Visceral white fat remodeling contributes to intermittent hypoxia-induced atherogenesis

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ABSTRACT

Objective: Obstructive sleep apnea is a highly prevalent disease characterized by repetitive upper airway collapse during sleep leading to intermittent hypoxia. Cardio-metabolic complications of sleep apnea have been mostly attributed to intermittent hypoxia. These consequences could be mediated through intermittent hypoxia-related alterations of the visceral white fat, as it is recognized for playing an important role in inflammation, atherogenesis and insulin resistance.

Methods: Epididymal adipose tissue alterations were investigated in twenty-week-old non obese male apolipoprotein E-deficient mice exposed to intermittent hypoxia (21-5% FiO2, 60s cycle, 8h/day) or air for 6 weeks. These adipose tissue alterations, as well as metabolic alterations and aortic atherosclerosis, were then assessed in lipectomized or sham-operated mice exposed to IH or air for 6 weeks.

Results: Intermittent hypoxia induced morphological (shrunken adipocytes), functional (increased UCP-1 expression) and inflammatory (increased macrophage recruitment and secretion of IL-6 and TNF-α) remodeling of epididymal adipose tissue. Hypoxic mice presented more severe dyslipidemia and atherosclerosis lesions, and developed insulin resistance. Epididymal lipectomy attenuated both intermittent hypoxia-induced dyslipidemia and atherogenesis, but did not improve insulin sensitivity.

Conclusion: Our results confirmed that the dyslipidemic and proatherogenic effects of intermittent hypoxia are partly mediated through morphological, functional and inflammatory remodeling of visceral white fat, regardless of any obesity.

Keywords: Atherosclerosis, Inflammation, Intermittent hypoxia, Sleep apnea, Visceral white adipose tissue
INTRODUCTION
Obstructive sleep apnea (OSA) is a public-health problem as it affects at least 10% of the middle aged men, and represents a main cause of cardiovascular morbidity and mortality [1]. Repetitive upper airway collapses during sleep result in intermittent hypoxia (IH), thought to be responsible for cardiovascular complications such as atherosclerosis [2]. In OSA patients, increased carotid intima-media thickness (IMT), an early sign of atherosclerosis, correlates with nocturnal oxygen desaturation, independently of other cardiovascular risk factors [3] [4]. The rodent sleep apnea model (i.e. rodents exposed to IH) confirmed the detrimental role of hypoxia in OSA-associated vascular alterations; IH increases aortic IMT in C57BL6 mice [5] [6] and accelerates the development of atherosclerotic lesions in C57BL6 mice on a high cholesterol diet [7] and in atherosclerosis-prone apolipoprotein E-deficient mice (ApoE<sup>−/−</sup>) [8] [9]. However the direct relationship between sleep apnea or IH and its cardiovascular consequences is not fully elucidated. We and others have evidenced a role for hemodynamic alterations [5], inflammation [6] and metabolic dysregulations [10] [11]. OSA is also frequently associated with obesity which is the main confounder for detrimental consequences of OSA [12]. Indeed, obesity is an independent risk factor for diabetes and cardiovascular events, and is directly associated with increased mortality [13]. In addition to its function as the largest energy storage compartment of the body, the adipose tissue has emerged as a hormonal organ actively modulating metabolism and inflammation through the release of numerous cytokines and adipokines [14]. In obesity, excessive visceral white adipose tissue (WAT) contributes to metabolic dysregulation, vascular lesions and chronic inflammation through autocrine and paracrine regulations [15]. For example, WAT of obese mice exhibits increased TNF-α expression and secretion which is known to mediate insulin resistance [15]. Additionally, a recent study showed that atherosclerosis-prone mice transplanted with inflamed epididymal WAT (EWAT, i.e. the largest and easily harvested visceral WAT depot in mice) developed significantly more atherosclerosis, and that reduction in EWAT inflammation reduced atherosclerosis [16]. Therefore, inflammation of visceral WAT seems to be an important factor for vascular complications and may represent a link between obesity and vascular disease [15].

