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Human adipocytes are highly sensitive to intermittent hypoxia induced NF-kappaB activity and subsequent inflammatory gene expression

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ABSTRACT

Introduction: Intermittent hypoxia (IH)-induced activation of pro-inflammatory pathways is a major contributing factor to the cardiovascular pathophysiology associated with obstructive sleep apnea (OSA). Obesity is commonly associated with OSA although it remains unknown whether adipose tissue is a major source of inflammatory mediators in response to IH. The aim of this study was to test the hypothesis that IH leads to augmented inflammatory responses in human adipocytes when compared to cells of non-adipocyte lineages.

Methods and results: Human primary subcutaneous and visceral adipocytes, human primary microvascular pulmonary endothelial cells (HUMEC-L) and human primary small airway epithelial cells (SAEC) were exposed to 0, 6 or 12 cycles of IH or stimulated with tumor necrosis factor (TNF)-α. IH led to a robust increase in NF-κB DNA-binding activity in adipocytes compared with normoxic controls regardless of whether the source of adipocytes was visceral or subcutaneous. Notably, the NF-κB response of adipocytes to both IH and TNF-α was significantly greater than that in HUMEC-L and SAEC. Western blotting confirmed enhanced nuclear translocation of p65 in adipocytes in response to IH, accompanied by phosphorylation of I-κB. Parallel to p65 activation, we observed a significant increase in secretion of the adipokines interleukin (IL)-8, IL-6 and TNF-α with IH in adipocytes accompanied by significant upregulation of mRNA expression. PCR-array suggested profound influence of IH on pro-inflammatory gene expression in adipocytes.

Conclusion: Human adipocytes demonstrate strong sensitivity to inflammatory gene expression in response to acute IH and hence, adipose tissue may be a key source of inflammatory mediators in OSA.

1. Introduction

Intermittent hypoxia (IH) characterized by repeated episodes of hypoxia interspersed with periods of reoxygenation, is increasingly recognized as a major pathophysiological factor in various disease processes with distinct cell and molecular responses [1]. For example, IH appears to play a key role in promoting resistance to radio- and chemotherapy in solid tumors [2] and is encountered in a wide range of respiratory and cardiac disorders such as chronic obstructive pulmonary disease and congestive cardiac failure [3].

Most notably, IH is the hallmark feature of obstructive sleep apnea (OSA). OSA is a highly prevalent disorder affecting about 14% of men and 5% of women [4]. It is characterized by repetitive episodes of upper airway obstruction usually associated with recurrent hypoxemia and typically terminated by brief arousals. The major health burden in patients with OSA is the strong risk of cardiovascular diseases. Large-scale epidemiological studies have demonstrated an independent relationship between OSA and cardiovascular disorders, such as systemic hypertension, ischemic heart disease, congestive cardiac failure and stroke [5].

The pathophysiological mechanisms of cardiovascular diseases in OSA remain incompletely understood but are likely to be multifactorial involving sympathetic excitation, endothelial dysfunction mediated by oxidative stress and systemic inflammation, and metabolic dysregulation [5,6]. There is increasing evidence that IH plays a key role in this process. We have previously shown, that IH preferentially activates pro-inflammatory, nuclear factor (NF)-κB mediated pathways over adaptive, hypoxia-inducible factor (HIF)-1 dependent pathways in contrast to sustained hypoxia, where adaptive responses predominate [7]. NF-κB is a key driver of inflammatory and innate immune responses and when chronically activated contributes to atherosclerosis through driving

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production of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-8 [8]. These mediators have been found to be upregulated in OSA patients compared to matched controls, with effective CPAP therapy significantly lowering their levels [9,10] supporting the key role of NF-κB as driver of inflammation in OSA.

