**Abstract**—Obstructive sleep apnea leads to chronic intermittent hypoxia (CIH) and is associated with atherosclerosis. We have previously shown that C57BL/6J mice exposed to CIH and a high-cholesterol diet develop dyslipidemia, atherosclerosis of the aorta, and upregulation of a hepatic enzyme of lipoprotein secretion, stearoyl coenzyme A desaturase 1 (SCD-1). We hypothesized that (1) SCD-1 deficiency will prevent dyslipidemia and atherosclerosis during CIH; and (2) human OSA is associated with dyslipidemia and upregulation of hepatic SCD. C57BL/6J mice were exposed to CIH or normoxia for 10 weeks while being treated with either SCD-1 or control antisense oligonucleotides. Obese human subjects underwent sleep study and bariatric surgery with intraoperative liver biopsy. In mice, hypoxia increased hepatic SCD-1 and plasma very-low-density lipoprotein cholesterol levels and induced atherosclerosis lesions in the ascending aorta (the cross-section area of 15651±57408 μm²), and descending aorta (7.0±2.5% of the total aortic surface). In mice exposed to CIH and treated with SCD-1 antisense oligonucleotides, dyslipidemia and atherosclerosis in the ascending aorta were abolished, whereas lesions in the descending aorta showed 56% reduction. None of the mice exposed to normoxia developed atherosclerosis. In human subjects, hepatic SCD mRNA levels correlated with the degree of nocturnal hypoxemia (r=0.68, P=0.001). Patients exhibiting oxyhemoglobin desaturations at night showed higher plasma triglyceride and low-density lipoprotein cholesterol levels, compared to subjects without hypoxemia. In conclusion, CIH is associated with dyslipidemia and overexpression of hepatic SCD in both humans and mice alike; SCD-1 deficiency attenuates CIH-induced dyslipidemia and atherosclerosis in mice. (*Circ Res.* 2008;103:1173-1180.)

**Key Words:** atherosclerosis ■ hypercholesterolemia ■ hypoxia ■ lipoproteins ■ obstructive sleep apnea

Obstructive sleep apnea (OSA) is characterized by chronic intermittent hypoxia (CIH) during sleep.1 OSA occurs in 9% of women and 24% of men in the United States, but the prevalence exceeds 30% to 50% in the obese population.2,3 OSA poses significant cardiovascular risk, which has been attributed to the high prevalence of atherosclerosis in patients with OSA.4–6 OSA is associated with dyslipidemia, lipid peroxidation, and vascular inflammation, all of which induce atherosclerosis.7–10 Recent studies have shown independent associations between the hypoxic stress of OSA and increased carotid artery intima–media thickness11,12 that is reversed by treatment with continuous positive airway pressure.13

Our group has previously explored relationships between CIH and atherosclerosis using a mouse model of CIH that mimics the oxygen profile in patients with severe OSA.14,15 We have shown that CIH induces atherosclerosis in C57BL/6J mice on a high-cholesterol diet.16 We have also found that the development of atherosclerosis during CIH was associated with increases in lipoprotein secretion, plasma very-low-density lipoprotein VLDL levels, and expression of hepatic stearoyl coenzyme A desaturase 1 (SCD-1).16–18 Four isoforms of SCD have been described in mice, of which SCD-1 is the best characterized.19 SCD converts saturated fatty acids, 18:0 and 16:0, into monounsaturated fatty acids (MUFAs), 18:1 and 16:1. Increased availability of MUFAs facilitates cholesterol ester and triglyceride biosynthesis and enhances lipoprotein secretion leading to hyperlipidemia.20,21 SCD-1 expression is regulated by sterol SREBP-1c, a key transcription factor of lipid biosynthesis.22 SCD-1...
has been implicated in the pathogenesis of obesity, fatty liver, and insulin resistance in rodents, but the role of SCD-1 in atherosclerosis has not been explored. Two human SCD genes have been identified with high homology to the murine SCD-1.19 Expression of hepatic SCD in human subjects has not been evaluated.

In this study, we explored a hypothesis that SCD-1 is involved in the pathogenesis of dyslipidemia and atherosclerosis during CIH. We examined our hypothesis by administering antisense SCD-1 oligonucleotides (ASOs) or control ASOs to C57BL/6J mice fed a high-cholesterol diet and exposed to CIH and measuring plasma lipid levels and aortic atherosclerotic lesions. We also tested the relevance of our findings for human OSA by examining hepatic SCD and plasma lipid levels in obese patients who underwent bariatric surgery and correlating these measurements with anthropometric and polysomnographic parameters.

Materials and Methods
A total of 24 wild-type, 8-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Me). The study was approved by the Johns Hopkins University Animal Care and Use Committee. Mice were fed a high-cholesterol diet and subjected either to CIH during the 12-hour light phase with an FiO2 nadir of 5%, 60 times per hour, or intermittent air (IA) for 10 weeks. Animals from each exposure group were treated either with SCD-1 ASOs or control ASOs.

