Intermittent hypoxia induces NFκB-dependent endothelial activation via adipocyte-derived mediators

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Running title: Adipocyte- endothelial cell interaction by IH

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**Abstract:**

Aberrant release of adipocytokines from adipose tissues dysregulates cardiometabolic functions. The present study hypothesizes that chronic intermittent hypoxia (IH) present in obstructive sleep apnea leads to adipose tissue dysfunction, which in turn contributes to vascular pathogenesis. The effect of IH was evaluated in adipose depots and aortic tissues in lean rats *in vivo*. Further, the cellular and molecular mechanisms underlying pathophysiological interactions between adipocytes and endothelial cells were investigated *in vitro*. The *in vivo* results showed that IH induced upregulation of IL-6 and MCP-1 in subcutaneous and peri-aortic adipose tissues; and downregulated phosphorylation of endothelial nitric oxide synthase [eNOS (ser1177)] in the aorta with activation of Erk and p38 MAPK. In support, cultured adipocytes demonstrated IH-induced elevations of NADPH oxidase (NOX) 4, phosphorylation of Erk, NFκBp65 and inducible NOS (iNOS); and increased expression of IL-6 and MCP-1. Likewise, endothelial EA.hy926 (EA) cells exposed to IH showed eNOS (ser1177) and [cGMP] reduction, while MCP-1 and iNOS expression were upregulated. Treatment of EA cells with conditioned media derived from IH-exposed cultured adipocytes caused nuclear translocation of NFκBp65 and elevation of MCP-1, which were prevented by addition of neutralizing IL-6 antibodies to the conditioned media. Recombinant IL-6 in addition to IH induced further MCP-1 release and iNOS protein expression in EA cells, which were prevented by pharmacological inhibition of Erk, p38 and NFκB. These findings suggest that IH could induce adipose tissue inflammation, which may cross-talk with endothelial cells via adipocyte-derived mediators such as IL-6, and promote NFκB-dependent endothelial dysfunction. (247 words)

Keywords: Intermittent hypoxia, adipose inflammation, endothelial function, NFκB
Introduction

Obstructive sleep apnea (OSA) is highly prevalent in adults of various ethnicities globally (29). It is characterized by repetitive episodes of complete or partial upper airway collapse during sleep, with direct consequences of recurrent drops in arterial oxygenation (intermittent hypoxia, IH). Obesity is a major risk factor for the development and severity of OSA (3). Increasing evidence demonstrates an association between OSA and augmented risk of cardiovascular morbidity and mortality (3, 18, 27). Thus, OSA represents another insult increasing the total burden of cardiovascular diseases (CVD). The endothelium plays an important role in the regulation of vascular tone and the maintenance of vascular homeostasis (27, 39). Endothelial dysfunction, characterized in vivo by a reduced flow-mediated vasodilatation due to decreased production of nitric oxide (NO), predates atherosclerosis and predicts future CVD (17, 25). In OSA subjects, decreased circulating nitric oxide levels and impaired endothelium-dependent relaxations, independent of obesity, have been demonstrated (24, 27). Chronic IH during sleep may result in a number of pathological responses such as oxidative stress, inflammation and sympathetic activation, all of which may contribute to endothelial dysfunction (23, 24).

Adipose tissues produce an array of bioactive molecules that regulate metabolic function systemically, and, under certain pathological conditions, adipose tissues become dysfunctional and invoke downstream vascular and metabolic pathogenesis (12, 40). In animal models of diet-induced obesity, adipocytes and adherent macrophages in depot-specific adipose tissues, mainly visceral and subcutaneous fat, exhibit a state of chronic low grade inflammation (40). Adipokines/cytokines produced in the adipose tissues may enter into the systemic circulation to affect peripheral target organs including the endothelium (4, 32). In particular, perivascular adipose tissue that anatomically surrounds blood vessels, participates in the maintenance of vasomotor tone and the regulation of endothelial functions (5, 6). This paracrine contributory role of perivascular adipose tissue in the pathogenesis of vascular disease in humans is becoming increasingly apparent (5, 6, 8).

The present study tested the hypothesis that IH induces dysregulated release of inflammatory mediators in adipose tissues and has a pathological impact on endothelial cell activation. The release of depot-specific adipose tissues-derived pro-inflammatory mediators and the status of aortic activation were assessed using an in vivo lean IH-exposed rat model. The possible signaling mechanisms involved in the IH-induced activation of adipocytes and endothelial cells and their interactions were
investigated using an in vitro approach.