The main source organ of the increased systemic levels of inflammatory mediators in OSA remains unknown. However, there is increasing evidence for interaction of OSA and obesity in the development of cardiovascular diseases. Obesity is strongly associated with OSA – the prevalence of OSA in obese subjects exceeds 30% and at least 60% of OSA patients are obese [11]. White adipose tissue (WAT) is a highly active endocrine organ secreting multiple factors, termed adipokines [12]. This includes a variety of pro-inflammatory mediators such as TNF-α and IL-6 that may be a critical link between obesity and obesity-induced cardiovascular diseases. There is emerging evidence that hypoxia is a key factor in modulating the production of inflammatory adipokines in obesity [13]. As IH represents a stronger inflammatory stimulus than sustained hypoxia this process may be potentiated in diseases associated with IH. Hence, we hypothesized that IH may lead to augmented inflammatory pathway activation in adipose cells. The aim of the current study is to test this hypothesis in cultured cells.

2. Methods

2.1. Cell culture and intermittent hypoxia treatment

Human primary white preadipocytes derived from the subcutaneous adipose tissue (PromoCell, Heidelberg, Germany) and human white visceral preadipocytes derived from the omentum (Zen-Bio, Research Triangle Park, NC) were cultured and differentiated into mature adipocytes according to the manufactures' instructions. Adipocyte maturity (typically at day 14 post-differentiation) was confirmed by observing the accumulation of lipid droplets by light microscopy. Human primary small airway epithelial cells (SAEC) and human lung microvascular endothelial cells (HMVEC-L) were obtained from Lonza (Basel, Switzerland) and cultured according to the supplier's recommendations.

Cells were exposed to IH as previously described [7]. The actual pO_2 values at the cell monolayer were monitored by fluorescence quenching oxymetry (Oxylite 2000, Oxford Optronix, U.K.) with the model resulting in a stable fluctuating pattern between 1 and 13 kPa [7]. Where indicated, control cells were exposed to 4 h of normoxia (atmospheric pO_2 20 kPa), 24 h of sustained hypoxia (atmospheric pO_2 2.67 kPa) or were stimulated with TNF-α (10 ng/ml) for 10 min.

2.2. p65- and HIF-1 DNA-binding assay

Nuclear and cytosolic extracts were prepared immediately following treatment using a nuclear extract kit (Active Motif, Carlsbad, CA). For the DNA binding assays, either the TransAM p65 activation assay kit or the HIF-1 kit (Active Motif) was used according to the manufacturer's instructions.

2.3. Western blot analysis

Nuclear or cytosolic extracts, normalized for protein content (DC protein assay, Bio-Rad), were separated by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted as described previously [14]. Primary antibodies against p65 (Santa Cruz biotechnology), HIF-1α (BD Biosciences), Phospho-I-κB (Ser 32/36), Lamin (Cell Signaling Technology) and β-Actin (Sigma Aldrich) were used, as well as species-specific HRP-conjugated secondary antibodies. ImageJ 1.47v was used to quantify the Western blot signals.

2.4. Real-time PCR

Total RNA was collected and purified using TRIzol (Invitrogen). The RNA concentration was measured by using a NanoDrop apparatus (NanoDrop Technology, Inc.) 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). Real time quantification of cDNA was carried out on the ABI Prism 7900HT sequence detection system, normalized to 18S rRNA and ΔCT values were recorded.

2.5. PCR array

cDNA was prepared as above and incubated on an inflammatory cytokine & receptor RT^2 qPCR array (Cat. No PAHS-011Z, SABiosciences, Frederick, MD) according to the manufacturer's instructions. This array profiles the expression of 84 key genes mediating the inflammatory response. Target genes were normalized to control 18S expression and expressed as fold-change relative to normoxia treatment.

2.6. Measurement of TNF-α, IL-8 and IL-6 by ELISA

The release of the inflammatory cytokines TNF-α, IL-8 and IL-6 by ELISA were measured in cell culture media after 24 h of incubation using commercial ELISA kits (TNF-α: Invitrogen (Camarillo, CA), IL-6 and IL-8: R&D (Abingdon, UK)). The assays were conducted in 96-well microplates according to the manufacturer's instructions. The sensitivity of each of the ELISAs was: TNF-α, <0.09 pg/ml; IL-8, <3.5 pg/ml; IL-6, <0.70 pg/ml.