Nineteen consecutive patients (18 women and 1 man) without history of diabetes, cardiovascular or liver disease, or dyslipidemia were recruited from the Johns Hopkins Bayview Medical Center Bariatric Surgery Clinic. The protocol was approved by the Western Institutional Review Board and the subjects gave informed consent. A detailed description of the experimental methods is available in the online data supplement at http://circres.ahajournals.org.

Table. Effects of CIH and SCD-1 ASOs on Metabolic Characteristics in C57BL/6J Mice

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control ASO</th>
<th>CIH</th>
<th>SCD-1 ASO</th>
<th>CIH</th>
<th>Effect of SCD-1 (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>Starting age, wk</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>N/A</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>23.1±0.3</td>
<td>22.9±1.9</td>
<td>23.1±0.2</td>
<td>23.5±0.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Day 30</td>
<td>23.5±0.4</td>
<td>20.8±1.9*</td>
<td>24.0±0.6</td>
<td>21.8±0.7***</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Day 70</td>
<td>24.2±0.5</td>
<td>23.7±0.7*</td>
<td>24.3±0.4</td>
<td>22.2±0.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>2.2±0.1</td>
<td>2.3±0.1</td>
<td>2.4±0.3</td>
<td>2.6±0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>2.11±0.07</td>
<td>1.89±0.08</td>
<td>1.90±0.11</td>
<td>1.80±0.10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Liver weight/body wt, %</td>
<td>8.85±0.49</td>
<td>8.01±0.34</td>
<td>7.85±0.52</td>
<td>8.12±0.89</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Epididymal fat, g</td>
<td>0.28±0.02</td>
<td>0.29±0.03</td>
<td>0.20±0.01</td>
<td>0.14±0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Epididymal fat/body wt, %</td>
<td>1.15±0.07</td>
<td>1.22±0.14</td>
<td>0.83±0.06</td>
<td>0.62±0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>167±14</td>
<td>163±14</td>
<td>165±18</td>
<td>172±6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fasting plasma insulin, mg/mL</td>
<td>0.39±0.05</td>
<td>0.42±0.04</td>
<td>0.51±0.10</td>
<td>0.55±0.12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fasting plasma total cholesterol, mg/dL</td>
<td>224±7</td>
<td>270±12†</td>
<td>202±9</td>
<td>218±10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fasting plasma triglycerides, mg/dL</td>
<td>14.4±3.6</td>
<td>13.2±5.8</td>
<td>16.7±5.3</td>
<td>13.9±2.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fasting plasma free fatty acids, mmol/L</td>
<td>0.31±0.06</td>
<td>0.28±0.08</td>
<td>0.41±0.04</td>
<td>0.36±0.06</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Liver cholesterol, mg/g</td>
<td>9.60±0.11</td>
<td>9.75±0.27</td>
<td>7.80±1.17</td>
<td>9.36±0.24</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Liver triglyceride, mg/g</td>
<td>13.4±0.8</td>
<td>13.5±2.0</td>
<td>7.91±1.88</td>
<td>9.84±1.63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liver free fatty acids, μmol/g</td>
<td>7.20±1.46</td>
<td>8.49±2.73</td>
<td>5.66±1.61</td>
<td>5.90±1.36</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*P<0.05 between day 30 and day 70; **P<0.01 between day 0 and day 30; ***P<0.001 between day 0 and day 30; †P<0.01 between IA and CIH.
Results

Effects of SCD-1 ASOs and CIH on Hepatic SCD-1 Levels and Activity in Mice

SCD-1 ASOs induced marked decreases in liver SCD-1 mRNA levels in both normoxic mice (∆∆Ct=2.9) and hypoxic mice (∆∆Ct=3.1) (Figure 1A). Given efficiencies of real-time PCR for SCD-1 and 18S (see the online data supplement), SCD-1 ASOs led to 84% to 87% depletion of SCD-1 mRNA in the liver (Figure 1A). SCD-1 ASOs decreased hepatic SCD-1 protein levels (Figure 1B and 1C), as well as 16:1/16:0 and 18:1/18:0 plasma fatty acid ratios (Figure I in the online data supplement), which indicated reduced enzymatic activity.

CIH led to a 71.6% elevation in SCD-1 mRNA and greater than a 2-fold increase in SCD-1 protein levels in the liver of mice receiving control ASOs (Figure 1). CIH also raised plasma 16:1/16:0 and 18:1/18:0 fatty acid ratios by 17% and 22%, respectively (supplemental Figure I). In contrast, animals injected with SCD-1 ASOs did not exhibit significant changes in SCD-1 mRNA, protein levels, or activity in response to CIH (Figure 1 and supplemental Figure I).

Effects of SCD-1 ASOs and CIH on Body Weight and Plasma Lipids in Mice

CIH caused significant weight loss by day 30 (Table), which was consistent with our previous observations.17 Mice treated with control ASOs from day 30 to day 70 of CIH regained weight by day 70, whereas SCD-1 ASO–treated mice did not. As a result, 10 weeks of CIH caused weight loss only in the SCD-1 ASO–treated mice but not in mice receiving control ASOs. SCD-1 ASOs did not affect liver weight, fasting blood glucose, or insulin levels but induced a significant loss of epididymal fat and declines in hepatic cholesterol, triglyceride, and free fatty acid content (Table). CIH had no impact on any of these parameters.

