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Abstract—Obstructive sleep apnea, a syndrome leading to recurrent intermittent hypoxia (IH), has been associated previously with hypercholesterolemia, independent of underlying obesity. We examined the effects of experimentally induced IH on serum lipid levels and pathways of lipid metabolism in the absence and presence of obesity. Lean C57BL/6J mice and leptin-deficient obese C57BL/6J-*Lep*^{ob} mice were exposed to IH for five days to determine changes in serum lipid profile, liver lipid content, and expression of key hepatic genes of lipid metabolism. In lean mice, exposure to IH increased fasting serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, phospholipids (PLs), and triglycerides (TGs), as well as liver TG content. These changes were not observed in obese mice, which had hyperlipidemia and fatty liver at baseline. In lean mice, IH increased sterol regulatory element binding protein 1 (SREBP-1) levels in the liver, increased mRNA and protein levels of stearoyl-coenzyme A desaturase 1 (SCD-1), an important gene of TG and PL biosynthesis controlled by SREBP-1, and increased monounsaturated fatty acid content in serum, which indicated augmented SCD-1 activity. In addition, in lean mice, IH decreased protein levels of scavenger receptor B1, regulating uptake of cholesterol esters and HDL by the liver. We conclude that exposure to IH for five days increases serum cholesterol and PL levels, upregulates pathways of TG and PL biosynthesis, and inhibits pathways of cholesterol uptake in the liver in the lean state but does not exacerbate the pre-existing hyperlipidemia and metabolic disturbances in leptin-deficient obesity. (*Circ Res.* 2005;97:698-706.)

Key Words: obstructive sleep apnea ■ cholesterol homeostasis ■ lipids ■ hypoxia ■ mouse ■ gene expression

Obstructive sleep apnea (SA) is the most common form of sleep-disordered breathing and is characterized by recurrent collapse of the upper airway during sleep, leading to periods of intermittent hypoxia (IH) and sleep fragmentation.¹ SA is present in 2% of women and 4% of men in the general US population, but it is more common in obese individuals.^{2,3} SA is an independent risk factor for increased cardiovascular morbidity and mortality.⁴⁻⁷ It has been postulated that metabolic dysfunction in SA may provide an intermediate step linking IH and sleep disturbances to cardiovascular disease. Although several recent studies have focused on the effects of SA on dysregulating glucose and insulin metabolism,^{2,8-10} little information is available about the impact of SA on lipid metabolism. Abnormalities in lipid regulation that occur in response to SA may act to increase the cardiovascular risk in an already susceptible population. Although obesity is one of the risk factors for elevations in total cholesterol (TC) and low-density lipoprotein (LDL) cholesterol levels,¹¹ recent clinical studies indicate that SA may also contribute to hypercholesterolemia.¹²⁻¹⁵ Thus, obesity and IH may interact to alter lipid metabolism in SA.

Key steps of lipid metabolism, including lipid biosynthesis, lipoprotein secretion, and reverse cholesterol transport, occur in the liver. Hypercholesterolemia may develop during IH as a consequence of accelerated lipid biosynthesis. Lipid biosynthesis in the liver is regulated by a family of transcription factors: sterol regulatory element binding proteins (SREBPs), which include SREBP-1a, SREBP-1c, and SREBP-2.¹⁶⁻¹⁸ SREBP-1a and SREBP-1c preferentially regulate enzymes of fatty acid synthesis, including acetyl coenzyme A (CoA) carboxylase,¹⁹ fatty acid synthase (FAS),²⁰ and stearoyl-CoA desaturase 1 (SCD-1).²¹ SREBP-2 regulates cholesterol biosynthesis and uptake, in particular, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and LDL receptor (LDLR).^{16-18,22,23} Although no previous studies have addressed whether SA can affect SREBP pathways, it is possible that IH may directly activate SREBP transcription factors in the liver to produce hyperlipidemia.

IH may also affect lipoprotein secretion and cholesterol clearance in the liver. Lipid transport into circulation is mediated mainly by apolipoprotein B (apoB).^{24,25} In turn, apoB traffic is facilitated by microsomal triglyceride (TG)

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TABLE 1. Body Weight, Daily Food Intake, Fasting Bilirubin, Leptin, Insulin, and Blood Glucose in Lean (C57BL/6J) and Obese (C57BL/6J-*Lep^{ob}*) Mice After Exposure to IH or IA for Five Days

	Lean Mice		Obese Mice	
	IA	IH	IA	IH
n	24	24	8	8
Age, weeks	13±1	13±1	14±0	14±0
Body weight, g	Day 0: 28.1±0.4 Day 5: 25.8±0.3*	Day 0: 28.0±0.3 Day 5: 25.8±0.3*	Day 0: 56.8±1.5 Day 5: 56.2±1.2	Day 0: 57.9±1.2 Day 5: 55.5±1.2*
Daily food intake, g	Day 0: 3.6±0.1 Day 5: 2.6±0.1*	Day 0: 3.6±0.1 Day 5: 2.7±0.1*	Day 0: 4.8±0.1 Day 5: 3.3±0.2*	Day 0: 4.6±0.1 Day 5: 3.2±0.1*
Serum bilirubin, mg/dL	0.48±0.07	0.34±0.12	0.39±0.11	0.42±0.08
Serum leptin, ng/mL	0.69±0.11	1.9±0.3†	NA	NA
Serum insulin, ng/mL	0.46±0.05	0.45±0.04	11.6±3.6	11.2±2.8
Blood glucose, mg/dL	158±11	172±11	233±21	290±28

Results presented show the mean±SEM. Statistical significance of the difference between day 0 and day 5 within each group of animals or between groups of animals exposed to IH and IA was determined by paired or unpaired *t* test, respectively. NA indicates not applicable.

**P*<0.01 for difference between day 0 and day 5 in the same group; †*P*<0.001 for difference between IH and IA.

transfer protein (MTP).^{26,27} Cholesterol clearance from circulation occurs via lipoprotein receptors. A key molecule of cholesterol uptake is the scavenger receptor B1 (SR-B1), which is an high-density lipoprotein (HDL) cholesterol receptor.²⁸ The effects of IH on lipoprotein secretion and cholesterol clearance pathways are unknown.

The purpose of the current study was to examine the effects of IH on lipid metabolism in the absence and presence of obesity. We hypothesized that IH would increase serum lipid levels, and that pre-existent hyperlipidemia in obesity may modify the response. We used a previously validated mouse model of IH and examined: (1) IH-induced changes in fasting serum lipid levels in lean and genetically obese mice; (2)

changes in liver lipid content in lean and obese mice exposed to IH; and (3) changes in lipid biosynthesis and lipoprotein secretion pathways and lipoprotein receptor expression in the livers of lean and obese mice exposed to IH.

Materials and Methods

A total of 80 wild-type, male, lean C57BL/6J mice (lean) and 16 male obese C57BL/6J-*Lep^{ob}* (obese) mice from Jackson Laboratory (Bar Harbor, Maine) were used in the study. The study was approved by the Johns Hopkins University animal use and care committee and complied with the American Physiological Society Guidelines for Animal Studies. A detailed description of the experimental methods is available in the online data supplement at <http://circres.ahajournals.org>.

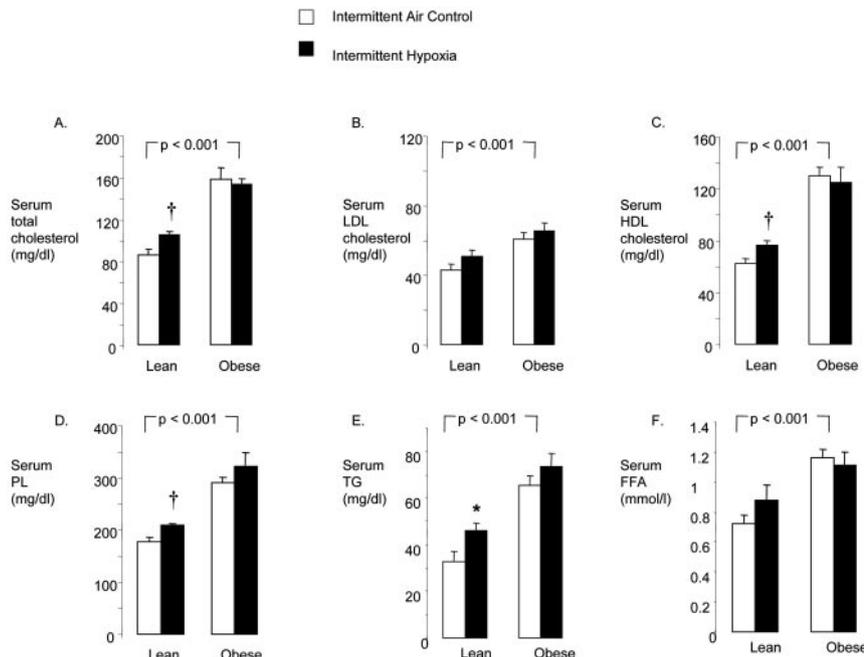


Figure 1. The effect of five days of IH or IA on fasting serum TC, LDL-C and HDL-C, PL, TG, and FFA levels in C57BL/6J (lean mice) and C57BL/6J-*Lep^{ob}* (obese mice). Results presented show the mean±SEM. Statistical significance of the difference between lean and obese mice was determined by general linear model ANOVA. Statistical significance of the difference between animals exposed to IH or IA was derived with unpaired *t* test. **P*<0.05 and †*P*<0.01 denote the difference between IH and IA within a strain.