2.7. Statistical analysis

Data are expressed as mean ± SEM for at least $n = 3$ independent experiments. Analysis of variance (ANOVA) followed by Tukey's post hoc analysis or Student's t-test were used to compare data. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using a commercial software package (SPSS Version 20, Chicago, IL).

3. Results

3.1. IH and TNF-α lead to higher p65 DNA binding activity in human adipocytes than in other human primary cells

Here, we tested the hypothesis that IH leads to augmented inflammatory pathway activation in human adipocytes. We firstly investigated the impact of IH on the DNA-binding activity of the transcriptionally active NF-κB subunit p65 in human adipocytes and we compared the response to two other, non-adipocyte human primary cells (SAEC, HMVEC-L). IH led to a 3.8-fold increase in p65 DNA-binding activity in adipocytes in comparison to normoxia ($p = 0.009$), significantly greater than the response to IH observed in the alternative cells (Fig. 1A). The increase in p65 DNA-binding activity was not different in cells derived from subcutaneous versus visceral adipose tissue ($p = 0.575$). We next assessed the response of adipocytes versus both other primary cells to TNF-α and observed the same enhanced sensitivity of adipocytes as seen with IH treatment. There was a strong increase in p65 DNA-binding activity in IH-treated adipocytes over treatment with normoxia

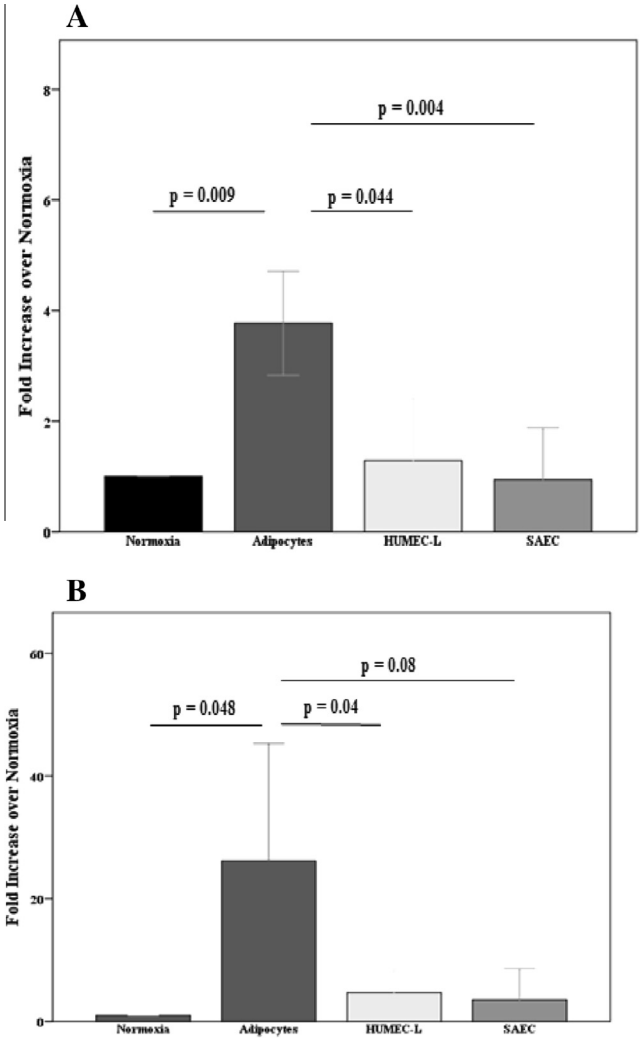

Fig. 1. Enhanced p65 DNA binding activity in response to IH in human primary adipocytes in comparison to HMVEC-L and SAEC. Cells were treated with 4 h normoxia or 12 cycles or IH (A) or stimulated with TNF-α 10 ng/ml for 10 min (B). Nuclear lysates were analyzed for p65 DNA binding activity by TransAM Elisa. (Data are presented as mean ± SEM and expressed as relative fold-change over normoxia treatment.)

($p = 0.048$) and again, this increase was greater than seen in HMVEC-L or SAEC (Fig. 1B).