SCD-1 ASO administration resulted in a significant decrease in fasting plasma total cholesterol levels, especially during exposure to CIH (Table). High-performance liquid chromatography (HPLC) revealed that cholesterol-lowering effect of SCD-1 ASOs occurred in the VLDL cholesterol (VLDL-C) fraction (Figure 2). SCD-1 ASOs induced a 38% decrease in VLDL-C at normoxic conditions, from $116.8 \pm 3.6$ mg/dL in mice receiving control ASOs to $71.9 \pm 7.1$ mg/dL ($P < 0.001$), and a 36% decrease in VLDL-C at hypoxic conditions, from $135.4 \pm 3.6$ mg/dL to $86.5 \pm 7.7$ mg/dL ($P < 0.001$), which was consistent with the previously described role of SCD-1 in secretion of VLDL particles by the liver.19,21 SCD-1 ASOs also increased plasma levels of high-density lipoprotein cholesterol (HDL-C), especially at normoxic conditions, from $62.0 \pm 2.0$ mg/dL in mice receiving control ASOs to $74.7 \pm 3.6$ mg/dL ($P < 0.01$) (Figure 2). CIH resulted in a significant increase in fasting plasma total cholesterol levels in mice treated with control ASOs (Table) with the majority of the increase occurring in the VLDL fraction (Figure 2). In mice receiving control ASOs, CIH reduced HDL-C levels. SCD-1 ASO deficiency essentially abolished detrimental effects of CIH on the cholesterol profile (Figure 2). Neither SCD-1 ASOs nor CIH impacted plasma triglycerides and free fatty acid levels.
Effects of SCD-1 ASOs and CIH on the Aorta in Mice

In the absence of the hypoxic stimulus, male C56BL/6J mice did not exhibit any evidence of atherosclerosis in the aortic origin, regardless of the type of ASO treatment (Figure 3A and 3C). In contrast, all mice exposed to CIH and treated with control ASOs developed extensive atherosclerosis in the aortic origin. The aortic lesions had the appearance of mature atherosclerotic plaques, with extensive lipid deposits in the intima and media, thickening of the aortic wall, mononuclear infiltration, and a necrotic acellular core (Figure 3B). The plaques varied in size from 26 726 to 560 836 μm², with an average cross-section area of 156 514 ± 57 408 μm². Interestingly, none of the mice exposed to CIH and SCD-1 ASOs exhibited atherosclerotic plaques in the aortic origin, suggesting that SCD-1 ASOs opposed atherogenic effects of CIH. Two of 6 animals in this group revealed a small amount of oil red O–positive subintimal lipid deposits (Figure 3D), whereas in 4 others, the proximal aorta was intact and similar in appearance to mice exposed to IA.

The en face preparation showed no lipid accumulation in the thoracic and abdominal aorta of mice exposed to IA (Figure 4A and 4C). In contrast, all mice exposed to CIH exhibited bright red Sudan IV–positive areas in the intima. Consistent with findings in the aortic origin, hypoxic mice treated with SCD-1 ASOs exhibited atherosclerotic plaques in the aortic origin, suggesting that SCD-1 ASOs opposed atherogenic effects of CIH. Two of 6 animals in this group revealed a small amount of oil red O–positive subintimal lipid deposits (Figure 3D), whereas in 4 others, the proximal aorta was intact and similar in appearance to mice exposed to IA.

Metabolic and Sleep Characteristics and Hepatic SCD Expression in Patients With Severe Obesity

The patients were divided in 2 groups according to the median values for body mass index (BMI) and the severity of obstructive sleep apnea, which was measured during routine polysomnography as the apnea–hypopnea index (AHI) and the magnitude of oxygen desaturation (ΔSaO₂; supplemental Table I). BMI, AHI, and indices of nocturnal hypoxemia were not associated with free fatty acid levels. The AHI and nocturnal oxyhemoglobin desaturation were similar in individuals with higher and lower BMI (supplemental Table I), whereas more severe obesity was associated with a trend to higher SCD gene expression (P<0.053). Nonetheless, a correlation between BMI and SCD mRNA levels was not significant. The AHI had no relationship with SCD levels. In contrast, patients showing severe intermittent hypoxemia (ΔSaO₂, ≥5%) exhibited significantly greater levels of hepatic SCD gene expression than patients experiencing mild intermittent hypoxemia (ΔSaO₂ <5%; supplemental Table I). Furthermore, there was a strong positive correlation between the magnitude of nocturnal oxygen desaturation and SCD mRNA levels in the liver (r=0.68, P<0.001, Figure 5A). Consequently, individuals with severe nocturnal oxygen desaturation showed higher SCD protein levels in the liver (Figure 5 B). The patients with severe nocturnal hypoxemia also exhibited a 17% increase in total plasma cholesterol and an 89% increase in plasma triglyceride levels compared to the nonhypoxic patients (supplemental Table I). The increase in plasma triglycerides occurred predominantly in the VLDL fraction and to a lesser extent in the LDL fraction, whereas the increase in plasma cholesterol levels was entirely attributable to LDL-C (Figure 6).
Discussion

