# Intermittent Hypoxia in Obstructive Sleep Apnoea mediates Insulin Resistance through Adipose Tissue Inflammation

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>European Respiratory Journal</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>ERJ-01731-2016.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>15-Dec-2016</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Murphy, Aoife; University College Dublin, School of Public Health, Physiotherapy & Sports Science  
Thomas, Amandine; Joseph Fourier University, Inserm U1042 / HP2 laboratory  
Crinion, Sophie; St. Vincent's University Dublin, Sleep Research Laboratory  
Kent, Brian; Guy's and Saint Thomas' NHS Foundation Trust  
Tambuwala, Murtaza M.; University of Ulster  
Fabre, Aurelie; St. Vincent's University Dublin, Department of Pathology  
PEPIN, Jean-Louis  
Roche, Helen; University College Dublin, School of Public Health, Physiotherapy & Sports Science  
ARNAUD, Claire; Joseph Fourier University, Inserm U1042 / HP2 laboratory  
Ryan, Silke; St. Vincent's University Dublin, Sleep Research Laboratory; University College Dublin, School of Medicine |
| Key Words: | obstructive sleep apnea, intermittent hypoxia, insulin resistance, adipose tissue inflammation |
Intermittent Hypoxia in Obstructive Sleep Apnoea mediates Insulin Resistance through Adipose Tissue Inflammation

Aoife M. Murphy1, Amandine Thomas2, Sophie J Crinion3, Brian D Kent4, Murtaza M Tambuwala5, Aurelie Fabre6, Jean-Louis Pepin2, Helen M Roche1, Claire Arnaud2 and Silke Ryan3,7*

1 Nutrigenomics Research Group, School of Public Health, Physiotherapy & Sports Science, The Conway Institute, University College Dublin, Ireland, 2 Université Grenoble Alpes, HP2, Inserm, U1042, CHU de Grenoble, Laboratoire EFCR, Pôle THORAX et VAISSEAUX Grenoble, France, 3 Pulmonary and Sleep Disorders Unit, St. Vincent’s University Hospital, Dublin, Ireland, 4 Pulmonary and Sleep Disorders Unit, Guy’s and St. Thomas’ Hospital, London, UK, 5 School of Pharmacy and Pharmaceutical Science, Ulster University, Coleraine, Northern Ireland, 6 Department of Pathology, St. Vincent’s University Hospital, Dublin, Ireland, 7 School of Medicine, The Conway Institute, University College Dublin, Ireland

*Corresponding author: Dr. Silke Ryan MD, PhD,
Dept. of Respiratory Medicine
St. Vincent’s University Hospital,
Elm Park,
Dublin 4, IRELAND.
Tel: 353-1-221 3702
Fax: 353-1-269 7949.
E-mail: silke.ryan@ucd.ie

Summary: Intermittent hypoxia induces an inflammatory phenotype of adipose tissue leading to insulin resistance in sleep apnoea.
Abstract

Obstructive sleep apnoea (OSA) is increasingly associated with insulin resistance (IR). The underlying pathophysiology remains unclear but intermittent hypoxia (IH)-mediated inflammation and subsequent dysfunction of the adipose tissue has been hypothesized to play a key role. We tested this hypothesis employing a comprehensive translational approach using a murine IH model of lean and diet-induced obese mice, an innovative IH system for cell cultures and a tightly-controlled patient cohort.

IH led to the development of IR in mice, corrected for the degree of obesity, and reduced insulin-mediated glucose uptake in 3T3-L1 adipocytes, associated with inhibition of the insulin-signalling pathway and down-regulation of insulin-receptor substrate (IRS)-1 mRNA. Providing mechanistic insight, IH induced a pro-inflammatory phenotype of visceral adipose tissue in mice with pro-inflammatory M1 macrophages polarization correlating with the severity of insulin resistance. Complimentary in vitro analysis demonstrated, that IH led to M1 polarization of THP1-derived macrophages. In subjects without comorbidities (n=186), OSA was independently associated with IR. Furthermore, we found an independent correlation of OSA severity with the M1 macrophage inflammatory marker sCD163.

This study provides evidence that IH induces a pro-inflammatory phenotype of the adipose tissue, which may be a crucial link between OSA and the development of IR.

Key words: intermittent hypoxia, obstructive sleep apnoea, insulin resistance, adipose tissue inflammation
Introduction

Obstructive sleep apnoea (OSA) represents a major public health burden due to its high prevalence [1] and substantial association with cardiovascular morbidity and mortality [2, 3]. Furthermore, there is emerging evidence of a relationship between OSA and metabolic perturbations, and in particular with alterations in glucose metabolism leading to insulin resistance (IR) and type 2 diabetes (T2D) [4-8].

Intermittent hypoxia (IH) likely plays a key role in the pathophysiology of cardiometabolic processes in OSA [9]. We have previously shown that IH preferentially activates pro-inflammatory, nuclear factor (NF)-κB mediated pathways leading to systemic inflammation in OSA patients [10, 11] and mice [12]. However, the tissues responsible for generating pro-inflammatory mediators in response to IH remain unknown. White adipose tissue (WAT) has emerged as an attractive possibility, given the strong link between OSA and obesity. A hallmark feature of metabolically dysfunctional obese WAT is the infiltration of immune cells, particularly macrophages with polarization towards a pro-inflammatory M1-phenotype and formation of crown-like structures (CLS), which generate a constant low-grade secretion of pro-inflammatory mediators, thereby driving cardiovascular and metabolic disease processes [13]. Emerging evidence suggests that local WAT hypoxia is a key factor in triggering inflammatory adipokine expression in obesity [14, 15] and in OSA, IH may potentiate this action. In support of this hypothesis we have demonstrated that primary human adipocytes develop a significantly greater inflammatory response to IH exposure than primary non-adipocyte cells [16]. Furthermore, IH leads to increased secretion of pro-inflammatory adipokines from adipose tissue in apolipoprotein E-deficient mice, associated with the development of more severe atherosclerotic lesions than seen in control mice [17].