3.2. IH leads to nuclear translocation of p65 and phosphorylation of I-κB in human primary adipocytes

We next aimed to confirm NF-κB activation by IH in adipocytes by investigating nuclear localization of the transcriptionally active p65 subunit. As shown in Fig. 2A, IH led in both subcutaneous and visceral adipocytes to nuclear translocation of p65 with the results indicative of a dose-dependent response. We have previously shown using HeLa cells that the IH-dependant NF-κB-activation follows the canonical pathway leading to Ser32/36 phosphorylation of I-κB [14]. Here, we confirm IH-dependent Ser32/36 phosphorylation of I-κB in human primary adipocytes (Fig. 2B).

3.3. Short-term IH does not lead to activation of HIF-1

In contrast to chronic IH which has been demonstrated to activate HIF-1 [15,16], this acute model does not lead to HIF-1 activation in keeping with our previously reported results [7]. This is indicated by a lack of increase of HIF-1 DNA-binding activity in adipocytes but also in SAEC and HMVEC-L. 24 h of sustained hypoxia as control treatment led to a robust response in adipocytes confirmed by western blotting of nuclear extracts obtained following indicated treatments (Supplementary Fig. 1).

3.4. Regulation of NF-κB-dependent adipokine secretion by IH

We next examined the secretion of well-characterized NF-κB-dependent pro-inflammatory mediators into cell culture medium in response to IH. We first investigated the secretion of IL-8, which we have previously shown to be upregulated in OSA subjects in comparison to matched controls [9], during IH-exposure of human adipocytes again in comparison to human primary endothelial and epithelial cells. We observed a significant increase in IL-8 secretion in adipocytes in response to IH which was greater than in the non-adipocyte primary cells although it failed to reach statistical significance in case of comparison to SAEC (Fig. 3). We also identified a statistically significant increase in IH-dependent TNF-α and IL-6 secretion in adipocytes (20% and 35% relative increase, respectively) without observed difference to the other cells.

3.5. Regulation of inflammatory adipokine gene expression in response to IH

The effect of IH on inflammatory-gene expression was next examined, focusing initially on TNF-α, IL-8 and IL-6. For all three genes, there was a substantial increase in mRNA levels in response to IH in adipocytes (TNF-α: mean fold increase over normoxia: 6.2, $p = 0.029$; IL-8: 2.4, $p = 0.004$, IL-6: 5.2, $p = 0.008$).

Finally, we investigated the effect of IH on global inflammatory cytokine gene expression. cDNA generated from adipocytes following 0 and 12 cycles of IH was assayed on a PCR array with 84 genes encoding inflammatory cytokines and receptors. As indicated in Table 1, IH regulates numerous genes on this assay including IL-8 and TNF-α. A number of genes differentially regulated in response to IH were selected for validation of the array. These genes included vascular endothelial growth factor (VEGF), the chemokine CCL-2 (MCP-1) and the pro-inflammatory cytokine IL-1β and for all a significant increase in mRNA expression in response to IH was confirmed (VEGF: mean fold increase over normoxia: 4.8, $p = 0.011$; CCL-2: 14.6, $p = 0.019$, IL-1: 3.8, $p = 0.005$).

4. Discussion

IH is the key driver of cardiovascular disease processes in OSA [5]. There is widespread evidence that systemic inflammation induced by IH plays a pivotal role in this process and the transcription factor NF-κB is central in mediating the response [6]. The main source organ of the IH-dependent release of inflammatory mediators in OSA remains unknown, but adipose tissue is a very attractive candidate given the close link between OSA and the presence of obesity. Numerous studies have detected an acceleration of atherosclerosis, deterioration in vascular function and worsening of glucose dysfunction in OSA patients in comparison to matched obese controls [5,9,17–19]. Moreover, although still poorly investigated, there is increasing evidence of an interaction of OSA and obesity in mediating cardiovascular and metabolic consequences and various studies reported that cardiovascular consequences of OSA may only occur in the presence of obesity [20–22].