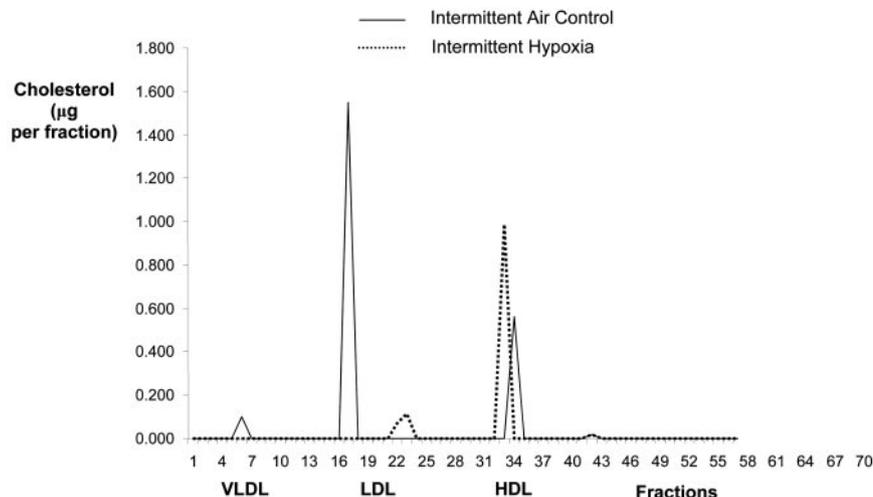


Figure 2. Characterization of serum lipoproteins in C57BL/6J (lean) mice after exposure to five days of IH or IA. Lipoproteins were examined by FPLC using AKTA prime (Amersham Bioscience), followed by cholesterol measurement by gas chromatography in each fraction. Each profile represents pooled serum from eight mice.

Results

Serum Lipid Levels in Lean and Obese Mice Exposed to IH

Exposure to IH led to weight loss and a decrease in food intake in lean and obese mice (Table 1). In lean mice, IH resulted in increases in fasting serum TC, HDL cholesterol (HDL-C), phospholipid (PL), and TG levels compared with weight-matched control animals exposed to intermittent air (IA); whereas changes in fasting levels of LDL cholesterol (LDL-C) and free fatty acids (FFAs) did not reach statistical significance (Figure 1). In obese leptin-deficient mice, fasting serum TC, LDL-C, HDL-C, PL, TG, and FFA levels were significantly higher than in lean

mice but were not affected by IH (Figure 1). IH did not increase serum total bilirubin levels, indicating that cholestasis did not play a role in the rise in serum cholesterol and PLs (Table 1). The fast protein liquid chromatography (FPLC) profile in pooled serum (Figure 2) confirmed that IH caused an increase in serum HDL-C, as determined by enzymatic assay (Figure 1), in lean mice. In IA control animals, LDL-C levels were determined by enzymatic assay and FPLC (Figures 1 and 2). In contrast, exposure to IH led to detection of a very small LDL-C peak by FPLC, which was right shifted, consistent with the hypoxia/reoxygenation of IH oxidizing LDL-C and masking its detection by FPLC²⁹ (Figure 2). IH also induced a 3-fold

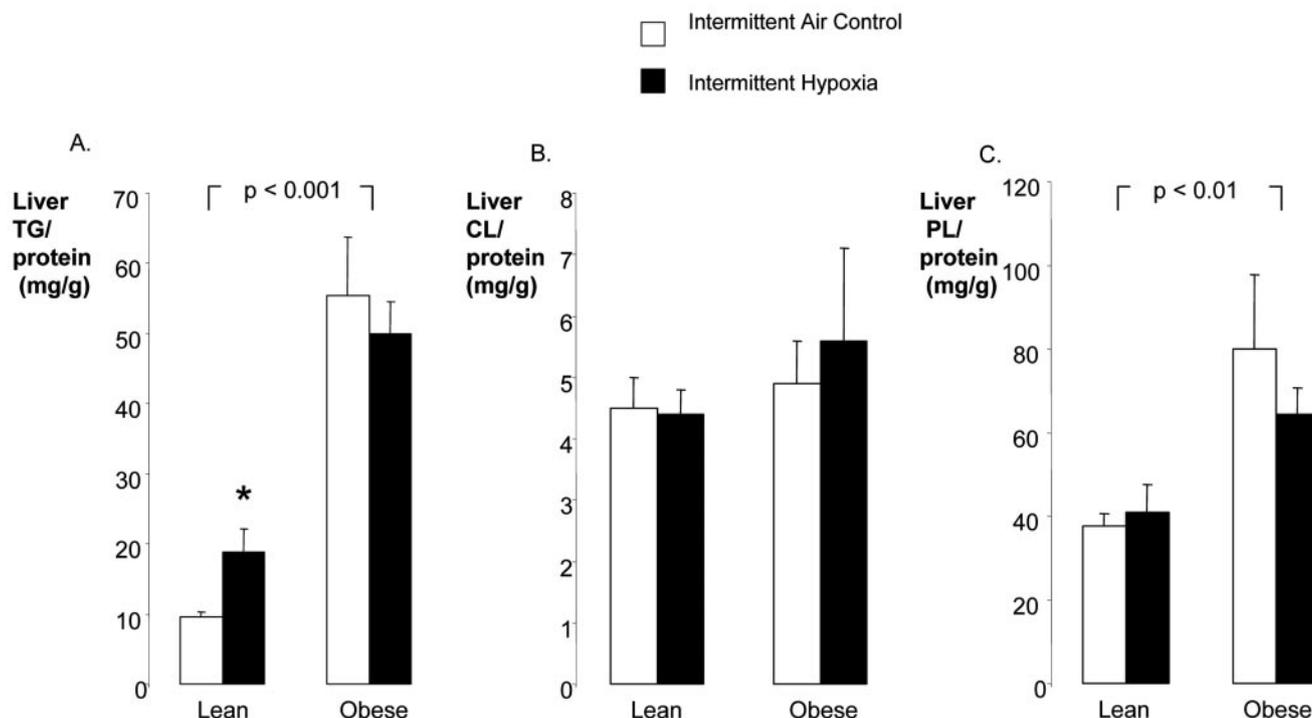


Figure 3. Liver TGs, cholesterol (CL), and PL content were normalized per protein concentration in the liver extract from C57BL/6J (lean) and C57BL/6J-*Lep*^{ob} (obese) mice exposed to either IH or IA for five days. Results presented show the mean ± SEM. Statistical significance was determined as in Figure 1.

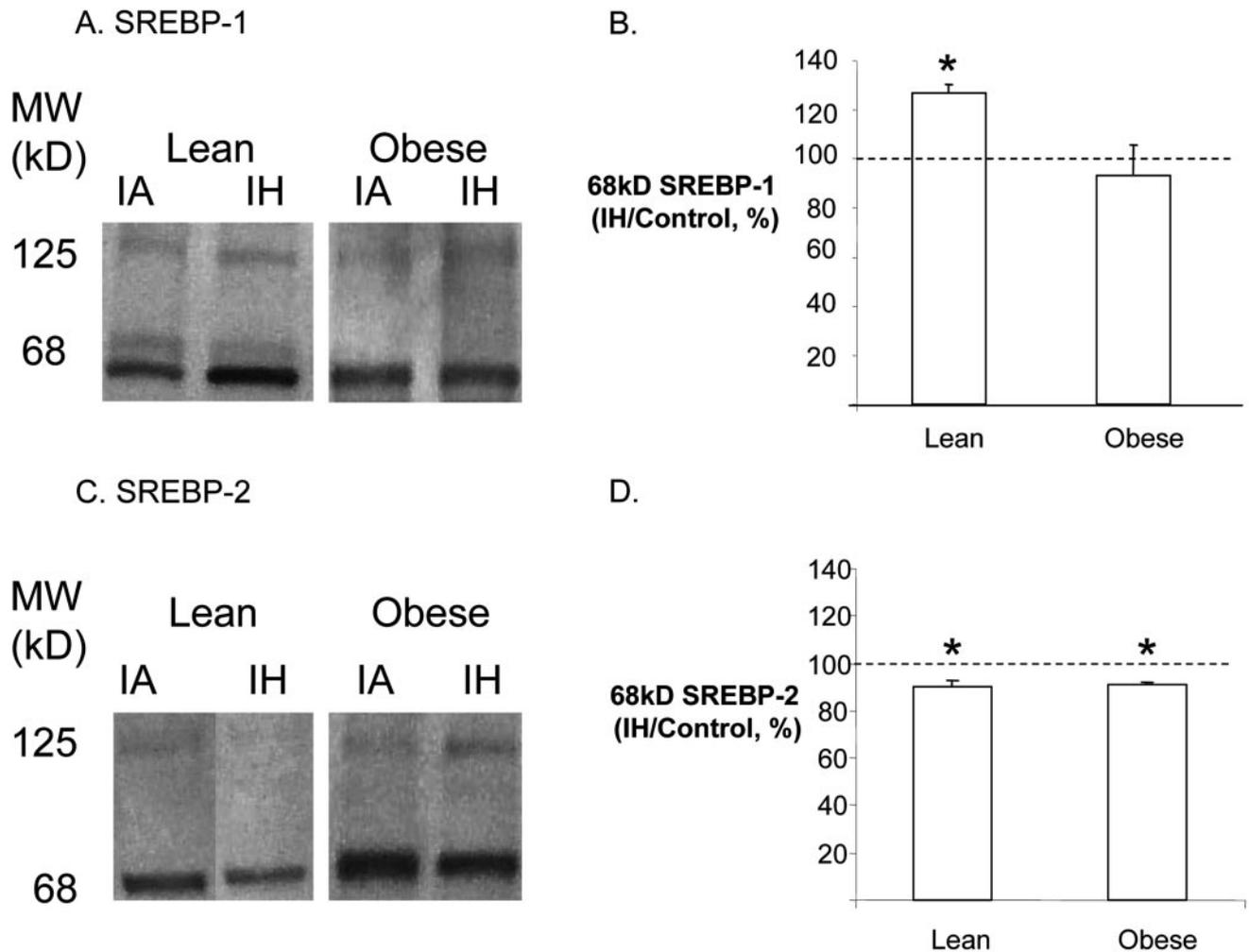


Figure 4. SREBP-1 (A and B) and SREBP-2 (C and D) in the whole cell lysate of the livers of lean and obese mice exposed to IH or IA for 5 days by Western blot. A and C, A representative sample from a control lean mouse (IA; lane 1) is compared with a representative sample from a lean mouse exposed to IH (lane 2). A representative sample from a control obese mouse (IA; lane 3) is compared with a representative sample from an obese mouse exposed to IH (lane 4). B and D, The ratios of optical density (OD) of 68-kDa bands of SREBP-1 (B) and SREBP-2 (D) per the same amount of total protein (70 μ g) were calculated between IH and pair-fed and weight-matched control mice as follows: SREBP (IH/control; %) = $100 \cdot \text{OD}_{\text{hypoxic mouse}} / \text{OD}_{\text{weight-matched control mouse}}$. Results presented show the mean \pm SEM. Statistical significance of the difference between animals exposed to IH or IA within a group was derived with unpaired *t* test. **P* < 0.05 for the difference between mice exposed to IH and IA within a strain.

increase in serum leptin levels in lean mice, whereas fasting serum insulin and glucose levels were unchanged in both strains (Table 1).

