline characteristics of C57BL/6J mice on a high-fat, high-cholesterol diet exposed to intermittent hypoxia for 6 mo

	Control	Chronic Intermittent Hypoxia	P
n	8	8	
Starting age, wk	7	7	
Body weight, g			
Day 0	24.0±0.4	24.0±0.4	>0.05
Day 180	30.2±0.4*	24.6±0.5	<0.001
Liver weight, g	2.31±0.06	2.01±0.08	<0.05
Liver/body weight, %	7.7±0.2	8.3±0.5	>0.05
Epididymal fat pads, g	0.65±0.07	0.32±0.03	<0.01
Epididymal fat pads/body weight, %	2.1±0.2	1.3±0.1	<0.05
Fasting blood glucose, mg/dl	155±4	113±3	<0.001
Fasting serum insulin, ng/ml	0.16±0.04	0.11±0.02	>0.05
Fasting serum total cholesterol, mg/dl	219±12	252±22	>0.05
Fasting serum total triglycerides, mg/dl	39±3	29±6	>0.05
Serum total bilirubin, mg/dl	0.09±0.04	0.25±0.15	>0.05
Serum albumin, g/dl	4.3±0.03	5.1±0.05	<0.001
Liver triglyceride content, mg/g	13.1±0.3	15.8±1.3	=0.09
Liver cholesterol content, mg/g	7.8±0.8	10.2±0.7	<0.05

Values are means ± SE; n, number of animals. \**P* < 0.001 vs. day 0 within a group.

hepatocytes, in mice subjected to CIH (Fig. 2, C and D). Biochemical measurements confirmed high triglyceride content in the liver tissue (Table 1), which exceeded previously reported levels in mice on a regular diet (32, 53). In mice exposed to CIH, liver cholesterol content significantly increased, and there was a trend toward an increase in liver triglyceride content.

Animals exposed to CIH exhibited lobular inflammation without strong zonal patterns, which was not evident in control mice (Fig. 2, A and B). There was little or no portal inflammation in either group of mice. PAS stains typically showed more glycogen accumulation in the hypoxic group compared with control animals (Fig. 2, E and F).

The sections showed no evidence for congestive hepatopathy of the sort seen with heart failure and also showed no coagulative necrosis in zone 3, which is typically seen in severe hypoxic injury to the liver. Masson trichrome stain

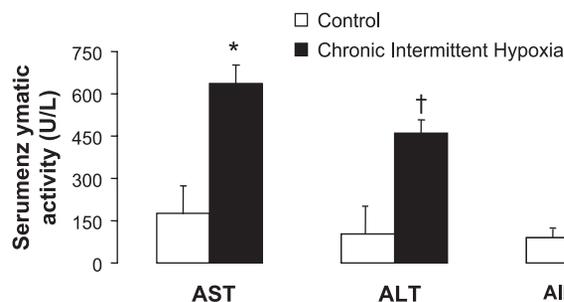


Fig. 1. Effect of chronic intermittent hypoxia (CIH) on serum enzymatic activity of ALT, AST, and alkaline phosphatase. \**P* < 0.05 for difference between CIH and control.

