

# Intermittent hypoxia inhibits clearance of triglyceride-rich lipoproteins and inactivates adipose lipoprotein lipase in a mouse model of sleep apnoea

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Aims	Delayed lipoprotein clearance is associated with atherosclerosis. This study examined whether chronic intermittent hypoxia (CIH), a hallmark of obstructive sleep apnoea (OSA), can lead to hyperlipidaemia by inhibiting clearance of triglyceride rich lipoproteins (TRLP).
Methods and results	Male C57BL/6J mice on high-cholesterol diet were exposed to 4 weeks of CIH or chronic intermittent air (control). FIO <sub>2</sub> was decreased to 6.5% once per minute during the 12 h light phase in the CIH group. After the exposure, we measured fasting lipid profile. TRLP clearance was assessed by oral gavage of retinyl palmitate followed by serum retinyl esters (REs) measurements at 0, 1, 2, 4, 10, and 24 h. Activity of lipoprotein lipase (LpL), a key enzyme of lipoprotein clearance, and levels of angiopoietin-like protein 4 (Angptl4), a potent inhibitor of the LpL activity, were determined in the epididymal fat pads, skeletal muscles, and heart. Chronic intermittent hypoxia induced sig- nificant increases in levels of total cholesterol and triglycerides, which occurred in TRLP and LDL fractions ( $P < 0.05$ for each comparison). Compared with control mice, animals exposed to CIH showed increases in REs throughout first 10 h after oral gavage of retinyl palmitate ( $P < 0.05$ ), indicating that CIH inhibited TRLP clearance. CIH induced a >5-fold decrease in LpL activity ( $P < 0.01$ ) and an 80% increase in Angptl4 mRNA and protein levels in the epididymal fat, but not in the skeletal muscle or heart.
Conclusions	CIH decreases TRLP clearance and inhibits LpL activity in adipose tissue, which may contribute to atherogenesis observed in OSA.
Keywords	Intermittent hypoxia • Sleep apnoea • Dyslipidemia • Lipoprotein clearance • Atherosclerosis

## Introduction

Obstructive sleep apnoea (OSA) is a common condition characterized by chronic intermittent hypoxia (CIH) and frequent arousals from sleep.<sup>1</sup> Recent evidence suggests that OSA is directly linked to high cardiovascular mortality and morbidity.<sup>2,3</sup> Mechanisms by which OSA leads to poor cardiovascular outcomes are not completely understood. Metabolic dysfunction may provide an

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intermediate step linking CIH and OSA to cardiovascular disease. Although several studies have focused on the effects of OSA on blood pressure, endothelial dysfunction and dysregulation of glucose and insulin,<sup>4-6</sup> little information is available about the impact of OSA on dyslipidemia<sup>7,8</sup> and lipid metabolism.<sup>9</sup> Previous reports from our group suggest that CIH induces hyperlipidaemia, up-regulates genes of lipid biosynthesis in the liver and increases lipoprotein secretion in mice.<sup>10-12</sup> However, a distinct pattern of an increase in triglycerides and very low-density lipoprotein (VLDL) cholesterol suggests that CIH may also impair the clearance of triglyceride-rich lipoproteins (TRLP).<sup>13</sup> TRLP, which include liver-synthesized VLDL and dietary chylomicrons (CM), are cleared from the circulation via a multi-step process beginning with lipoprotein lipase (LpL). LpL is a key enzyme in lipoprotein metabolism that is preferentially expressed in the adipose tissue, skeletal muscle and heart.<sup>14,15</sup> It is conceivable that CIH may lead to hyperlipidaemia by inhibiting LpL activity. Interestingly, sustained hypoxia at 1% O<sub>2</sub> up-regulates a powerful LpL inhibitor. angiopoietin-like protein 4 (Angptl4) in human pulmonary artery endothelial cells, cardiomyocytes, and adipocytes.<sup>16-18</sup> We hypothesize that CIH inactivates LpL, thereby inhibiting the clearance of CM and VLDL. In order to explore this hypothesis, we exposed C57BL/6J mice to CIH for 4 weeks and measured CM clearance and LpL activity.