Liver Lipid Content in Lean and Obese Mice Exposed to IH

In lean mice, TG content of the liver was significantly elevated in response to hypoxia (18.8 ± 3.3 mg/g of protein versus 9.6 ± 0.7 mg/g of protein in control group; *P* < 0.05; Figure 3A), whereas cholesterol and PL content were not affected (Figure 3B and 3C). In obese mice, TG and PL content of the liver at baseline were significantly higher than in lean mice, whereas cholesterol content at baseline was similar to the level observed in lean mice (Figure 3). IH had no effect on lipid content in the livers of the obese animals (Figure 3).

Lipid Biosynthesis Pathways in the Liver of Lean and Obese Mice Exposed to IH

IH resulted in a significant increase of the 68-kDa active isoform of SREBP-1 in the livers of lean mice (Figure 4A). Compared with the control levels in lean mice, IH raised SREBP-1 levels by $26.7 \pm 4.0\%$ (*P* < 0.001; Figure 4B). In obese mice, IH did not affect SREBP-1 protein levels. Exposure to IH for 5 days led to a small but statistically significant decrease in the levels of the active isoform of SREBP-2 in the livers of lean mice ($9.6 \pm 2.6\%$ decrease; *P* < 0.01) and obese mice ($9.3 \pm 1.4\%$ decrease; *P* < 0.001; Figure 4C and 4D). The 125-kDa SREBP precursors were present at low levels in both strains of mice and were not affected by IH.

Expression analysis in the liver tissue revealed that a number of enzymes of lipid biosynthesis were upregulated in obese mice compared with lean mice under hypoxic and

TABLE 2 The Effect of IH on the Expression of Genes of Lipid Metabolism by Real-Time PCR in the Livers of Lean (C57BL/6J) and Obese (C57BL/6J-*Lep^{ob}*) Mice After Exposure to IH or IA for Five Days

Gene	Gene of Interest/18S Ratio					
	Lean Mice			Obese Mice		
	IA	IH	<i>P</i>	IA	IH	<i>P</i>
SREBP-1	0.49±0.09	0.58±0.05	>0.05	0.39±0.08	0.31±0.02	>0.05
FAS	0.09±0.02	0.11±0.02	>0.05	1.7±0.4*	2.0±0.3*	>0.05
Acetyl CoA synthetase	0.16±0.01	0.22±0.05	>0.05	0.91±0.10*	1.1±0.2*	>0.05
Malic enzyme	0.11±0.02	0.11±0.02	>0.05	0.72±0.13*	0.67±0.16*	>0.05
SCD-1	18.1±3.6	38.5±7.8	<0.05	54.1±3.5*	52.4±3.7	>0.05
GPAT	0.77±0.06	1.23±0.14	<0.01	0.79±0.09	1.62±0.17	<0.001
SR-B1	0.37±0.02	0.50±0.06	>0.05	0.14±0.02*	0.18±0.01*	>0.05
LDLR	0.14±0.01	0.17±0.01	>0.05	0.19±0.02	0.25±0.03	>0.05
SREBP-2	0.07±0.01	0.08±0.01	>0.05	0.09±0.02	0.10±0.01	>0.05
HMG-CoA reductase	0.18±0.04	0.20±0.04	>0.05	0.45±0.12*	0.44±0.08*	>0.05
ApoB	51.5±3.4	75.6±6.3	<0.01	67.1±8.2	76.3±9.2	>0.05
MTP	10.6±1.0	21.6±2.2	<0.001	20.9±1.9*	20.5±2.7	>0.05

Results presented show the mean±SEM. Statistical significance of the difference between day 0 and day 5 within each group of animals or between animals exposed to hypoxia and control conditions was derived with paired or unpaired *t* test, respectively.

**P*<0.05 for difference between obese and lean mice at the same conditions.

control conditions (Table 2). These genes included FAS, acetyl-CoA synthetase, malic enzyme, and HMG-CoA reductase. The increase in active SREBP-1 in the livers of lean mice exposed to IH was associated with upregulation of the important genes of TG and PL biosynthesis such as mitochondrial glycerol-3-phosphate acyltransferase (GPAT) and SCD-1 (Table 2). A >2-fold increase in SCD-1 mRNA in lean mice exposed to IH was particularly striking, given that IH did not affect SCD-1 expression in obese mice (Table 2). Furthermore, in lean mice, IH led to a 2-fold increase in SCD-1 protein levels (Figure 5A and 5E), whereas, in obese mice, SCD-1 protein levels were unchanged. In contrast to SCD-1, IH increased mitochondrial GPAT mRNA levels not only in lean but also in obese mice (Table 2). However, protein levels of GPAT in the mitochondrial fraction of the liver (Figure 5B and 5E) and GPAT activity were not affected by IH (supplemental Figure S2, available online at <http://circres.ahajournals.org>). IH did not affect expression of the key genes of the SREBP-2 pathway, SREBP-2, and HMG-CoA reductase in either lean or obese mice (Table 2).

Fatty Acid Biosynthesis and Composition

In a separate series of experiments, we examined fatty acid biosynthesis *in vivo* in lean mice after administration of D₂O. Gas chromatography–mass spectrometry (GC-MS) detected incorporation of deuterium (D) in serum fatty acids (Figure 6A). There was no difference in serum levels of deuterated fatty acids 16:0D₂, 18:0 D₂, and 18:0D₃ between IH and IA groups, suggesting that IH did not affect fatty acid biosynthesis *de novo*. In lean mice, IH changed the composition of circulating fatty acids increasing serum levels of monounsaturated species 16:1 and 18:1 (Figure 6B and 6C). In obese mice, IH did not affect serum levels of 16:1 fatty acid (Figure

6B); 18:1 levels were not determined because of technical limitations of the study.

Lipoprotein Receptors and Pathways of Lipoprotein Secretion in the Livers of Mice Exposed to IH

In lean mice, IH augmented expression of genes controlling lipoprotein secretion, MTP, and apoB (Table 2). MTP mRNA levels in the liver were increased 2-fold, and apoB mRNA levels were increased 1.5-fold. However, IH did not have a significant effect on lipid secretion, as had been demonstrated by Triton WR-1339 administration²⁵ (supplemental Figure S3). In obese mice, expression of MTP was significantly higher than in lean mice, and MTP and apoB were not altered by IH (Table 2).

In obese mice, baseline levels of SR-B1 mRNA were lower than in lean mice (Table 2). IH did not have a significant effect on expression of SR-B1 and LDLR mRNA in either strain of mice. IH induced a small but significant decrease in SR-B1 protein levels in the livers of lean mice (by 17.5±2.7%; *P*<0.001; Figure 5C and 5F) and did not affect SR-B1 protein levels in obese mice. IH increased LDLR protein levels in obese mice but not in lean mice (Figure 5D and 5F).

Discussion

SA is emerging as a major cardiovascular risk factor,^{4–7,30} but the mechanisms of increased cardiovascular morbidity and mortality in patients with SA are still unknown. The purpose of this study was to assess the impact of IH, a key clinical manifestation of SA, on serum lipid levels and the pathways of lipid metabolism in the presence and absence of obesity. Several new findings resulted from the study. First, exposure