Materials and Methods
Animals and IH Exposure

Twelve healthy male Sprague-Dawley rats (~200 g; six-week old) were randomly divided into two groups and fed with standard chow diet ad libitum. One rat died during the run-in period. Rats receiving treatment of intermittent normoxia (IN) and intermittent hypoxia (IH) were housed in two identical commercially designed chambers (Oxycycler model A84; Biospherix, Redfield, NY, USA) which allowed for the application of IN or IH for six hours daily, during 9:00am to 3:00pm, for six weeks. The oxygen (O₂) concentration in the chamber was continuously measured by an O₂ analyzer during the exposure. Air-exchange cycles of two minutes of 21% oxygen (room air) and four minutes of 10% oxygen were applied, resulting in alternating intervals of 90 seconds of 21% O₂, gradually lowering to a trough of 10% O₂ maintained for about 90 seconds towards the end of each cycle (20). This hypoxic profile was associated with arterial oxyhemoglobin saturations (SpO₂) which fluctuated between a peak of 90-95%, gradually decreasing to a nadir of 60-75%, for ten times per hour, as measured using a pulse oximeter (MouseVentG500; Kent Scientific; Torrington, CT, USA). The achieved SpO₂ pattern was similar to the reported levels in similar experimental settings (7, 13).

At the end of the six-week exposure, all rats were sacrificed by an intraperitoneal overdose (100 mg/kg) of pentobarbitone. To determine whether or not chronic exposure to IH causes adipokine dysregulation in various depot-specific adipose tissues, subcutaneous, epididymal and peri-aortic adipose tissues were collected and dissected in cold phosphate-buffered saline (PBS) for the determination of protein expression of pro-inflammatory biomarkers. Aortae with intact endothelium, which may be influenced by either circulating adipose tissues-derived factors or their peri-vascular adipose tissue, were snap-frozen and stored at -70°C until detection of the protein expression of endothelial NOS (eNOS; phosphorylated at ser1177), pro-inflammatory Erk/p38 MAPK signaling components and inducible NOS (iNOS).

The animal care and experimental protocol for the present study were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATR 2371-11). The research works were performed in accordance with the APS Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.
**IH exposure and cell cultures**

**IH exposure**

To evaluate the direct impact of IH on adipocytes and endothelial EA.hy926 (EA) cells, these cells were subjected separately to the exposure of IH as described (21). Briefly, cells were maintained at 37°C at 5% CO₂ in a chamber (Oxycycler model A42, Biospherix) in which O₂ levels were alternated between 21% for five minutes and 1% for ten minutes, for a total of 64 cycles (18 hours). The percentage of O₂ in the cell culture medium fluctuated between 18% (normoxic phase) and a nadir of 4.5-5% (hypoxic phase) per cycle. Cells in the control group were maintained under normoxic condition (IN: 21% and 5% CO₂) throughout the experiments.

**Adipogenic differentiation of preadipocytes:** Primary human preadipocytes-subcutaneous (HPA-s; passages 3 to 5) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA; Catalog no.: 7220) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma-Aldrich; St. Louis, MO, USA) supplemented with 20% heat inactivated fetal bovine serum (FBS) (Gibco; Grand Island, NY, USA). After reaching 70-80% confluence in T25 culture flask, adipogenic differentiation was induced by replacing the culture medium with a differentiation cocktail containing 10 µg/mL insulin (Gibco), 500 µM isobutylmethylxanthine (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich) and 100 µM indomethacin (Sigma-Aldrich) in IMDM supplemented with 10% FBS for 3 days. This was followed by 2 days of maintenance in IMDM supplemented with 10% FBS and 10 µg/mL insulin. After 6 cycles of differentiation and confirmed by lipid staining [Oil Red O staining (data not shown)], the cells are referred to as mature adipocytes (adipocytes). Before the experiment, adipocytes were cultured in fasting medium (3 mL IMDM containing 1% FBS and 10 µg/mL insulin) overnight before being subjected to IH. After IH exposure, cells were collected for protein extraction and evaluation of protein expressions for NOX4, Erk, p38, NFκBp65 and iNOS. The adipocyte-derived conditioned media after IH exposure or under normoxic condition were collected, sterile-filtered and frozen at -70°C until future experiments. Levels of IL-6 and MCP-1 release in the conditioned media were measured.