Wild-type mice on a regular chow diet have low levels of VLDL and low-density lipoprotein (LDL) cholesterol,<sup>19</sup> while transgenic mice with high LDL levels (ApoE deficient or LDL receptor-deficient animals) have impaired lipoprotein clearance at base-line.<sup>20,21</sup> Therefore, we used C57BL/6J mice on an atherogenic diet, which markedly raises VLDL levels,<sup>19</sup> while lipoprotein clearance is not perturbed.

## Methods

## **Experimental animals**

A total of 79 male C57BL/6J mice from Jackson Laboratory (Bar Harbor, Maine), 6–8 weeks of age at the beginning of the experiments, were used in the study. Twenty animals were used for all reported metabolic measurements, 19 mice were used in the CM clearance experiment, 20 mice were used for epididymal fat separation into the stromal vascular fraction (SVF) and adipocyte fraction, and 20 mice were used for plasma LpL activity determination. The same animals were used for several biochemical and physiological assays. Mice were fed a high-fat, high-cholesterol diet (4 kcal/g, 15.8% fat, and 1.25% cholesterol; TD#94059, Harlan Teklad, Madison, WI) and exposed to CIH or chronic intermittent air (CIA). The CIA group was weight matched to the CIH group by varying food intake as previously described.<sup>22</sup> 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.

### Mouse model of intermittent hypoxia

We have developed a mouse model of CIH that mimics the oxygen profile observed in patients with OSA.<sup>22</sup> Briefly, a gas control delivery system was designed employing programmable solenoids and flow regulators, which controlled the flow of air, nitrogen, and oxygen into cages. During each cycle of intermittent hypoxia, the FiO<sub>2</sub> decreased from ~21 to ~6.5% over a 30 s period, followed by a rapid return

to ~21% over the subsequent 30 s period. This regimen of intermittent hypoxia induces oxyhaemoglobin desaturations from 99 to ~70% 60 times/h.<sup>22</sup> A control group was exposed to an identical regimen of CIA. Chronic intermittent hypoxia and CIA were administered during the light phase (9 am – 9 pm) to coincide with the mouse sleep cycle and the duration of exposure was 4 weeks. For tissue harvesting, mice were sacrificed under 1–2% isoflurane anaesthesia after a 4 h fast. Epididymal white adipose tissue, skeletal muscle, and the heart were immediately snap-frozen in liquid nitrogen.

## Plasma lipids and lipoproteins, insulin, blood glucose

Blood was collected after a 4 h fast between 12 p.m. and 1 p.m. Plasma total cholesterol, triglycerides, and free fatty acids (FFA) were measured with kits from Wako Diagnostics, Inc. (Richmond, VA). Plasma glycerol was measured with a kit from Biovision (Mountain View, CA). Plasma lipoproteins were subjected to gel filtration HPLC on two tandemly connected TSK-Gel Lipopropak XL columns  $(300 \times 7.8 \text{ mm})$  with simultaneous measurement of triglycerides and cholesterol using an on-line dual detection system, according to Lipo-SEARCH technology (Skylight Biotech, Inc., Tokyo, Japan) as we have previously described.<sup>12,23</sup> The TRLP fraction included both CM (>80 nm) and VLDL (30-80 nm). Low-density lipoprotein were defined as lipoproteins measured 16-30 nm in diameter and HDL were defined as lipoproteins measured 8-16 nm in diameter. Plasma insulin was measured with an ELISA kit from Millipore (Billerica, MA) and corticosterone was determined by ELISA Kit from R&D Systems, Inc. (Minneapolis, MN). Plasma epinephrine and norepinephrine were measured by ELISA (Labor Diagnostika Nord GmbH&Co, Nordhorn, Germany). Blood glucose was measured using Accu-Chek<sup>®</sup> Comfort Curve<sup>™</sup> kit from Roche Diagnostics, Inc. (Indianapolis, IN).