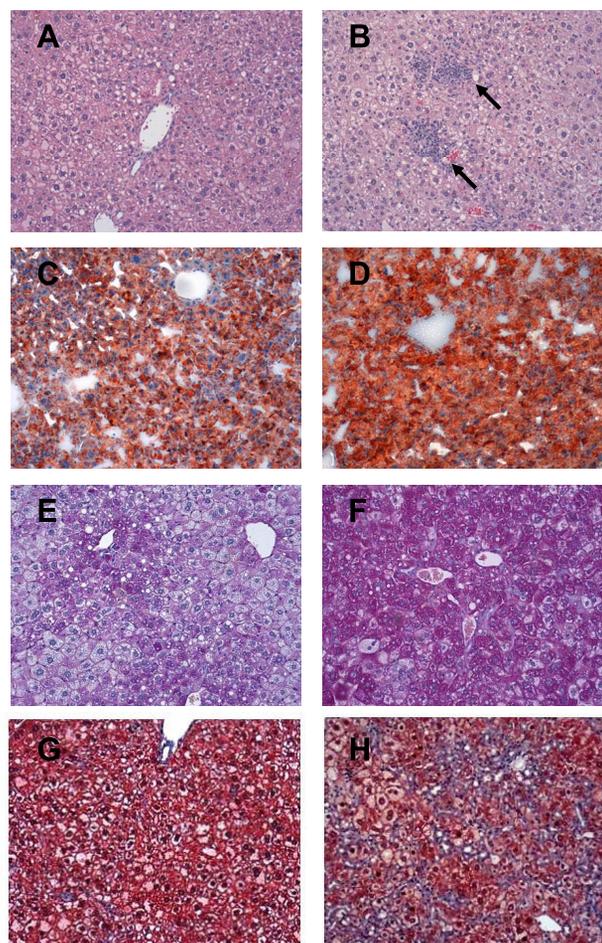


Fig. 2. Livers of control mice showed no significant lobular inflammation (A, hematoxylin and eosin stain, ×200 original magnification), whereas hypoxic mice showed increased lobular inflammation (B, hematoxylin and eosin stain, ×200 original magnification). Arrows point toward inflammatory infiltrates. Oil red O staining showed macrovesicular steatosis in both sets of animal livers (C and D, ×200 original magnification). Normoxic mice exhibited a diffuse lobular distribution of fat, with a decrease of fat deposition in zone 3 (C), whereas hypoxic animals showed a mild increase in fat deposition, predominantly in zone 3 near central veins (D). Livers of normoxic mice [E, periodic acid Schiff stain (PAS), ×400 original magnification] showed less PAS-positive glycogen accumulation compared with hypoxic mice (F, PAS stain, ×400 original magnification). Livers of control mice showed no evidence of fibrosis (G, Masson's trichrome, ×400 original magnification), whereas the hypoxic mice showed extensive collagen depositions, stained blue (H, Masson's trichrome, ×400 original magnification).

revealed abundant collagen depositions in the liver of mice exposed to CIH but not in control animals (compare Fig. 2, G and H for blue staining of collagen).

**Markers of oxidative stress and inflammation in liver tissue.** CIH resulted in a 20% increase in lipid peroxidation levels in the liver, whereas serum lipid peroxidation was increased greater than fourfold (Fig. 3 A and B). Also evidence of upregulation of oxidative stress in liver tissue was the 23% elevation of MPO levels in the liver (Fig. 3C). Along with cellular infiltration in hepatic lobules, an increase in MPO indicates that CIH leads not only to liver injury but also to inflammation. Further confirmation of proinflammatory effects of CIH was obtained by measuring levels of proinflammatory cytokines in liver tissue. CIH induced a 37% increase in

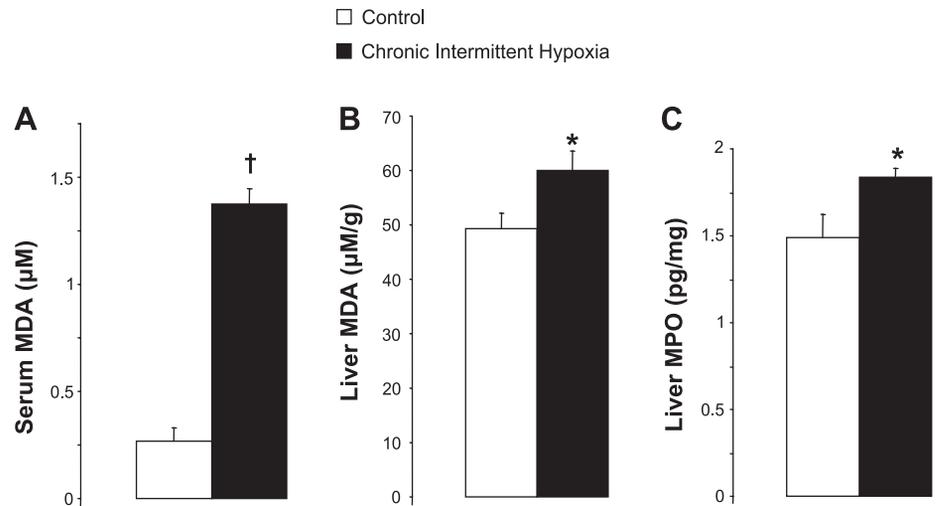


Fig. 3. Selected markers of lipid peroxidation (LPO) and oxidative stress in mice exposed to CIH. LPO in serum (A) and liver tissue (B) was determined by malondialdehyde (MDA) levels. Myeloperoxidase (MPO) levels in liver were determined by ELISA (C). \* $P < 0.05$  and † $P < 0.001$  for difference between CIH and control.