However, the detailed mechanisms of the interaction between OSA and obesity and the specific role of WAT in the development of metabolic conditions such as IR in OSA
remain poorly understood. Thus, we hypothesized that IH contributes to the pathogenesis by inducing morphological and functional changes of the adipose tissue promoting an inflammatory response and we tested the hypothesis using a comprehensive translational approach employing a murine model of IH, a state-of-the-art IH system for cell cultures and a large human study including well-phenotyped subjects free of co-morbidities.

**Methods**

Detailed methods are described in the online supplement.

**Mice**

Male C57Bl/6 mice (5-weeks old) were either fed a high-fat diet or matched low-fat diet for 14 weeks before randomization to IH (21-5% FiO2, 60-s cycle, 8hrs/d) as previously described [17] or control for 6 weeks. Upon completion of the protocol, intraperitoneal insulin tolerance test (ITT) was performed [17]. Epididymal adipose tissue (EAT) was harvested, processed immediately for isolation of stromal vascular fraction, culture or ex vivo insulin stimulation or stored for further analysis. Flow cytometry of the stromal vascular fraction was performed to quantify the M1 and M2 fraction of the adipose macrophage population, while F4/80 immunohistochemistry was performed to determine the crown-like structure (CLS) density. The study was approved by the Institutional Animal Care and Use Committee (#02256.02) and mice were maintained in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

**Human studies**

186 male subjects without significant co-morbidities were recruited from St. Vincent’s University Hospital, Dublin, Ireland and University Hospital, Grenoble, France. The study was approved by both local ethics committees and all participants gave written informed consent. Polysomnography (PSG) was performed as previously described [10].
Fasting glucose, insulin and lipid profile were determined and circulating levels of sCD163 were measured by ELISA (R&D, Abingdon, UK).

**In vitro model of IH**

Previous cell culture models of IH have been limited by requiring prolonged soak times, reduced cycle numbers and inadequate control treatment. Here, we have developed a state-of-the-art model using a custom-built system (Coy Laboratories, Grass Lake, MI, USA) overcoming these limitations. Cells were grown on a semi-permeable membrane (Lumox®, Sarstedt, Nuernbrecht, Germany) to allow rapid gas exchange. IH and control treatment were achieved in 2 separate closed cabinets with gas flow simultaneously regulated by an automated external controller (Coy). The IH protocol consisted of alternating cycles of 40 sec of 16% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2} and 40 sec of 3%O\textsubscript{2}/5%CO\textsubscript{2}/balance N\textsubscript{2}. The protocol was applied for 8 hours/day for 3 consecutive days. The actual pO\textsubscript{2} values at the cell monolayer were continuously monitored by fluorescence quenching oxymetry (Oxylite 2000, Oxford Optronix, UK). The control chamber was simultaneously cyclically gassed with 16% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2}. Both chambers were kept in a closed glove box, maintaining temperature at 37°C with separate heat exchangers ensuring constant temperature in the IH and control cabinets.

**Cell Culture**

3T3-L1 cells were differentiated into mature adipocytes according to the manufacturers’ instructions. NIH-3T3 cells (Pomics, Cambridge, UK) stably transfected with a NF-κB-luciferase reporter construct were maintained in complete DMEM supplemented with hygromycin (100 µg/ml) (Roche, Clare, Ireland). Human monocytic THP1 cells (ATCC) were differentiated into macrophages using 5ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 hrs before resting in medium for 72hrs. Then, cells were
either left unstimulated or incubated with interferon γ (20 ng/ml, R&D) or LPS (1 ng/ml, Sigma) for 24 hrs.

**Statistical analysis**

All data are expressed as mean ± SD or mean ± SEM as indicated. Group comparison was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison. To assess the possible confounding effect of weight on IR in the mice experiment, a General Linear Model to assess for Analysis of Covariance (ANCOVA) was employed. To identify potential independent predictors of HOMA-IR or sCD163, we used a stepwise linear regression model with HOMA-IR or sCD163 as the dependent variables and baseline demographic, anthropometric, and PSG variables as covariates. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using a commercial software package (SPSS Version 20, Chicago, IL, USA).

**Results**

**IH induces insulin resistance in lean and obese mice and impedes insulin-mediated glucose uptake in adipocytes**

Previous studies have linked IH to IR in mice but limited data are available in the setting of obesity. Here, using the murine diet-induced obesity (DIO) model, we exposed lean and obese mice to IH or control conditions. IH had a clear adverse effect upon IR in lean and weight-matched obese mice as demonstrated by ITT (Figure 1A-C). In obese mice, as expected, IH treated animals lost significantly more weight than controls (Table 1). Therefore, all of the IR measures took account of this confounding effect and results are presented for the whole cohort of obese mice (Figure 1A) and separately for weight-matched animals (Figure 1B). Weight matching (WM) was performed by excluding mice with highest and lowest body weights and matching was performed prior to statistical analysis by an
investigator blinded to any obtained results (MT). Furthermore, using an ANCOVA with body weight as covariate, IH treatment remained a significant predictor of IR (p<0.001).

Obese mice displayed higher fasting insulin levels and HOMA index and demonstrated impaired glucose tolerance in comparison to lean mice (p<0.05) but this was not further altered by IH treatment.

In order to gain mechanistic insight into IH-induced IR, we utilized a state-of-the-art in vitro model of IH. This model results in rapid fluctuations of cellular pO2 values closely mimicking the IH pattern experienced by OSA patients (Supplementary figure E1). In cultured 3T3-L1 adipocytes, IH treatment significantly attenuated insulin-mediated glucose uptake in comparison to control treatment (Figure 1D).

Taken together, these results indicate that IH leads to decreased insulin sensitivity in lean and obese mice as well as cultured adipocytes.