### **Chylomicrons clearance**

Chylomicrons clearance was assessed by oral gavage of retinyl palmitate, which was performed after an overnight fast. After the oral administration of retinyl palmitate, retinyl esters (REs) are incorporated into CM in the intestine and enter the bloodstream; the disappearance of REs from the plasma reflects the rate of CM clearance.<sup>24</sup> Mice were given 5000 IU of retinyl palmitate (all-trans) by oral gavage  $(50 \mu l)$ <sup>24</sup> Blood samples were taken immediately before gavage and 1, 2, 4, 10, and 24 h later. Plasma levels of REs were measured by HPLC on a Beckman Ultrasphere C18 column  $(4.6 \times 250 \text{ mm})$ . Retinyl esters were separated in a mobile phase consisting of acetonitrile-methanol-dichloromethane (70:15:15 v/v) at a flow rate of 1.8 mL/min and detected by UV absorbance at 325 nm with a Waters 996 photodiode array detector. Retinyl acetate was used as an internal standard. Retinyl esters standards were synthesized from authentic all-trans retinol and the corresponding fatty acyl chloride.<sup>25</sup> The reported plasma REs concentrations represent the sum of individual REs concentrations (retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate). All extraction and HPLC procedures were carried out under N<sub>2</sub> and reduced light to prevent oxidation of the compounds. Plasma samples (25-200 µl) were denatured with an equal volume of absolute ethanol containing known amounts of retinyl acetate and then extracted into hexane. Following phase separation, the hexane extract was evaporated under a stream of N<sub>2</sub>, and the residue was resuspended in benzene for injection onto the HPLC column.

## Lipoprotein lipase activity in plasma and tissues

Pre-heparin plasma was obtained after a 4 h fast by retroorbital puncture under isoflurane anaesthesia. Fasting post-heparin plasma was obtained 5 min after heparin injection (200 U/kg body weight injected retroorbitally) by contralateral retroorbital puncture. Total lipase activities were assayed using an artificial glycerol-[1-<sup>14</sup>C]trioleate (Sigma T-7140)-containing lipid emulsion as previously described. Lipoprotein lipase activity was distinguished from hepatic lipase activity by suppression with 1 M NaCl. Lipoprotein lipase activity in heparin eluates from the epididymal fat pad fragments, skeletal muscle (gastrocnemius), and the heart were performed according to Nilsson-Ehle and Schotz<sup>26</sup> with minor modification.<sup>27</sup> One unit of LpL activity was defined as the release of 1  $\mu$ mol of FFA in 1 h per gram of tissue.

#### Western blot

The tissue was homogenized in the whole lysate buffer for Angptl4 and LpL measurements. SDS-PAGE and western Blot were performed using BioRad pre-cast gel system. Twenty micrograms of protein was applied per lane. For LpL measurements, we used affinity purified chicken IgY (#219) raised against bovine LpL (0.2 mg/mL diluted 1:1000) and rabbit anti-chicken-HRP (diluted 1:6000, Sigma, Saint Louis, USA). For Angptl4 measurements, we used primary rabbit polyclonal antibody against mouse Angptl4 (a gift from Prof. Paul Trayhurn, University of Liverpool, UK) and goat anti-rabbit-HRP (KPL, Gaithersburg, Maryland). Actin was detected with mouse monoclonal anti-actin antibody from Sigma (A3853) and goat anti-mouse-HRP (KPL). Densitometry was performed using Chemi-Doc XRS system from Bio-Rad (Hercules, CA) and UN-SCAN-IT Gel Automated Digitizing System, version 5.1 software (Silk Scientific, Orem, UT). The results were expressed as ratios of optical density of the bands representing LPL or Angptl4 to actin (Sigma-Aldrich, St Louis, MO). In a subset of mice, epididymal fat pads were collected and digested with collagenase (Liberase Blendzyme 2, Roche, Indianapolis, IN) in M199 media (GIBCO, Grand Island, NY) with 1% BSA at 37°C for 45 min. The resulting cell suspension was washed, filtered, and then spun at 300 g for 5 min to separate floating adipocytes from the SVF pellet. Western blot was performed in the cellular fractions as described above with antibody against mouse Angptl4, actin, and mouse thrombomodulin (R&D Systems, Minneapolis, MN). Mouse thrombomodulin, an endothelial cell marker,<sup>28</sup> was used to identify the SVF.