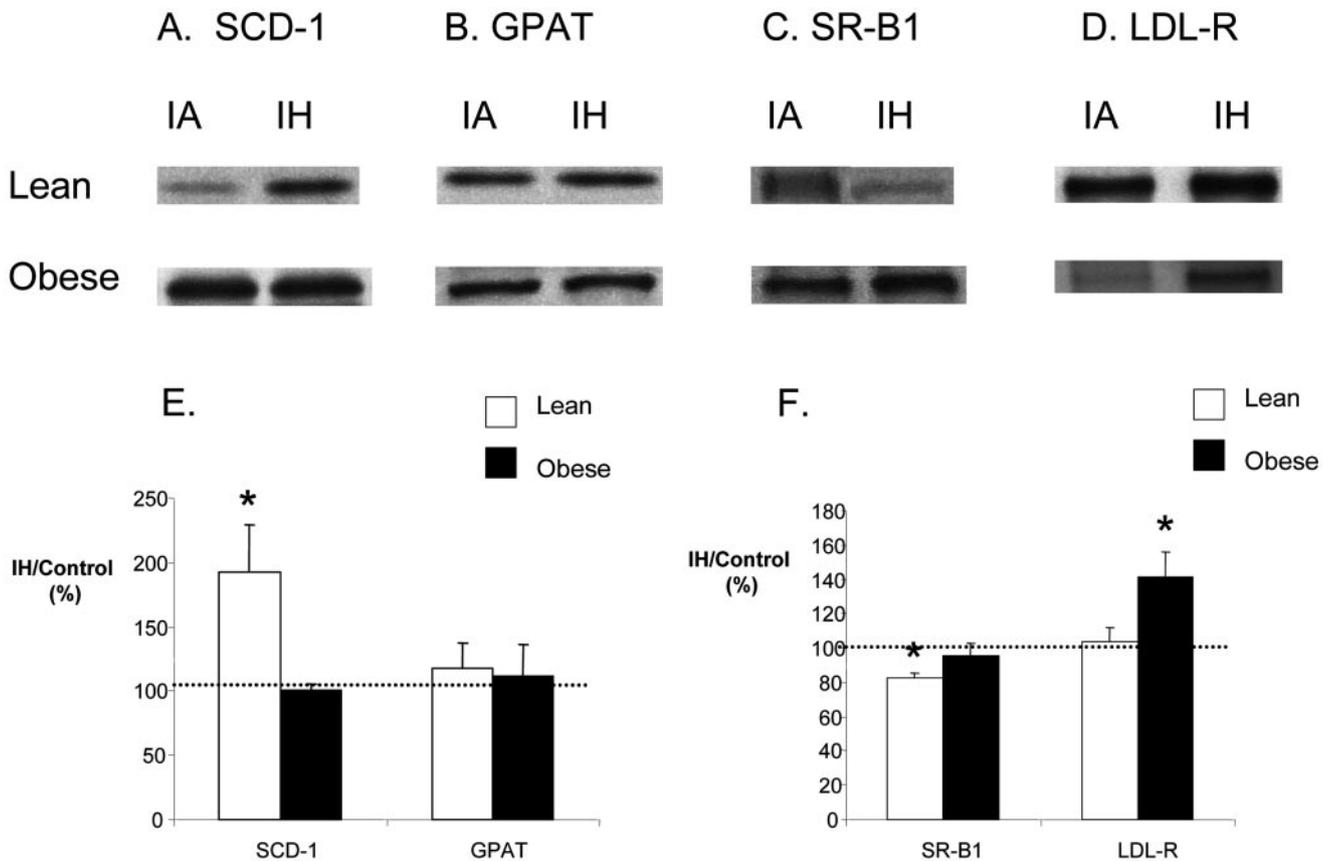


Figure 5. SCD-1 was determined in the microsomal fraction of the liver; mitochondrial GPAT was determined in the mitochondrial fraction of the liver (SR-B1); and LDLR was determined in the whole cell liver lysate. Western blot (see online supplement for the details). A through D, A representative sample from a control mouse (IA; lane 1) is compared with a representative sample from a mouse exposed to IH (lane 2). E and F, The ratios of optical density (OD) of the SCD-1 and GPAT bands (E), SR-B1, and LDLR per the same amount of total protein (70 μ g) were calculated between IH and pair-fed and weight-matched control mice as described for Figure 5.

to IH led to increases in fasting serum TC, HDL-C, PL, and TG levels in lean mice but not in leptin-deficient obese mice with hyperlipidemia at baseline. Second, exposure to IH led to increases in the liver TG content in lean mice but not in obese leptin-deficient mice. Third, in lean mice, but not in obese mice, IH increased protein levels of a key transcription factor of lipid biosynthesis in the liver: SREBP-1. Furthermore, in lean but not in obese mice, IH increased mRNA and protein levels as well as activity of SCD-1, an SREBP-1-regulated enzyme of lipid biosynthesis. Fourth, in lean but not in obese mice, IH decreased protein levels of the HDL receptor SR-B1, which is a regulator of cholesterol uptake by the liver. In this discussion, we explore the relationships and putative pathways linking lipid metabolism and IH and discuss the clinical implications of our work.

IH and Pathways of Lipid Biosynthesis

The enzymes that control lipid synthesis in the liver are coordinated by a family of SREBP transcription factors that act as master regulators.^{16–18,22} However, an increase in SREBP-1 levels in lean mice was not associated with increases in expression of FAS (Table 2) nor with an increase in fatty acid biosynthesis *de novo* (Figure 6A). Increases in GPAT mRNA (Table 2) induced by IH were not accompanied by increases in protein levels (Figure 5B and 5E) nor

enzymatic activity (supplemental Figure S2) and therefore did not carry physiological significance. On the other hand, upregulation of SREBP-1 in lean mice, a phenomenon not observed in obese mice, was coupled with increases in SCD-1 mRNA and protein levels (Table 2; Figure 5A and 5E). Furthermore, IH increased the proportion of monounsaturated fatty acids (MUFAs) in murine serum in lean but not in obese mice (Figure 6B and 6C), consistent with an increase in SCD-1 activity.^{31,32} MUFA is a biological substrate for synthesis of TG and PL^{31–33}; therefore, it is conceivable that increases in the liver TG and serum PL levels in lean mice exposed to IH are related to upregulation of SCD-1. Indeed, hepatic steatosis in *ob/ob* mice has been linked to increased SCD-1 expression and activity,³⁴ which is confirmed by our current data (Figure 6B; Table 2). On the other hand, the SCD-1 mutation leads to low liver TG, despite normal activity of other enzymes of lipid biosynthesis.³³ Thus, IH may upregulate lipid biosynthesis in the livers of lean mice via SCD-1. The pre-existing hepatosteatosis and high SCD-1 levels in *ob/ob* mice may offset any effects of IH.

The mechanisms of SREBP-1 and SCD-1 activation by IH are unclear. One possible mechanism of SREBP-1 activation is through insulin.³⁵ In the current study, serum insulin levels were unchanged, a finding that does not support the role of insulin in upregulation of SREBP-1 and SCD-1 in response to

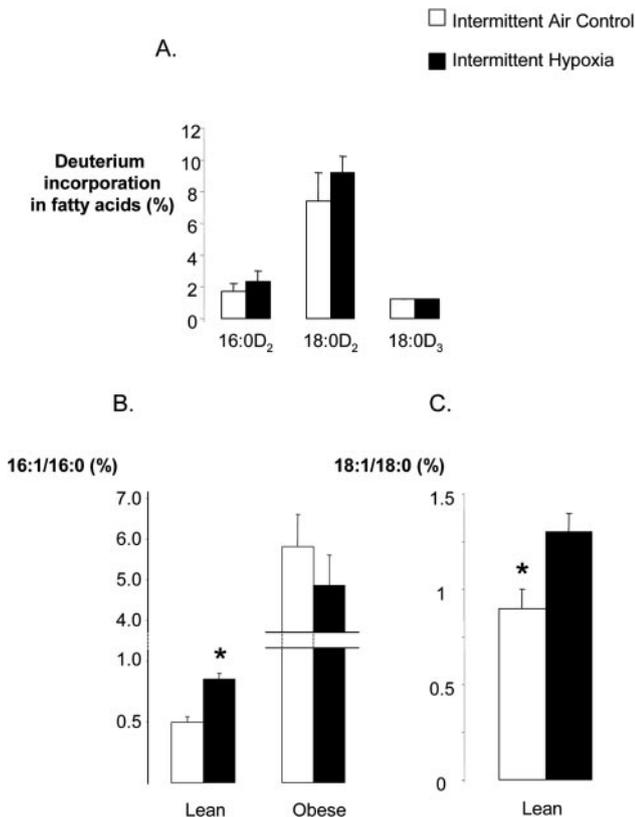


Figure 6. Fatty acid molecular species were identified via combined GC-MS analysis. A, GC-MS was performed in serum of lean C57BL/6J mice exposed to IH or IA control conditions for 5 days. Deuterated water (1 mL) was administered intraperitoneally to the mice, which were then fasted and water deprived for 5 hours. Incorporation of deuterium (D) in fatty acids was calculated as the ratio of deuterated higher molecular weight (MW) species of fatty acids to the native isoforms. Results presented show the mean \pm SEM. Statistical significance of the difference between animals exposed to IH or IA was examined by a two-way ANOVA. B and C, GS-MS was performed in serum of fasted lean C57BL/6J mice and obese C57BL/6J-*Lep^{ob}* exposed to IH or IA control conditions for 5 days. B, The 16:1/16:0 ratio of fatty acids was calculated for each strain and each condition. C, The 18:1/18:0 ratio was calculated in lean mice only. Results presented show the mean \pm SEM. Statistical significance of the difference between animals exposed to IH or IA within a group was derived by an unpaired *t* test.

IH. SREBP-1c can also be induced by elevated glucose levels, independent of insulin,³⁶ but our data did not show significant changes in blood glucose after IH (Table 1).

Another potential mechanism of SREBP-1 and SCD-1 induction by IH would be a decrease in leptin because SREBP-1c overexpression is associated with low leptin levels.³⁷ High levels of SCD-1 expression in leptin-deficient *ob/ob* mice are suppressed by leptin administration.³⁴ However, our current and previously published findings³⁸ indicate that IH increases serum leptin levels, which may be ascribed to the direct stimulating effects of hypoxia during the hypoxic phase³⁹ or to the relative hyperoxia during the reoxygenation phase.⁴⁰

Finally, SREBP-1 may be directly activated by hypoxia as it has been shown recently for SREBP homologs in yeast.⁴¹ Summarizing all of the above, we propose that IH enhances

TG biosynthesis in the liver of lean mice via an SREBP-1-mediated increase in SCD-1 expression.

In contrast to TG biosynthesis, our data do not support a role for IH in increasing cholesterol biosynthesis. Hepatic cholesterol synthesis is predominantly regulated by the SREBP-2 transcription factor.^{16–18,22,23} However, hypercholesterolemia in lean mice exposed to IH was not accompanied by increases in SREBP-2 or HMG-CoA reductase levels in the liver. Therefore, it is likely that IH leads to hypercholesterolemia via mechanisms other than biosynthesis *de novo*.