**IH inhibits the insulin signalling pathway and down-regulates insulin receptor substrate (IRS)-1 in mice and cultured adipocytes**

Having established the direct link between IH and IR, we next investigated potential upstream events. In obesity, down-regulation of insulin-receptor substrate (IRS)-1 mRNA with subsequent alteration in IRS-1 tyrosine phosphorylation and diminished phosphorylation of Akt have been recognized as important components of IR [18] and here, we examined the impact of IH on these events. In both, murine epididymal visceral fat and 3T3-L1 adipocytes, IH reduced IRS-1 mRNA, although this did not reach statistical significance in lean mice (p=0.06) (Figure 2A-B). Murine IRS-1 mRNA correlated significantly with insulin sensitivity as determined by ITT (r=0.436, p=0.021). Furthermore, IH led to decreased insulin-induced tyrosine-phosphorylation of the insulin receptor (IRβ) and IRS and phosphorylation of Akt (Figure 2C) in adipocytes. In addition, in adipose explants of obese mice, *ex vivo* insulin
stimulation also resulted in attenuated Akt phosphorylation (Figure 2D) (given the limited amount of adipose tissue this experiment was not performed in lean mice).

Thus, IH leads to decreased functionality of the adipose tissue with inhibition of the insulin signalling pathway and down-regulation of IRS-1 mRNA.

**IH induces a pro-inflammatory phenotype in visceral adipose tissue in vivo and polarizes THP1 cells into M1 pro-inflammatory macrophages**

In order to further understand the mechanisms responsible for the development of the adipose IR phenotype in response to IH, we next examined the effect of IH on pro-inflammatory adipose-tissue macrophage (ATM) alteration. Notably, IH was associated with increased M1 (pro-inflammatory) ATM infiltration in lean and obese mice, and M1 ATM numbers correlated significantly with the level of IR as assessed by ITT (Figure 3A-B). Percentages of M2 (anti-inflammatory) macrophages were significantly reduced with HF diet, but this was not further altered by IH treatment (Figure 3C and supplementary figure E2). Overall, there were no changes in total ATM infiltration between diets and treatments (LF-N: 141±61/mm² vs LF-IH: 102±56 vs HF-N: 169±141 vs HF-IH: 176±200; ANOVA: p=0.118) as determined by automated analysis of F4/80 immunohistochemistry. Another typical characteristic feature of obese, metabolically dysfunctional adipose tissue is the formation of crown-like structures (CLS), which consist of macrophages surrounding necrotic adipocytes [13]. Visceral adipose tissue in lean animals treated with IH demonstrated an increased amount of CLS in comparison to controls. Furthermore, there was a trend toward increased CLS in weight-matched obese mice, however this failed to reach statistical significance (Figure 3D-E). In terms of understanding the impact of IH on the adipose inflammatory phenotype, we found that conditioned media from adipose tissue explants from obese mice treated with IH induced greater activation of NF-κB compared with explants from control mice (Figure 3F), and there was also a trend towards greater NF-κB activation with IH in lean
mice (p=0.097). To further understand whether macrophage polarization is directly caused by IH, we treated THP1-derived macrophages with IH or control in vitro. The classical protocol for M1 polarization involves macrophage incubation in the presence of IFNγ or LPS and to measure the expression of several classical M1 characterizing genes such as TNF-α, IL-8 or IL-6 [19, 20]. IH under basal conditions and in the presence of these stimuli increased mRNA expression of all three of these genes (Figure 3G). These data support the findings obtained from the mice studies indicating that the adipose macrophage polarization observed in vivo is directly been caused by IH.

Thus, these in vivo and in vitro data demonstrate that IH induces a pro-inflammatory phenotype in adipose tissue which in obesity is known to correlate with metabolic dysfunction.

**OSA is independently associated with IR**

As a next step, we investigated the potential role of the findings obtained from the murine and in vitro models of IH for patients with OSA. Several studies have suggested an association of OSA and IR, however uncertainty remains if this relationship persists in obese subjects. To gain further insight into this subject, we employed a cross-sectional cohort of carefully selected subjects with a specific focus on the interaction of OSA with obesity. 186 male subjects without pre-existing cardiovascular and metabolic disorders were recruited from the Pulmonary Sleep disorders unit of St. Vincent’s University Hospital, Dublin, Ireland (n=152) and from the Sleep Laboratory at the University of Grenoble, France (n=34). Subjects were classified into 3 different weight groups (group 1: lean/overweight [body mass index (BMI) <30 kg/m²]; group 2: obesity class 1 [30≤BMI<35], group 3: obesity class ≥2 [BMI≥35]) and within these groups into subjects without (A) and with (B) OSA (Table 2). While BMI and waist/hip ratio (WHR) were similar between OSA and controls within all three weight categories, HOMA-IR values were higher in OSA patients than controls,
although in group 3 this difference failed to reach statistical significance (p=0.07) (Figure 4).

Pearson’s correlation analysis showed that HOMA-IR correlated most strongly with BMI (r=0.539, p<0.001), oxygen desaturation index [ODI] (r=0.406, p<0.001) and apnoea/hypopnoea index [AHI] (r=0.405, p<0.001). Stepwise multiple linear regression analysis identified BMI (β=0.421, p<0.001) and the OSA-severity markers AHI (β=0.228, p=0.004) and ODI (when this latter value was entered instead of AHI, β=0.239, p=0.004) as independent predictors of HOMA-IR.

Thus, OSA contributes to IR in men without cardiovascular and metabolic co-morbidities independently of the degree of obesity.

The circulating marker of macrophage activation and polarization sCD163 correlates significantly and independently with OSA severity

As outlined above, we demonstrated that IH induces ATM polarization towards a M1 phenotype in lean and obese mice and also in cultured THP-1 derived macrophages. To investigate the potential role of these findings for subjects with OSA, we next measured serum levels of CD163, an established marker of macrophage activation and M1 polarization, in our patient cohort (n=149). We found a significant correlation between sCD163 and OSA severity (Figure 5) and in linear regression, adjusted for anthropometric and demographic parameters, the AHI (or ODI when substituted for AHI) remained the only significant predictor of this response (β=0.369, p<0.001).