## Magnetic beads isolation and flow cytometry

In a separate experiment, we pooled SVF pellets from mice exposed to CIH (n = 5) and CIA (n = 5) and isolated CD11b+ adipose tissue macrophages using magnetic cell sorting CD11b+ microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Following positive selection, a small fraction of CD11b+ cells was separated to assess purity using flow cytometry. Cells were incubated with CD11b-APCeFluor780 (47-0112-82, Ebioscience) and F4/ 80-PE-Cy5 (15-4801-82, Ebioscience) antibodies for 15 min after 10 min incubation with Fc blocking antibody (CD16/CD32, 553142, BD Pharmingen). Flow cytometry was performed with an FACSAria (Becton Dickinson) and CellDiva software (Becton Dickinson), and analysed with FlowJo software (Tree Star). CD11b+ cells and the unbound to the microbeads cells (CD11b - cells) were lysed and subjected to western blot with Angptl4, actin, thrombomodulin (see above), CD31 [platelet endothelial cell adhesion molecule (PECAM-1)] antibodies (Santa Cruz) and perilipin antibodies (Progen, Heidelberg, Germany). Platelet endothelial cell adhesion molecule-1 was used as an additional endothelial cell marker.<sup>29</sup> Perilipin was used as a preadipocyte marker in the SVF.<sup>30</sup>

#### **Real-time polymeric chain reaction**

Total RNA was isolated using Trizol reagent (Life Technologies, Rockville, MD) with additional RNA clean-up using the RNAeasy (Qiagen, Valencia, CA) purification kit. cDNA was produced from total RNA using Advantage RT for PCR kit from Clontech (Palo Alto, CA). cDNA was used for real-time PCR using Applied Biosystems 7300 machine with primers and probes and 18s as a house-keeping gene as we have previously done.<sup>11,12</sup> The mRNA expression levels were normalized to 18S rRNA concentrations using the following formula: gene of interest/18S =  $2^{Ct(18S) - Ct(gene of interest)}$  and then expressed as a ratio of hypoxic conditions to control.

### Statistical analysis

Statistical analyses were performed using Minitab Statistical Software, release 15 (State College, PA). All values are reported as means  $\pm$  SEM after confirming that all continuous variables were normally distributed using the Kolmogorov–Smirnov test. The comparison of continuous variables between mice in two groups was performed using an unpaired *t*-test. Statistical significance for REs was determined by repeated measures analysis of variance. All tests were two-sided and the significance level was established at P < 0.05.

## Results

## **Basic characteristics**

Chronic intermittent hypoxia resulted in loss of epididymal fat, whereas liver mass increased, which was consistent with our previous observations.<sup>31</sup> CIH also increased fasting blood glucose levels, whereas plasma insulin levels were unchanged. CIH did not affect plasma epinephrine and norepnephrine levels. Unexpectedly, CIH decreased plasma corticosterone level, although in both hypoxic and control animals the values remained within the normal physiological range for the light phase in unstressed animals (*Table 1*).<sup>32,33</sup>

## Plasma lipid levels in mice exposed to chronic intermittent hypoxia

Compared to CIA, CIH induced a 40% increase in fasting total cholesterol levels exclusively affecting TRLP and LDL fractions. In fasting animals, TRLP were almost exclusively VLDL particles. TRLP was increased nearly 3-fold and LDL was increased by 50% (*Figure 1A*). Fasting triglycerides levels were increased nearly 2-fold and the increases were also predominantly localized to TRLP fractions (*Figure 1B*). Chronic intermittent hypoxia did not affect FFA levels, whereas there was a strong trend to an increase in glycerol (*Table 1*).

## **Chylomicrons clearance**

Figure 2 shows the time course of REs concentrations after oral gavage of retinyl palmitate. Compared with control, REs were higher in CIH at 2, 4, and 10 h. The area under the curve for the REs concentrations was significantly larger in mice exposed to CIH compared with the CIA group (461.7  $\pm$  65.1 vs. 260.0  $\pm$ 

Table I	Basic characteristics of C57BL/6J mice on a
high-chole	esterol high-fat diet exposed to chronic
intermitte	ent air or hypoxia for 4 weeks.

