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

ONLINE DATA SUPPLEMENT

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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.
(version 12, Aperio Technologies, Inc.). 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. [³H]glucose (0.1mM 2-deoxyglucose+ 0.5 μCi/ml [³H]deoxyglucose [Perkin-Elmer Analytical Sciences, Dublin, Ireland]) was added for 30 min before washing with PBS. Cells were lysed in RIPA buffer, and [³H] 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 (mU/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 ΔΔC_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


