Chronic Intermittent Hypoxia Induces Atherosclerosis

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Rationale: **Obstructive sleep apnea, a condition leading to chronic intermittent hypoxia (CIH), is associated with hyperlipidemia, atherosclerosis, and a high cardiovascular risk. A causal link between obstructive sleep apnea and atherosclerosis has not been established.**
Objectives: **The objective of the present study was to examine whether CIH may induce atherosclerosis in C57BL/6J mice.**
Methods: **Forty male C57BL/6J mice, 8 weeks of age, were fed either a high-cholesterol diet or a regular chow diet and subjected either to CIH or intermittent air (control conditions) for 12 weeks.**
Measurements and Main Results: **Nine of 10 mice simultaneously exposed to CIH and high-cholesterol diet developed atherosclerotic lesions in the aortic origin and descending aorta. In contrast, atherosclerosis was not observed in mice exposed to intermittent air and a high-cholesterol diet or in mice exposed to CIH and a regular diet. A high-cholesterol diet resulted in significant increases in serum total and low-density lipoprotein cholesterol levels and a decrease in high-density lipoprotein cholesterol. Compared with mice exposed to intermittent air and a high-cholesterol diet, combined exposure to CIH and a high-cholesterol diet resulted in marked progression of dyslipidemia with further increases in serum total cholesterol and low-density lipoprotein cholesterol (124 ± 4 vs. 106 ± 6 mg/dl; $p < 0.05$), a twofold increase in serum lipid peroxidation, and up-regulation of an important hepatic enzyme of lipoprotein secretion, stearoyl-coenzyme A desaturase-1.**
Conclusions: **CIH causes atherosclerosis in the presence of diet-induced dyslipidemia.**

Keywords: obstructive sleep apnea; lipids; hypoxia; mouse; stearoyl-coenzyme A desaturase

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Obstructive sleep apnea is associated with atherosclerosis, and the severity of atherosclerosis correlates with the severity of hypoxia. The causal relationships between sleep apnea and atherosclerosis are unknown.

What This Study Adds to the Field

Chronic intermittent hypoxia, similar to that observed in patients with sleep apnea, causes atherosclerotic lesions in the aorta of male C57BL/6J mice, which are resistant to the disease in the absence of hypoxia.

Obstructive sleep apnea (OSA) is characterized by recurrent collapse of the upper airway during sleep, leading to chronic intermittent hypoxia (CIH) (1). OSA has been associated with an increased risk of hypertension, type II diabetes, angina, myocardial infarction, congestive heart failure, stroke, and fatal cardiovascular events, independent of underlying obesity (2–5).

Poor cardiovascular outcomes may be related to the high prevalence of atherosclerosis in patients with OSA. Studies have shown independent associations between hypoxic stress of OSA and increased carotid artery intima–media thickness (6) as well as progressive narrowing of the coronary artery lumens (7). Advanced atherosclerotic disease in patients with OSA is probably related to specific metabolic disturbances exacerbated by sleep apnea. OSA is associated with hypercholesterolemia independent of adiposity (8), and continuous positive airway pressure, which is the therapy of choice for OSA, leads to a decrease in total cholesterol and low-density lipoprotein cholesterol (LDL-C) (9, 10) without any change in body weight. OSA is also linked to increased lipid peroxidation in the serum (11) and elevated levels of oxidized LDL (12). Finally, patients with OSA develop systemic inflammation with increased levels of circulating tumor necrosis factor (TNF)-α and IL-6, along with other cytokines and adhesion molecules with known proatherogenic properties (9, 10, 13, 14). Thus, OSA is associated with atherosclerosis and conditions predisposing to atherosclerosis, including dyslipidemia, lipid peroxidation, and systemic inflammation.

Causal relationships between CIH of OSA and lipid metabolism have been studied in rodent models of intermittent hypoxia (IH). We have previously demonstrated in mice that IH induces hypercholesterolemia by increasing lipoprotein secretion via upregulation of a key hepatic enzyme, stearoyl-coenzyme A desaturase-1 (SCD-1) (15, 16). Other investigators reported that IH increases lipid peroxidation in myocardial tissue of rats (17) and activates inflammatory pathways *in vitro* (18). One study demonstrated that IH increased severity of underlying atherosclerosis (19), but the study was performed in rabbits, which are susceptible to atherosclerosis, and used sustained hypoxia with occasional interruptions, rather than CIH similar to OSA. Thus, CIH may predispose to atherosclerosis via multiple mechanisms. However, despite abundant evidence on causal links between OSA, CIH, and atherosclerosis, it remains unknown whether CIH can directly lead to atherosclerosis in a resistant host.

We hypothesized that CIH may cause atherosclerosis in wild-type mice, but only in the presence of other risk factors, such as an atherogenic diet. We subjected male C57BL/6J mice, which are usually resistant to atherosclerosis, to CIH in combination with either a regular or high-cholesterol diet and examined (*1*)

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CIH-induced pathology in the aortic origin and descending aorta, (*2*) CIH-induced changes in serum lipid profile, (*3*) CIH-induced changes in hepatic SCD-1 mRNA and protein levels, and (*4*) CIH-induced changes in serum lipid peroxidation, serum IL-6, and serum and hepatic TNF-α levels.

METHODS

Forty wild-type, 8-week-old male, lean C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) were used in this study. The study was approved by the Johns Hopkins University (Baltimore, MD) Animal Care and Use Committee and complied with the American Physiological Society (Bethesda, MD) Guidelines for Animal Studies. For blood sample collection, surgical procedures, and tissue collection anesthesia was induced and maintained with 1–2% isoflurane administered through a facemask. Twenty mice were fed a regular Purina chow diet (3.3 Cal/g, 4% fat). Twenty mice were fed a high-cholesterol diet (TD88051 [Harlan Teklad, Madison, WI]: 4 kcal/g, 15.8% fat, and 1.25% cholesterol).