We have previously shown that CIH induces atherosclerosis in the aorta of C57BL/6J mice on a high-cholesterol diet in association with hypercholesterolemia, increased lipoprotein secretion, and upregulation of a key hepatic enzyme of lipoprotein secretion, SCD-1.16 The purpose of this study was to explore the role of SCD-1 in CIH-induced dyslipidemia and atherosclerosis. Several novel findings resulted from the study. Our main finding was that CIH-induced atherosclerosis was completely abolished by SCD-1 ASOs in the ascending aorta and decreased by 56% in the descending aorta. Secondly, SCD-1 ASOs abolished CIH-induced increases in hepatic SCD-1 mRNA and protein levels, as well as an increase in plasma 16:1 and 18:1 MUFAs. Third, SCD-1 ASOs reversed a CIH-induced increase in plasma VLDL-C. Finally, we demonstrated that patients with OSA exhibit an increase in hepatic SCD expression in direct proportion to the severity of nocturnal intermittent hypoxemia and that SCD overexpression in human subjects with OSA was associated with marked increases in plasma triglyceride and LDL-C levels. In the discussion below, we explore relationships between CIH, SCD-1, and atherosclerosis and elaborate on the clinical implications of our work.

CIH Increases SCD Expression in the Liver

We have previously demonstrated that, in mice, CIH upregulated hepatic SCD-1 mRNA and protein levels, consistent with our earlier reports.16–18,27 Other investigators found that hypoxia augments SCD expression in cell culture of human macrophages in association with increased intracellular lipid droplets.28 A novel finding of the present study was that SCD expression in the liver significantly correlated with the depth of oxyhemoglobin desaturation in human subjects with OSA, suggesting that SCD is upregulated by intermittent hypoxia (IH) in humans. We have previously reported that IH enhances expression of SREBP-1c and SREBP cleavage–activating protein (SCAP),18,27,29 which mediates posttranscriptional modifications of SREBP from the immature form to the active nuclear form.30,31 Because SREBP-1c regulates SCD-1 expression,22 it is conceivable that IH affects SCD-1 through the SREBP-1 pathway, either directly or through hypoxia inducible factors (HIFs).32–36 HIF-1, HIF-2, and HIF-3 consist of O2-regulated HIF-1α, HIF-2α, and HIF-3α subunits and a constitutively expressed HIF-1β subunit.33,35,37–40 IH upregulates HIF-1α both in vitro and in vivo,33,35,41,42 whereas effects of IH on HIF-2α and HIF-3α are unknown. HIF-1α activation has a direct proinflammatory effect and may promote atherogenesis by increasing macrophagal infiltration, angiogenesis, and lipid content in the atherosclerotic plaque.43,44 Transgenic mice with partial deficiency of HIF-1α exhibit significantly attenuated increases in serum

Figure 5. Relationships between nocturnal oxyhemoglobin desaturation (ΔSaO2) and hepatic levels of SCD in patients undergoing bariatric surgery; ΔSaO2 is a difference between baseline SaO2 and an average nocturnal SaO2 nadir during disordered breathing events (average low SaO2). A, mRNA levels measured by real-time RT-PCR; Ct indicates the critical threshold cycle; ΔCt is the difference between 18S and SCD Ct values. Each data point represents 1 patient. B, SCD protein by immunoblot. Left, SCD and β-actin bands in representative samples of patients with mild (ΔSaO2 <5%) and severe (ΔSaO2 ≥5%) oxyhemoglobin desaturation. Right, Mean optical density of SCD-1 bands normalized to β-actin. Solid bars indicate ΔSaO2 <5%; open bars, ΔSaO2 ≥5%.

Figure 6. The HPLC profile of serum triglycerides (A) and cholesterol (B) in patients undergoing bariatric surgery separated in 2 groups based on the median value of ΔSaO2, which is a difference between baseline nocturnal SaO2 and average low SaO2 during disordered breathing events. *P<0.05 and †P<0.01 for the difference between the groups.
lipids, hepatic SREBP-1, SCAP, and SCD-1 during IH. Based on these findings, we have previously formulated a hypothesis that IH can induce hepatic SCD-1 and dyslipidemia via sequential upregulation of HIF-1 and SREBP-1 (see Figure 7 in the report by Li et al).}

