Intermittent Hypoxia Alters Gene Expression in Peripheral Blood Mononuclear Cells of Healthy Volunteers

Vsevolod Y. Polotsky1*, Shannon Bevans-Fonti1, Dmitry N. Grigoryev2*, Naresh M. Punjabi1,3

1 Department of Medicine, Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 2 Department of Medicine, Division of Allergy and Clinical Immunology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, 3 Department of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, United States of America

* Current address: Department of Genetics and Genomics, Icahn School of Medicine at Mount Sinai, New York, New York, United States of America

* vpolots1@jhmi.edu

Abstract

Obstructive sleep apnea is associated with high cardiovascular morbidity and mortality. Intermittent hypoxia of obstructive sleep apnea is implicated in the development and progression of insulin resistance and atherosclerosis, which have been attributed to systemic inflammation. Intermittent hypoxia leads to pro-inflammatory gene up-regulation in cell culture, but the effects of intermittent hypoxia on gene expression in humans have not been elucidated. A cross-over study was performed exposing eight healthy men to intermittent hypoxia or control conditions for five hours with peripheral blood mononuclear cell isolation before and after exposures. Total RNA was isolated followed by gene microarrays and confirmatory real time reverse transcriptase PCR. Intermittent hypoxia led to greater than two fold up-regulation of the pro-inflammatory gene toll receptor 2 (TLR2), which was not increased in the control exposure. We hypothesize that up-regulation of TLR2 by intermittent hypoxia may lead to systemic inflammation, insulin resistance and atherosclerosis in patients with obstructive sleep apnea.

Introduction

Obstructive Sleep Apnea (OSA) is characterized by recurrent collapse of the upper airway during sleep leading to intermittent hypoxemia and recurrent arousals from sleep. [1] It is now well established that OSA is associated with incident hypertension, cardiovascular disease, and all-cause mortality.[2–4] Although underlying mechanisms that explicate the increase in cardiovascular risk are not well defined, a commonly accepted hypotheses is that recurrent exposure to intermittent hypoxemia induces low grade systemic inflammation [5–9] that, in turn,
can promote atherosclerosis and the development of insulin resistance. However, the putative links between intermittent hypoxemia and systemic inflammation are not well defined.

Over the last decade, gene microarray analyses have provided novel insights into intermediate mechanisms for a number of chronic conditions. In fact, utilizing whole blood or peripheral blood mononuclear cells (PBMC) RNA, gene microarrays have demonstrated up-regulation of pathways of oxidative stress and inflammation in adult and pediatric OSA samples.[10–12] While pioneering, the available studies to date are limited by inevitable substantial variability in the phenotype across patients with OSA. Genomic studies that have used cell culture and animal models have shown that exposure to intermittent hypoxia up-regulates signaling pathways for cardiac hypertrophy in the myocardium, [13] induces genes responsible for lipid biosynthesis in the liver [14] and activates pro-inflammatory genes in immortal cell lines [5] and in the endothelium.[8,15] In contrast, the acute effects of intermittent hypoxia on gene expression in humans have not been previously examined. The current study sought to evaluate PBMC gene expression profiles induced by intermittent hypoxia in healthy volunteers. To identify changes specifically induced by intermittent hypoxia, gene expression was examined in a cohort of normal subjects before and after exposure to intermittent hypoxia or air (control conditions). Moreover, to distinguish the effects of intermittent hypoxia from sleep fragmentation, the focus of the current study was to assess the effects during wakefulness and not sleep. It was hypothesized that exposure to intermittent hypoxia for as short as 5-hours will induce pro-inflammatory genes.

### Methods

#### Human subjects

Healthy adult men (n = 8) were recruited from local community as previously described.[16] Exclusion criteria included a history of any respiratory illness, hypertension, hepatic, renal, cardiovascular, neurologic or a hematologic disorder, habitual sleep duration < 7 h, any circadian sleep disorder and current smoking.[16] In addition, a full montage polysomnogram excluded volunteers with undiagnosed obstructive sleep apnea. A mean age was 24.3 years (range 18–35 yr) and the body mass index was 25.8 ± 1.7 kg/m², which is considered overweight.[17] Since 68.8% of the adult U.S. population is overweight or obese, [18] our volunteers adequately represented an average healthy U.S. adult. The study has been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consents have been obtained from the participants. The consent forms signed by the participants have been scanned and stored on the secure drive. The study and the consent procedure have been approved by the Johns Hopkins University Institutional Review Board (IRB) 5.