IH with an $F_{I_{O_2}}$ of 5% was delivered 60 times per hour. Ten mice on a regular diet and 10 mice on a high-cholesterol diet were placed in the IH chamber for 12 consecutive weeks. Ten mice on a regular diet and 10 mice on a high-cholesterol diet were placed in an identical chamber, but received intermittent air (IA) at the identical flow rate. The IH and IA states were induced during the 12-hour light phase alternating with 12 hours of constant room air during the dark phase. *See* the online supplement for a detailed description of the experimental design.

Mice were fasted for 5 hours before bleeding and sacrifice. Arterial blood (about 1 ml) was obtained by direct cardiac puncture under 1–2% isoflurane anesthesia. The heart and aorta were dissected. The atria with ascending aorta were separated and frozen in Sakura Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA). The descending aorta was fixed in 10% paraformaldehyde. Livers were surgically removed and immediately frozen at –80°C for future analysis.

Atherosclerotic lesions were examined in cross-sections of the aortic origin, using oil red O stain according to Zhang and coworkers (20) and in *en face* preparations of the descending aorta according to Tangirala and coworkers (21), in a blinded fashion. *See* the online supplement for a detailed description.

Serum total cholesterol, LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), phospholipids, and triglycerides were measured with kits from Wako Diagnostics, Inc. (Richmond, VA). Lipids were extracted from the liver with chloroform–methanol, according to the Bligh-Dyer procedure, and measured with kits from Wako Diagnostics. Serum IL-6 and TNF-α were measured with ELISA kits purchased from R&D Systems, Inc. (Minneapolis, MN). Serum lipid peroxidation was assessed by malondialdehyde (MDA) levels with a Bioxytech MDA-586 assay kit from OXIS Health Products, Inc. (Portland, OR). Fast protein liquid chromatography (FPLC) was performed as previously described (15). *See* the online supplement for descriptions of real-time polymerase chain reaction, ELISA, and immunoblot of liver tissue.

All values are reported as means ± SEM. Statistical significance of the effects of hypoxia and diet was determined by two-way analysis of variance (ANOVA). Comparisons between Day 0 and Day 84 were performed by repeated-measures ANOVA followed by the Tukey *post hoc* test. A p value of less than 0.05 was considered significant.

RESULTS

Serum Lipid Levels, Markers of Oxidative Stress and Inflammation

C57BL/6J mice gained a significant amount of weight throughout the exposure (Table 1, repeated-measures ANOVA; $p < 0.001$). *Post hoc* analysis demonstrated that weight gain was present in all groups, except for animals exposed to CIH and a high-cholesterol diet simultaneously. CIH and a high-cholesterol diet had an independent lowering effect on body weight throughout the exposure (Table 1). Food intake in grams was decreased by a high-cholesterol diet, but not by CIH, whereas caloric food intake was not affected by either hypoxia or diet. We speculate that weight loss in the presence of preserved caloric intake occurred because of upregulation of leptin by hypoxia and dietary fat with a subsequent increase in metabolic rate (22–24). CIH led to hyperglycemia in a setting of decreased serum insulin levels, suggesting insulin deficiency, which was particularly notable in mice on a regular diet (Table 1). Relatively low blood

TABLE 1. EFFECTS OF CHRONIC INTERMITTENT HYPOXIA AND HIGH-CHOLESTEROL DIET ON METABOLIC CHARACTERISTICS AND SERUM CYTOKINES IN C57BL/6J MICE

	Regular Chow Diet		High-Cholesterol Diet		Effect of CIH (p Value)	Effect of Diet (p Value)
	IA	CIH	IA	CIH		
n	10	10	10	10	N/A	N/A
Age, wk						
Day 0	8	8	8	8	N/A	N/A
Day 84	20	20	20	20	N/A	N/A
Body weight, g						
Day 0	23.9 ± 0.5	24.5 ± 0.3	23.6 ± 0.4	23.2 ± 0.3	> 0.05	> 0.05
Day 84	26.6 ± 0.6*	25.7 ± 0.3†	25.9 ± 0.4*	23.5 ± 0.4	< 0.05	< 0.01
Daily food intake						
g	3.0 ± 0.2	2.7 ± 0.2	2.4 ± 0.3	2.1 ± 0.2	> 0.05	0.001
kcal	9.9 ± 0.7	8.9 ± 0.7	9.6 ± 1.2	8.4 ± 0.8	> 0.05	> 0.05
Fasting blood glucose, mg/dl	159 ± 8	210 ± 11	141 ± 17	155 ± 7	< 0.01	< 0.01
Fasting serum insulin, ng/ml	0.71 ± 0.12	0.32 ± 0.02	0.36 ± 0.05	0.27 ± 0.01	< 0.01	< 0.01
Serum IL-6, pg/ml	9.6 ± 3.0	8.5 ± 2.5	11.9 ± 1.8	9.2 ± 1.3	> 0.05	> 0.05
Serum TNF-α, pg/ml	3.4 ± 1.7	2.1 ± 1.2	5.3 ± 2.9	4.2 ± 2.6	> 0.05	> 0.05
Liver weight, g	1.2 ± 0.1	1.1 ± 0.03	1.9 ± 0.07	1.9 ± 0.06	> 0.05	< 0.001
Liver weight/body weight, %	4.3 ± 0.3	4.2 ± 0.1	7.7 ± 0.4	8.0 ± 0.3	> 0.05	< 0.001
Liver cholesterol, mg/g	1.0 ± 0.1	1.2 ± 0.1	5.1 ± 0.5	5.7 ± 0.4	> 0.05	< 0.001
Liver triglyceride, mg/g	10.6 ± 0.6	9.5 ± 0.7	15.5 ± 1.6	12.6 ± 2.0	> 0.05	< 0.01
Liver phospholipids, mg/g	8.9 ± 0.3	8.4 ± 0.3	10.8 ± 0.6	9.4 ± 0.3	> 0.05	< 0.01
Liver free fatty acids, μmol/g	1.9 ± 0.3	1.7 ± 0.1	5.3 ± 0.5	3.4 ± 0.6	> 0.05	< 0.001