**Endothelial cells:** Endothelial EA cells were originally obtained from American type Culture Collection (ATCC, Manassas, US; catalog no.: CRL-2922™) and were cultured in Dulbecco's Modified Eagle Medium (DMEM GlutaMax) (Gibco) supplemented with 10% FBS. Experiments were performed using cells at passages 6 to 11. Cells in six-well plates were cultured in fasting medium (1 mL DMEM containing 1% FBS) overnight before IH exposure. Treated cells were collected for
protein extraction and evaluation of protein expressions for p-eNOS, eNOS, p-Erk, Erk, p-p38, p38 and iNOS. Levels of IL-6 and MCP-1 release in the EA cells-derived conditioned media were measured.

**Influence of adipocyte-derived factors on endothelial cells:**
To investigate the potential impact of adipocyte dysfunction on endothelial cells, conditioned media derived from cultured adipocytes under hypoxic (IH) and normoxic (IN) conditions were collected for treatment of EA cells *in vitro*. The conditioned media were diluted 1:1 with DMEM containing 1% FBS before treatment. Since all organs would be simultaneously vulnerable to IH exposure *in vivo*, all experiments were done on EA cultures under both IH or IN conditions. NFκBp65 nuclear translocation and MCP-1 release were studied. To investigate the possible effect of IL-6 as an adipocyte-derived cytokine on endothelial cell activation, EA cells were pre-treated with anti-human IL-6 antibodies (0.2 µg/mL; R&D; Minneapolis, MN, USA) or the respective control (IgG) before incubation with adipocyte-derived conditioned media. Furthermore, recombinant human IL-6 (rIL6; R&D), at a concentration similar to that detected in IH-stimulated adipocyte-derived conditioned media, was added to the endothelial cells for evaluation of cyclic guanosine monophosphate (cGMP), MCP-1 release and iNOS protein expression. The possible signaling mechanisms involved in endothelial cell activation by rIL-6, illustrating the effect of adipocyte-derived IL-6, were determined with the use of various selective inhibitors. Inhibitors of MAPK/ERK (PD98059, 10 µM; Merck Millipore; Darmstadt, Germany), p38 MAPK (SB203580, 10 µM; Cell Signaling; Danvers, MA, USA) and NFκB/IKB kinase (SC514, 10 µM; Tocris; Bristol, UK) were administered to the EA cells 45 minutes prior to IH exposure.

**Intracellular cGMP measurement**
To investigate the influence of IH on nitric oxide bioavailability, intracellular cGMP ([cGMP]i) levels were measured in duplicate by immunoassay (Biomedical Technologies Inc.; Stoughton, MA, USA) in treated EA cells, according to the manufacturer’s instructions. Values were calculated as picomole (pmol) per milligram cellular protein and expressed as relative fold-changes compared to normoxic group.

**Assessment of pro-inflammatory mediator expression in adipose tissues or cell cultures**
Tissue lysates from adipose tissues were prepared in ice-cold lysis buffer (Thermo Scientific; Waltham, MA, USA) containing a cocktail of protease and phosphatase inhibitors (Thermo Scientific); and 1 mM phenylmethylsulfonyl fluoride (Thermo
Scientific). The protein concentration was determined using the Bradford Assay. Tissue lysates from subcutaneous, epididymal and peri-aortic adipose tissues were then analyzed in duplicate for the protein expression of IL-6, MCP-1 (ELISA kits, BD Biosciences; San Jose, CA, USA) and the content of angiotensin II (Ang II) (EIA kit, Cayman; Ann Arbor, MI, USA) in peri-aortic adipose tissues from both IN- and IH-treated groups. For in vitro experiments, the protein release of IL-6 and MCP-1 in conditioned media derived from cultured adipocytes and EA cells were analyzed in duplicate (ELISA kits, BD Biosciences) according to the manufacturer’s instructions.

**Western blotting**

Total protein lysates (30 μg) from adipose tissues, aortae, cultured adipocytes and EA cells or cytoplasmic (30 μg) and nuclear (5 μg) protein extracted from EA cells were used. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes which were incubated overnight with the antibodies against phosphorylated (p)-eNOS (ser1177) (BD-612393), eNOS (BD-610297), iNOS (1:1000; BD-610432; BD Biosciences), p-p38 (CS-4511), p38 (CS-9212), p-Erk1/2 (CS-4370) and NFκBp65 (CS-4764) (1:1000; Cell Signaling); NADPH oxidase 4 (NOX4; SC-30141; 1:500) and Erk1 (SC-94; 1:1000; Santa Cruz; Dallas, TX, USA) at 4°C. This was followed by incubation with HRP-labeled secondary antibodies (Novus; Littleton, CO, USA) prior to image detection by enhanced chemiluminescence (Amersham Biosciences Inc; Piscataway, NJ, USA). A control housekeeping protein, GAPDH, was used to demonstrate the levels of sample loading.