Unfortunately, there are many potential confounding variables that limit the ability of human studies to further clarify the interactions of OSA and obesity. Animal studies allow studying the impact of IH as a single pathophysiological trigger and have greatly enhanced our knowledge in this field. IH has been shown to

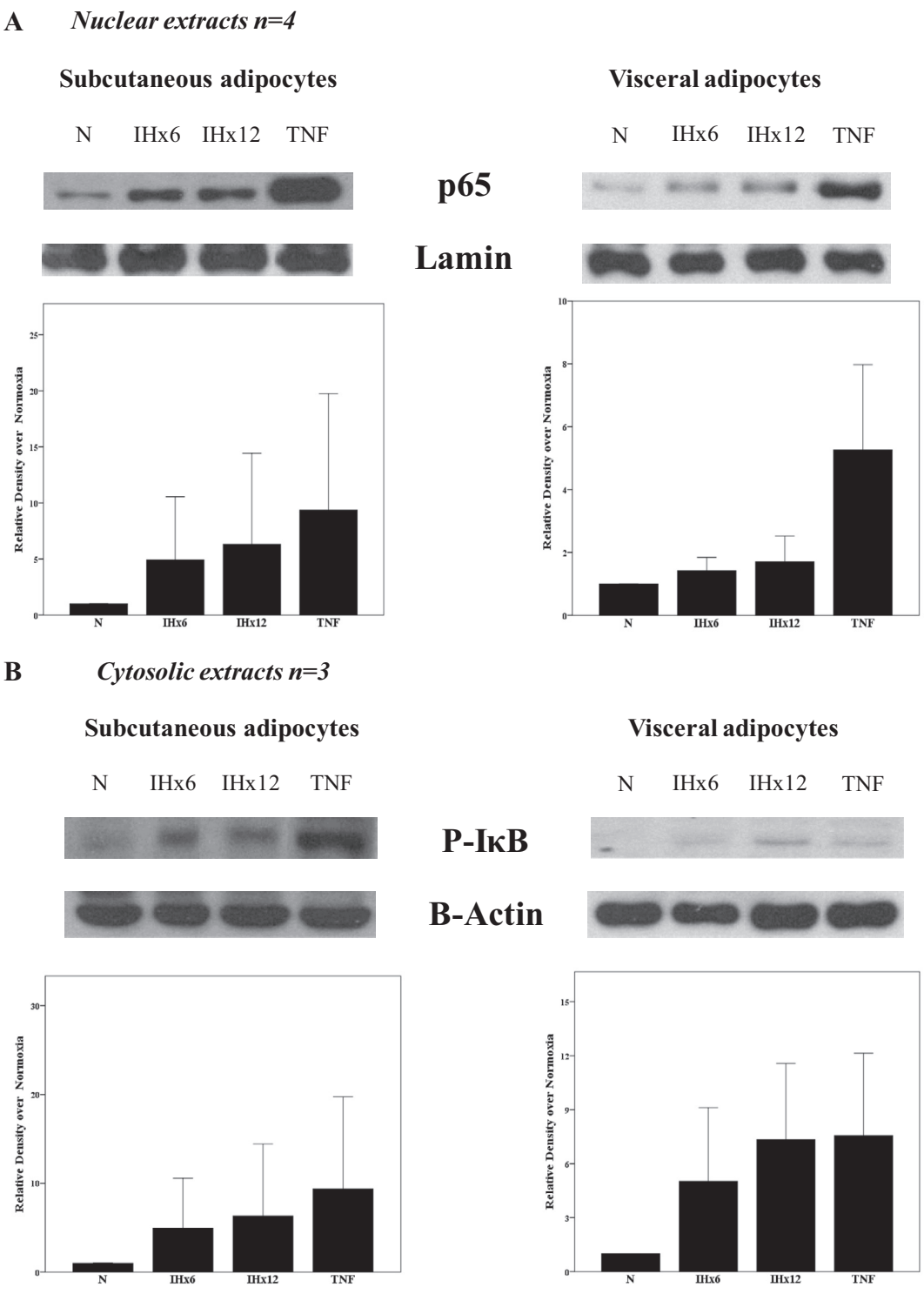

Fig. 2. IH leads to nuclear translocation of p65 and phosphorylation of I-κB in human primary adipocytes. Subcutaneous and visceral adipocytes were treated with 4 h normoxia, 6 or 12 cycles of IH or stimulated with TNF-α 10 ng/ml for 10 min. Nuclear and cytosolic extracts were prepared immediately following treatment. Nuclear extracts were immunoblotted for p65 (A) and cytosolic extracts for I-κB (B).