Definition of abbreviations: CIH = chronic intermittent hypoxia; IA = intermittent air; N/A = not applicable; TNF-α = tumor necrosis factor-α.

* $p < 0.001$ between Day 0 and Day 84.

† $p < 0.05$ between Day 0 and Day 84.

glucose and serum insulin levels in mice on a high-cholesterol diet could be attributed to low body weight of these animals.

As expected, feeding with a high-cholesterol diet resulted in a 2.5- to 3-fold increase in fasting serum total cholesterol levels, altering the lipoprotein profile from HDL to LDL predominance (Figure 1). The high-cholesterol diet also led to decreases in fasting serum phospholipid and triglyceride levels. Exposure to CIH induced significant increases in fasting serum levels of total cholesterol, LDL-C, HDL-C, and phospholipids in both dietary groups, whereas serum triglycerides were not affected (Figure 1). Significant increases in cholesterol levels across all classes of lipoproteins by CIH were also shown by FPLC in mice on a high-cholesterol diet (Figure 2). The most dramatic increase was evident in the very low-density lipoprotein (VLDL) fractions, indicating that an enzymatic assay might have measured both LDL and VLDL cholesterol. In addition, lipid peroxidation induced by CIH could mask LDL-C detection by FPLC through alterations in the elution profile of oxidized LDL (25).

Neither CIH nor dietary manipulations affected serum levels of IL-6 and TNF-α (Table 1). In contrast, both CIH and a high-cholesterol diet significantly increased TNF-α gene expression in the liver, suggesting proinflammatory effects of both stimuli (Figure 3A). CIH nearly doubled TNF-α protein levels in the liver of mice on a high-cholesterol diet, whereas mice on a regular diet with low baseline TNF-α were resistant to the hypoxic stimulus, revealing a proinflammatory interaction between CIH and dietary lipids ($p = 0.01$; Figure 3B). An MDA assay showed a twofold increase in serum lipid peroxidation, which was notable in both dietary groups (Figure 4). Diet did not impact serum lipid peroxidation. The high-cholesterol diet resulted in hepatomegaly with increases in liver cholesterol, triglyceride, free fatty acid, and phospholipid content (Table 1). CIH had no effect on liver weight or lipid content in either dietary group.

Effects of CIH and High-Cholesterol Diet on Atherosclerosis in Mouse Aorta

Oil red O–hematoxylin staining of the cross-sections of the aortic origin from 10 mice subjected to IA exposure and fed a regular diet for 12 weeks, 10 mice subjected to CIH exposure and fed a regular diet for 12 weeks, and 10 mice subjected to control exposure to intermittent air (IA) and fed a high-cholesterol diet for 12 weeks was negative for lipids, showing no evidence of atherosclerosis (Figures 5A–5C). In contrast, 9 of 10 mice exposed to both CIH and a high-cholesterol diet exhibited 2 or 3 atherosclerotic lesions in the ascending aorta (Figure 5D), which varied in size between 4,170 and 145,240 μm^2, with an average cross-sectional area of 23,010 ± 13,323 μm^2. Lesions in the aortic origin were polymorphic, varying from lipid deposition in the intima (fatty streaks; Figure 5D, thin arrow) to more mature atherosclerotic plaques with extensive lipid deposition in the intima and media, thickening of the aortic wall, and a necrotic core (Figure 5D, *thick arrow*).

Findings on the *en face* preparation of thoracic and abdominal aorta were consistent with cross-sections of the aortic origin. Neither mice exposed to CIH and a regular diet, nor mice exposed to control conditions and a high-cholesterol diet, exhibited any evidence of lipid deposition in the intima (Figures 6A–6C and 6E). Pale pink staining in the *en face* preparation of the aorta in mice exposed to IA and a high-cholesterol diet represented adipose accumulation in the adventitia (Figure 6C). Nine of 10 mice subjected to CIH and a high-cholesterol diet for 12 weeks exhibited 1–5 atherosclerotic lesions, which appeared as bright red lipid-positive areas in the intima (Figures 6D and 6F). Sudan IV–positive lesions were small in size, covering only 0.70 ± 0.23% of the total aortic surface (Figure 6D). *En face* preparation did not allow us to distinguish between fatty streaks and mature plaques, but a higher magnification showed that atherosclerotic lesions occasionally protruded into the vascular lumen, suggestive of plaque formation (Figure 6F).