**Statistical analysis**

All data are shown as mean±S.E.M. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc; San Diego, CA, USA). Comparisons between groups were performed using independent sample t-test where appropriate. Comparisons of more than two groups were carried out with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. P values less than 0.05 were considered to indicate statistically significant differences.
Results

Basal and stimulated levels (IH exposure) of pro-inflammatory mediators from adipose tissues in rats

The basal levels of IL-6 and MCP-1 protein expression in peri-aortic adipose tissue were significantly higher (greater than ten-fold) compared to that of epididymal and subcutaneous adipose samples. Exposure of the animals to IH for six weeks significantly enhanced the protein expression of IL-6 and MCP-1 in subcutaneous and peri-aortic but not in epididymal adipose tissues (Fig. 1A-B). Peri-aortic adipose tissue displayed a significant upregulation of Ang II and iNOS after IH (Fig. 1C-D). However, the circulating levels of IL-6 and Ang II were below detection limits of the assays used (data not shown).

Endothelial NOS, Erk, p38 and iNOS in rat aorta after exposure to IH

The rat aorta, corresponding to the site of peri-aortic fat depot studied, was selected for evaluation of the vasculature after IH exposure. The protein expression of one of the phosphorylation sites, ser1177 that positively regulates eNOS activity, was studied and was significantly downregulated after IH exposure (Fig. 2A). In addition, IH also induced activation of the inflammatory signaling components, p-Erk 1/2 and p-p38 (Fig. 2B-C) in aortic tissue compared to that of IN-treated rats. The protein expression of total iNOS in aorta was not different between IN- and IH-exposed groups (Fig. 2D).

Activation of cultured adipocytes and endothelial EA cells after exposure to IH

Exposure to IH, compared to normoxia, increased the protein expression of NOX4 in cultured adipocytes significantly (Fig. 3A). At the same time, IH also augmented the protein expressions of p-Erk 1/2, p65 and iNOS in cultured adipocytes (Fig. 3B-D). The p38 MAPK signaling component was not changed after IH (data not shown). The levels of IL-6 and MCP-1 secreted in conditioned media from cultured adipocytes were also augmented by IH exposure (Fig. 3E-F), in agreement with the in vivo findings in adipose tissues (Fig. 1A-B).

In EA cells, the protein expression of p-eNOS (ser1177) was downregulated (Fig. 4A). The bioavailability of nitric oxide, estimated from the [cGMP], level in these cells was significantly reduced after IH (Fig. 6A). The phosphorylation of Erk 1/2, p38 (Fig. 4B-C) and the release of pro-inflammatory mediators, MCP-1 (Fig. 4D), and iNOS protein expression (Fig. 4E) were significantly elevated after IH exposure. The cellular and secretory levels of IL-6 in EA cells were below detectable limits.
Activation of EA cells by IH-stimulated adipocyte-derived conditioned media

Treatment with IH-stimulated adipocyte-derived conditioned media (IL-6 level: 996±96 pg/ml and MCP-1 level 744±87 pg/ml after 1:1 dilution in the medium) followed by either IN or IH exposure caused a comparable nuclear translocation of NFκBp65 in EA cells (Fig. 5A and 5B) without affecting the cytoplasmic p65 protein expression. IN-stimulated adipocyte-derived conditioned media did not produce significant difference in p65 nuclear translocation in EA cells under either IH or IN condition. IH-stimulated adipocyte-derived conditioned media also significantly increased MCP-1 release in EA cells compared to IN-stimulated adipocyte-derived conditioned media but the level did not differ when the cells were further exposed to IH (Fig. 5C). The nuclear translocation of NFκBp65 and the elevation of MCP-1 release were blunted when the cells were pre-incubated with neutralizing antibodies against IL-6. There was no significant difference in MCP-1 release when the cells were incubated with IN-stimulated adipocyte-derived conditioned media with or without IL-6 neutralizing antibodies (Fig. 5C).

Activation of EA cells by rIL-6 treatment and/or IH

Recombinant IL-6 (rIL-6) instead of conditioned media derived from cultured adipocytes was used to treat EA cells. Treatment with rIL-6 (18 hours) alone caused downregulation of [cGMP], level in EA cells, consistent with that observed after IH exposure. Similarly, treatment of IH-exposed EA cells with rIL-6 caused significant further reduction in the [cGMP], level compared to IH alone. Nevertheless, there was no further reduction in the rIL-6 plus IH combination group compared to rIL-6 treatment alone (Fig. 6A). On the other hand, MCP-1 release from the cells after rIL-6 treatment or IH alone was significantly increased. Incubation with rIL-6 of IH-exposed EA cells caused additional induction of MCP-1 release compared to either rIL-6 or IH alone (Fig. 6B).