SCD-1 ASOs Reverse Dyslipidemia During CIH

SCD-1 ASOs prevented hypoxia-induced increases in 16:1/16:0 and 18:1/18:0 fatty acid ratios (supplemental Figure I). Given that all experimental animals consumed diet with the same MUFA content and that SCD-1 is the main mechanism of MUFA biosynthesis in the liver, our data provide solid evidence that CIH raises palmitoleate and oleate levels via the SCD-1. Relative abundance of palmitoleate and oleate induces biosynthesis of triglyceride and cholesterol esters in the liver, augmenting lipoprotein secretion and leading to hypercholesterolemia and hypertriglyceridemia. SCD-1 deficiency results in low plasma levels of cholesterol and triglycerides in Asebia mice and SCD-1−/− transgenic mice fed regular chow. Our murine data showed that the CIH-induced increase in plasma total cholesterol levels occurred exclusively in the VLDL fraction and was entirely abolished by SCD-1 ASOs. This increase in VLDL cholesterol is likely attributable to cholesterol esters, the synthesis of which is regulated by SCD. SCD-1 ASOs abolished this decrease (Figure 2). The latter is consistent with the reports that SCD-1 inhibits reverse cholesterol transport, destabilizing ATP-binding cassette transporter A1, and that SCD-1 deficiency increases plasma HDL-C levels and SCD-1 ASOs abolished this decrease (Figure 2). The latter is consistent with the reports that SCD-1 inhibits reverse cholesterol transport, destabilizing ATP-binding cassette transporter A1, and that SCD-1 deficiency increases plasma HDL-C. Surprisingly, in our study, neither CIH nor SCD-1 ASOs affected plasma triglyceride levels in mice that could be ascribed to low baseline levels after a prolonged fast. In contrast, SCD-1 ASOs significantly decreased hepatic lipid content and the amount of epididymal fat, which is consistent with the previous observations in SCD-1−/− deficient mice and attributable to downregulation of lipoprotein secretion and upregulation of fatty acid oxidation. Human studies showed that hypoxic upregulation of SCD in the liver was associated with a 2-fold increase in plasma triglycerides, predominantly in the VLDL fraction, implying that intermittent hypoxemia could augment lipoprotein secretion via the SCD mechanism as it occurs in mice. Summarizing all of the above, our data demonstrate that CIH causes dyslipidemia with elevation of VLDL, which was attenuated by SCD-1 ASOs.

SCD-1 ASOs Attenuate Atherosclerosis During CIH

We have reproduced our recently reported results and have again shown that a combination of CIH with a high-cholesterol diet leads to atherosclerosis in C57BL/6j mice, whereas mice exposed to the dietary fat alone did not exhibit atherosclerotic lesions. The main finding of the present study is that atherosclerosis in the mouse aorta was associated with upregulation of hepatic SCD-1 and was attenuated by SCD-1 ASOs. Thus, our data clearly demonstrate that SCD-1 inhibition has a therapeutic effect for atherosclerotic lesions induced by CIH.

SCD-1 upregulation in CIH may be proatherogenic. What are the mechanisms by which SCD-1 can lead to atherosclerosis? The most obvious pathway would be dyslipidemia resulting from upregulation of VLDL secretion and downregulation of reverse cholesterol transport. However, CIH led only to modest changes in VLDL-C and HDL-C levels (Figure 2), which are not likely to cause such a dramatic effect on atherosclerotic lesions, unless other pathways are involved. One of the potential mechanisms is SCD-1 upregulation in macrophages of the aortic intima, which may result in accelerated foam cell formation, similar to that previously described in human macrophages exposed to hypoxia in vitro. Accelerated lipid accumulation in the vascular wall may also activate the nuclear factor κB pathway and induce local production of proinflammatory cytokines, leading to vascular inflammation, necrosis, and fibrosis. Finally, CIH leads to systemic oxidative stress with serum lipid peroxidation and high levels of oxidized LDL. Lipid peroxidation may exacerbate proatherogenic effects of SCD-1-induced dyslipidemia. We hypothesize that CIH-induced upregulation of SCD-1 may accelerate atherogenesis by a number of mechanisms, including dyslipidemia, oxidative stress, and vascular inflammation.

Conclusions, Clinical Implications, and Limitations of the Study

Our study has shown that, in C57BL/6j mice, CIH upregulates a key hepatic enzyme of lipoprotein secretion, SCD-1, and induces dyslipidemia and atherosclerosis, which are markedly attenuated by SCD-1 depletion. The major limitation of the study is that ASOs inhibited SCD-1 expression in CIH-exposed mice to the levels, which were significantly lower than in control animals. Thus, our study has shown that SCD-1 depletion has an antiatherogenic effect during CIH but does not necessarily suggest that SCD-1 is responsible for CIH-induced atherosclerosis. We have also shown that, in human OSA, oxyhemoglobin desaturation is associated with upregulation of hepatic SCD and dyslipidemia, consistent with augmented lipoprotein secretion. Our data imply that SCD-1 ASOs could be a potential candidate for therapeutic use in patients with OSA, dyslipidemia, and atherosclerosis.

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Disclosures

S.B. is an employee of Isis Pharmaceuticals and has a commercial interest in the development of SCD-1 anti-sense oligonucleotides.
References


Dyslipidemia and Atherosclerosis Induced by Chronic Intermittent Hypoxia Are Attenuated by Deficiency of Stearoyl Coenzyme A Desaturase

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METHODS

Animal Studies: Anti-sense oligonucleotides (ASO) Selection

ASOs were produced as previously described with modifications. Briefly, rapid throughput screens with ~80 ASOs against mouse SCD-1 were performed and reduction of target gene expression was analyzed with real-time quantitative RT-PCR after transfection of the cells with different concentrations of the ASOs for 24h. Based on IC50 values, 3-4 potent ASOs were selected and their in vivo activity was confirmed in lean mice. The final selection of the SCD-1 ASO (ISIS 185222) was based on the maximal reduction of hepatic SCD-1 mRNA levels in lean mice. A negative control ASO (ISIS141923), which has the same chemical composition as ISIS185222 but does not exhibit perfect complementarity to any known gene sequence in public databases, was also included in the studies. Both ASOs were synthesized as 20-base phosphorothioate chimeric ASOs, where bases 1-5 and 16-20 had a 2'-O-(2-methoxyethyl) modification. This chimeric design provides increased nuclease resistance and mRNA affinity, while maintaining the RNAase H terminating mechanism, as described previously.