We have shown that IH induced vascular and systemic inflammation in non-obese C57BL6 [6] and ApoE<sup>−/−</sup> [9] mice, and noted that IH also induced inflammatory alterations of EWAT and morphological changes with histological features of brown adipose tissue [17]. Therefore, we hypothesized that IH itself could induce EWAT remodeling that could contribute to
metabolic and vascular disorders, independently of any obesity. The aims of the present study were to first characterize IH-induced functional and structural alterations of EWAT using a non-obese mouse model of IH, then to determine the contribution of this fat remodeling to IH-induced metabolic and vascular alterations using EWAT lipectomized animals.
METHODS

Animals
Thirty five 20-week-old male ApoE^{-/-} mice (C57BL6 background) fed on a standard-chow diet were used. They were weighed throughout the experiments, and food intake was measured daily. The study was conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986), and to the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Intermittent hypoxia
IH was performed as previously described [6]. The animals were exposed during daytime (n=10 per cage, 8h/day, cyclic 21-5% FiO_{2}, 60s cycle (60 events/h), lowest blood oxygen saturation up to 60%) for 6 weeks. FiO_{2} was measured with a gas analyzer (ML206, ADInstruments) throughout the experiment. Control animals (normoxic mice, N) were exposed to air in similar cages to reproduce similar noise and turbulences to those of the IH stimulus. Ambient temperature was maintained at 20-22°C.

Adipose tissue alterations
The day following the last exposure period, animals were anesthetized by intraperitonal ketamine-xylazine mixture (100 mg/kg-10 mg/kg) for bilateral EWAT collection. The tissue was either snap frozen in liquid nitrogen then stored at -80°C for mRNA study, or fixed in 90% ethanol for immuno-histochemistry, or incubated for cytokine determinations.

Immuno-histochemical study
Ethanol-fixed, paraffin-embedded EWAT were sectioned (3.5 μm), deparaffinized in toluene and rehydrated in descending ethanol series, then stained with hematoxylin-eosin to assess tissue morphology. Adipocyte size was measured from photographs (10x40 magnification) using the NIS-Elements microscope imaging software (Nikon).
For macrophage staining, sections were incubated overnight at 4°C with a rat anti-F4/80 (1:10, eBiosciences). Endogenous peroxidase activity was blocked with hydrogen peroxide. Non-specific binding was blocked with normal goat serum (Vector). Specific staining was detected using the N-Histofine Simple Stain Mouse Max-PO ® (Nichirei Biosciences Inc.)
followed by hematoxylin counterstaining. Macrophage infiltration was calculated as the ratio of nuclei of F4/80 positive cells to total nuclei counted in eight fields for each animal (n=5 mice per group).

**UCP-1 and MCP-1 mRNA expression**
Uncoupling protein (UCP)-1, the hallmark of brown adipocytes, and monocyte chemoattractant protein (MCP)-1 mRNA levels were determined by reverse transcription followed by quantitative Real-Time reverse transcription polymerase chain reaction (qRT-PCR). Total mRNA was extracted from epididymal fat of IH and N mice (n=7-11 per group), isolated with Tri-reagent (Invitrogen) and analyzed by qRT-PCR (ABI-7500 Applied Biosystems, or LightCycler Roche Diagnostics). Each sample was analyzed in duplicate, and 36B4 or TATA-box Binding Protein (TBP) mRNA level was simultaneously analyzed as a housekeeping gene (Table 1).

**Cytokine secretion**
EWAT was isolated from mice and weighed. Each pad was divided into two equal pieces and incubated at 37°C with mild shaking in RPMI medium only (Invitrogen) or with the beta-adrenergic agonist isoprenaline (4.10^{-5} M, Hospira Enterprises BV). Basal condition was defined as the cytokine secretion in the absence of the lipolytic agent isoprenaline. After 120 minutes of incubation, interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured in the supernatants using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (R&D System). Cytokine concentrations were expressed as ng/ml for 1g of adipose tissue. Plasmatic levels of IL-6, TNF-α and adiponectin were measured using an ELISA (R&D System). Circulating leptin level was evaluated using MILLIPLEX™ MAP Mouse Adipokine Panel based on multiplex technology (Millipore).