Thus, OSA severity correlates significantly with sCD163, suggesting M1 macrophage polarization in OSA patients.

Discussion

In this report we provide evidence from cell culture, murine and human studies that IH in OSA contributes independently to the development of insulin resistance (IR). We also demonstrate for the first time that IH induces obesity-like morphological pro-inflammatory
changes in adipose tissue, which correlate with IR in lean and obese mice and may contribute to the pathogenesis of IR in patients with OSA.

The results of our study are in keeping with previous reports [4-6, 8, 21] supporting an independent association of OSA with IR. These findings are strengthened by the exclusion of subjects with significant confounding co-morbidities, and the recruitment of participants across the spectrum of weight categories. We believe that the lack of statistical significance in HOMA-IR between OSA and controls in group 3 is due to the low number of controls, subjects which are especially difficult to recruit, and overall, our data indicate that OSA impacts on IR independently of the degree of obesity.

The pivotal role of IH in the underlying pathogenesis is supported by the mouse experiments demonstrating diminished insulin sensitivity with IH. Previous studies have mainly been performed in lean mice [17, 22-25]. Drager et al. also reported impaired insulin sensitivity in diet-obese mice [23] using the HOMA-IR index as a surrogate marker which remained unaffected by IH in our experiments. This parameter reflects the balance between hepatic glucose output and insulin secretion, which is maintained by a feedback loop between the liver and β-cells, unlike the ITT which also assesses the peripheral insulin sensitivity of the target tissues, i.e. adipose tissue and skeletal muscle [26]. In rodents, HOMA-IR index is less sensitive to changes in insulin sensitivity [27] and whereas Drager et al measured fasting glucose and insulin directly after cessation of IH, we performed these measurements after overnight cessation of the stimulus, which may explain the discrepancy between the two studies.

Although the detailed pathophysiological sequences underlying IH-mediated metabolic dysfunction still need to be defined, our study provides novel insight into the mechanisms by which IH contributes to IR. We reported previously that IH induces morphological and inflammatory remodelling of visceral adipose tissue in lean mice [17] and
a profound pro-inflammatory response in cultured adipocytes [16] and we demonstrate here that these events lead to IR associated with down-regulation of the insulin signalling pathway. In obesity, changes in the cellular composition of the stromal-vascular fraction towards a pro-inflammatory phenotype are a key component of metabolically dysfunctional adipose tissue. The polarization of macrophages towards a M1, pro-inflammatory subtype leading to the generation of multiple pro-inflammatory factors plays a pivotal role in this setting [13]. We demonstrate that even in lean mice, IH induces typical obesity-like morphological changes of the adipose tissue, with significant increases of M1 macrophages, confirming data by Carreras et al. using a similar protocol [22]. In obese mice, IH potentiates these pro-inflammatory changes, despite IH leading to significant weight loss in these animals. Differences in insulin sensitivity only became significant after adjustment for IH-related weight loss, perhaps reflecting the effect of obesity on IR in other target organs, such as skeletal muscle. Weight loss in response to IH is a well-described confounding finding [23, 28] and is due to a combination of decreased food intake and altered energy expenditure. Pair-feeding would only partly resolve this issue and hence, provides limited benefit. Supporting the conclusion that the adipose tissue changes were due to the direct effects of IH treatment are the changes seen in lean mice, where no difference in body and adipose tissue weight was observed between mice exposed to IH and controls, and from the cell culture studies demonstrating M1 macrophage polarizations of THP1 cells in response to IH.

As a novel finding of our study, we established a significant correlation of OSA severity with sCD163 as emerging marker of M1 macrophage polarization in humans. Although a definitive direct connection between the increased sCD163 levels observed in OSA patients and the adipose tissue as source cannot be drawn at this stage, the results from the animal model nonetheless suggest that this may be the case. In support of this hypothesis, a recent study demonstrated a significant correlation of sCD163 with CD163 expression in
adipose tissue [29]. CD163 is expressed on M2 macrophages but to become soluble requires cleavage by ADAM-17 which is dependent on M1 macrophages [30]. Hence, sCD163 has increasingly been linked to adipose tissue inflammation in insulin resistance and T2D, and is an emerging pro-inflammatory biomarker of various cardiovascular diseases [31-34]. This is the first study investigating sCD163 in OSA and strongly supports evidence of macrophage polarization in this condition. Its detailed role in OSA and the link directly to metabolic and cardiovascular disease processes will, however, require further targeted evaluation including large prospective studies.

Our study has significant strengths including the comprehensive translational nature of the study, which includes a carefully selected patient cohort, an animal model that includes the evaluation of lean and obese mice, and a state-of-the art in vitro model of IH. Previous cell culture models of IH have been limited by requiring prolonged soak times, reduced cycle numbers and inadequate control treatment. Here, we are able to closely resemble the oxygen desaturation patterns observed in OSA and thus, this model is far superior to previous reported models. Campillo et al have recently described a similar model [35]. However, we acknowledge that the IH pattern may differ substantially in various tissues. One study using a murine model suggested that the oxygen fluctuations of IH are attenuated in adipose tissue [36]. However, how this relates to human adipose tissue, is unknown and it is likely that there will be significant local differences within tissue depending on the relative distance to the circulatory system. Notably, our findings obtained in vitro closely resembled the animal data supporting the suitability of this model for adipocytes, however further targeted studies are required.

One potential limitation of our study is the sole focus on the adipose tissue but we acknowledge that other organs are likely involved in the pathogenesis of IR. IH also contributes to steatohepatitis [37, 38] and may also have detrimental effect on β-cell function
Further studies will need to define the detailed contribution of these adverse actions in the glucose metabolic dysfunction. Moreover, we did not investigate the potential contribution of other WAT compartments in mice other than epididymal adipose tissue (EAT), such as the subcutaneous and mesenteric fraction. In cell culture, human primary subcutaneous and visceral adipocytes have similar pro-inflammatory potential in response to IH [16], but if other parts of WAT behave similar to EAT in vivo remains unknown. A further potential limitation is the exclusion of female OSA patients. We designed the study specifically in this way to avoid gender differences which could influence the analysis. However, as a consequence, our data cannot be extrapolated to women.