Role of Erk, p38 and NFκB in rIL-6/IH-induced pro-inflammatory response in EA cells

The additive effect of rIL-6 and IH on elevation of MCP-1 release and iNOS protein expression was abolished after pre-incubation with PD98059 (MAPK/Erk inhibitor; 10 μM), SB203580 (p38 MAPK inhibitor; 10 μM) and SC514 (NFκB/IκB kinase inhibitor; 10 μM) (Fig. 7A and B). Under normoxic condition, there were no significant inhibitory effects of the various inhibitors on rIL-6-induced MCP-1 release and iNOS protein expression, except for a significant reduction in iNOS protein expression in the PD98059-treated group.
Discussion

The present study demonstrates that IH exposure of lean rats induced a pro-inflammatory phenotype in subcutaneous and peri-aortic adipose tissues as well as an impaired eNOS activity with inflammatory activation in the aorta. In support of a regulatory role of adipose tissues on the endothelium, IH-exposed cultured adipocytes showed activation of NOX4 and Erk/NFκB signaling pathway, while its conditioned media, when added to endothelial cell cultures, evoked NFκB activation and secretion of pro-inflammatory mediators from endothelial cells. Consequently, adipocyte-derived IL-6 was demonstrated to be a mediator in this interaction.

IH and adipose tissue inflammation

The pathological impact of IH on adipose tissues has been suggested to contribute to functional alterations and upregulation of pro-inflammatory mediators, such as IL-6, MCP-1, TNF-α and the oxidative product, malondialdehyde, in fat tissues of experimental animals (11, 30, 31). However, most earlier experiments focused on the evaluation of this impact on visceral fat while there is limited information on other fat depots (11, 30, 31), probably because visceral fat may be more relevant for cardiometabolic diseases in humans (1, 30, 32). The present study in lean rats shows that IH induces the production of pro-inflammatory components (IL-6, MCP-1) in subcutaneous fat depot. Our in vitro findings on cultured subcutaneous adipocytes also demonstrate a similar augmented pro-inflammatory response as evidenced by the levels of IL-6, MCP-1 and iNOS, in line with previous in vitro studies (22, 37).

NADPH oxidase 4 (NOX4), a constitutive active enzyme responsible for the generation of reactive oxygen species (ROS), is expressed in various cell types including adipocytes (19). A significant upregulation of NOX4 protein expression was seen in cultured adipocytes after IH exposure, consistent with an increased oxidative load in IH. In line with the upregulation of NOX4 expression as the possible upstream component activating Erk/NFκB (19, 35), the current findings demonstrate a significant increase in Erk phosphorylation and NFκBp65 protein expressions as well as elevation of downstream pro-inflammatory mediators in IH-exposed adipocytes. It is proposed that this IH-induced adipose tissue inflammation may then exert pathological effect on the vascular endothelium.

Another notable finding relates to the differential effect of IH on various adipose tissue depots in the in vivo rat model. In the context of adipose tissue biology, the most abundant depot in human body is subcutaneous white fat, which plays an initial role in the response to external stimuli such as a positive caloric balance (1, 16).
When the subcutaneous fat can no longer respond to the stimulation and becomes dysfunctional, visceral fat may be affected (1, 16). Angiopoietin-like 4, one of the key players during atherogenesis, is present in subcutaneous adipose tissue and its level correlated with the severity of nocturnal hypoxemia in patients with sleep apnea (9), suggesting that subcutaneous adiposity may not be entirely an innocent bystander in cardiometabolic pathogenesis in the context of IH. The absence of increase in IL-6 and MCP-1 in visceral epididymal fat in the current findings may be related to the relatively mild IH profile used. Perivascular fat tissue, with its intimate anatomic relationship with the blood vessels, is recognized as an important visceral fat tissue (38) with a distinct physiological role in the regulation of vascular function and disease (6, 8). The present data demonstrate that, when compared to other adipose tissues including visceral epididymal and subcutaneous fat, the basal and IH-induced secretion of pro-inflammatory mediators (IL-6 and MCP-1) is higher (by more than ten-fold) in the peri-aortic depot. These findings are in agreement with other studies under different pathological conditions including obesity, suggesting a regulatory role of perivascular adipose tissues in vascular inflammation (6, 8). In addition, the elevated protein expression of Ang II in peri-aortic fat depots after IH might play a modulatory role. Besides its important role in the regulation of arterial blood pressure, Ang II induces inflammation and vascular injury (2, 28), and co-localizes with IL-6 in human coronary atherosclerotic plaques (33).