accelerate the atherosclerotic process, which is associated with increased vascular and systemic inflammation [23,24]. In both, genetically and diet-induced obese mice, IH promotes insulin resistance and glucose intolerance associated with increased hepatic inflammation and pro-inflammatory cytokine levels in comparison to mice exposed to control conditions while having only a minimal impact on these parameters in lean mice [25,26]. There are only sparse data addressing the impact of IH directly on the adipose tissue in mediating inflammatory responses. In support of our hypothesis, two independent studies investigated the effect of IH on murine-derived adipocytes and reported activation of NF-κB and decreased secretion of the key anti-inflammatory adipokine adiponectin [27,28]. Recently, Poulain et al. investigated the effect of IH on atherosclerotic-prone apolipoprotein E-deficient mice and

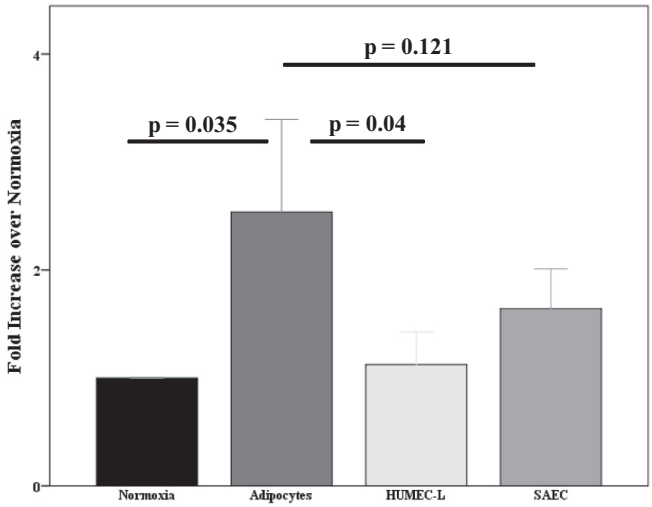

Fig. 3. Effect of IH on the release of the pro-inflammatory NF-κB-dependent chemokines IL-8 from human adipocytes in comparison to HMVEC-L and SAEC. Cells were treated with 4 h normoxia or 12 cycles of IH. Following incubation for 24 h supernatants were collected and concentration of IL-8 was measured by ELISA. (Data are presented as mean ± SEM and expressed as relative fold-change over normoxia treatment.)

Table 1
Genes of PCR array whose expression was up-or downregulated by at least 2-fold following exposure of human adipocytes to IH. Relative expression is IH/normoxia.

Gene name	Relative expression
Chemokine (C–C motif) ligand 2 (Monocyte chemotactic protein-1)	9.6
Chemokine (C–C motif) ligand 20 (Macrophage inflammatory protein-3)	54.8
Chemokine (C–C motif) ligand 3 (Macrophage inflammatory protein-1α)	3.5
Chemokine (C–C motif) ligand 7 (Monocyte-specific chemokine 3)	12.6
Chemokine (C–X-C motif) ligand 2 (Macrophage inflammatory protein-2α)	2.6
Chemokine (C–X–C motif) ligand 3 (Macrophage inflammatory protein-2β)	2.4
Chemokine (C–X–C motif) ligand 1	2.0
Chemokine (C–X–C motif) ligand 6	2.0
Interleukin 8	2.6
Interleukin 1β	2.6
Tumor necrosis factor α	2.0
Vascular endothelial growth factor	3.9
Nicotinamide phosphoribosyltransferase	2.0
Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	−2.7
Complement component 5	−2.1
Chemokine (C–C motif) ligand 5	−2.7
C–C chemokine receptor type 1	−2.2
Chemokine (C–X–C motif) ligand 5	−3.1
Interleukin 5	−2.4
Interleukin 7	−2.5
Tumor necrosis factor receptor superfamily member 11B	−2.2
Tumor necrosis factor (ligand) superfamily member 4	−2.0

found that IH led to remodeling of the adipose tissue associated with higher secretion of IL-6 and TNF-α and also more severe atherosclerotic lesions than mice treated with intermittent air [29].