In conclusion, this study provides evidence of pro-inflammatory changes of the adipose tissue in response to IH which may be a crucial link between OSA and the development of IR.

Acknowledgement

This work was supported by the Health Research Board of Ireland (SR), the Science Foundation of Ireland (HMR & AMM) and by Agir pour les maladies chroniques (JLP).
References


Table 1. Baseline characteristics of lean (LF) and diet-induced obese (HF) mice (whole cohort and weight-matched) treated with intermittent hypoxia (IH) or control (* = vs. HF control; † = vs. LF control and LF-IH)

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF (whole cohort)</th>
<th>HF (Weight-matched)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IH</td>
<td>Control</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Body weight (g) day 0 (non-fasting)</td>
<td>29.7±3.6</td>
<td>30.3±2.3</td>
<td>45.0±5.4†</td>
</tr>
<tr>
<td>Body weight (g) day 42 (fasting)</td>
<td>25.2±2.9</td>
<td>25.6±1.7</td>
<td>42.0±5.1†</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>0.446±0.124</td>
<td>0.534±0.139</td>
<td>2.002±0.556†</td>
</tr>
</tbody>
</table>
Table 2. Baseline characteristics of human population (¹ = body mass index; ² = waist/hip ratio; ³ = pack years; ⁴ = Epworth Sleepiness Scale; ⁵ = apnoea/hypopnoea index; ⁶ = oxygen desaturation index; ⁷ = mean nocturnal oxygen saturation; ⁸ = per cent of total sleep time < 90%; * = vs. group 1A+B; † = vs. group 2A+B; ‡ = vs. group 1A; § = vs. control; £ = vs group 1B, †† = vs. group 2A, ‡‡ = vs all other groups)
<table>
<thead>
<tr>
<th>Group</th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1B</td>
<td>2A</td>
<td>2B</td>
<td>3A</td>
<td>3B</td>
</tr>
<tr>
<td>BMI&lt;30</td>
<td>BMI&lt;30</td>
<td>BMI&gt;30,&lt;35</td>
<td>BMI&gt;30,&lt;35</td>
<td>BMI&gt;35</td>
<td>BMI&gt;35</td>
</tr>
<tr>
<td>Control</td>
<td>OSA</td>
<td>Control</td>
<td>OSA</td>
<td>Control</td>
<td>OSA</td>
</tr>
<tr>
<td>No</td>
<td>35</td>
<td>42</td>
<td>23</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>Age</td>
<td>44±10</td>
<td>45±9</td>
<td>40±7</td>
<td>43±8</td>
<td>38±8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.7±2.0</td>
<td>27.0±2.3</td>
<td>32.1±1.4*</td>
<td>32.2±1.3*</td>
<td>38.6±3.9*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.94±0.06</td>
<td>0.94±0.06</td>
<td>1.01±0.05*</td>
<td>1.01±0.05*</td>
<td>1.03±0.05*</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>6 (18%)</td>
<td>5 (12%)</td>
<td>4 (17%)</td>
<td>5 (17%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Ex</td>
<td>5 (15%)</td>
<td>18 (44%)</td>
<td>9 (39%)</td>
<td>10 (34%)</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>py³</td>
<td>4±8</td>
<td>6±10</td>
<td>8±11</td>
<td>8±12</td>
<td>9±9</td>
</tr>
<tr>
<td>ESS^4</td>
<td>8±6</td>
<td>10±6</td>
<td>10±6</td>
<td>12±5</td>
<td>9±7</td>
</tr>
<tr>
<td>AHI^5</td>
<td>4.0±2.8</td>
<td>28.5±17.7*</td>
<td>4.0±2.8</td>
<td>37.7±21.9*</td>
<td>3.1±1.3</td>
</tr>
<tr>
<td>ODI^6</td>
<td>2.3±2.1</td>
<td>22.3±14.5°</td>
<td>3.4±2.3</td>
<td>33.0±20.4°</td>
<td>2.8±1.1</td>
</tr>
<tr>
<td>Basal SaO₂^6</td>
<td>95±1</td>
<td>94±4</td>
<td>94±2</td>
<td>94±1°</td>
<td>94±2</td>
</tr>
<tr>
<td>Min SaO₂</td>
<td>90±3</td>
<td>83±6°</td>
<td>88±2</td>
<td>79±8°</td>
<td>88±4</td>
</tr>
<tr>
<td>TST&lt;90 (%)</td>
<td>0±1</td>
<td>5±10</td>
<td>0±1</td>
<td>8±15</td>
<td>1±2</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>5.0±0.4</td>
<td>5.0±0.5</td>
<td>5.2±0.7</td>
<td>5.8±2.1°</td>
<td>5.3±0.7</td>
</tr>
<tr>
<td>Fasting Insulin (mU/l)</td>
<td>6.8±4.3</td>
<td>10.2±9.2°</td>
<td>13.1±4.9°</td>
<td>17.3±11.7°</td>
<td>20.2±8.4°°</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.1±1.6</td>
<td>3.5±1.7</td>
<td>4.9±0.6°</td>
<td>4.9±0.8°</td>
<td>5.3±1.0°</td>
</tr>
<tr>
<td>HDL-Cholesterol (mmol/L)</td>
<td>1.24±0.31</td>
<td>1.32±0.36</td>
<td>1.03±0.18°</td>
<td>1.09±0.27°</td>
<td>1.11±0.13</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol/L)</td>
<td>2.44±1.37</td>
<td>1.73±1.51</td>
<td>3.12±0.58°</td>
<td>3.03±0.71°</td>
<td>3.41±0.91°</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.63±1.82</td>
<td>1.30±0.64</td>
<td>1.67±0.74</td>
<td>1.79±1.02</td>
<td>1.67±0.45</td>
</tr>
</tbody>
</table>
Legend-Figures