**Role of the epididymal adipose tissue**
**Epididymal lipectomy**
In another series of mice, the animals were either lipectomized or sham-operated under intraperitoneal ketamine-xylazine anesthesia (100 mg/kg/10 mg/kg). Bilateral EWAT was carefully removed to keep the gonads intact. Sham animals underwent the same surgical protocol without removing the fat. Surgery sites were closed with 4-0 nylon absorbable monofilament (Ethicon, Inc.) and mice received buprenorphine (0.1 mg/kg, intraperitoneal).
After three days during which body weight was daily measured, the animals were exposed to IH or N for 6 weeks, therefore constituting four experimental groups (lipectomized IH and N mice, sham IH and N mice, n = 7-10 per group). On the day following the last exposure period, the animals were anesthetized as described above. Blood was collected by cardiac puncture. Thoraco-abdominal aorta including the aortic roots was removed and snaps frozen in liquid nitrogen and stored at -80°C until analysis.

**Metabolic alterations**

**Lipid measurements**
Total- and HDL-cholesterol and triglycerides were measured in plasma by a colorimetric enzymatic reaction using the Modular P® analyzer (Roche Diagnostics). LDL-cholesterol was calculated using the Friedewald formula.
Plasma levels of glycerol and non-esterified fatty acids (NEFA) were measured by a colorimetric enzymatic reaction using the Free Glycerol Reagent (Sigma-Aldrich) and the NEFA-HR(2) assay (Wako Diagnostics), respectively.

**Intraperitoneal insulin tolerance test (IpITT)**
During IH exposure, we used IpITT to assess global insulin sensitivity. Mice were fasted for 10 hours then weighted before blood was collected from the tail tip for baseline glucose determination (t=0). Blood glucose was measured using the OneTouch® Ultra® glucometer. Insulin (0.75 IU/kg body weight, Novo Nordisk A/S) was injected intraperitoneally, followed by further blood glucose measurements at 15, 30, 60 and 90 minutes after the injection. The lowest blood glucose level (nadir) and its timing following insulin administration were calculated.

**Assessment of aortic lesions**

**Atherosclerotic lesion size**
Lesions of the thoraco-abdominal aorta and aortic roots were analyzed by Oil-red-O staining (n=7-10 per group). For each aortic root, we quantified lipid deposition from 5 sections (8 µm thickness), separated by 80 µm from each other. Quantitative analysis of lipid deposition was performed using computer image analysis (MetaMorph 7 software, Zeiss Microscope) after manual thresholding (thoraco-abdominal lesion) or contouring (aortic roots).
**Atherosclerotic lesion inflammation**

Macrophage and T-cell infiltration in the atherosclerotic lesions was assessed on immunostained aortic roots using anti-mouse macrophage (Mac-3, 1:100) and CD3 (1:1000, DakoCytomation) antibodies. Anti-α-smooth muscle actin antibody (1:50, Abcam) was used to stain smooth muscle cells. Non-specific binding was blocked with normal goat serum (Vector). Primary antibodies were detected using the N-Histofine Simple Stain Mouse Max-PO ® (Nichirei Biosciences Inc.) followed by hematoxylin counterstaining. All stainings were assessed by computer-assisted image analysis (MetaMorph 7 software, Zeiss Microscope). Content of macrophages, smooth muscle cells and collagen were expressed as percentage of plaque size.

**Statistical analysis**

Results were expressed as mean ± standard errors of the means. Differences between two groups over time were determined by a two-way analysis of variance (ANOVA) for repeated measures, with a subsequent Tukey’s post hoc test. For comparison between independent groups, Student’s t test or Mann-Whitney U-test were used according to normality and variance homogeneity. Statistical significance was set at $p<0.05$. 
RESULTS

**IH induces morphological and functional remodeling of epididymal fat**
EWAT from IH mice exhibited shrunken adipocytes (Figure 1A) with thicker extracellular matrix (Figure 1B) and increased expression of UCP-1 (Figure 1C).

**IH induces inflammatory remodeling of epididymal fat**
The hypoxic WAT exhibited increased macrophage (F4/80 positive cells) infiltration (Figure 2A), organized in crown-like structures around individual adipocytes (Figure 2B), and increased expression of MCP-1 mRNA (Figure 2C). This inflammatory phenotype was confirmed by TNF-α and IL-6 secretion from EWAT. At baseline, IL-6 and TNF-α were not modified by IH (Figures 2D,E). After isoprenaline stimulation, both cytokine increased in IH animals, significantly for IL-6 but without reaching significance for TNF-α (p=0.08). Whereas we observed localized inflammation in adipose tissue, we did not evidence systemic inflammation, as plasmatic cytokines (IL-6 and TNF-α) were undetectable, and adipokines (adiponectin and leptin) were not different between the four groups.