Animal Studies: Experimental Design

A total of 24 wild-type, 8 week-old male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, Maine) 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 blood sample collection, surgical procedures, and tissue collection anesthesia was induced and
maintained with 1-2 % isoflurane administered through a facemask. Mice were fed a high cholesterol diet (TD88051, Teklad WI, 4 kcal/g, 15.8% fat, and 1.25% cholesterol).

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. During each period of IH, the FIO2 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. The use of multiple inputs into the cage produced a uniform nadir FIO2 level throughout the cage.

Twelve mice were placed in the IH chamber for ten consecutive weeks. Twelve mice on were placed in an identical chamber, but received intermittent air (IA) at the identical flow rate. During the last six weeks of exposure, six mice from the CIH group and six mice from the IA group were treated with SCD-1 ASO. Six mice from the CIH group and six mice from the IA group were treated with scrambled control ASO i.p. at a dose of 25/mg/kg twice a week. Animals were kept in a controlled environment (22-24 °C with a 12 h : 12 h light : dark cycle; lights on at 09.00) with free access to food and water. The IH and IA states were induced during the 12 h light phase alternating with 12 h of constant room air during the dark phase.

Mice fasted for 6 hrs prior to bleeding and sacrifice. Arterial blood (~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 OCT Compound (Sakura Finetek USA, Inc. Torrance, CA). The descending aorta was
fixed in 10% paraformaldehyde. Livers were surgically removed and immediately frozen at -80°C for future analysis.

Animal Studies: Histopathology

Serial 7-10 µm-thick sections were prepared from the proximal 1 mm of the aortic origin. Ten-fifteen sections were collected at 50 µm intervals starting from the appearance of the aortic valves, thaw-mounted on slides and air dried. After fixation in 4% paraformaldehyde (pH =7), the sections were stained with Meyer's hematoxylin and oil red-O (Sigma-Aldrich, USA) as previously described 3. Images of the aorta were recorded using Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024x768 pixels. Total area of the lesion was measured in each slide by using Image-Pro Discovery, version 5.1 software from Media Cybernetics, Inc. (Silver Spring, MD) in a blinded fashion. ROS production in the aorta was assessed by the DHE stain 4. Four frozen sections representing every eighth serial section were incubated with 10 μM of DHE (Molecular Probes Inc., Eugene, OR) for 5 min, rinsed, mounted, observed with an Olympus BX41 fluorescent microscope, using excitation at 535 nm an emission at 610, and quantified using NIH Image software.

Animal Studies: Enface Preparation and Oil Red-O staining of the aorta

The whole descending aorta fixed in 4% formaldehyde was separated from adventitia and surrounding adipose and connective tissues and processed as previously described 5 with minor modifications. Briefly, the dissected aorta was rinsed in 70% ethanol, immersed in a filtered solution containing 0-5% Sudan IV (Sigma Chemical Co),
35% ethanol, and 50% acetone, and destained in 80% ethanol. The Sudan-stained aortas were pinned out on a black wax surface, and the size of lesions in the aortas was quantified by using images of aortas captured with a Q-Color 3 color digital video camera (Olympus America, Canada) linked to the microscope. The captured images were analyzed and the lesion areas were measured with Image-Pro Discovery, version 5.1 software from Media Cybernetics, Inc. in a blinded fashion by one of the authors (VS).

**Human Studies: Experimental Design**

Nineteen consecutive patients (eighteen women and one man) were recruited from the Johns Hopkins Bayview Medical Center (JHBMC) Bariatric Surgery Clinic. The protocol was approved by the Western Institutional Review Board. All subjects gave informed consent. Patient inclusion criteria were age > 21yrs and a body mass index of > 35 kg/m². Exclusion criteria included: previous treatment of OSA within the prior 3 months, sleep disorders other than OSA (i.e., narcolepsy or periodic leg movements), previous upper airway surgery, recent weight loss of ≥10%, diabetes mellitus type 2 (defined by prior clinical diagnosis or use of hypoglycemic agents), therapy with any lipid lowering medications, current history of alcohol abuse, known chronic liver disease including non-alcoholic steatohepatitis, chronic viral hepatitis (B or C), iron overload (hemochromatosis), or cirrhosis of any etiology, history of HIV disease and/or current anti-HIV therapy, current systemic use of steroids, unstable cardiovascular disease (congestive heart failure, myocardial infarction or revascularization procedures, and unstable arrhythmias), uncontrolled hypertension (blood pressure > 190/110 mm Hg), significant lung disease with daytime hypoxemia or hypercapnea, supplemental oxygen...
use, renal failure on dialysis, pregnancy, or bleeding disorders. One to three months prior to the bariatric surgery, patients were admitted to the Johns Hopkins Bayview General Clinical Research Center for a full-night sleep study. At the termination of the sleep study at 7 am, fasting blood samples were obtained for blood glucose, insulin levels and the lipid profile, and body anthropometry was performed. The AHI (number of episodes/hr) was computed as the number of apneas and hypopneas that occurred per hour of sleep. Prevalent OSA was defined by an AHI of >10 episodes/hr. OSA severity was defined by both the AHI and the average fall in oxyhemoglobin saturation ($\Delta\text{SaO}_2$) during apneic/hypopneic episodes. A wedge liver biopsy was obtained by the surgeon the gastric bypass procedure and immediately frozen at -80°C for future analysis.