**IH and endothelial inflammation**

Exposure to IH has been consistently reported to result in direct activation of the endothelium and/or accelerates atherosclerosis (9, 14, 21, 23, 24, 26, 41). In the present experiments, endothelial dysfunction was observed after exposure to IH both in vivo and in vitro, as evidenced by the reduced protein expression of phosphorylated ser1177 eNOS (aorta and cultured EA cells) and the reduced cyclic GMP level (cultured EA cells) which is another well-established marker for NO bioavailability (10). Activation of the MAPK signaling pathway (Erk and p38) in aortic tissue with intact endothelium in vivo indicates initiation of proatherogenic responses within the vasculature (15, 26, 36). The initiation of pathological events such as upregulation of NFκB-dependent monocyte chemotactic activity due to reduced NO bioavailability (42) may be anticipated, given the IH-induced decrease in p-eNOS and elevation of MCP-1. Despite an increase in protein expression of iNOS in cultured endothelial cells after IH, the level of iNOS expressed in aorta was comparable between IH and control groups, in contrast to that reported in a previous animal study (14), and may be due to differences in IH duration and severity.
Adipose tissue–endothelium interactions

Several studies have reported the negative impact of IH on the endothelium as discussed (9, 14, 21, 23, 24, 26, 41). However, there are limited data regarding the regulatory impact of adipose tissues on the endothelium in the context of IH. The acceleration of atherosclerotic lesion formation in atherosclerotic-prone ApoE−/− mice after IH exposure can be improved after epididymal lipectomy (30). This observation suggests a possible contribution of adipose tissue dysfunction in IH-induced atherogenesis. In agreement, we found an elevation of NFκBp65 nuclear translocation and MCP-1 release in endothelial cells after direct incubation with IH-exposed adipocyte derived conditioned media. It has been reported previously that IL-6 participates in endothelial dysfunction and vascular pathogenesis via inflammatory activation of NFκB signaling pathway (33, 34). The attenuation effect produced by neutralizing antibodies against IL-6 strongly supports a mediating role of IL-6 in the adipocyte-derived conditioned medium. In confirmation, the application of rIL-6 produced similar activation as IH-exposed adipocyte-derived conditioned media, which was inhibited after pre-treatment with the selective inhibitors for Erk, p38 MAPK and NFκB, providing a mechanistic pathway for the interaction between adipocytes and endothelial cells.

Of note, the direct pro-inflammatory action of IH on endothelial cells was diminished in the presence of adipocyte-derived conditioned media. In IH-exposed endothelial cells, an additive increase in MCP-1 release was seen on treatment with rIL-6, but this was not observed when the cells were treated with IH-stimulated adipocyte-derived conditioned media. This suggested the possible presence of anti-inflammatory factors in adipocyte-derived conditioned media, and could reflect a physiologic response to mitigate the adverse impact of IH in vivo. The biologic system is equipped with various homeostatic mechanisms, so it is likely that multiple mediators, pathogenetic or protective, would be at play in IH-associated atherogenesis.

Limitations and Significance

Several caveats should be noted in the interpretation of the current findings. Firstly, using the IH model as a surrogate for OSA carries inherent limitations. The literature on IH models comprises diverse IH profiles, and this could result in different metabolic responses making comparisons of data between studies difficult. However, this may be analogous to the possible human scenario of OSA which encompasses a wide range of IH profiles which may even vary from night to night within the same individual. There is further limitation in replicating the IH profile of the in vivo model for in vitro experiments. Nevertheless, our in vitro model can achieve swings in
partial oxygen pressure in the medium for each IH cycle. Furthermore, IH is only one factor, albeit a prominent one, in OSA which carries multiple pathophysiological factors including hypercapnia, pressure swings, sympathetic activation, cerebral arousals and sleep fragmentation. Secondly, in the present study, we have only used cultured subcutaneous adipocytes \textit{in vitro} for further interrogation of adipose tissue-endothelium interactions. We have also assumed that adipocytes form the predominant cell type in adipose tissue in the lean rat, and account for the major effects of such tissues. We did not exclude the contribution of activated macrophages (especially in the context of obesity) in adipose tissue inflammation. Finally, the present study selected IL-6 and MCP-1 as the potential adipocyte-derived candidates for initiation of endothelial dysfunction in the context of IH. Nevertheless, it is plausible that other adipose tissue-derived mediators may also be involved.