Characteristics	Chronic intermittent air	Chronic intermittent hypoxia	Р
nª	40	39	
Mean food intake (g/day)	2.45 ± 0.08	2.61 ± 0.11	0.294
Body weight (g)	$25.5\pm0.3$	$25.4\pm0.3$	0.831
Liver weight (g)	$1.19\pm0.05$	$1.35\pm0.05$	0.042
Liver weight (% body weight)	4.5 ± 0.2	5.2 ± 0.2	0.027
Epididymal fat (g)	$0.54\pm0.02$	$0.38\pm0.02$	0.000
Epididymal fat (% body weight)	2.1 ± 0.1	1.5 ± 0.1	0.000
Fasting serum insulin (ng/mL)	0.55 ± 0.09	0.34 ± 0.11	
Fasting blood glucose (mg/dL)	84 <u>+</u> 4	103 ± 5	0.011
Fasting plasma norepinephrine (ng/mL)	0.15 ± 0.05	0.15 ± 0.03	0.985
Fasting plasma epinephrine (ng/ mL)	1.07 ± 0.39	1.10 ± 0.38	0.960
Fasting plasma corticosterone (ng/mL)	79.0 ± 9.4	40.4 ± 5.6	0.007
Fasting plasma free fatty acids (mmol/ L)	0.32 ± 0.06	$0.28\pm0.07$	0.094
Fasting plasma glycerol (mmol/L)	0.52 ± 0.06	$0.71\pm0.07$	0.059
Plasma pre-heparin lipase activity (μmol/mL/h)	1.03 ± 0.21	0.90 ± 0.16	0.612
Plasma post-heparin LpL activity (μmol/mL/h)	19.8 ± 0.8	18.8 ± 0.4	0.273

<sup>a</sup>Liver, epididymal fat and biochemical measurements were not performed in mice subjected to retinyl palmitate gavage as well as in mice used for plasma LpL activity and epididymal fat fractionation experiments.

57.2  $\mu mol \times h, \ \ensuremath{\textit{P}} = 0.035),$  suggesting that CIH delayed RE clearance.

## Lipoprotein lipase

Chronic intermittent hypoxia did not affect plasma pre- and postheparin LpL activity (*Table 1*). In contrast, CIH resulted in a decrease of LpL gene expression in adipose tissue by 39% and a trend to a decrease in LpL mRNA in the heart, whereas the skeletal muscle was not affected (*Figure 3A*). The corresponding decrease in the protein level of LpL was also observed in the adipose tissue (CIA: 0.98  $\pm$  008 vs. CIH: 0.66  $\pm$  0.05; *P* = 0.003; *Figure 3B*). CIH induced a 5-fold decrease in LpL activity in the



**Figure I** Cholesterol and triglyceride profile after 4 weeks of chronic intermittent air (CIA, open bars) or chronic intermittent hypoxia (CIH, shaded bars). n = 8 per group. TC, total cholesterol; TRLP, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.







**Figure 3** The effect of chronic intermittent air (CIA, open bars) and chronic intermittent hypoxia (CIH, shaded bars) on lipoprotein lipase (LpL). The tissue was collected after 4 h fasting. n = 10 per group. (A) Lipoprotein lipase mRNA levels in the epididymal fat, skeletal muscle, and heart. (B) Lipoprotein lipase protein levels in the epididymal fat. The left panel shows the lipoprotein lipase and actin bands in representative samples. The right panel shows the mean optical density of lipoprotein lipase bands normalized to actin. (C) Lipoprotein lipase activity in the epididymal fat, skeletal muscle and heart.

epididymal fat (CIA: 11.46  $\pm$  1.77 vs. CIH: 2.58  $\pm$  0.81 µmol FFA/ g/h; *P* = 0.0003), whereas LpL activity in the skeletal muscle and heart was not affected (*Figure 3C*). Chronic intermittent hypoxia did not affect hepatic mRNA levels of Apo-CIII, a potent LpL inhibitor (CIA: 1.00  $\pm$  0.20 vs. CIH: 0.99  $\pm$  0.10, *P* = 0.991).