Effects of CIH and High-Cholesterol Diet on Hepatic SCD-1 Expression

We have previously demonstrated that short-term IH leads to hypercholesterolemia in mice on a regular diet, possibly because of an increase in SCD-1, a hepatic enzyme up-regulating lipoprotein secretion. Exposure to CIH for 12 weeks caused a greater

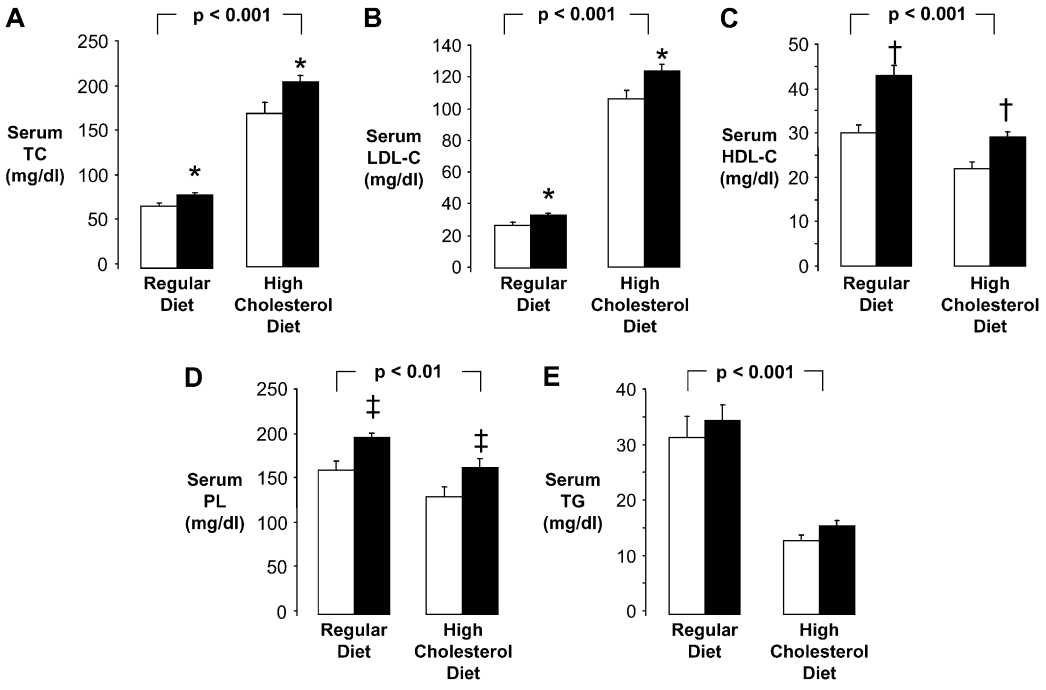

Figure 1. Effect of chronic intermittent hypoxia (CIH) or intermittent air (IA) on fasting serum levels of (*A*) total cholesterol (TC), (*B*) low-density lipoprotein cholesterol (LDL-C), (*C*) high-density lipoprotein cholesterol (HDL-C), (*D*) phospholipids (PL), and (*E*) triglycerides (TG) in C57BL/6J mice on regular chow and a high-cholesterol diet. *Solid bars*, CIH; *open bars*, IA control. *$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$, for the difference between IH and IA.

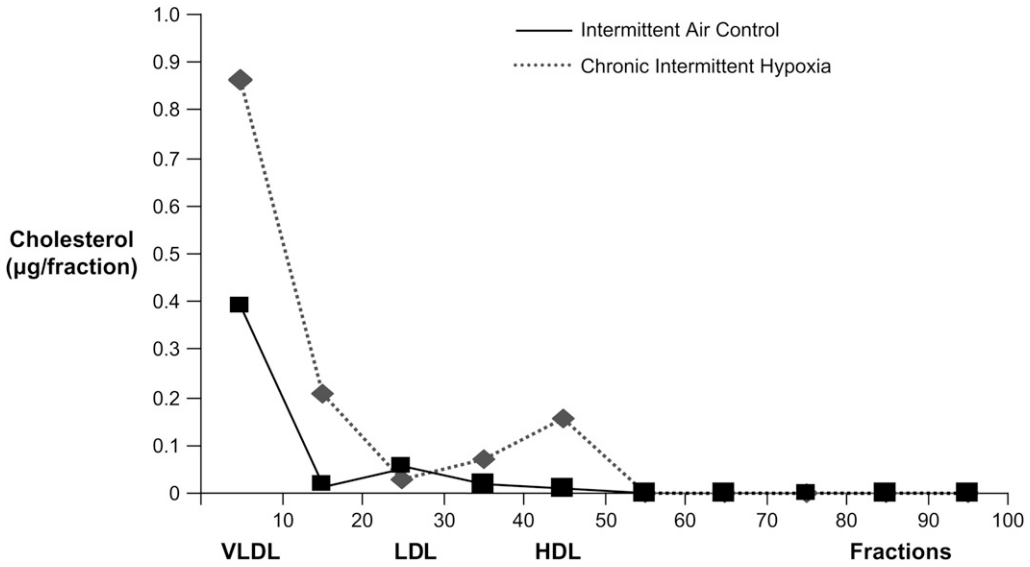

Figure 2. Characterization of serum lipoproteins in C57BL/6J mice on a high-cholesterol diet after exposure to chronic intermitten hypoxia or intermittent air. Lipoproteins were examined by fast protein liquid chromatography, using ÄKTAprime system (GE Healthcare Life Sciences, Piscataway, NJ) followed by cholesterol measurement by gas chromatography in each fraction. Each profile represents pooled serum from eight mice. HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein.

than twofold increase in hepatic SCD-1 mRNA expression in both mice on regular and high-cholesterol diets (Figure 7A). Results of immunoblot in liver tissue mirrored real-time polymerase chain reaction data with significant hypoxia-induced increases in SCD-1 protein levels in both dietary groups (Figures 7B and 7C). Mice fed a high-cholesterol diet exhibited higher levels of SCD-1 mRNA and protein than did mice on a regular diet.