In summary, the present study demonstrates a pathological impact of IH on adipose tissue biology, and the possible crosstalk between adipocytes and endothelial cells (Figure 8). Adipose tissue may exert its effect on the endothelium either from a distance through the blood circulation, or from a peri-vascular depot through paracrine effects. It is conceivable that IH-induced adipose cellular dysfunction may contribute to a proatherogenic milieu and further promote inflammatory endothelial activation in the context of clinical conditions with intermittent hypoxia such as sleep apnea.
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Disclosure

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author contributions

Conception and design of work: MYKL, JCWM, MSMI. Acquisition, analysis, and interpretation of data: MYKL, YW. Drafting and finalization of work for important intellectual content: MYKL, JCWM, MSMI.
Figure Legends:

Figure 1. The protein expression of pro-inflammatory mediators in different depots of adipose tissues after IH. The protein expression of (A) IL-6 and (B) MCP-1 in epididymal (n=3), subcutaneous (n=3) and peri-aortic (n=5) depots; (C) Angiotensin II (Ang II; n=3&4 for IH and IN groups, respectively) and (D) iNOS (n=4&5 for IH and IN groups, respectively) in peri-aortic depot. Values were normalized as milligram protein for IL-6, MCP-1 and Ang II determination. Protein expression for iNOS was normalized to GAPDH. Bars: mean ± S.E.M. *P<0.05 when compared to the corresponding control, intermittent normoxia (IN).

Figure 2. Phosphorylation of eNOS (ser1177), Erk and p38 in rat aorta after IH. The protein expression of (A) p-eNOS (ser1177) (n=4&5 for IH and IN groups, respectively), (B) p-Erk 1/2 (n=5), (C) p-p38 (n=5) and (D) iNOS (n=5) in rat aorta. Bars: mean ± S.E.M. *P<0.05, **P<0.01 when compared to the corresponding control, intermittent normoxia (IN). All results were normalized to the respective total protein or GAPDH for Western blot experiments.

Figure 3. Activation of cultured adipocytes after IH. Protein expressions of (A) NOX4 (n=5), (B) p-Erk 1/2 (n=5), (C) NFκBp65 (p65; n=4) and (D) iNOS (n=3) in cultured adipocytes exposed to IH for 64 cycles. Levels of adipocyte-derived pro-inflammatory mediators (E) IL-6 (n=6) and (F) MCP-1 (n=5) in conditioned medium were measured by ELISA. Bars: mean ± S.E.M. *P<0.05, **P<0.01 when compared to the corresponding controls, IN. All results were normalized to the respective total protein or GAPDH and expressed as relative fold-change with respective to control in IN condition for Western blot experiments.

Figure 4. Activation of endothelial EA cells after IH. Protein expressions of (A) p-eNOS (ser1177) (n=4), (B) p-Erk 1/2 (n=4), (C) p-p38 (n=4), (D) MCP-1 release (n=4) and (E) protein expression of iNOS (n=3) in cultured EA cells exposed to IH for 64 cycles; Bars: mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 when compared to the corresponding controls, IN. All results were normalized to the respective total protein or GAPDH and expressed as fold-change with respective to control in IN condition for Western blot experiments.

Figure 5. Cytoplasmic and nuclear protein expression of NFκBp65 and MCP-1 release in cultured EA cells with IN/IH-stimulated adipocyte-derived conditioned media (ADIPO-IN/-IH) treatments. (A-B) Cytoplasmic and nuclear protein expression of NFκBp65 (p65) (n=3) and (C) MCP-1 release (n=3&4 for IH and IN groups,
respectively, for MCP-1 measurement) in cultured EA cells with ADIPO-IN/-IH treatment followed by IH exposure (18 hours) in the absence or presence of neutralizing antibody for IL-6 (IL-6). Bars: mean±S.E.M. § P<0.01 when compared to ADIPO-IN in IN condition; *P<0.05, **P<0.01 when compared to ADIPO-IH in IH condition. Ɨ P<0.05 when compared to the control, ADIPO-IN in IH condition. All results were normalized to GAPDH or lamin A/C and expressed as fold-change with respective to control in IN/IH condition for Western blot experiments.

Figure 6. [cGMP]i level and MCP-1 release in cultured EA cells after rIL-6 and/or IH treatment. (A) Intracellular cGMP ([cGMP]i) level in EA cells was analyzed. Cells were also treated with rIL-6 (1 ng/mL) in the absence or presence of IH exposure. Level was normalized as milligram protein and expressed as relative fold-change for [cGMP]i level. n=4&6 for IH and IN groups, respectively. (B) The release of MCP-1 in the same experimental setting was measured. n=4&5 for IH and IN groups, respectively. Bars: mean ± S.E.M. *P<0.05, ***P<0.001 when compared to IN; Ɨ P<0.05 when compared to IH; § P<0.05 when compared to rIL-6.