IH and Pathways of Cholesterol Uptake and Lipoprotein Secretion

We have shown that IH led to significant increases in serum TC and HDL-C levels. The elevation in circulating HDL-C was demonstrated by enzymatic assays and FPLC (Figures 1 and 2). IH did not affect serum LDL-C according to the enzymatic assay results (Figure 1), whereas FPLC was unable to detect a relevant peak for LDL-C in mice exposed to IH (Figure 2). The inability to detect significant LDL-C may be related to the presence of a largely oxidized form of LDL-C in mice exposed to IH,^{42–44} which would alter the FPLC profile for LDL-C.²⁹ One possible explanation for the increase in HDL-C is that the clearance of cholesterol from the circulation was impaired by IH. A major pathway of cholesterol clearance from the bloodstream is the SR-B1, originally described as an HDL receptor.²⁸ SR-B1 affinity for HDL is mediated via cholesterol esters²⁸ and apoA-I⁴⁵ in HDL particles. The SR-B1-deficient mouse has an elevation of HDL-C levels.⁴⁶ In the current study, we observed that IH induced a decline in SR-B1 protein levels in the livers of lean mice without any change in LDL-R levels (Figure 5C, 5D, and 5F). Notably, SR-B1 can also mediate reuptake of PLs,⁴⁷ which may contribute to an increase in circulating PLs in an analogous manner to cholesterol. Thus, a decrease in SR-B1 levels in response to IH would be consistent with our observed increase in circulating levels of HDL-C.

In *ob/ob* mice, IH induced an increase in LDLR protein levels without any change in SR-B1. Upregulation of LDLR did not induce any decrease in circulating cholesterol, probably because of low baseline expression of SR-B1 (Table 2).⁴⁸ The low expression of SR-B1 in leptin-deficient mice results in a phenotype of elevated HDL and LDL-C,^{48,49} which was confirmed by our data (Figure 1). Thus, pre-existing downregulation of SR-B1 in *ob/ob* mice may offset any suppressive effects of IH on cholesterol reuptake by the liver.

IH also increased the expression of key genes of lipoprotein secretion: apoB and MTP (Table 2). However, IH did not affect the rate of lipid secretion into the bloodstream (supplemental Figure S3), indicating that upregulation of genes of lipoprotein secretion did not have physiological significance. Our data are consistent with the previous observations that lipoprotein secretion could be regulated at the post-transcriptional level.⁵⁰ Thus, hypercholesterolemia that we observed in lean mice exposed to IH may be related to perturbations in the mechanisms of cholesterol clearance rather than lipoprotein secretion.

Clinical Implications

Our data suggest that the stimulus of IH that characterizes SA may contribute to the development of hypercholesterolemia. Consequently, the increases in cardiovascular morbidity and mortality in SA may be related, in part, to elevation in serum cholesterol levels. Unlike clinical studies, suggesting that SA is associated with decreases in HDL-C,^{12,13,15} we found that IH can lead to elevations in HDL cholesterol in mice, which may be related to physiological differences in cholesterol processing between species. However, IH may change properties of lipoprotein and convert HDL from anti-inflammatory antiatherogenic to proinflammatory proatherogenic factors.^{43,51} Hypercholesterolemia in patients with SA may be particularly detrimental because SA leads to increased lipid peroxidation.^{42,43,52} Oxidized LDL and PLs are taken up by macrophages more readily leading to macrophage foaming and progression of atherosclerosis.^{53,54}

Conclusion

We have shown that IH increases fasting serum cholesterol, TG, and PL levels as well as liver TG content in lean mice, but that obesity and baseline hypercholesterolemia can mask the effects of IH on lipid metabolism. Furthermore, our data suggest that hyperlipidemia after IH may be related to changes in pathways of lipid biosynthesis and cholesterol uptake in the liver.

Acknowledgments

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References

- Gastaut H, Tassinari CA, Duron B. [Polygraphic study of diurnal and nocturnal (hypnic and respiratory) episodic manifestations of Pickwick syndrome]. *Rev Neurol (Paris)*. 1965;112:568–579.
- Punjabi NM, Sorkin JD, Katzell LI, Goldberg AP, Schwartz AR, Smith PL. Sleep-disordered breathing and insulin resistance in middle-aged and overweight men. *Am J Respir Crit Care Med*. 2002;165:677–682.
- Young T, Palta M, Dempsey J, Skatrud J, Weber S, Badr S. The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med*. 1993;328:1230–1235.
- Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD, Pickering TG. Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study. *J Am Med Assoc*. 2000;283:1829–1836.
- Peppard PE, Young T, Palta M, Skatrud J. Prospective study of the association between sleep-disordered breathing and hypertension. *N Engl J Med*. 2000;342:1378–1384.
- Shahar E, Whitney CW, Redline S, Lee ET, Newman AB, Javier NF, O'Connor GT, Boland LL, Schwartz JE, Samet JM. Sleep-disordered breathing and cardiovascular disease: cross-sectional results of the Sleep Heart Health Study. *Am J Respir Crit Care Med*. 2001;163:19–25.
- Moore T, Franklin KA, Holmstrom K, Rabben T, Wiklund U. Sleep-disordered breathing and coronary artery disease. Long-term prognosis. *Am J Respir Crit Care Med*. 2001;164:1910–1913.
- Harsch IA, Schahin SP, Radespiel-Troger M, Weintz O, Jahreiss H, Fuchs FS, Wiest GH, Hahn EG, Lohmann T, Konturek PC, Ficker JH. Continuous positive airway pressure treatment rapidly improves insulin sensitivity in patients with obstructive sleep apnea syndrome. *Am J Respir Crit Care Med*. 2004;169:156–162.
- Ip MS, Lam B, Ng MM, Lam WK, Tsang KW, Lam KS. Obstructive sleep apnea is independently associated with insulin resistance. *Am J Respir Crit Care Med*. 2002;165:670–676.
- Punjabi NM, Ahmed MM, Polotsky VY, Beamer BA, O'Donnell CP. Sleep-disordered breathing, glucose intolerance, and insulin resistance. *Respir Physiol Neurobiol*. 2003;136:167–178.
- Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel Spaceiiqq) final report. *Circulation*. 2002;106:3143–3421.
- Newman AB, Nieto FJ, Guidry U, Lind BK, Redline S, Pickering TG, Quan SF. Relation of sleep-disordered breathing to cardiovascular disease risk factors: the Sleep Heart Health Study. *Am J Epidemiol*. 2001;154:50–59.
- Chin K, Shimizu K, Nakamura T, Narai N, Masuzaki H, Ogawa Y, Mishima M, Nakao K, Ohi M. Changes in intra-abdominal visceral fat and serum leptin levels in patients with obstructive sleep apnea syndrome following nasal continuous positive airway pressure therapy. *Circulation*. 1999;100:706–712.
- Robinson GV, Pepperell JC, Segal HC, Davies RJ, Stradling JR. Circulating cardiovascular risk factors in obstructive sleep apnea: data from randomized controlled trials. *Thorax*. 2004;59:777–782.
- Chin K, Nakamura T, Shimizu K, Mishima M, Nakamura T, Miyasaka M, Ohi M. Effects of nasal continuous positive airway pressure on soluble cell adhesion molecules in patients with obstructive sleep apnea syndrome. *Am J Med*. 2000;109:562–567.
- Shimano H. Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res*. 2001;40:439–452.
- Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc Natl Acad Sci U S A*. 1998;95:5987–5992.
- Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002;109:1125–1131.
- Magana MM, Lin SS, Dooley KA, Osborne TF. Sterol regulation of acetyl coenzyme A carboxylase promoter requires two interdependent binding sites for sterol regulatory element binding proteins. *J Lipid Res*. 1997;38:1630–1638.
- Magana MM, Osborne TF. Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty-acid synthase promoter. *J Biol Chem*. 1996;271:32689–32694.
- Tabor DE, Kim JB, Spiegelman BM, Edwards PA. Identification of conserved cis-elements and transcription factors required for sterol-regulated transcription of stearoyl-CoA desaturase 1 and 2. *J Biol Chem*. 1999;274:20603–20610.
- Sheng Z, Otani H, Brown MS, Goldstein JL. Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. *Proc Natl Acad Sci U S A*. 1995;92:935–938.
- Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. *J Clin Invest*. 1997;100:2115–2124.
- Stalenhoef AF, Malloy MJ, Kane JP, Havel RJ. Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in patients with familial dysbetalipoproteinemia. *J Clin Invest*. 1986;78:722–728.
- Li X, Catalina F, Grundy SM, Patel S. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J Lipid Res*. 1996;37:210–220.
- Bartels ED, Lauritsen M, Nielsen LB. Hepatic expression of microsomal triglyceride transfer protein and in vivo secretion of triglyceride-rich lipoproteins are increased in obese diabetic mice. *Diabetes*. 2002;51:1233–1239.
- Hussain MM, Iqbal J, Anwar K, Rava P, Dai K. Microsomal triglyceride transfer protein: a multifunctional protein. *Front Biosci*. 2003;8:s500–s506.
- Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996;271:518–520.
- Vedie B, Myara I, Pech MA, Maziere JC, Maziere C, Caprani A, Moatti N. Fractionation of charge-modified low density lipoproteins by fast protein liquid chromatography. *J Lipid Res*. 1991;32:1359–1369.