**IH induces insulin resistance and aggravates both dyslipidemia and atherosclerosis**
The pre-existing dyslipidemic pattern of the ApoE⁻/⁻ mouse strain was aggravated by IH, including higher plasma levels of total and LDL-cholesterol, triglycerides, glycerol and NEFA in sham hypoxic compared to normoxic animals (Table 2). Sham-operated hypoxic mice developed insulin resistance as shown by the lower glycemia decline following insulin administration (Figures 3A,B). IH also accelerated the development of atherosclerosis, as shown by the larger lesions in thoraco-abdominal aorta and aortic roots (Figures 4A-D). The atherosclerotic plaque composition was not affected by IH, as shown by the similar plaque content of macrophages (N, 5.40 ± 1.40 vs IH, 3.81 ± 0.56 % of plaque size), T cells (N, 0.25 ± 0.05 vs IH, 0.29 ± 0.04 % of plaque size) and smooth muscle cells (N, 4.5 ± 1.53 vs IH, 4.16 ± 1.20 % of plaque size).

**Epididymal lipectomy prevents IH-induced dyslipidemia and atherosclerosis**
Lipectomy prevented the IH-related disturbances in plasma cholesterol, whereas glycerol and NEFA levels remained similar to those of sham hypoxic animals (Table 2). The proatherogenic effect of IH was also prevented by lipectomy, as atherosclerotic plaque size in
the thoracoabdominal aorta was very similar to those of normoxic animals (Figures 4A,B). This effect was more modest in the aortic roots (Figures 4C,D). In contrast, plaque quality was not modified by lipectomy, as shown by the plaque contents of macrophages (N, 3.02 ± 0.62 vs IH, 3.99 ± 0.66 % of plaque size), T cells (N, 0.29 ± 0.07 vs IH, 0.26 ± 0.05 % of plaque size) and smooth muscle cells (N, 2.83 ± 1.10 vs IH, 5.0 ± 1.96 % of plaque size).

Regarding glucidic metabolism, lipectomy did not prevent the IH-induced insulin resistance. In the other hand and in agreement with the buffering role of WAT to maintain glucose homeostasis, lipectomy induced insulin resistance in normoxic animals (Figures 3A,B).
DISCUSSION

The pathophysiology of OSA-induced atherogenesis and metabolic alterations remains poorly understood. Here, we showed in non-obese ApoE-deficient mice that 6 weeks of IH led to morphologic, functional and inflammatory remodeling of the visceral WAT, and this remodeling contributed to IH-induced dyslipidemia and atherosclerosis. This study is the first to provide evidence that a physiological amount of visceral WAT exposed to IH becomes pathological, behaving like excess fat in obesity.

Methodological considerations

The IH experimental model has been developed more than 2 decades ago and reproduces one of the major components of OSA which is considered as the most detrimental factor contributing to sleep apnea complications [2]. However, in the absence of additional factors (obesity, high fat diet, etc), IH needs to be severe enough to induce measurable and reproducible detrimental effects [2]. Therefore, and as previously published by our group and others, the hypoxic stimulus used in this study mimics severe sleep apnea [2] [5] [6].

IH induces EWAT remodeling

In lean mice, IH induced moderate EWAT inflammation, including macrophage infiltration, increased MCP-1 expression and proinflammatory cytokine release (IL-6 and TNF-α). Fat inflammation is well known in obesity and results at least in part from hypoxia, as adipocytes become too distant from blood vessels with fat expansion [18]. Through the repetitive hypoxia-reoxygenation sequence, IH generates free radical stress and inflammation. IH also increases the sympathoadrenergic activity which in turn exacerbates the oxidative stress [19], and the adipose expression of MCP-1 [20] which is a key chemokine regulating macrophage tissue infiltration [21]. We used isoprenaline to mimic the sympathetic overactivity occurring in OSA patients during sleep, which resulted in enhanced proinflammatory cytokine release. Collectively, our data suggest chronic low-grade EWAT inflammation with intermittent exacerbation during sympathetic activation.