Figure 7. MCP-1 release and iNOS protein expression in the presence of specific selective inhibitors in cultured EA cells after rIL-6 and/or IH treatment. EA cells were pre-treated with MAPK/Erk inhibitors, PD98059 (PD, 10 μM), p38 MAPK inhibitor, SB203580 (SB, 10 μM); and NFκB/IκB kinase inhibitor, SC514 (SC, 10 μM) for 45 minutes before challenged with rIL-6 (1 ng/mL) in the absence or presence of IH. (A) MCP-1 release (n=5&4 for IN and IH groups, respectively) and (B) iNOS protein expression (n=3) in EA cells was measured. Bars: mean ± S.E.M. *P<0.05 when compared to the control, IN-CTRL. § P<0.05 when compared to IH-CTRL; Ɨ P<0.05, # P<0.01 and ## P<0.001 when compared to rIL-6-treated cells with IH exposure. # P<0.05 when compared to rIL-6-treated cells with IN exposure. All results were normalized to GAPDH and expressed as fold-change with respective to control in IN condition for Western blot experiments.

Figure 8. Schematic diagram proposing the cellular interactions between adipocytes and endothelial cells in the context of intermittent hypoxia. Intermittent hypoxia (IH) increases the protein expression of NADPH oxidase 4 (NOX4), a reactive oxygen species-generating enzyme, and activates the downstream pro-inflammatory Erk/NFκB signaling pathway in adipocytes, which result in aberrant release of adipocytokines. Some of these IH-induced adipocyte-derived secretory products (e.g. IL-6) may exert a pathological impact on endothelial cells. Likewise, IH causes inflammatory activation of the Erk/p38 MAPK/NFκB signaling
pathway in endothelial cells, leading to upregulation of iNOS protein expression and monocyte chemoattractant protein 1 (MCP-1), an important chemokine involved in the sub-endothelial recruitment of monocytes as a proatherogenic event. Endothelial dysfunction is evidenced by reduced levels of phosphorylated eNOS (ser1177) and cGMP. These endothelial inflammatory responses are further augmented by IL-6, a pro-inflammatory mediator secreted by adipocytes on IH exposure, illustrating IH-induced cross-talk between adipocytes and endothelial cells. PD, PD98059, selective inhibitor of Erk; SB, SB203580, selective inhibitor of p38 MAPK; and SC, SC514, selective inhibitor of NFκB/IκB kinase.
Reference:


26. Li RC, Haribabu B, Mathis SP, Kim J, Gozal D. Leukotriene B4 receptor-1...


Figure 1

A

IL-6 (ng/mg protein)

Epididymal
Subcutaneous
Peri-aortic

IN
IH

* * *

B

MCP-1 (ng/mg protein)

Epididymal
Subcutaneous
Peri-aortic

IN
IH

* *

C

peri-aortic Ang II (pg/mg protein)

IN
IH

* *

D

iNOS
GAPDH

peri-aortic iNOS/GAPDH

IN
IH

* *

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Figure 2

A. p-eNOS (ser1177), eNOS, GAPDH

B. p-Erk1/2, Erk 1, GAPDH

C. p-p38, p38, GAPDH

D. iNOS, GAPDH

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Figure 4

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Figure 5

A

Cyto p65
GAPDH

B

Nuclear p65
Lamin A/C

C

MCP-1 release (pg/mL)

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Figure 6

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Figure 7

A

![Graph showing MCP-1 release (pg/mL) with different conditions]

B

![Western blot images of iNOS and GAPDH]

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Intermittent hypoxia

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Adipocytes

- \(\uparrow\)NOX 4
- \(\uparrow\)Erk
- \(\uparrow\)IL-6
- \(\uparrow\)NF\(\kappa\)B
- \(\uparrow\)iNOS
- \(\uparrow\)MCP-1

Endothelial cells

- \(\downarrow\)p-eNOS
- \(\uparrow\)Erk
- \(\uparrow\)p38
- \(\downarrow\)NO
- \(\downarrow\)cGMP
- \(\uparrow\)NF\(\kappa\)B
- \(\uparrow\)MCP-1
- \(\uparrow\)iNOS

Intermittent hypoxia leads to endothelial dysfunction.

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