hepatic levels of IL-1 $\beta$ , >70% increases in hepatic levels of IL-6 and MIP-2 (Fig. 4, A–C), and a trend toward an increase in TNF- $\alpha$  levels ( $P = 0.08$ ; Fig. 4D). CIH also caused a >2.7-fold upregulation of  $\alpha_1$ (I)-collagen gene expression (Fig. 5), which was consistent with hepatic fibrosis shown by histopathology (Fig. 2, G and H).

#### DISCUSSION

There is a growing body of evidence in the clinical literature that OSA is associated with NASH. However, a direct causal link between OSA and NASH has not been established. We

have previously shown that CIH, one of the key physiological mechanisms of OSA, leads to chronic liver injury and liver lipid peroxidation in C57BL/6J mice without underlying liver abnormalities (53). The findings in this study extend these observations by demonstrating that simultaneous exposure of C57BL/6J mice to CIH and a high-fat, high-cholesterol diet for 6 mo led to a marked elevation in serum ALT and AST activities, inflammatory infiltration and fibrosis of the liver, and significant increases in levels of proinflammatory cytokines IL-1 $\beta$ , IL-6, and MIP-2 in liver tissue, whereas control animals on the same diet exhibited liver steatosis without any evidence of inflammation. Several additional observations can also be made. First, CIH significantly upregulated serum and liver lipid peroxidation, which was consistent with our previous observations. Second, CIH induced a significant increase in liver MPO levels, which could contribute to oxidative stress. Third, CIH increased glycogen deposition in the liver, which was also consistent with our prior findings in mice on a regular diet. In the discussion below, we will explore the relationships and putative pathways linking CIH and NASH and discuss clinical implications of our work.

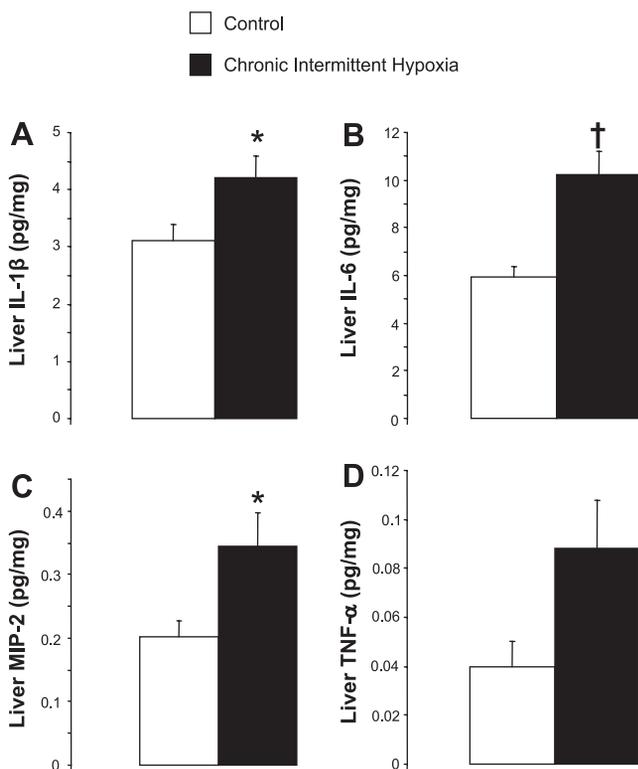


Fig. 4. Levels of proinflammatory cytokines IL-1 $\beta$  (A), TNF-1 $\alpha$  (B), IL-6 (C), and chemokine macrophage-inflammatory protein-2 (MIP-2; D) in liver tissue of mice exposed to CIH and control conditions. \* $P < 0.05$  and † $P = 0.001$  for difference between CIH and control.