DISCUSSION

There is a growing body of evidence in the clinical literature that OSA is associated with a high prevalence of atherosclerosis (6). However, a causal link between OSA and atherosclerosis has never been established. We have previously shown that IH, one of the key physiological mechanisms of OSA, leads to hyperlipidemia (15, 16). The purpose of the current study was to explore whether CIH can cause atherosclerosis. The main finding of the study was that exposure to CIH for 12 weeks led to the development of atherosclerotic lesions in the aortic origin and descending aorta in male C57BL/6J mice on a high-cholesterol diet, whereas control mice on a high-cholesterol diet and mice exposed to CIH and a regular diet were free of the disease. Several additional findings resulted from the study. First, exposure to CIH exacerbated hyperlipidemia in mice on a high-cholesterol diet, despite already elevated baseline levels of total and LDL cholesterol. CIH also raised serum levels of total cholesterol and LDL-C in mice on a regular diet, but the levels of total and LDL cholesterol remained low and HDL-C levels were high, compared with the animals on a high-cholesterol diet. Second, hypoxia-induced hypercholesterolemia was associated with significant increases in hepatic SCD-1 mRNA and protein levels in both dietary groups, but SCD-1 levels were significantly higher in mice on a high-cholesterol diet. Third, CIH induced a twofold increase in serum lipid peroxidation, regardless of diet. Fourth, CIH increased hepatic levels of a proinflammatory cytokine, TNF-α, exclusively in mice on a high-cholesterol diet, whereas serum cytokine levels were not altered. In this discussion we explore the relationships and putative pathways linking CIH and atherosclerosis and discuss clinical implications of our work.

CIH and Atherosclerosis of Aorta in Male C57BL/6J Mice

The principal finding of our study is that CIH leads to atherosclerosis in male C57BL/6J mice on a high-cholesterol diet. These data are particularly striking because male wild-type mice are usually resistant to atherosclerosis (26). Early atherosclerotic lesions (fatty streaks) are observed in female C57BL/6J mice fed high-fat, high-cholesterol Western diets for long periods of time (at least 14 weeks) (27).

Wild-type mice initially develop lesions in the aortic origin, which can be examined by serial cross-sections. Paigen and coworkers (27) reported that after 14 weeks of a high-cholesterol diet, 71% of female C57BL/6J mice developed atherosclerosis in the aortic origin with an average lesion size of 660–2,700 μm^2, whereas only 27% of male mice had evidence of the disease, with an average lesion size of 290–1,500 μm^2. We report that combined exposure to CIH and high-cholesterol diet for 12 weeks induced fatty streaks and atherosclerotic plaques in the aortic origin of 90% of male C57BL/6J mice, with an average lesion area 10- to 20-fold greater than previously reported in susceptible female mice (27), whereas none of the mice fed a high-cholesterol diet under control IA conditions had any evidence of atherosclerosis (Figure 5). Remarkably, the extent of atherosclerosis in the aortic origin after the combined exposure to CIH and an atherogenic diet for 12 weeks was comparable to that of female C57BL/6J mice fed a high-cholesterol diet for 1 year (21).

Atherosclerosis of the aortic tree is usually undetectable in male C57BL/6J mice on a high-cholesterol diet for 14 weeks, whereas female mice develop lesions covering 1.1% of the aortic surface (27). According to Tangirala and coworkers (21), exposure to a high-cholesterol diet for 1 year led to atherosclerotic lesions in the aortic tree only in two of nine exposed female mice, with the lesion area less than 0.5% of the aortic surface. In contrast, we report that combined exposure to CIH and a high-cholesterol diet for 12 weeks induced lesions in 90% of male C57BL/6J mice, covering 0.7% of the aortic surface (Figure 6).

Mature atherosclerotic plaques develop in transgenic mice with mutations in crucial antiatherogenic genes, including LDL receptor (28), scavenger receptor-B1 (29), and apolipoprotein E (ApoE) (20). In wild-type mice, fatty streaks usually do not progress to atherosclerotic plaques with a fibrous cap (27). In contrast, we observed not only fatty streaks, but also mature plaques in C57BL/6J mice exposed to both CIH and a high-cholesterol diet (Figures 5D and 6F).

CIH did not cause atherosclerosis in C57BL/6J mice on a regular diet, suggesting that preexistent or coexisting dyslipidemia