30. Gami AS, Howard DE, Olson EJ, Somers VK. Day-night pattern of sudden death in obstructive sleep apnea. *N Engl J Med*. 2005;352:1206–1214.
31. Ntambi JM. Regulation of stearyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J Lipid Res*. 1999;40:1549–1558.
32. Ntambi JM, Miyazaki M. Regulation of stearyl-CoA desaturases and role in metabolism. *Prog Lipid Res*. 2004;43:91–104.
33. Miyazaki M, Kim YC, Gray-Keller MP, Attie AD, Ntambi JM. The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearyl-CoA desaturase 1. *J Biol Chem*. 2000;275:30132–30138.
34. Cohen P, Miyazaki M, Socci ND, Hagge-Greenberg A, Liedtke W, Soukas AA, Sharma R, Hudgins LC, Ntambi JM, Friedman JM. Role for stearyl-CoA desaturase-1 in leptin-mediated weight loss. *Science*. 2002;297:240–243.
35. Foretz M, Pacot C, Dugail I, Lemarchand P, Guichard C, Le Liepvre X, Berthelie-Lubrano C, Spiegelman B, Kim JB, Ferre P, Foufelle F. ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol Cell Biol*. 1999;19:3760–3768.
36. Guillet-Deniau I, Pichard AL, Kone A, Esnous C, Nieruchalski M, Girard J, Prip-Buus C. Glucose induces de novo lipogenesis in rat muscle satellite cells through a sterol-regulatory-element-binding-protein-1c-dependent pathway. *J Cell Sci*. 2004;117:1937–1944.
37. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature*. 1999;401:73–76.
38. Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP. Intermittent hypoxia increases insulin resistance in genetically obese mice. *J Physiol*. 2003;552:253–264.
39. Ambrosini G, Nath AK, Sierra-Honigmann MR, Flores-Riveros J. Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. *J Biol Chem*. 2002;277:34601–34609.
40. Barazzone-Argiroffo C, Muzzin P, Donati YR, Kan CD, Aubert ML, Piguat PF. Hyperoxia increases leptin production: a mechanism mediated through endogenous elevation of corticosterone. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L1150–L1156.
41. Hughes AL, Todd BL, Espenshade PJ. SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. *Cell*. 2005;120:831–842.
42. Barcelo A, Miralles C, Barbe F, Vila M, Pons S, Agusti AG. Abnormal lipid peroxidation in patients with sleep apnea. *Eur Respir J*. 2000;16:644–647.
43. Tan KC, Chow WS, Lam JC, Lam B, Wong WK, Tam S, Ip MS. HDL dysfunction in obstructive sleep apnea. *Atherosclerosis*. In press.
44. Rydberg EK, Krettek A, Ullstrom C, Ekstrom K, Svensson PA, Carlsson LM, Jonsson-Rylander AC, Hansson GI, McPheat W, Wiklund O, Ohlsson BG, Hulten LM. Hypoxia increases LDL oxidation and expression of 15-lipoxygenase-2 in human macrophages. *Arterioscler Thromb Vasc Biol*. 2004;24:2040–2045.
45. Xu S, Laccotripe M, Huang X, Rigotti A, Zannis VI, Krieger M. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J Lipid Res*. 1997;38:1289–1298.
46. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A*. 1997;94:12610–12615.
47. Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem*. 1995;270:16221–16224.
48. Lundasen T, Liao W, Angelin B, Rudling M. Leptin induces the hepatic high density lipoprotein receptor scavenger receptor B type I (SR-BI) but not cholesterol 7 α -hydroxylase (Cyp7a1) in leptin-deficient (ob/ob) mice. *J Biol Chem*. 2003;278:43224–43228.
49. Silver DL, Jiang XC, Tall AR. Increased high density lipoprotein (HDL), defective hepatic catabolism of apoA-I and apoA-II, and decreased apoA-I mRNA in ob/ob mice. Possible role of leptin in stimulation of HDL turnover. *J Biol Chem*. 1999;274:4140–4146.
50. Ko C, O'Rourke SM, Huang LS. A fish oil diet produces different degrees of suppression of apoB and triglyceride secretion in human apoB transgenic mouse strains. *J Lipid Res*. 2003;44:1946–1955.
51. Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, La Du BN, Fogelman AM, Navab M. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest*. 1995;96:2758–2767.
52. Lavie L, Vishnevsky A, Lavie P. Evidence for lipid peroxidation in obstructive sleep apnea. *Sleep*. 2004;27:123–128.
53. Palinski W, Witztum JL. Immune responses to oxidative neopeptides on LDL and phospholipids modulate the development of atherosclerosis. *J Intern Med*. 2000;247:371–380.
54. Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugli B, Fox PL, Hoff HF, Salomon RG, Hazen SL. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J Biol Chem*. 2002;277:38503–38516.

INTERMITTENT HYPOXIA INDUCES HYPERLIPIDEMIA IN LEAN MICE

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On-line Supplement

METHODS

Animals

A total of 80 wildtype, male, lean C57BL/6J mice (lean) and 16 male obese C57BL/6J-*Lep*^{ob} (obese) mice from Jackson Laboratory (Bar Harbor, ME, USA) were used in the study. The study was approved by the Johns Hopkins University Animal Use and Care Committee and complied with the American Physiological Society Guidelines for Animal Studies. For all blood samples, injections and surgical procedures, anesthesia was induced and maintained with 1-2 % isoflurane administered through a facemask.

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 it was previously described¹. A maximum of three lean mice or two obese mice were housed continuously in a single customized cage (dimensions 27 X 17 X 17 cm) with constant access to food

and water. Animals were acclimated to the cage for a week prior to the exposure, and lean mice and obese mice were always housed separately. A series of programmable solenoids and flow regulators altered the inspired oxygen fraction (FIO₂) over a defined and repeatable profile that simulated the timing and magnitude of arterial oxygen desaturation changes seen in SA patients². During each period of intermittent hypoxia (IH), the FIO₂ was reduced from 20.9 to 4.8-5.0 % over a 30 s period and then rapidly reoxygenated to room air levels in the subsequent 30 s period (Fig. S1). The use of multiple inputs into the cage produced a uniform nadir FIO₂ level throughout the cage.

Twenty four lean mice and eight obese mice were placed in the IH chamber for five consecutive days. Food intake and body weight were monitored daily for each animal. All animals were kept in a controlled environment (22-24 °C with a 12 h : 12 h light : dark cycle; lights on at 09.00) on a standard chow diet with free access to water. In a separate series of animals, twenty four lean and eight obese mice were exposed to intermittent room air (IA, control groups) for 5 days in identical chambers and were weight-matched to the IH group daily during the experiment by varying food intake (Table 1). IA was administered with the intermittent flow of air at identical rates to the IH exposure every 30 sec with FIO₂ remaining at 20.9% throughout the exposure. Weight-matching was conducted in pairs. 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

All serum and tissue samples were obtained during exposure to IH or control conditions. Mice fasted for 5 hrs prior to bleeding and sacrifice, from 9am until 2pm on the 6th day of exposure. Arterial blood (1 ml) was obtained by direct cardiac puncture exposure under 1-2 % isoflurane anesthesia in all mice. Serum was separated and frozen at -80° C. After blood withdrawal, the animals were euthanized with pentobarbital (60 mg, intraperitoneally). In thirty two lean mice (sixteen mice subjected to IH and sixteen control mice) and all obese mice, livers were surgically removed, dissected in four separate aliquots and immediately frozen for future analysis.

Serum and Liver Lipids, Blood Glucose, Serum Insulin and Leptin

Serum total cholesterol, LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), phospholipids (PL), free fatty acids (FFA), triglycerides (TG) were measured in all mice, and bilirubin was measured in sixteen lean (eight mice subjected to IH and eight control mice) and all obese mice using test kits from Wako Diagnostics, Inc. (Richmond, VA). The first portion of the liver was homogenized using Omni EZ Connect Homogenizer (Omni International, Warrington, VA); cholesterol, PLs and TG were extracted in a chloroform-methanol mixture (2:1) as described by Yokode et al.³ and measured using kits from Wako Diagnostics. Glucose was measured in blood of all mice using Accu-Chek® Comfort Curve™ kit from Roche Diagnostics, Inc. (Indianapolis, IN). Serum leptin levels were measured in all mice with a mouse leptin radioimmunoassay kit from

Linco Research, Inc. (St. Charles, MO). Serum insulin levels were measured in all mice with a rat insulin ultrasensitive radioimmunoassay kit (cross reactivity with mouse insulin 100%) from Linco Research, Inc. (St. Charles, MO).

Fast Protein Liquid Chromatography (FPLC)

Serum samples from lean mice fasted for 5 hrs were pooled in the following manner: the IH group (n = 8, sample 1), the IA control group (n = 8, sample 2). Lipoproteins were isolated by fast protein liquid chromatography (FPLC) using the AKTA prime (Amersham Bioscience, Piscataway , NJ) as previously described⁴. In brief, 400 μ L of serum was loaded on two Superose 6 (Amersham Bioscience. Piscataway , NJ) columns in series and eluted with PBS (Phosphate Buffer Saline) pH 7.4 to which EDTA 0.02 % was added at a flow rate of 400 μ L/min. The column eluate were detected on line at 280 nm and collected in fractions of 500 μ L. Total cholesterol concentration was measured using the gas chromatographer GC-2010 (Shimadzu, Japan) in each column fraction concentrated by Bligh- Dyer extraction⁵.

Immunoblot in the Liver Tissue

The second portion of the liver was homogenized in 150 mM NaCl 20 mM Tris, pH 7.2 1% Triton X-100 1 mM DTT to obtain the whole cell lysate. The third portion of the liver was homogenized in 250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT , and separated in the nuclear, mitochondrial, and microsomal fractions as previously described⁶. Total protein was measured using a D_C kit from BioRad (Hercules, CA). Protein gel electrophoresis of the whole cell lysate, mitochondrial, and

microsomal fractions of the liver was performed using 4-15% SDS-PAGE from BioRad (Hercules, CA) loaded with 70 µg of total protein per a lane and was followed by Western blot. SREBP-1 and SREBP-2 were determined in the whole cell lysate by Western blot using rabbit anti-mouse polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), SCD-1 was determined in the microsomal fraction using goat antibodies from Santa Cruz , GPAT was determined in the mitochondrial fraction using rabbit antibodies kindly donated by Dr. Sandra Schreyer (AstraZeneca, Göteborg, Sweden), LDL-R was determined in the whole cell lysate using rabbit antibodies kindly donated by Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX), and SR-B1 was measured using rabbit anti-mouse polyclonal antibodies from Novus Biologicals (Littleton, CO). An anti-SREBP-1 antibody had affinity for both SREBP-1a and SREBP-1c. Goat anti-rabbit-HRP conjugate was from BioRad, bovine anti-goat-HRP conjugate was from SantaCruz, and ECL system was from Amersham (Piscataway, NJ). Densitometry was performed using Kodak DC290 ZOOM digital camera 2,150,000 pixels (2 Megapixel) and UN-SCAN-IT gel Automated Digitizing System, VERSION 5.1 software from Silk Scientific Corporation (Orem, VT). For the semi-quantitative analysis, optical density of the band from a mouse subjected to IH was normalized to the band from the weight-matched control mouse, which was assumed to be 100%.