Figure 1

Intermittent hypoxia leads to insulin resistance in lean and diet-obese mice and impedes insulin-mediated glucose uptake in adipocytes. Lean (LF) and diet-induced obese (HF) mice were treated with IH or control (N) for 6 weeks. Intraperitoneal insulin tolerance test (ITT) was performed following a 6-hours fasting. (A) ITT represented for the whole, un-matched cohort (n=10-13 per group) and (B) ITT represented for the cohort with obese mice matched for body weight (n=7 per group) and (C) Area under the curves (AUC). (Data are presented as mean ± SD, *p<0.05; p<0.05 † = vs. LF-N, ¶ = vs. LF-IH). Next, we investigated the impact of IH on cultured adipocytes. 3T3-L1 adipocytes were exposed to alternating cycles of 40 sec of 16%O\textsubscript{2}/5%CO\textsubscript{2}/balance N\textsubscript{2} and 40 sec of 3%O\textsubscript{2}/5%CO\textsubscript{2}/balance N\textsubscript{2} for 8hrs/d for 3 consecutive days (IH) or an identical regimen of 16%O\textsubscript{2}/5%CO\textsubscript{2}/balance N\textsubscript{2} (Control). Insulin-stimulated \textsuperscript{3}H-glucose transport was subsequently monitored. Fold-change relative to basal glucose uptake is presented (D). (n=4, data are presented as mean ± SD, *=p<0.05).

Figure 2

IH down-regulates insulin-receptor substrate (IRS)-1 mRNA in mice and cultured adipocytes and inhibits the insulin signalling pathway. mRNA of epididymal adipose tissue of lean (n=6 per group) and obese mice (n=8 per group) (A) and cultured 3T3-L1 adipocytes (n=4) (B) following treatment with IH or control was harvested and IRS-1 expression was measured by real-time PCR. (Data are presented as mean ± SEM, *=p<0.05). 3T3-L1 adipocytes treated with IH or control were stimulated ± insulin 100 nM for 30 min and whole-cell lysates were blotted for the indicated antibodies (C) (n=3, shown is a representative blot and densitometry analysis, mean ± SEM, *=p<0.05). Adipose explants of obese mice treated with IH or control were stimulated ± insulin (100 nM) for 15 min and lysates were blotted for P-Akt, total Akt and β-Actin (D) (n=4 per group, shown is a representative blot and densitometry analysis for P-Akt/total Akt, mean ± SEM, *=p<0.05)
Figure 3

IH induces a pro-inflammatory phenotype in visceral epididymal adipose tissue (EAT) in lean (LF) and obese (HF) mice and polarizes THP1-derived macrophages towards a M1 pro-inflammatory phenotype. Lean (LF) and diet-induced obese (HF) mice were treated with IH or control (N) for 6 weeks. Stromal vascular fraction of the EAT was obtained (LF: n=6, HF: n=10 per group) and recruitment of adipose-tissue macrophages (ATM) were determined by flow cytometry. Cells double positive (F4/80+/CD11B+) were characterized as ATM. Of these, percentage of M1 macrophages (F4/80+/CD11B+/CD11C+/Cd206dim) was monitored (A) and correlated to the level of insulin resistance as determined by insulin tolerance test (B). Percentage of M2 macrophages (F4/80+/CD11B+/CD11C+/Cd206bright) was also evaluated (C). (Data are presented as mean ± SD, p<0.05, *=vs LF-N, ¶=vs LF-IH, †=vs HF-N). Furthermore, we investigated the presence of Crown-like structures (CLS) by F4/80 immunohistochemistry (D) (shown are representative images, arrows indicate CLS). CLS density was determined by counting of CLS in 10 random high-power fields (20x) (E) and results are represented for un-matched and weight-matched animals (HF mice). (Data are presented as mean ± SEM, *=p<0.05 vs LF-N). Next, we investigated the pro-inflammatory signature of the adipose tissue. Adipose explants (50mg) obtained following treatment with IH or control were cultured ex-vivo for 24 hrs. Media was harvested and incubated with NIH-3T3-NF-κB luciferase cells for 16 hrs at 37°C before luminescence was measured (F) (Data are presented as mean ± SD, p<0.05, *=vs LF-N, ¶=vs LF-IH, †=vs HF-N, LF: n=7, HF: n=10-12 per group). In order to test the direct effect of IH on macrophage polarization, THP1-derived macrophages were treated with IH or control and for the last 24 hrs were incubated in RPMI medium (basal) or stimulated with IFNγ (20ng/ml) or LPS (1µg/ml). mRNA was harvested and reverse
transcribed before real-time PCR of M1-characteristic genes. (Data are presented as mean ± SEM, *p<0.05 IH vs Control, n=3-6) (G).

Figure 4

**OSA is independently associated with insulin resistance.** *HOMA-IR* index in subjects of three different weight categories without (group 1A, 2A and 3A) and with OSA (group 1B, 2B and 3B) free of any cardiovascular or metabolic co-morbidities were calculated. (Data are presented as mean ± SEM, p<0.05 $=$ vs. control, ¶= vs. group 1A, * = vs. group 1A+B; † = vs. group 2A, £=vs group 2A+B).