## Angiopoietin-like protein 4

Chronic intermittent hypoxia induced an 80% increase in Angptl4 gene expression in epididymal fat with a corresponding increase in protein level (*Figure 4A* and *B*). The adipose tissue fractionation localized Angptl4 protein to the SVF, but not to adipocytes, and CIH induced an increase in SVF Angptl4 (*Figure 5A, B*). In the attempt to determine what cells in SVF express Angptl4, we isolated the adipose tissue macrophages using CD11b+ magnetic beads. More than 90% of CD11b+ cells stained positive for a



**Figure 4** The effect of chronic intermittent air (CIA, open bars) and chronic intermittent hypoxia (CIH, shaded bars) on angiopoietin-like protein 4 (Angptl4) in epididymal fat. The tissue was collected after 4 h fasting. n = 9 per group. (A) Angiopoietin-like protein 4 mRNA levels. (B) Angiopoietin-like protein levels. The left panel shows the angiopoietin-like protein 4 and actin bands in representative samples. The right panel shows the mean optical density of angiopoietin-like protein 4 bands normalized to actin.

macrophage marker F4/80 by flow cytometry. Subsequent western blot showed that these cells did not express Angptl4. The western blot remained negative even when CD11b+ cells from hypoxic and normoxic mice were pooled together (n = 10) to increase the yield (Figure 6). In contrast, unbound to magnetic beads CD11b- cells expressed Angptl4 and levels of this protein were increased by CIH. Thrombomodulin and PECAM-1 were undetectable in both CD11b+ and CD11b- cells (not shown) indicating that endothelial cells were lost from the SVF during magnetic beads isolation and that Angptl4 expression in CD11b- cells was not attributable to endothelial cells.<sup>28,29</sup> The CD11b- cells were positive not only for Angptl4, but also for perilipin suggesting that this subfraction of SVF was rich in preadipocytes.<sup>30</sup> Perilipin was undetectable in the CD11b+ cells (Figure 6). Angptl4 was undetectable in skeletal muscle and heart regardless of experimental conditions.

## Discussion

To the best of our knowledge, this is the first study to examine the impact of CIH, a hallmark of OSA, on clearance of TRLP, providing important mechanistic insights to the pathophysiology of OSA-induced metabolic dysregulation and atherosclerosis. The main finding of our study was that CIH markedly delayed clearance of TRLP. We have also found that CIH induced a >5-fold suppression of adipose LpL activity, the enzyme that



**Figure 5** The effect of chronic intermittent air (CIA, open bars) and chronic intermittent hypoxia (CIH, shaded bars) on angiopoietin like protein 4 (Angptl4) in fractions of the epididymal fat [stromal vascular fraction (SVF) and adipocytes]. The tissue was collected after a 4 h fasting. n = 10 per group. (A) Thrombomodulin, angiopoietin-like protein 4, and actin bands in representative samples. (B) The mean optical density of angiopoietin-like protein 4 bands normalized to actin.



**Figure 6** Angipoietin-like protein 4 (Angptl4) in pooled CD11b+ and CD11b- subfractions of the stromal vascular fraction of epididymal fat. CD11b- cells were pooled from mice in the chronic intermittent air (CIA, n = 5) and chronic intermittent hypoxia (CIH, n = 5) groups. CD11b+ cells were pooled together from the chronic intermittent air and chronic intermittent tent hypoxia groups (n = 10).

catalyzes the first step of TRLP clearance.<sup>14,15</sup> Therefore, our data suggest that CIH increases CM and VLDL levels by inhibiting LpL. Finally, inactivation of LpL occurred only in the adipose tissue and was out of proportion to a moderate decrease in the LpL mRNA and protein levels suggesting a post-translational effect in addition to transcriptional effects. Our concurrent finding that CIH up-regulates Angptl4 in the adipose tissue, a potent post-translational inhibitor of LpL,<sup>34,35</sup> suggests a novel mechanism of dyslipidemia in CIH. In the discussion below, we will elaborate on potential mechanisms of dyslipidemia in CIH and clinical implications of our work.

We confirmed our previous findings that CIH exacerbates dyslipidemia induced by high-fat high-cholesterol diet selectively increasing TRLP.<sup>23</sup> We have previously shown that the increase in TRLP was due to up-regulation of VLDL secretion by the liver.<sup>12</sup> We have also shown that CIH specifically up-regulates a pivotal enzyme of triglycerides and cholesterol esters biosynthesis, stearoyl coenzyme A desaturase 1,<sup>11,23</sup> which may occur due to activation of hypoxia inducible factor 1 (HIF-1).<sup>36</sup> In the present study, we have shown that CIH delays TRLP clearance, which may lead to both fasting (Figure 1) and post-prandial hyperlipidaemia (Figure 2). The decrease in TRLP clearance was evident from the results of retinyl palmitate oral gavage. After the oral administration of retinyl palmitate, REs are incorporated into CM in the intestine and enter the bloodstream; the disappearance of REs from the plasma reflects the rate of TRLP clearance.<sup>24</sup> Of note, LpL plays a principal role in hydrolysis of CM REs, an event that is important for uptake of this retinoid in extrahepatic tissues.<sup>24</sup>