The ratios of optical density (OD) per the same amount of total protein (70 µg) were calculated as follows:

$$\text{IH/control (\%)} = 100 * \text{OD}_{\text{hypoxic mouse}} / \text{OD}_{\text{weight-match control mouse}}$$

Real time PCR in the Liver Tissue

The fourth portion of the liver was used for total RNA extraction by Trizol method (Life Technologies, Rockville, MD) with subsequent cDNA synthesis using Advantage RT for PCR kit from Clontech (Palo Alto, CA) and real time reverse transcriptase PCR (RT-PCR) with primers from Invitrogen (Carlsbad, CA) and Taqman probes from Applied Biosystems (Foster City, CA, see Table S1)). The threshold cycle (Ct) was determined for every sample. The mRNA expression levels were normalized to 18S rRNA concentrations using the following formula:

$$\text{Gene of interest}/18\text{S} = 2^{\text{Ct}(18\text{S})-\text{Ct}(\text{Gene of Interest})}$$

Fatty Acid Biosynthesis in Vivo

In a separate series of experiments male lean C57BL/6J mice (13-14 weeks old, 27-28 g of weight) were exposed to IH (n = 8) or IA control conditions with daily weight matching (n = 8) for five days. Prior to bleeding, mice were fasted and water-deprived for 5 hrs. Deuterated water (D₂O, Cambridge Isotope Laboratories, Andover MA, 1 ml) was administered intraperitoneally 5 hrs prior to bleeding. The mice were removed from IH or IA environment, bled by cardiac puncture under 1-2% isoflurane anesthesia, and euthanized. Serum was separated and analyzed by gas chromatography and mass spectroscopy (GCMS).

Gas Chromatography and Mass Spectroscopy(GC-MS)

Palmitoleic acid (16:1), palmitic acid (16:0), oleic acid (18:1) and stearic acid (18:0) standards were from Sigma (St. Louis, MO), trideuterated-stearic acid (18:0 D₃)

was from Cambridge Isotope Laboratories (Andover, MA), hexane was from Fisher Scientific (Pittsburgh, PA), acetonitrile, dodecane and N-O-(bistrifluoromethylfluoro)-acetamide (BSTFA) were from Aldrich (Milwaukee, WI).

Serum (5.0 μ l) was mixed with fatty acid standards, 1N HCl (0.5 ml) and hexane (0.75 ml) and centrifuged at 7500 g for 5'. The upper (organic) phase was dried under a stream of N₂. The trimethylsilyl (TMS) ester was synthesized by adding BSTFA (50 μ l) and acetonitrile (25 μ l). TMS esters were dissolved in dodecane (10 μ l) and analyzed by GC-MS using a Varian 3400 for GC and a Finnigan MAT® TSQ700 for MS. The molecular species were monitored for detection of the fatty acids are listed in Table S2. Incorporation of deuterium in fatty acids was calculated as a ratio of higher molecular weight species of fatty acids to the native isoforms after subtraction of background.

Mitochondrial GPAT Activity

GPAT activity was determined as previously described⁶. All reagents were from Sigma, unless indicated otherwise. Briefly, the assay buffer contained 75 mM Tris, pH 7.5, 4 mM MgCl₂, 8 mM NaF, 100 μ mol palmitoyl-CoA, 500 μ mol ¹⁴C glycerol-3-phosphate (Amersham), and 2 mg/ml BSA was prepared. The mitochondrial fraction of the liver (15 μ g) was mixed with 2 mM of N-ethylmaleimide (Sigma) and incubated for 15 min at 4C. The volume of the sample was brought to 0.2 ml with the assay buffer. After 20 min incubation in a shaker at room temperature, water-saturated butanol was added (1 ml). The sample was vortexed vigorously. The butanol phase was saved. The extraction with butanol was repeated three times. The combined butanol phase was counted in the

scintillation counter Beckman LS6000 SC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Triglyceride Secretion

Triglyceride secretion was determined as previously described^{7,8}. Briefly, in a separate experiment, lean male C57BL/6J mice (13 weeks old, 24-27 g) were exposed to IH (n = 8) or IA control conditions with daily weight matching (n = 8) for five days. At the end of the exposure, mice were fasted for 5 hrs, bled by a retro-orbital sinus puncture (50 μ l) under 1-2% isoflurane anesthesia and then injected into the tail vein with Triton WR-1339 (Sigma), 12.5 mg in 100 μ l of 0.9% NaCl. After the injection, mice were returned to the IH or control chamber. Mice were bled by a retro-orbital sinus puncture (50 μ l) 1 hr, 2, 3, and 4 hrs after the Triton injection and then euthanized. Serum TG levels were measured using test kits from Wako Diagnostics, Inc. (Richmond, VA).

Statistical Analyses

All values are reported as mean \pm standard error of the mean (SEM). Comparisons within and between the IH and IA groups of obese and lean mice were performed using ANOVA, paired *t-test* (between day 0 and day 5 within a group) or unpaired *t-test* (between groups). A repeated measures ANOVA was performed in the Triton experiment. A p-value of less than 0.05 was considered significant.

Reference List

1. Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP. Intermittent hypoxia increases insulin resistance in genetically obese mice. *J Physiol.* 2003;552:253-264.
2. Tagaito Y, Polotsky VY, Campen MJ, Wilson JA, Balbir A, Smith PL, Schwartz AR, O'Donnell CP. A model of sleep-disordered breathing in the C57BL/6J mouse. *J Appl Physiol.* 2001;91:2758-2766.
3. Yokode M, Hammer RE, Ishibashi S, Brown MS, Goldstein JL. Diet-induced hypercholesterolemia in mice: prevention by overexpression of LDL receptors. *Science.* 1990;250:1273-1275.
4. Marz W, Siekmeier R, Scharnagl H, Seiffert UB, Gross W. Fast lipoprotein chromatography: new method of analysis for plasma lipoproteins. *Clin Chem.* 1993;39:2276-2281.
5. Bligh, E. G. and W.J.Dyer. A rapid method of total lipid extraction and purification. *Can.J.Biochem.Physiol.* 37, 911-917. 1959.
6. Linden D, William-Olsson L, Rhedin M, Asztely AK, Clapham JC, Schreyer S. Overexpression of mitochondrial GPAT in rat hepatocytes leads to decreased fatty acid oxidation and increased glycerolipid biosynthesis. *J Lipid Res.* 2004;45:1279-1288.
7. Li X, Catalina F, Grundy SM, Patel S. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J Lipid Res.* 1996;37:210-220.
8. Riddle TM, Schildmeyer NM, Phan C, Fichtenbaum CJ, Hui DY. The HIV protease inhibitor ritonavir increases lipoprotein production and has no effect on lipoprotein clearance in mice. *J Lipid Res.* 2002;43:1458-1463.