Figure 5

**OSA severity as determined by the apnoea/hypopnoea index (AHI) correlates significantly and independently with serum levels of sCD163.** To determine the potential role of ATM polarization towards a M1 pro-inflammatory phenotype in patients with OSA, we measured circulating serum levels of CD163, an established marker of macrophage activation and M1 polarization, in OSA patients and controls via ELISA (n=149). Correlation of sCD163 with OSA severity as determined by apnoea/hypopnoea index (AHI) is demonstrated.
Figure 2

A

IRS-1

Fold Change over LF-N

<table>
<thead>
<tr>
<th>Condition</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
<td>0.062</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

IRS-1

Fold Change over Control

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Control

Insulin: - - + + +

P-Tyr (IRS)

P-Tyr (IRβ)

P-Akt (Ser43)

Total Akt

β-Actin

IH

Insulin: - - + + +

P-Akt (Ser43)

Total Akt

β-Actin

D

Insulin: N - + IH - +

P-Akt/Akt

Fold Increase with Insulin over basal (AU)

<table>
<thead>
<tr>
<th>Condition</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fold Increase with Insulin over basal (AU)

<table>
<thead>
<tr>
<th>Condition</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3**

**A** M1

Percentage of M1 Macrophages

<table>
<thead>
<tr>
<th>Condition</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.001

**B**

Percentage of M1

<table>
<thead>
<tr>
<th>AUC ITT</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r=0.691

**C** M2

Percentage of M2 Macrophages

<table>
<thead>
<tr>
<th>Condition</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p=0.103

**D**

NF-κB Activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p=0.097

**E**

Mean CLS n/20x

<table>
<thead>
<tr>
<th>Condition</th>
<th>LF-N</th>
<th>LF-IH</th>
<th>HF-N</th>
<th>HF-IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>6-7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p=0.03

**F**

Gene of interest (fold change relative to Control basal)

| Condition | Basal IFN-γ LPS Basal IFN-γ LPS Basal IFN-γ LPS |
|-----------|---------|---------|---------|---------|
|           |         |         |         |         |

* p<0.001
Figure 4

HOMA-IR

BMI<30
Control
BMI<30
OSA
BMI>30,<35
Control
BMI>30,<35
OSA
BMI>35
Control
BMI>35
OSA

GROUP: 1A 1B 2A 2B 3A 3B

p=0.07
Intermittent Hypoxia in Obstructive Sleep Apnoea mediates Insulin Resistance through Adipose Tissue Inflammation

ONLINE DATA SUPPLEMENT

Aoife M. Murphy, Amandine Thomas, Sophie J Crinion, Brian D Kent, Murtaza M Tambuwala, Aurelie Fabre, Jean-Louis Pepin, Helen M Roche, Claire Arnaud and Silke Ryan
Methods

Study Design

To investigate the impact of IH on IR we employed a comprehensive translational approach using a murine model of IH, a state-of-the-art IH system for cell cultures and a tightly-selected patient cohort. Primary objective of the human study was to evaluate the effect of OSA on HOMA-IR in subjects of different weight categories. The expected difference in HOMA-IR between groups which might be clinically important and the pooled standard deviation were specified on the basis of previous published studies and of in-house pilot investigations. The required sample size to detect a difference in HOMA-IR of 1.3 between OSA subjects and controls with 80% power at the 5% significance level was 31 subjects in each group.

The number of animals used in the mice studies was determined by power analysis on the basis of pertinent literature and pilot studies and the number of animals used in each experiment is listed in the figure legend. Animals were randomly assigned to diets and treatment groups.

In vitro experiments were routinely repeated a minimum of three times.

Diets

Male C57Bl/6 mice (5-weeks old) were purchased from Janvier Labs (France). Mice were either fed a high-fat diet (HF, 20 kcal/100 kcal protein, 20 kcal/100 kcal carbohydrate, 60 kcal/100 kcal fat, Research Diets, New Brunswick, NJ, USA) or matched low-fat diet (LF, 20 kcal/100 kcal protein, 70 kcal/100 kcal carbohydrate, 10 kcal/100 kcal fat, Research Diets) for 14 weeks.

Intraperitoneal glucose and insulin tolerance test

Mice were fasted for 6 hours and were injected intraperitoneally with 15% (wt/vol) glucose (2g/kg) for glucose tolerance test (GTT) or insulin (0.5 IU/kg, Novo Nordisk A/S,
Bagsvaerd, Denmark) for insulin tolerance test (ITT), respectively. Blood glucose was monitored at indicated time points via tail vein blood sampling using a glucometer (LifeScan, Issy-Les-Moulineaux, France).

**Stromal vascular fraction isolation and flow cytometry**

Epididymal fat pads were minced and adipocytes and stromal vascular fractions were separated by collagenase (2 mg/ml) digestion prior to centrifugation. Stromal vascular cells were filtered, blocked with PBS/2% BSA, and stained with fluorescently labelled antibodies: F4/80-FITC, CD11C-RPE, Cd206-Alexa Flour 647 (ABD Serotec, Kidlington, UK) or CD11B-PerCP Cy5.5 (BD Biosciences, Franklin Lakes, NJ, USA). Unstained, single stains and fluorescence minus one controls were used for setting compensations and gates. Flow cytometry was performed and analysed on a BD Accuri™ C6 platform (BD Biosciences) (E1). Cells double positive (F4/80+/CD11b+) were classified as macrophages and of them, CD11C+/Cd206dim cells were categorized as M1 and CD11C+/Cd206bright cells as M2 macrophages, respectively.

**F4/80 Immunohistochemistry**

Formalin-fixed, paraffin-embedded adipose tissue samples were deparaffinized and hydrated using xylene and alcohol. Heat antigen retrieval was performed for 20 min at 97 degrees in pH 6 buffer (Dako, Carpinteria, CA, USA). Sections were incubated in 3% H2O2/methanol for 10 min before being processed using the Dako Envision FLEX Rabbit Linker kit (Dako) according to the manufacturer’s instructions. Sections were incubated with primary F4/80 antibody (Abcam, Cambridge, UK) for 30 min at room temperature (1:500), followed by polyclonal rabbit anti-rat secondary antibody (1:500). Sections were detected with DAB and counterstained with hematoxylin. For quantification of F4/80 positive cells, slides were digitalized using ScanScope® XT (Aperio, Vista, CA, USA) and automatically analysed using Spectrum analysis algorithm package and ImageScope Analysis software.
Crown-like structure (CLS) density was determined by counting of CLS in 10 random high-power fields (20x). Immunohistochemistry analysis was performed by an expert pathologist (AF) blinded to the randomization and diets.