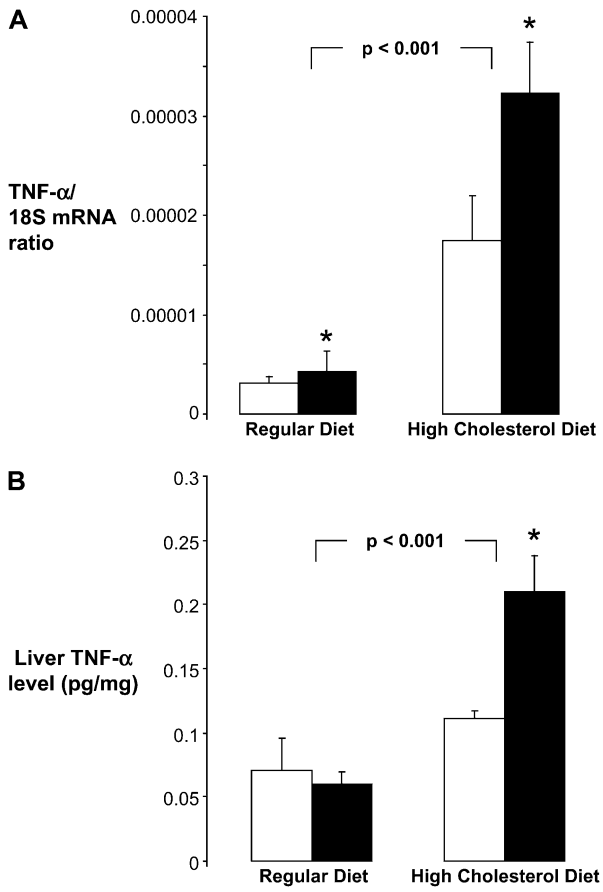

Figure 3. Tumor necrosis factor (TNF)-α in the livers of C57BL/6J mice on a regular chow diet or on a high-cholesterol diet and exposed to chronic intermittent hypoxia (CIH) or intermittent air (IA) for 12 weeks. (*A*) Hepatic TNF-α mRNA levels by real-time reverse transcription–polymerase chain reaction; (*B*) TNF-α protein levels by ELISA with total liver lysate. *Solid bars* = CIH; *open bars* = IA control. *$p < 0.05$ for the difference between CIH and IA.

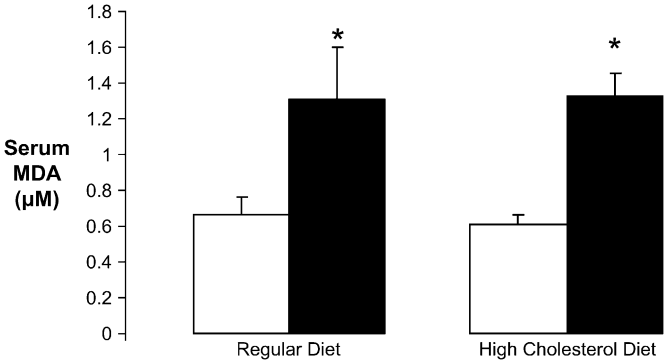

Figure 4. Effect of chronic intermittent hypoxia (CIH) on serum lipid peroxidation, using the malondialdehyde (MDA) assay. *Solid bars* = CIH; *open bars* = intermittent air control. *$p < 0.05$ for the difference between CIH and intermittent air control.

due to either genetic or environmental factors is necessary for expression of atherogenic properties of CIH.

CIH, Dyslipidemia, and Atherosclerosis

Our data showed that a high-cholesterol diet increased serum total cholesterol and altered the lipoprotein profile, decreasing HDL-C levels and increasing LDL-C, which was consistent with previous reports (26). Dyslipidemia induced by a high-cholesterol diet is implicated in atherogenesis, but the presence of hypoxic exposure was necessary for atherosclerotic lesions to develop. Mice exposed to both CIH and a high-cholesterol diet exhibited further increases in total cholesterol, VLDL-C, and LDL-C in comparison with mice exposed only to a high-cholesterol diet, which might have contributed to atherogenesis.

Our present data are consistent with our previous observations that both short-term exposure to IH and CIH in C57BL/6J mice on a regular diet led to hypercholesterolemia (15, 16). We have shown earlier that hypercholesterolemia during IH can be attributed to induction of hypoxia-inducible factor-1 in the liver, which activates sterol regulatory element–binding protein-1 (SREBP-1) and SCD-1 (30), a gene transcriptionally regulated by SREBP-1. SCD-1, an enzyme catalyzing conversion of saturated fatty acids to monounsaturated fatty acids, induces lipoprotein secretion by increasing levels of cholesterol esters and triglycerides, important components of VLDL (31, 32). We now show that, in mice on a high-cholesterol diet, SCD-1 is increased not only by dietary cholesterol, which is a well-described phenomenon (33), but also by CIH. Our current data reveal that an increase in hepatic SCD-1 coincided with significant elevations in VLDL-C (Figure 2), suggesting that CIH may lead to atherosclerosis by up-regulating lipoprotein secretion in the liver via the SCD-1 mechanism.

CIH, Lipid Peroxidation, and Atherosclerosis

Oxidative stress is an established mechanism of atherosclerosis in mice. Deficiency of a key enzyme of oxidative stress, NADPH oxidase, and treatment with antioxidant vitamin E alleviated atherosclerosis in $ApoE^{-/-}$ mice (34, 35). Clinical trials of antioxidants failed to show any benefit on atherosclerosis in adults (36, 37), but improved endothelial function in children with hyperlipidemia (38), which suggests that antioxidants may be effective for primary prevention of atherosclerosis in humans.

Our data indicate that lipid peroxidation is a putative mechanism of atherogenesis in CIH. Indeed, CIH led to a twofold increase in serum MDA levels, reflecting enhanced oxidation of polyunsaturated fatty acids (PUFAs). The oxidation of PUFAs leads to the formation of aldehydes that modify lysine residues in apolipoprotein B-100, resulting in oxidized LDLs (39). Oxidized LDLs are taken up by macrophages more readily via scavenger receptors SR-A and CD36, leading to macrophage foaming and progression of atherosclerosis (40). It has been previously reported *in vitro* that hypoxia induces lipid accumulation in smooth muscle cells loaded with LDL (41) and that IH increases lipid loading in human macrophages (42) via oxidative stress mechanisms. We have now shown *in vivo* that atherogenesis during CIH occurred concurrently with enhanced serum lipid peroxidation and hypercholesterolemia. Atherosclerotic lesions were not observed in normoxic mice on a high-cholesterol diet, exhibiting hypercholesterolemia without excessive serum lipid peroxidation, or in mice subjected to CIH and a regular diet, exhibiting increased serum lipid peroxidation without hypercholesterolemia. Thus, atherogenesis during CIH is caused by interaction of CIH-induced lipid peroxidation and dyslipidemia, when preexisting hyperlipidemia is present.