EWAT from IH mice had shrunken adipocytes and UCP-1 overexpression. UCP-1 is a hallmark of brown adipocytes (thermogenic fat) and is also expressed in adipogenic cells gaining characteristics of brown adipocytes (phenotyping switch into "brite" adipocytes, i.e. brown-like adipocytes). Again, beta-adrenergic stimulation may account for both alterations as it leads to lipolysis (NEFA release from adipocytes) and induces the appearance of brite
adipocytes in WAT [22]. However a full characterization is required to confirm the browning of the white fat, as well as the involvement of the sympathoadrenergic system which could be a common denominator of the various WAT remodeling due to IH. Moreover, the meaning of this adipose plasticity, in particular regarding the energy imbalance under IH, remains to be elucidated.

**IH induces insulin resistance, dyslipidemia and atherosclerosis**
As previously, we used ApoE-deficient mice to assess the atherogenic and metabolic effects of IH, as they are atherosclerotic-prone animals and develop dyslipidemia and insulin resistance. We confirmed the atherogenic and dyslipidemic effects of IH, hypoxic mice exhibited larger aortic atherosclerotic lesions and higher plasmatic levels of cholesterol (total and LDL) and triglycerides. Lipid alterations, constituting an early step of atherogenesis [23], have been previously described both in OSA-patients and in mice exposed to IH [24]. IH also increased plasmatic glycerol and NEFA, and aggravated the insulin resistance. WAT constitutes the main source of NEFA, also called free fatty acids (FFA), and excessive circulating FFA contributes to insulin resistance [25] [26]. The relationship between fat inflammation, in particular macrophage-related inflammation in WAT, and insulin resistance is now largely described in the literature [14]. Our results are in agreement with clinical studies showing a progressive worsening of insulin resistance and metabolic syndrome with OSA severity [24]. Exposure to IH also leads to decreased insulin sensitivity in healthy volunteers [27] and in lean mice [28]. Collectively, our data and those from the literature demonstrate that IH induces metabolic disorders independently of any obesity, and suggest that IH-induced adipose tissue alterations could represent one of the links between inflammation, insulin resistance and vascular consequences.

**EWAT lipectomy attenuates the dyslipidemic and atherogenic effects of IH**
We performed bilateral EWAT lipectomy before exposure to assess the specific role of EWAT in IH-induced cardiovascular and metabolic alterations. Lipectomized animals had smaller atherosclerotic lesions in thoraco-abdominal aorta whereas the preventive effect was lower in the aortic root. This suggests that, beyond WAT alterations, the atherogenic effect of IH involves additional mechanisms such as regional aortic susceptibility to develop atherosclerosis, e.g. aortic roots are prone to develop the largest lesions in particular due to hemodynamic factors.
From our results, the lower atherosclerosis progression after lipectomy appears to be related to the prevention of IH-induced dyslipidemia, as lipectomized hypoxic mice had similar levels of total and LDL cholesterol to normoxic animals. However the beneficial effect of lipectomy may also be related to the prevention of EWAT inflammation. We did not investigate the mechanisms underlying the beneficial effect on lipid levels as it was beyond the scope of this study. Because we found inflammatory alterations in EWAT, we chose to focus on proinflammatory cytokines but other signaling proteins such as the adipose angiopoietin-like 4, a potent lipoprotein lipase inhibitor, are likely involved in IH-associated atherosclerosis and may have contributed to the preventive effect of lipectomy [29]. In contrast, the IH-induced elevation in plasma glycerol and FFA remained unchanged. Lipectomy in this study was limited to the epididymal compartment which is the largest and most easily harvested visceral WAT depot in mice. However there are other WAT territories such as mesenteric, perirenal, subcutaneous WAT. Glycerol and FFA are two products of lipolysis occurring in the various adipose tissues, including epididymal and subcutaneous tissues. Although visceral WAT is predominantly associated with metabolic disease, the majority of circulating FFA originates from subcutaneous WAT [30].

Regarding insulin sensitivity, whereas it has been shown that removing visceral fat could prevent insulin resistance in obese or aging rats [31], we found that EWAT lipectomy induced insulin resistance in lean normoxic animals. These data confirm the major role of WAT in the homeostatic regulation of glucose; it is indeed one of the primary targets of insulin-stimulated glucose uptake, and it regulates insulin sensitivity through the release of numerous cytokines and adipokines. Therefore clinical situations with quantitative (excessive or overly reduced adipose mass) and/or qualitative alterations of the WAT may lead to insulin resistance [32]. We found that sham-operated hypoxic mice and lipectomized normoxic mice exhibited similar insulin resistance suggesting that EWAT in IH animals was functionally impaired for glucose buffering. As a consequence, lipectomy in hypoxic animals did not further aggravate the insulin resistance.