The present study, for the first time, investigates the effect of IH on human primary adipocytes towards inflammatory pathway activation and subsequent expression and secretion of pro-inflammatory adipokines. Our results indicate that adipocytes are significantly more sensitive to IH than other primary cells with the downstream consequence of increased expression of multiple inflammatory mediators known to play key roles in the pathogenesis of atherosclerosis and glucose metabolic dysfunction. Remarkably, adipocytes also demonstrated a greater inflammatory response to TNF-α stimulation and it is likely that this pathway will potentiate the IH-induced response. Given the limited lifespan of cell cultures, our model only allows studying the effects of short-term IH exposure. However, in many disease conditions, such as OSA, IH is experienced chronically and hence, this subject warrants further detailed investigation in vivo. Furthermore, inflammatory responses to IH in human adipose tissue will certainly depend on the degree of IH that the tissue is experiencing. Various studies using direct measurement of the oxygen partial pressure or immunohistostaining of hypoxia-probes have demonstrated that WAT in obese humans or obese rodents is more hypoxic than in lean controls and this is likely due to the poor vascularization of WAT [30,31]. Given the technical difficulties in the direct measurement of rapid fluctuations in oxygen concentrations in tissue there are currently no data on the potential additive effect of IH in OSA subjects. Reinke et al. investigated the oxygen profile in response to IH in a mouse model and suggested that the oxygen fluctuations of IH are attenuated in adipose tissue [32]. 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. The local proximity to the circulatory system and particularly the liver is also the main reason for visceral adipose tissue being more atherogenic than its subcutaneous counterpart [33]. Importantly, our results indicate that there are no differences in the cellular level of inflammatory pathway activation in response to IH between the cells of both compartments but how this relates to in vivo conditions remains to be investigated.

Data presented here indicate the regulation of multiple inflammatory genes by IH, suggesting a profound influence of IH on inflammation. A previous study reported global gene expression in response to IH in human aortic endothelial cells and similarly noted upregulation of certain pro-inflammatory genes such as IL-6 and IL-8 [34]. We also detected robust upregulation of VEGF mRNA by IH. VEGF is increasingly linked to IH [35,36] and has also been found to be elevated in plasma of patients with OSA versus controls [37]. Importantly, our results suggest that gene expression of VEGF is independent of HIF-1, however its activation is complex and can be regulated by a wide range of other transcription factors other than HIF-1, including specificity protein 1 (Sp1), specificity protein 3 (Sp3) and activating protein 2 (AP-2) [38].

The exact mechanisms of the higher sensitivity of human adipocytes remain to be determined. The fact that we observe this event in response to two different pro-inflammatory stimuli indicates, however, that adipocytes are generally susceptible and primed towards inflammatory pathway activation. The signaling mechanisms of IH-induced inflammation in general are still incompletely understood. Notwithstanding the fact that HIF-1 activation may occur in response to chronic IH exposure [15,16] the absence of same to acute IH treatment relative to significant NF-κB activation suggests different mechanisms than in response to sustained hypoxia. We and others have previously shown that the pathway is dependent upon activation of p38 mitogen-activated protein (MAP) kinase [14,39,40]. Increased production of reactive oxygen species (ROS) in IH has been proposed to be involved in the initial sensing events. However, the involvement of ROS in NF-κB signaling is controversial and pivotal experiments by Hayakawa et al. indicate that NF-κB is unlikely to be a sensor of oxidative stress and previous results have been influenced by cell type dependency and methodological pitfalls [41].

The present study provides evidence for high sensitivity of human primary adipocytes to the pro-inflammatory stimuli IH and TNF-α. This may be a crucial link in the understanding of the interaction between OSA and obesity in mediating cardiovascular

and metabolic morbidity but further translational studies will be required to determine the importance of these events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.062.

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