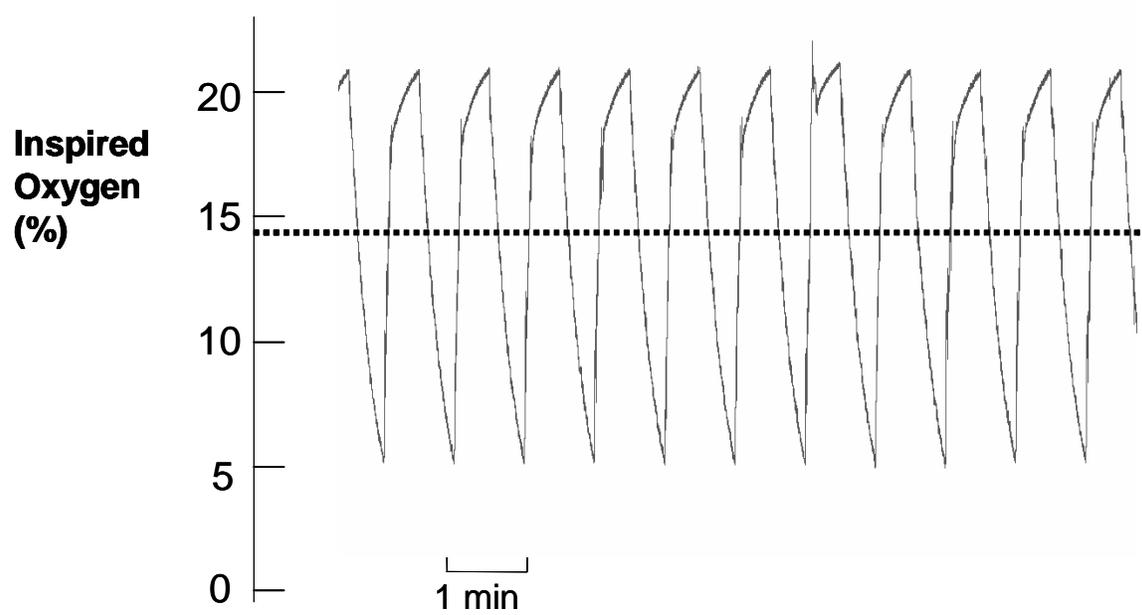
SUPPLEMENTAL DATA

FIGURE LEGENDS

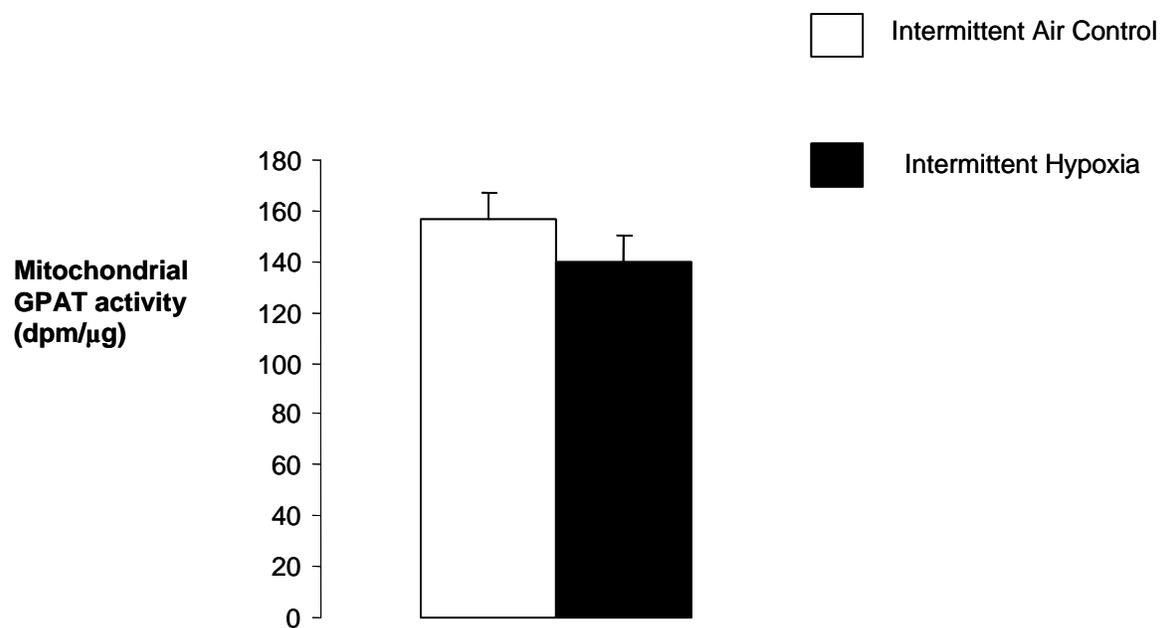
Online Figure S1. Repetitive Intermittent Hypoxia. During each period of intermittent hypoxia (IH), the FIO₂ was reduced from 20.9 to 4.8-5.0 % over a 30 s period and then rapidly reoxygenated to room air levels in the subsequent 30 s period. The use of multiple inputs into the cage produced a uniform nadir FIO₂ level throughout the cage. The mean FIO₂ during the 12 hr -light phase was 14.3% (a dotted line).

Online Figure S2. Enzymatic activity of mitochondrial glycerol-3-phosphate acyltransferase in the mitochondrial fraction of the livers of C57BL/6J (lean) mice exposed to either intermittent hypoxia (IH, n = 8) or intermittent air (IA, n = 8) for five days. Results presented show the mean \pm SEM. Statistical significance of the difference between animals exposed to IH or IA within a group was examined with unpaired *t*-test.

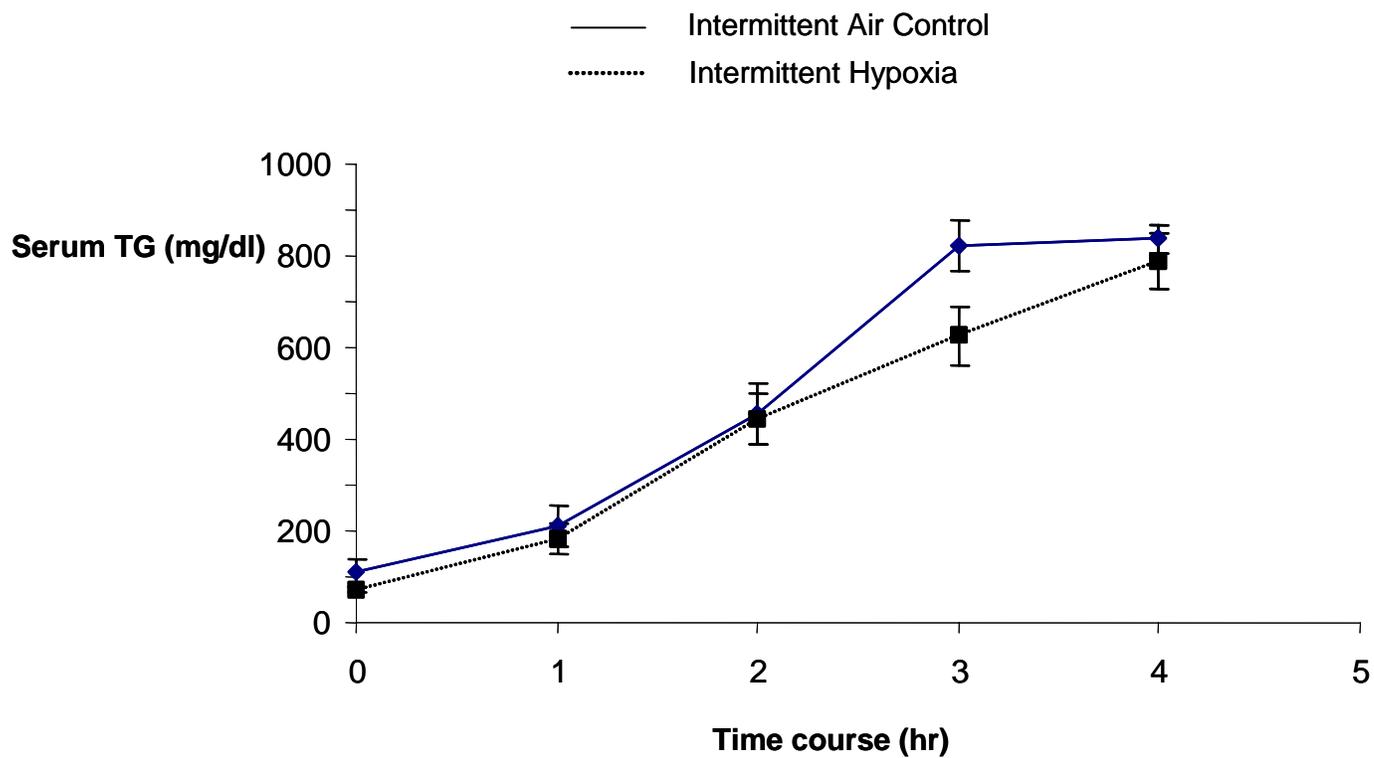
Online Figure S3. A time course of serum triglyceride (TG) levels after intravenous administration of Triton WR-1339 in C57BL/6J (lean) mice. Prior to the injection, mice were exposed to either intermittent hypoxia (n = 8) or intermittent air (n = 8) for five days. Results presented show the mean \pm SEM. Statistical significance of the difference between animals exposed to IH or IA within a group was examined with a repeated measures ANOVA.



Li et al. Online Supplement. Figure S1



Li et al. Online Supplement. Figure S2



Li et al. Online Supplement. Figure S3

Online Table S1. Primers and probes for real time PCR with cDNA derived from hepatic total RNA

Gene	GeneBank ID	Primers	Tagman Probe
18 S	K01364.1	5' CGCTCTCTGTTCCGCCTAGT 3' CCGTGCGTACTTAGACATGCA	ACCTGGTTGATCCTGCCA
SREBP-1	AI326423	5' CCACTAGAGGTCGGCATGGT 3' TCCCTTGAGGACCTTTGTCATT	TGCTTGTCAGGCTCACCCCTCTGG
FAS	AF127033	5' CCTGTTCTCGAGGAAGGCACTA 3' CAGAGAGTCCACCCACTGGAA	CACAGGAGAAACCTATC
Acetyl-CoA synthetase	NM_019811	5' TTGGCGACAAAGTTGCTTTTT 3' CGGAGAACATTGCTGAACTG	CACACCTGGACCAGGAGTTCA
Malic enzyme	NM_008615	5' AGCAGTGCTACAAGGTGACCAA 3' CTCCAGGGAACACGTAGGAATT	TGCAATCTTTGCCAGCGGCAGTC
SCD-1	NM_009127	5' CCCCTGCGGATCTTCCTTAT 3' AGGGTCGGCGTGTGTTTCT	CACCGCGCCCACCACAAGTTCT
GPAT	BC019201	5' CCTCTCAGTGGTAGTGGATACTCTGT 3' GTGACCTTCGATTATGCGATCAT	TCGTCATACCCGTGGGCATCTCG
SR-BI	BC004656.1	5' GGGAGCGTGGACCCTATGT 3' ACACGGTGTGCTTGTTCATTGA	CAGGGAGTTCAGACAAAAGGTC
LDL-R	BM207920	5' AGTCTGGGATTACTTTTGGTTTTTAGA 3' GAGGGATACACGCATGGTTTCT	CCACCTCTTCCTCTTG
SREBP-2	BF168632	5' CCGAGATGCAGGGCAAAG 3' GATGAAAGAACAATGAACAAGGCTTA	TCCCCTGTGCCTGAC
HMG-CoA reductase	M62766	5' CGCCCACGCAGCAAA 3' GGGACCACTGGCTTCCATTA	CACTGCTATCTACATCGC
ApoB	BC028880	5' TTTCTGCCTTTCTCCTACAAGA 3' CCCATCCGGTTGGTACCTT	ATCAACAGTCGCTTCTT
MTP	NM_008642	5' ACTCCGGTGGATGGTTTTGA 3' CAAGGGTCAGGCACGTCATA	TGGGTTCCCTTCCACC

Online Table S2. Species of fatty acids analyzed by gas chromatography and mass spectroscopy in the serum of mice subjected to intermittent hypoxia (IH) or intermittent air (IA) for five days

n	Molecular Weight	Structure	Number of deuterium atoms (D)
1	311	16:1	0
2	313	16:0	0
3	313	16:1	1
4	314	16:0	2
5	314	16:1	3
6	315	16:0	2
7	339	18:1	0
8	340	18:1	1
9	341	18:0	0
10	341	18:1	2
11	342	18:0	1
12	342	18:1	3
13	343	18:0	2
14	344	18:0	3