Lipoprotein lipase is a critical initial step in lipolysis of CM and VLDL, which is followed by hepatic uptake of TRLP remnants.<sup>14</sup> Lipoprotein lipase is synthesized in adipocytes, myocytes, and macrophages, then being secreted and transported to the luminal surface of blood vessels.<sup>14,15</sup> Genetic deficiency in LpL in humans results in the familial chylomicronemia syndrome with extremely high serum levels of triglycerides and very low levels of high-density lipoprotein (HDL).<sup>14</sup> Total LpL deficiency in mice is lethal.<sup>14</sup> Mice with LpL deficiency in the heart exhibit marked dyslipidemia,<sup>37,38</sup> while mice over-expressing LpL in muscle tissue have low levels of serum triglycerides.<sup>14</sup> Mice deficient in glycophosphatidyl-anchored HDL-binding protein 1, which anchors LpL to CM, develop severe chylomicronemia and atherosclerosis.<sup>39,40</sup> LpL activation in the muscle and adipose tissue plays an anti-atherogenic role due to a decrease in plasma triglycerides and increased HDL.<sup>41,42</sup> We have found that CIH induces a striking 5-fold decrease in the adipose LpL activity. Our previous work has shown that CIH leads not only to loss of epididymal fat, but also to hepatic steatosis.<sup>10,11,31</sup> The present results suggest that fat redistribution during CIH may be a result of inactivation of LpL in the adipose tissue. We have previously found that increased levels of TRLP in CIH cause atherosclerosis in C57BL/6| mice.<sup>31</sup> We have also previously linked CIH-induced atherosclerosis to up-regulated lipoprotein secretion.<sup>31</sup> We now hypothesize that impaired TRLP clearance due to inactivation of LpL in the adipose tissue can contribute to atherosclerosis in CIH.

Chronic intermittent hypoxia may affect LpL via multiple mechanisms. Chronic intermittent hypoxia up-regulates the sympathetic nervous system,<sup>43</sup> which may result in a decrease of LpL mRNA and protein levels and activity.<sup>44–47</sup> However, we did not observe increases in circulating catecholamine or corticosterone levels (*Table 1*) and LpL was affected predominantly posttranslationally and exclusively in the adipose tissue. Chronic intermittent hypoxia may inhibit LpL by modulating levels of various apolipoproteins. We measured hepatic expression of ApoCIII, a potent LpL inhibitor<sup>48,49</sup> and found no change.

Chronic intermittent hypoxia may lead to insulin resistance and insulin regulates LpL transcription via SREBP-1 and PPAR $\gamma$  transcription factors.<sup>32,50,51</sup> Chronic intermittent hypoxia increased fasting blood glucose, whereas fasting insulin levels did not change significantly, suggesting both a direct deleterious effect of CIH on insulin secretion and insulin resistance.<sup>52,53</sup> Thus, transcriptional down-regulation of LpL may be attributable to insulin resistance.

Chronic intermittent hypoxia dramatically down-regulated adipose LpL activity out of proportion to a rather modest effect on mRNA and protein levels, which suggests a predominant post-translational mechanism. Notably, the drastic decrease in adipose LpL activity was not accompanied by a decrease in plasma post-heparin LpL activity, suggesting a tissue-specific effect of CIH on LpL *in vivo*. One potential explanation would be Angptl4. Angiopoietin-like protein 4 is a powerful LpL inhibitor acting post-translationally by converting an active LpL dimer into inactive monomers.<sup>35</sup> Mice overexpressing Angptl4 in peripheral tissues showed reduction in the adipose tissue weight, increased levels of plasma triglycerides, impaired lipoprotein clearance without an effect on plasma post-heparin LpL activity, which, similarly to our data, suggested inactivation of LpL *in vivo* without reduction in total LpL levels.<sup>54</sup>