#### Study protocol

A cross-over study was conducted in healthy volunteers (n = 8) who were exposed to intermittent hypoxia or control conditions in a randomized fashion for 5 hours while awake as previously described (Fig 1).[16] Briefly, the study was performed in a single-blind fashion and consisted of two days with an interval of one week. On one of the days, the subject was exposed to intermittent hypoxia for 5 h, from 8 am to 1 pm, and on the other day the subject was exposed to ambient air for 5 h, from 8 am to 1 pm, using a similar set up as a control. A full face mask was applied, and the inspiratory valve was attached to a three-way Hans-Rudolph valve so that inspiration could be from one of two pressurized cylinders with ambient air (21% O₂) or hypoxic gas (95% N₂ and 5% O₂). Intermittent hypoxia was induced by inspiration of the hypoxic (95% N₂ and 5% O₂) gas until the oxyhemoglobin saturation dropped to 85% followed by reoxygenation to the baseline, resulting in ~25 hypoxic events/h. In control condition,
the hypoxic gas was substituted with air. Phlebotomy was performed in the antecubital fossa and whole blood (15 ml) was collected immediately prior and after the exposure.

PBMC isolation

Whole blood (15 ml) was anticoagulated with ethylenediaminetetraacetic acid (EDTA), mixed with phosphate-buffered saline (PBS) 1:1, layered over Ficoll-Hypaque solution (Pharmacia, Biotech AB, Uppsala, Sweden) (20 ml) and centrifuged at 400 x g for 40 min according to the manufacturer’s instructions. PBMCs were isolated, washed in ice-cold PBS and snap-frozen at -80°C.

Gene microarrays

Microarray studies were performed in four subjects before and after intermittent hypoxia or control exposures. Total RNA was isolated from PBMCs using the Trizol Reagent method (Invitrogen, Carlsbad, California 92008, cat. no. 15596–026) with subsequent RNEasy clean up (Qiagen, Valencia, CA 91355, cat. no. 74104). 0.5 μg of total RNA from each sample were labelled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX 78744–1832, cat. no. IL1791). RNA was converted into double-stranded cDNA using an oligo-dT primer containing the T7 RNA polymerase promoter. Single stranded RNA (cRNA) was from double-stranded cDNA in an in vitro transcription reaction. cRNA was labelled by incorporating biotin-16-UTP. 0.85 μg of biotin-labelled cRNA was hybridized (16 hours) to Illumina’s Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA 92121–1975, cat.no. 11201828). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantified with Illumina’s BeadStation 500GX Genetic Analysis Systems scanners. Preliminary
analysis of the scanned data was performed using Illumina BeadStudio software. Resulting digitized matrix was processed by modified for Illumina platform approach described previously.[19] The significant hybridization signals were defined based on the BeadStudio identification ratio IR > 0.95. The chip background and brightness were computed using high quartile and whole set of hybridization signals that fell below IR < 0.95. The expression data was stratified by experimental conditions and hybridization of each transcript was evaluated. The transcripts that were detectable by BeadStudio (IR > 0.95) and produced signal at least twice as high as that of background in at least 3 out of 4 hybridizations in any given experimental condition were considered truly expressed. The signal intensity values of these transcripts from each chip were increased by corresponding to a given chip background value (background adjustment) and divided by a chip brightness coefficient (normalization).

Real time PCR

Real-time reverse-transcriptase PCR (RT-PCR) was performed in all eight subjects before and after intermittent hypoxia and control exposures. Total RNA was extracted using Trizol and cDNA was synthesized using Advantage RT for PCR kit from Clontech (Palo Alto, CA). Real-time reverse-transcriptase PCR (RT-PCR) was performed with primers from Invitrogen (Carlsbad, CA). The sequences of primers and probes for 18s were previously described.[20] The sequences of primers were designed based on the GeneBank sequences: for toll receptor 2 (TLR-2)[NM_003264.3], forward 5’-CGGAAGTGCTGTCCTGTGACATTC[FAM]G-3’, reverse 5’-GCCAGCAAATTACCTGTGTGA-3’; for chemokine (C-C motif) receptor 2 (CCR2) [NM_000647], forward 5’-CGGCCTGAGTAACTGTGAAGC-3’, reverse 5’-CGCAAAGAGTCTCTGTACCTTG[FAM]G-3’; for hemoglobin alpha 1 (HBA1) [NM_000558], forward 5’-CGGGCCACCAAGACCTACTTC[G]G-3’, reverse 5’-CTTGCCGTGGCTAACCT-3’. The mRNA expression levels were normalized to 18s rRNA concentrations using the following formula: Gene of interest/18S = 2^([Ct(Gene of Interest) - Ct(18S)].