CIH and Systemic Inflammation

Exposure to CIH did not affect serum inflammatory markers. However, CIH up-regulated hepatic TNF-α in mice on a

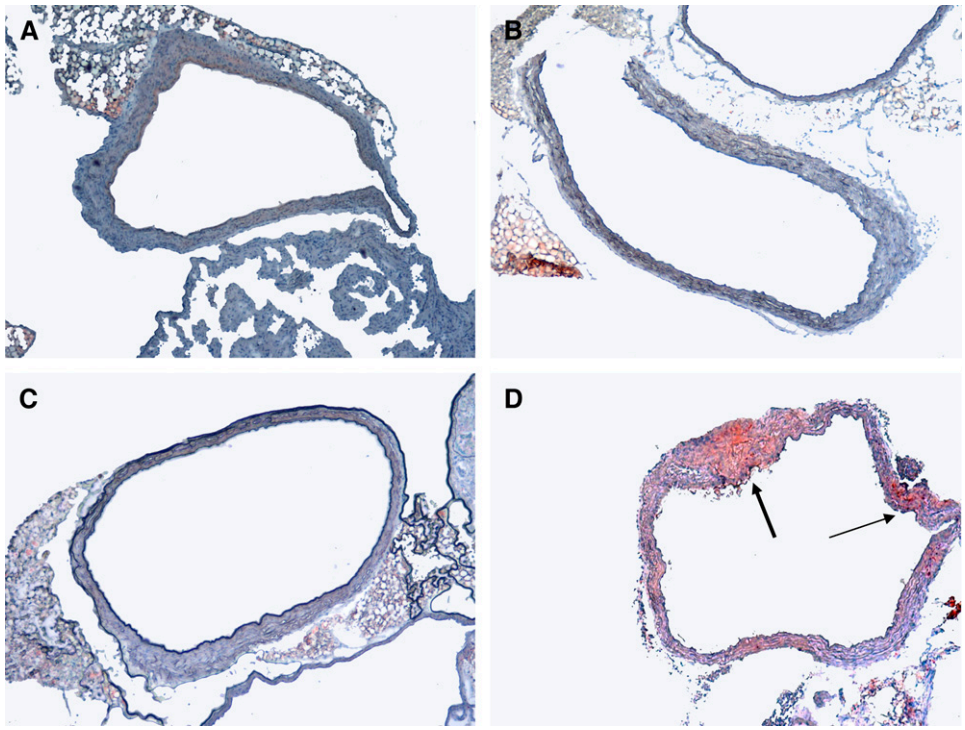

Figure 5. Representative cross-sections of the ascending aorta (sinus of Valsalva) in C57BL/6J mice exposed to (*A*) intermittent air (IA) control conditions and regular diet, (*B*) chronic intermittent hypoxia (CIH) and regular diet, (*C*) IA and a high-cholesterol diet, or (*D*) CIH and a high-cholesterol diet. Transverse frozen sections of the aorta were stained with oil red O and hematoxylin. Original magnification: ×100. The *thick arrow* points at the atherosclerotic plaque with a necrotic core. The *thin arrow* points at the fatty streak.

high-cholesterol diet with underlying hyperlipidemia (Figure 3), which could lead to the progression of atherosclerosis (43). It is unclear why an increase in proinflammatory cytokines in liver tissue was not accompanied by elevation in circulating cytokines. One explanation would be the relatively low sensitivity of the ELISA used in this study. Another possibility is that CIH-induced changes are time dependent and systemic inflammation could be implicated earlier in a time course of atherosclerosis. Indeed, we have previously shown that a 5-day exposure to IH increases serum IL-6 levels (30). In addition, a number of proatherogenic cytokines (IL-1β and IL-18), chemokines and adhesion molecules (P-selectin, E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1), and anti-atherogenic IL-10 (44–46) were not evaluated in this study. Thus, CIH and a high-cholesterol diet may interact to cause systemic inflammation, which could lead to atherosclerosis.

CIH and Other Potential Mechanisms of Atherogenesis

OSA is an established cause of hypertension (4). CIH leads to systemic hypertension in rats and mice (47, 48). We did not measure blood pressure in this study, but it is conceivable that CIH-induced hypertension contributed to atherogenesis in C57BL/6J mice on a high-cholesterol diet.