**Conclusion**

Although the causative mechanisms remain to be elucidated, we showed that IH had detrimental effects on EWAT of non-obese mice, and these contributed to the cardiovascular and metabolic complications induced by IH. EWAT exhibited inflammatory alterations that were exacerbated under beta-adrenergic activation, the latter mimicking the sympathetic
activation induced by sleep apnea events. Therefore, our original findings suggest novel therapeutic options for OSA patients as the compliance with the gold standard continuous positive airway pressure therapy is limited, and losing fat through weight reduction is applicable only in obese OSA patients. Targeting inflammation either using drugs or improving lifestyle (diet, exercise...), and buffering the nocturnal sympathetic activation could provide further therapeutic options for OSA patients.
ACKNOWLEDGMENTS

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REFERENCES


Table 1. Primers used for qRT-PCR analysis.
UCP-1: uncoupling protein-1; MCP-1: Monocyte chemoattractant protein-1; 36B4: acidic ribosomal phosphoprotein (ribosomal protein, large, P0: RPLP0); TBP: TATA box binding protein.

Table 1 Primers used for qRT-PCR analysis.

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<th>Gene</th>
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Table 2. Weight and biological characteristics in lippectomized and sham-operated apolipoprotein-E deficient mice exposed to intermittent hypoxia or normoxia.

Effects of epididymal lipectomy on weight and biological alterations induced by intermittent hypoxia (IH) or air (N); *p<0.05 vs N, †p<0.05 vs day 0.

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Figure 1. Intermittent hypoxia induces epididymal white adipose tissue morphological and functional remodeling.

(A) Quantification of adipocyte size after 6 weeks of intermittent hypoxia (IH) or air (N); *p<0.05 vs N; (7-8 per group). (B) H&E staining of adipose tissue (10x40 magnification) from IH or N mice; the scale bar represents 50µm. (C) Adipose tissue mRNA expression of UCP-1. Measurement is normalized to 36B4 mRNA; *p<0.05 vs N; (n=7-8 per group).
Figure 2. Intermittent hypoxia induces epididymal white adipose tissue inflammatory remodeling.

**Figure 2**

(A) Quantification of macrophage infiltration after 6 weeks of intermittent hypoxia (IH) or air (N); *p<0.05 vs N; (n=5 per group). (B) Representative F4/80 immunostaining with arrow showing macrophages organized as crown-like structure around adipocytes (10x40 magnification). (C) Adipose tissue mRNA expression of MCP-1. Measurement is normalized to Tata box Binding Protein (TBP) mRNA; *p<0.05 vs N; (n=8-11 per group). IL-6 (D) and TNF-α secretion (E) from epididymal adipose tissue at basal condition or after isoprenaline stimulation. *p<0.05 vs N; ‡p<0.05 vs basal condition; (n=13 per group).
Figure 3. Intermittent hypoxia induces insulin resistance.

(A) Intraperitoneal insulin tolerance test in epididymal lipectomized or sham-operated mice exposed to intermittent hypoxia (IH) or normoxia (N) for 6 weeks; *p<0.05 vs N, §p<0.05 vs N sham (n=6-15 per group).

(B) Lowest blood glucose level during the first 90 minutes (nadir); *p<0.05 vs N, §p<0.05 vs N sham (n=6-15 per group)
Figure 4. Epididymal lipectomy attenuates intermittent hypoxia-induced atherogenesis.

**Figure 4**

(A) Thoraco-abdominal aortic lesions expressed as percentage of total aorta surface in lipectomized or sham-operated mice exposed to intermittent hypoxia (IH) or normoxia (N) for 6 weeks; *p<0.05 vs N; (n=10 per group). (B) Representative photographs of thoraco-abdominal lesions. (C) Atherosclerotic lesions in aortic roots expressed in μm²; *p<0.05 vs N; (n=10 per group). (D) Representative photographs of aortic root lesions.