ELISA

The enzyme-linked immunosorbent assay (ELISA) was performed in EDTA plasma samples of all eight subjects before and after intermittent hypoxia and control exposures using a human interleukin 8 (IL-8) kit from R&D Systems (Minneapolis, MN).

Statistical analysis

Significance Analysis of Microarrays (SAM 2.20) was conducted using 1000 permutation of 4 control and 4 treated PBMC samples without application of arbitrary restrictions. Genes with 2.0 fold change and 5% false discovery rate (q) were considered significantly affected by intermittent hypoxia. All values obtained in PCR are reported as means ± SEM. Comparisons between baseline and 5 hrs data points after IH and control exposures were performed using repeated-measures ANOVA. A p-value ≤ 0.05 was considered significant.

Results

In four healthy human volunteers, exposure to intermittent hypoxia up-regulated 20 genes and down-regulated 7 genes out of 12291 genes, which were examined with the Illumina’s Sentrix HumanRef-8 Expression BeadChip (Table 1). In contrast, exposure of the same subjects to control conditions did not lead to marked induction of any genes (Table 2), and 29 genes were down-regulated. Among genes induced by intermittent hypoxia, we detected increases in pro-atherogenic chemokine receptor CCR-2 [21] and TLR-2 and a number of apoptotic factors
such as PDCD4 and APAF1 (Table 1). Among genes down-regulated by intermittent hypoxia were hemoglobin transcripts, including hemoglobin delta, alpha 1 and 2, HBD, HBA1 and HBA2, respectively (Table 1). However, the hemoglobin transcripts were similarly down-regulated after exposure to control condition suggesting that the changes in gene expression were not specific to the hypoxic stimulus (Table 2). Exposure to control conditions decreased expression of IL-8 and IL-8 receptor in PBMCs (Table 2), which did not occur during intermittent hypoxia (Table 1). However, plasma levels of IL-8 were undetectable, regardless of conditions.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [22,23] and are accessible through GEO series accession no. GSE71356 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71356.

Expression of selected genes has been examined in real-time PCR in eight healthy volunteers, including four volunteers, whose PBMC were studied by gene arrays. According to the PCR data, there were no differences in the levels of expression of CCR2 and HBA1 between baseline and IH (not shown). In contrast, 5 hours of intermittent hypoxia resulted in a 2.0 ± 0.3-fold increase in TLR2 mRNA levels, which did not occur at control conditions (Fig 2).

Table 1. Genes Differentially Regulated in Peripheral Blood Mononuclear Cells after Exposure to Intermittent Hypoxia for 5 hrs Compared to Baseline.