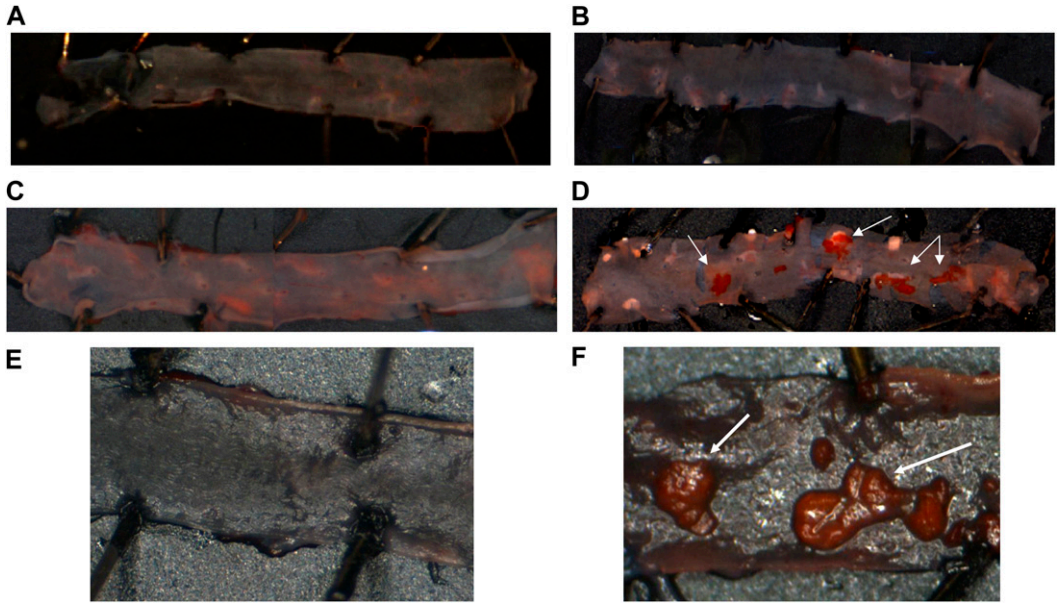

Figure 6. Representative images of the thoracic (aortic arch and descending aorta) and abdominal aorta in C57BL/6J mice by the *en face* method. Sudan IV staining; original magnification: (*A–D*) ×10, water immersion; (*E* and *F*) ×20, dry preparation. (*A*) Intermittent air (IA) control conditions and regular diet; (*B* and *E*) chronic intermittent hypoxia (CIH) and regular diet; (*C*) IA and high-cholesterol diet; (*D* and *F*) CIH and high-cholesterol diet. *Arrows* point at atherosclerotic lesions.

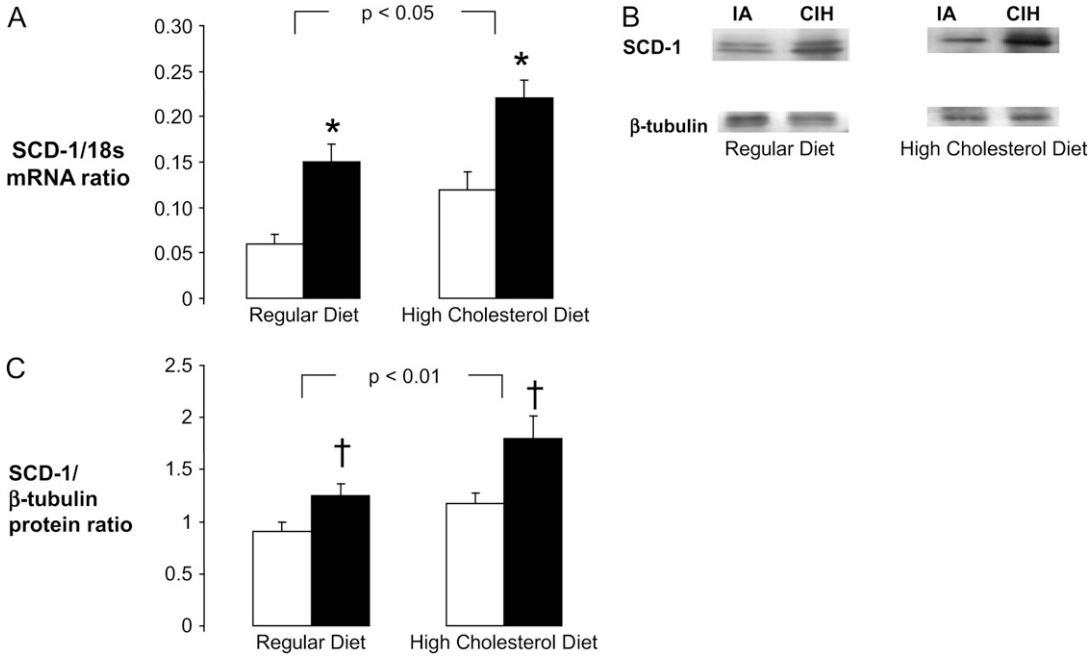

Figure 7. Analysis of stearoyl-coenzyme A desaturase-1 (SCD-1) in the livers of C57BL/6J mice on a regular chow diet and on a high-cholesterol diet and exposed to chronic intermittent hypoxia (CIH) or intermittent air (IA) for 12 weeks. n = 10 per group. (*A*) Hepatic SCD-1 mRNA levels by real-time reverse transcription–polymerase chain reaction; (*B* and *C*) SCD-1 protein levels by immunoblot with total liver lysate. (*B*) shows SCD-1 and β-tubulin bands in representative samples. (*C*) shows mean optical density of SCD-1 bands normalized to β-tubulin. (*A* and *C*) *Solid bars* = CIH; *open bars* = IA control. *$p < 0.05$ and †$p < 0.01$ for the difference between CIH and IA.

OSA is associated with insulin resistance and glucose intolerance (5). We have previously shown that CIH leads to insulin resistance and glucose intolerance in obese mice (24). Both hyperglycemia and insulin resistance are risk factors for atherosclerosis (49, 50). In our study, mice on a regular diet exhibited higher levels of blood glucose after CIH than did mice on a high-cholesterol diet (Table 1), whereas atherosclerosis was present only in mice on a high-cholesterol diet. CIH did not induce hyperinsulinemia. Thus, our data indicate that hyperglycemia and insulin resistance did not contribute to atherogenesis during CIH.

Conclusions, Clinical Implications, and Limitations of the Study

We have provided the first evidence that CIH causes atherosclerosis in the presence of preexisting hyperlipidemia. We hypothesize that atherogenesis in CIH occurs via two principal mechanisms: dyslipidemia and lipid peroxidation. Our data suggest that CIH of OSA can directly cause atherosclerosis and that CIH-induced atherosclerosis may contribute to increased cardiovascular morbidity and mortality in OSA.

Our study did not explore whether discontinuation of CIH or lowering dietary fat during CIH can reverse atherogenesis. The response to cessation of the hypoxic stimulus could be an important predictor of efficacy of continuous positive airway pressure for treating atherosclerosis in patients with OSA and should be a subject of future investigation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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