**Biochemical Analyses of Mouse and Human Samples**

Plasma total cholesterol, triglycerides, and FFA were measured with kits from Wako Diagnostics, Inc. (Richmond, VA). Lipids were extracted from the mouse liver with chloroform- methanol, according to Bligh-Dyer procedure and measured using kits from Wako Diagnostics. Mouse fasting blood glucose was measured with Accu-Chek® Comfort Curve TM kit from Roche Diagnostics, Inc. (Indianapolis, IN). Plasma insulin levels in mice were detected with ELISA kits from Linco Research, Inc. (St. Charles, MO). Plasma lipoproteins from mice and human subjects were subjected to gel filtration HPLC on two tandemly connected TSK-Gel Lipopropak XL columns (300 x 7.8 mm) with simultaneous measurement of TG and cholesterol using an on-line dual detection system, according to LipoSEARCH technology (Skylight Biotech, Inc., Tokyo, Japan).
Plasma total fatty acids were analyzed as previously described with minor modifications. Briefly, fatty acids were hydrolyzed, extracted with hexane, and derivatized with pentafluorobenzyl bromide. The resulting esters were resolved on capillary gas chromatography electron capture negative ion mass spectrometry (GC/MS). The analysis was performed on an Agilent 6890/5973 GC/MS operating in the negative ion mode using an SP-2560 capillary column (Supelco, Inc., Bellefonte, PA) and ammonia as the reagent gas. Each fatty acid was matched to the labeled internal standard of closest chain length, retention time, and concentration.

Real Time PCR in Mouse and Human Liver Tissue

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 for mouse SCD-1 and 18S were previously described. Efficiency of mouse SCD-1 real time PCR was 94.7 ± 3.4%, and efficiency of mouse 18S real time PCR was 99.9 ± 4.1%. The sequences of primers and probes for human SCD were designed based on the GeneBank sequence NM_005063, forward primer 5’- GCCCGCCAGTCA -3’, reverse primer 5’- TGACCGTGTCGGGTATTTCC-3’, and the probe 5’- TCGCACTTTGCCCC-3’. The sequences of primers and probes for human 18S were designed based the GeneBank sequence X03205, forward primer 5’- GCGGCTTTGGTGACTCTAGATAA -3’, reverse primer 5’- ACGGCGACTACCATCGAAAGT -3’, and the probe 5’-
ATTCGAACGTCTGCC -3’. Efficiency of human SCD-1 real time PCR was 98.6 ± 4.7%, and efficiency of human 18S real time PCR was 101.0 ± 5.9%. The mRNA expression levels were normalized to 18S rRNA concentrations using the following formula: ΔCt = 18SCt – SCD Ct. Given that SCD/18S = (2 x E_18S/ E_SCD)^Ct(18S)-Ct(Gene of Interest), where E_SCD and E_18S are SCD and 18S PCR efficiencies respectively, ΔΔCt of 1 in SCD-1/18S between two groups of mice corresponded to a 1.92 fold difference, and ΔΔCt of 1 in SCD/18S between two groups of patients corresponded to a 1.96 fold difference.

**Immunoblot in Mouse and Human Liver Tissue**

An aliquot of the liver tissue from each mouse was homogenized and the microsomal fraction was isolated as previously described 7. Aliquots (70µg of protein) were analyzed by 4-15% SDS-PAGE followed by immunoblot assays using goat polyclonal antibodies against mouse SCD-1 (sc-14719) and rabbit polyclonal antibodies against mouse actin (sc-10731) from Santa Cruz Biotechnology (Santa Cruz, CA). Human livers were processed in an identical fashion. Immunoblot was performed using mouse monoclonal antibodies against human SCD (sc-58420) and rabbit polyclonal antibodies against human actin (sc-10731). Goat anti-mouse-HRP conjugate and goat anti-rabbit conjugate from BioRad (Hercules, CA) or bovine anti-goat-HRP conjugate from Santa Cruz were used as needed. Immunoblot was developed using ECL system from Amersham (Piscataway, NJ). Densitometry was performed using ChemiDoc XRS system from BioRad (Hercules, CA) and UN-SCAN-IT Gel Automated Digitizing System, version 5.1 software (Silk Scientific Corporation, Orem, UT). The results were expressed as ratios of
optical density of the bands representing SCD to optical density of the band representing actin.