| N | Target ID   | Gene Name                                          | Fold Change | q-value (%) |
|---|-------------|----------------------------------------------------|-------------|-------------|
| 1 | GI_21361115-S | chondroitin sulfate proteoglycan 2 (versican) (CSPG2) | 3.26        | 0.216       |
| 2 | GI_13325059-S | cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) | 2.74        | 0.216       |
| 3 | GI_15451896-I | chemokine (C-C motif) receptor 2 (CCR2), transcript variant A | 2.55        | 0.216       |
| 4 | GI_31377777-S | serine/threonine kinase 38 (STK38)                | 2.47        | 0.216       |
| 5 | GI_34304340-A | programmed cell death 4 (PDCD4), transcript variant 1 | 2.44        | 0.216       |
| 6 | GI_42659661-S | macrophage expressed gene 1 (MPEG1)                | 2.44        | 0.216       |
| 7 | GI_29171722-A | G protein-coupled receptor 86 (GPR86), transcript variant 2 | 2.38        | 0.216       |
| 8 | GI_28416432-S | immunity associated protein 4 (HIMAP4)             | 2.32        | 0.216       |
| 9 | GI_32483626-A | apoptotic protease activating factor (APAF1), transcript variant 5 | 2.31        | 0.216       |
| 10| GI_32261305-S | IQ motif containing GTPase activating protein 1 (IQGAP1) | 2.24        | 0.216       |
| 11| GI_5730074-S | fibrinogen-like 2 (FGL2)                           | 2.16        | 0.216       |
| 12| GI_32307149-A | O-linked N-acetylgulcosamine (GlcNAc) transferase (OGT), transcript variant 2 | 2.15        | 0.216       |
| 13| GI_21536438-A | F-box and leucine-rich repeat protein 5 (FBXL5), transcript variant 1 | 2.13        | 0.216       |
| 14| GI_22027524-S | Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (ARHGEF6) | 2.12        | 0.216       |
| 15| GI_4506516-S | regulator of G-protein signalling 2, 24kDa (RGS2) | 2.11        | 0.216       |
| 16| GI_19718733-S | toll-like receptor 2 (TLR2)                        | 2.06        | 0.216       |
| 17| GI_35493781-A | ring finger protein 19 (RFN19), transcript variant 2 | 2.05        | 0.216       |
| 18| GI_3367050-S | polyposis locus protein 1 (DP1)                    | 2.04        | 0.216       |
| 19| GI_33589822-S | pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4) | 2.01        | 0.216       |
| 20| GI_32698701-S | HECT domain containing 1 (HECTD1)                  | 2.01        | 0.216       |
| 21| GI_21071025-S | histone 1, H1c (HIST1H1C)                          | -2.05       | 2.647       |
| 22| GI_28302128-S | hemoglobin, beta (HBB)                            | -2.16       | 0.216       |
| 23| GI_4557676-S | integrin, beta 3 (platelet glycoprotein IIa, antigen CD61) (ITGB3) | -2.17       | 0.730       |
| 24| GI_38455401-S | lipocalin 2 (oncogene 24p3) (LCN2)                 | -2.50       | 0.730       |
| 25| GI_6633803-S | hemoglobin, delta (HBD)                           | -2.57       | 0.216       |
| 26| GI_14043068-S | hemoglobin, alpha 2 (HBA2)                         | -3.07       | 0.216       |
| 27| GI_14456711-S | hemoglobin, alpha 1 (HBA1)                        | -3.23       | 0.216       |

doi:10.1371/journal.pone.0144725.t001
In the current study, healthy awake volunteers were exposed to acute intermittent hypoxia for 5 hours to mimic the oxygen profile of patients with moderate to severe OSA or to comparable control conditions. Gene expression profiles were examine in PBMCs before and after the exposure. The primary finding is that intermittent hypoxia caused a 2 fold induction of TLR2, an important gene of systemic and vascular inflammation.

Toll receptors are the family of pattern recognition receptors, which were initially described as the first line of defense against pathogens.[24] TLRs bind to different component of microorganisms including lipopeptide, double-stranded RNA, lipopolysaccharides, flagellin, etc. TLRs act via myeloid differentiation primary-response gene 88 (MyD88) to recruit downstream kinases and activate nuclear factor kappa B (NF-kB). TLR-driven induction of NF-kB results in production of pro-inflammatory cytokines, including TNF\(\alpha\), interleukin 1, interleukin 6 (IL-6), and IL-8, which play a role in the development of atherosclerosis and insulin resistance.[25–27] TLR2 deficiency inhibits the progression of atherosclerosis in ApoE-deficient mice and improves structural stability of the plaque, whereas TLR2 ligand specific activation