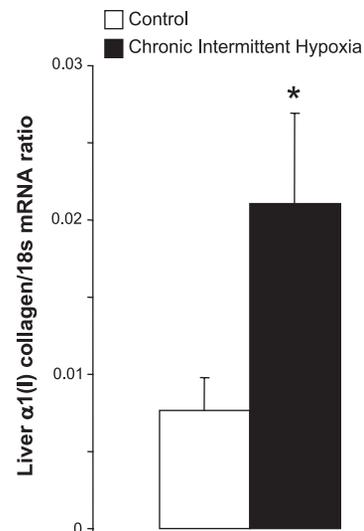


Fig. 5. Levels of  $\alpha_1$ (I)-collagen mRNA in liver tissue of mice exposed to CIH and control conditions. \* $P < 0.05$  for difference between CIH and control.

*CIH and inflammatory response in the liver.* OSA and NASH are common diseases, which frequently occur in obese individuals (2, 6, 20, 41, 48, 68). NASH is a major public health problem as one of the most common causes of liver cirrhosis (38, 43). Multiple conditions may lead to nonalcoholic hepatic steatosis, including obesity and insulin resistance (2, 6, 20, 41). Nevertheless, factors mediating the progression of hepatic steatosis to NASH remain incompletely characterized. Clinical literature suggests that there is an association between OSA and NASH and that the severity of NASH is directly proportional to the hypoxic stress of OSA (43, 60, 61). We have now shown that CIH induces inflammation in the liver of mice with diet-induced NAFLD. In combination with our previous report that CIH causes injury in the previously intact livers (53) but does not induce NASH, our new data imply that CIH may act as a "second hit," and coexisting hepatic steatosis is necessary for NASH to develop.

Effects of CIH on the liver appear to be distinct from sustained hypoxia and bear similarities to ischemia-reperfusion injury (9, 15, 18). Indeed, the liver is well adapted to hypoxia, because it normally functions at low oxygen tension (8, 54), and sustained hypoxia leads to liver injury only when the stimulus is very severe, for example in shock liver (19, 66). Prolonged hypoxemia may lead to right-side heart failure, which results in ischemia and congestive hepatopathy (14, 21, 22). However, sustained hypoxia does not lead to inflammatory changes in the liver. In contrast, ischemia-reperfusion injury, as in liver surgery, may cause hepatic inflammation (23). Studies *in vivo* demonstrated that ischemia-reperfusion injury of the liver leads to secretion of such proinflammatory cytokines as TNF- $\alpha$  and IL-1 $\beta$  by Kupffer cells (37). Studies in primary hepatocyte cell culture revealed that TNF- $\alpha$  induces mRNA expression and protein secretion of CXC chemokines, monocyte chemoattractant protein-1, and MIP-2 by hepatocytes (28). CXC chemokines have neutrophil chemotactic properties and increase neutrophilic infiltration in the liver (36, 62). Our current data show that, similarly to ischemia-reperfusion injury (23, 37), CIH increases levels of TNF- $\alpha$ , IL-1 $\beta$ , and MIP-2 and cellular infiltration in the liver. Moreover, an increase in liver MPO level suggests activation of neutrophils in liver tissue (55). Elevation of IL-6 by CIH may also have a deleterious effect on the liver, because long-term exposure to IL-6 activates apoptosis and inflammation in liver parenchyma (24). Thus hepatitis after CIH is likely to be induced by repeated cycles of hypoxia and reoxygenation, leading to activation of Kupffer cells and hepatocytes, which produce proinflammatory cytokines and CXC chemokines.

*CIH, oxidative stress, and NASH.* The molecular mechanisms of upregulation of proinflammatory cytokines in the liver by CIH are not fully understood, but oxidative stress may play a major role. We have found that CIH leads to a marked increase in serum and liver lipid peroxidation, which indicates that CIH increases production of reactive oxygen species (ROS) in the liver. Lipid accumulation in hepatic steatosis increases generation of ROS and lipid peroxidation in the liver (6, 57, 67), suggesting that preexisting or coexisting steatosis may be necessary for any inflammatory process to develop. Indeed, our previous data indicate that, in C57BL/6J mice on a regular diet and without underlying fatty liver, CIH leads to mild liver injury and does not cause significant inflammation

(53), in contrast to our current data in mice on a high-fat, high-cholesterol diet with underlying fatty liver.