Angiopoietin-like protein 4 is expressed primarily in fat and to a lesser degree in other organs and tissues, including the liver, lung, and heart.<sup>55,56</sup> We found that CIH induces a 2-fold increase in adipose Angptl4 levels, but not in the heart or skeletal muscle. One study reported that Angptl4 was induced by hypoxia in isolated cardiomyocytes in vitro via the HIF-1 mechanism.<sup>18</sup> The discrepancy between Belanger et al.<sup>18</sup> and our data are likely related to the severity of hypoxia. Belanger et al exposed cardiomyocytes to 1% O2 (or 7.1 mmHg) in vitro, whereas, in our model, minimal oxyhaemoglobin saturation was  $\sim$ 70% corresponding to PaO<sub>2</sub> 30-35 mmHg with rapid reoxygenation to 99%.<sup>22</sup> Of note, our unpublished data show that epididymal fat tissue, which has low O<sub>2</sub> tension in normoxic environment,<sup>57</sup> becomes profoundly hypoxic during intermittent hypoxia with PtO<sub>2</sub> in the 20–25 mmHg range. Wang et al.<sup>17</sup> reported that exposure of human adipocytes to 1% O2 in vitro induced a dramatic increase in Angptl4 mRNA. Human pulmonary endothelial cells and cardiomyocytes infected with adenovirus expressing a constitutively active  $O_2$ -sensing subunit  $\alpha$  of HIF-1, also showed robust up-regulation of Angptl4 mRNA.<sup>16,18</sup> We propose that selective induction of Angptl4 in the adipose tissue could be attributed to more severe tissue hypoxia and activation of HIF-1, although this hypothesis needs to be further explored.

Angptl4 was localized to the SVF (*Figure 5*). Interestingly, Deguchi *et al.*<sup>58</sup> have recently shown that chronic hypoxia (2%  $O_2$ ) activates human macrophages *in vitro*. However, our data showed that Angptl4 was absent in the CD11b+ F4/80+ mouse adipose tissue macrophages, regardless of exposure conditions. In contrast, Angptl4 was detected in CD11b- PECAM-1-perilipin+ subfraction of the SVF (*Figure 6*) implying that Angptl4

was expressed in preadipocytes.<sup>30,59</sup> Thus, our study suggests that an increase in Angptl4 in preadipocytes contributes to impaired TRLP clearance during CIH.

An increase in adipose Angptl4 may have metabolic sequelae other than inhibition of LpL activity, e.g. accelerated adipose tissue lipolysis.<sup>54</sup> Indeed, animals subjected to CIH had decreased epididymal fat mass and increased plasma glycerol levels (*Table 1*). The lack of the corresponding increase in plasma FFA could be related to rapid utilization of FFA for enhanced hepatic biosynthesis of TG and cholesterol esters at hypoxic conditions.<sup>10,12</sup>

Our study has direct clinical implications. TRLP are a significant risk factor for atherosclerosis and cardiovascular disease.<sup>60</sup> Delayed removal of CM from the circulation is an independent predictor of coronary artery disease and a marker of coronary artery disease severity.<sup>61</sup> Obstructive sleep apnoea leads to dyslipidemia and atherosclerosis,<sup>62–65</sup> but the role of TRLP clearance in OSA has not been elucidated. There is a single report that OSA decreases plasma LpL activity in proportion to the severity of OSA and that this decrease was reversed by continuous positive airway pressure treatment for 3 months.<sup>66</sup>

Our study has some limitations. First, TRLP clearance was assessed by oral gavage of lipids rather by intravenous injection. Delayed disappearance of REs from plasma could be attributed to either poor intestinal absorption or impaired clearance from the bloodstream. However, in the CIH mice, REs peaked earlier than in control animals (*Figure 2*) suggesting that intestinal absorption was not impaired. Second, the role of Angptl4 was not definitively probed by a mechanistic intervention.

In conclusion, CIH inhibits clearance of TRLP, which likely occurs due to LpL inactivation in the adipose tissue. We hypothesize that inactivation of LpL in the adipose tissue is caused by CIH-induced up-regulation of Angptl4, a potent LpL inhibitor. Our study in the mouse model of CIH suggests that the role of TRLP clearance and LpL in cardiovascular complications of OSA should be investigated.

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