**Insulin stimulation of adipose tissue explants**

Fresh adipose tissue explants (50 mg) from HF mice were placed in PBS+0.2% BSA before stimulation ± insulin (100 nM) for 15 min. Tissue was washed, lysed in RIPA buffer and homogenized using a tissue homogenizer (Precellys24, Bertin Technologies, France). Lysates were stored at -80°C for further analysis.

**Ex-vivo adipose tissue culture and NIH-3T3 NF-κB luciferase activity assay**

Adipose explants (50mg) were cultured in complete media for 24 hrs. Media was harvested and incubated with NIH-3T3 cells for 16 hrs at 37°C. Cells were washed with PBS and lysed, and luminescence was measured using a commercial assay kit (Promega, Madison, Wisconsin, USA).

**In vitro glucose uptake assay**

Insulin sensitivity of 3T3-L1 adipocytes following treatment was monitored as previously described (E2). Briefly, cells were serum starved for 24 hours, and glucose starved in glucose-free DMEM (ThermoFisher, Grand Island, NY, USA)/0.2% BSA for 30 min before stimulation ± insulin (100 nM) for 15 min. \(^{3}H\)glucose (0.1mM 2-deoxyglucose+ 0.5 µCi/ml \(^{3}H\)deoxyglucose [Perkin-Elmer Analytical Sciences, Dublin, Ireland]) was added for 30 min before washing with PBS. Cells were lysed in RIPA buffer, and \(^{3}H\) glucose uptake was measured by liquid scintillation counting. Fold increase in glucose uptake over basal (non-insulin-stimulated) is presented.

**Human studies**

Consecutive males with suspected OSA or a history of snoring, without prior diagnosis of cardiovascular or metabolic disorders and not commenced on regular medication...
were invited to participate. Each subject underwent detailed clinical assessment, testing for full blood count, liver and kidney function, and was assessed for cardiovascular risk factors. The presence of OSA was determined by an apnoea/hypopnoea index (AHI) of ≥ 10/hr. The homeostasis model assessment resistance index (HOMA-IR) was calculated by the equation: insulin (μU/l)*glucose (mmol/l)/22.5.

**General laboratory methods**

*Real-time PCR analysis:* RNA was extracted from adipose tissue, 3T3-L1 cells or THP1-derived macrophages using TRIzol (Invitrogen, Carlsbad, CA, USA). The RNA concentration was measured by using a NanoDrop apparatus (Wilmington, DE, USA) and RNA integrity was determined by agarose gel electrophoresis. Reverse transcription was carried out using SuperScript II (Invitrogen). Primers, probes and Taqman Universal Mastermix were purchased from Applied Biosystems (Foster City, CA, USA). Real time quantification of cDNA was carried out on the ABI Prism 7900HT sequence detection system, normalized to 18S rRNA or GAPHD for each sample and analysed according to the $\Delta\Delta C_T$ method (E3).

*Western blot analysis:* Whole cell extracts from 3T3-L1 adipocytes and murine adipose tissue lysates, normalized for protein content (DC protein assay, Bio-Rad, Hercules, CA, USA), were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted as described previously (E4). Primary antibodies against Phosphotyrosine (Upstate Biotechnology, Billerica, MA, USA), Phospho-Akt (Ser 473), total Akt (Cell Signaling Technology, Danvers, MA, USA) and β-Actin (Sigma Aldrich, St. Louis, Missouri, USA) were used, as well as species-specific HRP-conjugated secondary antibodies. Software ImageJ 1.47v was used to quantify the Western blot signals.

**Stepwise linear regression analysis**
To identify potential independent predictors of HOMA-IR and sCD163, respectively, we used a stepwise backward linear regression model with HOMA-IR and sCD163 as the dependent variables and age, BMI, waist-hip ratio, smoking status (pack years), total cholesterol, triglyceride, LDL-Cholesterol, HDL-Cholesterol, Epworth sleepiness scale (ESS) and apnoea/hypopnoea index (AHI) as independent factors. In a second analysis, the AHI was replaced by the oxygen desaturation index (ODI).
References


Supplementary Figures

Supplementary Figure E1

Atmospheric and cellular pO\textsubscript{2} values in the in vitro model of intermittent hypoxia (IH).

Here, we describe a novel, state-of-the-art model of IH for cell cultures. Cells were grown on a semi-permeable membrane (Lumox\textsuperscript{®}, Sarstedt, Nuermbrecht, Germany) to allow rapid gas exchange. The IH protocol consisted of alternating cycles of 40 sec of 16% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2} and 40 sec of 3%O\textsubscript{2}/5%CO\textsubscript{2}/balance N\textsubscript{2}. The protocol was applied for 8 hours/day for 3 consecutive days. The control chamber was simultaneously cyclically gassed with 16% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2}. Both chambers were kept in a closed glove box maintaining temperature at 37°C with separate heat exchangers ensuring constant temperature in the IH and control cabinets. A snapshot of measurements of atmospheric and cellular pO\textsubscript{2} values is demonstrated.

Supplementary Figure E2

Macrophage M1/M2 ratio in epididymal adipose tissue. Lean (LF) and diet-induced obese (HF) mice were treated with IH or control (N) for 6 weeks. Stromal vascular fraction of the EAT was obtained (LF: n=6, HF: n=10 per group) and recruitment of adipose-tissue macrophages (ATM) were determined by flow cytometry. Percentages of M1 and M2 were monitored and ratio of M1/M2 was calculated. (Data are presented as mean ± SD, p<0.05, *=vs LF-N, ¶=vs LF-IH, †=vs HF-N).
1 cycle consists of 40 sec of 16% atmospheric O2 and 40 sec of 3% O2.
Figure E2

M1/M2 ratio

- LF-N
- LF-IH
- HF-N
- HF-IH

p = 0.068

* ¶†