CIH may affect ROS generation via multiple mechanisms. Cycles of hypoxia-reoxygenation induce mitochondrial oxidative pathways, which form ROS through flavoprotein-mediated donation of electrons to molecular oxygen (6). CIH may also activate NADPH oxidase in Kupffer cells and xanthine oxidase in hepatocytes (13, 26). Our data indicate that CIH increases levels of MPO in the liver, suggesting that neutrophils could be an important source of oxidative stress in CIH-induced NASH. Enhanced generation of ROS and lipid peroxidation during CIH lead to depletion of ATP, DNA damage, and destruction of biological membranes, which may directly increase production of proinflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$ , and CXC chemokines and promote influx of inflammatory cells in the liver, leading to the progression of NASH (3, 6, 27, 49).

*CIH and regulation of glucose and lipid metabolism in the liver.* CIH induced hepatic inflammation in wild-type mice with diet-induced liver steatosis but did not markedly affect triglyceride accumulation in the liver or insulin resistance. In contrast, in leptin-deficient and insulin resistant *ob/ob* mice, CIH significantly exacerbated both insulin resistance and hepatic steatosis, as we reported earlier (31). The difference in the effects CIH on diet-induced and genetic fatty liver is likely related to the differences in leptin and is consistent with a previously described protective role of leptin in hepatic steatosis (34, 58).

Another finding of our study was that CIH increased liver cholesterol content, which could be a result of several different processes. Cholestasis would not be a likely mechanism of an increase in liver cholesterol, because CIH did not induce any changes in serum bilirubin and alkaline phosphatase levels or bile duct dilatation on liver histology. CIH could upregulate lipid biosynthesis in the liver. Indeed, short-term IH upregulates lipid biosynthesis in the liver via sterol regulatory-element binding protein (33). CIH may also increase liver cholesterol content by downregulating lipoprotein secretion or increasing reverse cholesterol transport from peripheral organs to the liver (5, 64). However, mechanism and biological significance of an increase in liver cholesterol content after CIH are not clear.

Finally, we have also found that, in mice on a high-fat, high-cholesterol diet, CIH caused an increase in glycogen content in the liver, which was similar to our previous report in mice on a regular diet (53). Glycogen accumulation in the liver during CIH resembles glycogenic hepatopathy in patients with diabetes mellitus type 1 and may be caused by suppression of glycogen phosphorylase and/or induction of glycogen synthase (63). CIH-induced glycogenic hepatopathy may contribute to liver injury, but the mechanism and implications of this condition remain unknown.

*Limitations of the study.* Our study had several limitations. First, both NASH and OSA are associated with obesity and insulin resistance (2, 6, 20, 41), whereas experimental CIH leads to weight loss (32). We have previously reported that animals on a high-fat diet appeared to be more sensitive to anorectic effects of CIH than mice on a regular diet (52), possibly due to synergistic upregulation of leptin by both stimuli (45, 65). As a result, CIH for 6 mo led to significant decreases in body weight and amount of visceral (epididymal) fat, with ensuing improvement in insulin sensitivity, compared

with the control animals on an identical high-fat diet (Table 1). In contrast, CIH increased lipid accumulation, inflammation, and fibrosis in the liver, which suggests that our murine model could be used to explore effects of CIH on fatty liver, despite concurrent weight loss.

Another potential concern is whether the severity of CIH is relevant for human OSA. The ABG measurements during CIH demonstrated that the severity of hypoxia in mice is comparable with severe OSA (1, 25). Human OSA also leads to nocturnal hypoventilation and hypercapnia, whereas in murine CIH hypercapnia does not occur. Finally, CIH leads to severe sleep disruption (46), and sequelae of CIH could be attributable to either hypoxia per se or sleep fragmentation. However, a combination of CIH and sleep fragmentation is a prominent feature of OSA and, therefore, could be interpreted as an advantage of our model. In summary, although murine models of CIH and fatty liver disease do not entirely replicate OSA and NAFLD in human subjects, pertinent conclusions could be derived from our study.

**Conclusions and clinical implications.** Our data indicate that CIH causes significant inflammation and liver injury in mice with diet-induced fatty liver, probably via oxidative stress mechanisms. Our data suggest that CIH of OSA may directly contribute to the progression of NAFLD from liver steatosis to NASH and that NASH could be another metabolic complication of OSA.

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