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

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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 ± 58 U/l vs. 103 ± 16 U/l in the control group; P < 0.001) and AST (637 ± 37 U/l vs. 175 ± 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-1β, IL-6, and CXC chemokine MIP-2; a trend toward an increase in TNF-α; and an increase in α1(I)-collagen mRNA. We conclude that CIH induces lipid peroxidation and inflammation in the livers of mice on a high-fat, high-cholesterol diet.

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**METHODS**

**Animals.** A total of 16 wild-type, 6- to 8-wk-old, male, 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 ± 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 isoflurane anesthesia.

**Liver.** Liver was surgically removed and weighed.

**Blood.** Arterial blood (1 ml) was obtained by direct cardiac puncture under isoflurane anesthesia. Blood draw was performed over a 60-s interval and reflected average values over a hypoxic cycle or a corresponding interval during normoxia.

**Histology.** Paraffin-embedded tissue was sectioned at 3–5 μm thickness and was stained with hematoxylin and eosin, periodic acid-Schiff, or Mason’s trichrome. OCT frozen tissue was sectioned at 7–10 μm 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).

**Biochemical analyses.** Total RNA was extracted from liver with 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 α1(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 α1(I)-collagen expression level was normalized to 18s rRNA by using the following formula: α1(I)-collagen/18s = 2(Ct(18s) – Ct(18s)-collagen).

**Histology.** Paraffin-embedded tissue was sectioned at 3–5 μm thickness and was stained with hematoxylin and eosin, periodic acid-Schiff, or Mason’s trichrome. OCT frozen tissue was sectioned at 7–10 μm 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 ± 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 ± 0.01, Paco2 of 29.1 ± 2.6 mmHg, and Paco2 of 51.7 ± 4.2 mmHg, which corresponds to oxyhemoglobin saturation (SaO2) ~ 80%, commonly seen in patients with severe OSA (1, 25). ABG during normoxia showed pH of 7.43 ± 0.01, Paco2 of 27.1 ± 2.0 mmHg, and Paco2 of 99.7 ± 2.1 mmHg, indicating that IH-induced hypoxemia resolves completely on cessation of the exposure and that IH does not significantly affect Paco2 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
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 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).

**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).

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

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

*Values are means ± SE; n, number of animals. *P < 0.001 vs. day 0 within a group.
hepatic levels of IL-1β, >70% increases in hepatic levels of IL-6 and MIP-2 (Fig. 4, A–C), and a trend toward an increase in TNF-α 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-1β, 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.
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-α and IL-1β by Kupffer cells (37). Studies in primary hepatocyte cell culture revealed that TNF-α 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 neutrophil infiltration in the liver (36, 62). Our current data show that, similarly to ischemia-reperfusion injury (23, 37), CIH increases levels of TNF-α, IL-1β, 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-α, IL-1β, 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 hypventilation 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.

GRANTS

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