Statistical Analyses

All values are reported as mean ± SEM. Statistical comparisons between four groups of mice (CIH-control ASO, IA-control ASO, CIH-SCD-1 ASO, IA-SCD-1 ASO) were performed by a general linear model ANOVA across two independent variables, hypoxia and ASO, followed by the Tukey’s post-hoc test. Comparisons between Day 0, Day 30 and Day 84 of mouse exposure were performed using repeated-measures ANOVA. Human subjects were divided in two groups according to a median BMI, AHI, or Δ SaO₂ and analyzed using an unpaired t-test. A p-value of less than 0.05 was considered significant. Linear regression analysis was used to assess relationships between Δ SaO₂ and SCD mRNA.

All authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Reference List

(1) McKay RA, Miraglia LJ, Cummins LL, Owens SR, Sasmor H, Dean NM.

Characterization of a potent and specific class of antisense oligonucleotide


**Online Table I.** Basic, sleep, and metabolic characteristics of obese patients undergoing bariatric surgery

<table>
<thead>
<tr>
<th></th>
<th>Body mass index (kg/m²)</th>
<th>Apnea-hypopnea index (events/hr)</th>
<th>Δ SaO₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 48.7</td>
<td>≥48.7</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Age</td>
<td>42.8±2.4</td>
<td>39.6±2.5</td>
<td>38.3±2.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>44.7±1.1</td>
<td>51.8±1.5‡</td>
<td>48.1±1.4</td>
</tr>
<tr>
<td>Neck (cm)</td>
<td>39.7±0.8</td>
<td>44.0±1.0†</td>
<td>41.2±1.1</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>132±10.8</td>
<td>140±3.3</td>
<td>140±9.5</td>
</tr>
<tr>
<td>AHI (events/hr)</td>
<td>24.6±8.5</td>
<td>27.5±10.4</td>
<td>6.8±1.3</td>
</tr>
<tr>
<td>Baseline SaO₂ (%)</td>
<td>95.8±0.7</td>
<td>95.6±0.4</td>
<td>96.3±0.3</td>
</tr>
<tr>
<td>Average low SaO₂ (%)</td>
<td>90.9±1.0</td>
<td>90.1±1.0</td>
<td>91.9±0.6</td>
</tr>
<tr>
<td>Δ SaO₂ (%)</td>
<td>4.9±0.5</td>
<td>5.5±0.6</td>
<td>4.4±0.4</td>
</tr>
<tr>
<td>SCD mRNA (ΔCt)</td>
<td>-14.2±0.5</td>
<td>-11.9±1.0</td>
<td>-13.4±0.7</td>
</tr>
<tr>
<td>Fasting plasma cholesterol (mg/dl)</td>
<td>195 ± 2.8</td>
<td>185 ± 10</td>
<td>186 ± 15</td>
</tr>
<tr>
<td>Fasting plasma triglycerides (mg/dl)</td>
<td>86.8 ± 10</td>
<td>111 ± 10</td>
<td>94.6 ± 22</td>
</tr>
<tr>
<td>Fasting free fatty acids (mmol/l)</td>
<td>0.63±0.07</td>
<td>0.44±0.04</td>
<td>0.44±0.08</td>
</tr>
</tbody>
</table>

SaO₂, oxyhemoglobin saturation; Δ SaO₂ – difference between baseline SaO₂ and average low SaO₂ during apnic events ; SCD, hepatic stearoyl coenzyme A desaturase; Ct, the critical threshold cycle; Δ Ct is the difference between 18S and SCD Ct values; *, †, and ‡ denote p < 0.05, p < 0.01, and p < 0.001, respectively, between two groups of patients.
Online Figure I. Fatty acid molecular species were identified via combined Gas chromatography–mass spectrometry (GC-MS) analysis. GC-MS was performed in plasma of C57BL/6J mice receiving SCD-1 or control ASO and exposed to CIH or IA for 10 weeks. The 16:1 (n-9)/16:0 (A) and the 18:1 (n-9)/18:0 (B) ratios of fatty acids were calculated for each condition. * denotes p < 0.05 for the difference between CIH and IA.

Online Figure II. Representative cross-sections of the ascending aorta (sinus of Valsalva) in C57BL/6J mice exposed to (A) intermittent air (IA) and control anti-sense oligonucleotides (ASO) injections, (B) chronic intermittent hypoxia (CIH) and control ASO, (C) IA and SCD-1 ASO, or (D) CIH and SCD-1 ASO. Transverse frozen sections of the aorta were stained with dihydroethidium (DHE) for 10 min. Original magnification: x100. (E) shows mean relative fluorescence of the aortic sections; * denotes p < 0.05 for the difference between CIH and IA.
Online Supplement

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A. Serum 16:1/16:0 fatty acid ratio (wt/wt)

- Control ASO
- SCD-1 ASO

B. Serum 18:1/18:0 fatty acid ratio (wt/wt)

- Control ASO
- SCD-1 ASO

* p < 0.001

Savransky et al. Online Figure I