| N  | Target ID       | Gene Name                                                                 | Fold Change | q-value (%) |
|----|-----------------|---------------------------------------------------------------------------|-------------|-------------|
| 1  | GI_42658769-S   | hypothetical gene supported by BC062364; BX647289 (LOC401457)             | -2.01       | 0.356       |
| 2  | GI_31982886-S   | GATA binding protein 2 (GATA2)                                           | -2.04       | 0.356       |
| 3  | GI_28610153-S   | interleukin 8 (IL8)                                                       | -2.05       | 0.356       |
| 4  | GI_4504152-S    | chemokine (C-X-C motif) ligand 1 (CXCL1)                                  | -2.08       | 0.356       |
| 5  | GI_5174662-S    | S100 calcium binding protein P (S100P)                                   | -2.10       | 0.356       |
| 6  | GI_7706179-S    | erythroid associated factor (ERAF)                                       | -2.16       | 0.356       |
| 7  | GI_38455401-S   | lipocalin 2 (oncogene 24p3) (LCN2)                                       | -2.17       | 0.356       |
| 8  | GI_13787192-S   | alkaline phosphatase, liver/bone/kidney (ALPL)                           | -2.19       | 0.356       |
| 9  | GI_7706149-A    | mitochondrial solute carrier protein (MSCP)                              | -2.24       | 0.356       |
| 10 | GI_23110992-S   | membrane-spanning 4-domains, subfamily A, member 3 (MS4A3)              | -2.24       | 0.356       |
| 11 | GI_31657130-S   | protease inhibitor 3, skin-derived (SKALP) (PI3)                        | -2.25       | 0.356       |
| 12 | GI_30581169-A   | chemokine (C-C motif) receptor 3 (CCR3), transcript variant 2            | -2.27       | 0.356       |
| 13 | GI_29171680-S   | interleukin 8 receptor, beta (IL8RB)                                    | -2.28       | 0.356       |
| 14 | GI_31563435-S   | chemokine-like factor super family 2 (CKLFSF2)                           | -2.28       | 0.356       |
| 15 | GI_4826835-S    | matrix metalloproteinase 9 (MMP9)                                        | -2.30       | 0.356       |
| 16 | GI_39753969-S   | cathelicidin antimicrobial peptide (CAMP)                                | -2.37       | 0.356       |
| 17 | GI_28302128-S   | hemoglobin, beta (HBB)                                                   | -2.37       | 0.356       |
| 18 | GI_12621916-S   | defensin, alpha 3, neutrophil-specific (DEFA3)                           | -2.42       | 0.356       |
| 19 | GI_21071007-S   | transcobalamin I (TCN1)                                                  | -2.50       | 0.356       |
| 20 | GI_4505042-S    | lactotransferrin (LTF)                                                    | -2.54       | 0.356       |
| 21 | GI_4557298-S    | aminolevulinate, delta-, synthase 2 (ALAS2)                              | -2.57       | 0.356       |
| 22 | GI_28302130-S   | hemoglobin, gamma A (HBG1)                                               | -2.64       | 0.356       |
| 23 | GI_29171679-S   | interleukin 8 receptor, alpha (IL8RA)                                   | -2.65       | 0.356       |
| 24 | GI_28302132-S   | hemoglobin, gamma G (HBG2)                                               | -2.66       | 0.356       |
| 25 | GI_13027808-A   | matrix metalloproteinase 25 (MMP25), transcript variant 2                | -2.67       | 0.356       |
| 26 | GI_21536462-I   | matrix metalloproteinase 25 (MMP25), transcript variant 1                | -3.08       | 0.356       |
| 27 | GI_6633803-S    | hemoglobin, delta (HBD)                                                  | -3.21       | 0.356       |
| 28 | GI_14043068-S   | hemoglobin, alpha 2 (HBA2)                                               | -3.83       | 0.356       |
| 29 | GI_14456711-S   | hemoglobin, alpha 1 (HBA1)                                               | -4.18       | 0.356       |

Discussion
In the current study, healthy awake volunteers were exposed to acute intermittent hypoxia for 5 hours to mimic the oxygen profile of patients with moderate to severe OSA or to comparable control conditions. Gene expression profiles were examine in PBMCs before and after the exposure. The primary finding is that intermittent hypoxia caused a 2 fold induction of TLR2, an important gene of systemic and vascular inflammation.

Toll receptors are the family of pattern recognition receptors, which were initially described as the first line of defense against pathogens.[24] TLRs bind to different component of microorganisms including lipopeptide, double-stranded RNA, lipopolysaccharides, flagellin, etc. TLRs act via myeloid differentiation primary-response gene 88 (MyD88) to recruit downstream kinases and activate nuclear factor kappa B (NF-kB). TLR-driven induction of NF-kB results in production of pro-inflammatory cytokines, including TNF\(\alpha\), interleukin 1, interleukin 6 (IL-6), and IL-8, which play a role in the development of atherosclerosis and insulin resistance.[25–27] TLR2 deficiency inhibits the progression of atherosclerosis in ApoE-deficient mice and improves structural stability of the plaque, whereas TLR2 ligand specific activation
accelerates atherosclerosis.\textsuperscript{[28]} TLR2 agonists induce insulin resistance in 3T3-L1 adipocytes.\textsuperscript{[29]} Furthermore, inhibition of TLR2 expression with antisense oligonucleotides improved insulin sensitivity in muscle and white adipose tissue in mice with diet-induced obesity.\textsuperscript{[30]} Thus, up-regulation of TLR2 may lead to systemic inflammation, insulin resistance and atherosclerosis.

OSA has been associated with systemic inflammation with increased circulating levels of pro-inflammatory cytokines, including TNF-\(\alpha\), IL-6 and IL-8, independent of obesity.\textsuperscript{[5–7,31]} Our data showing decreases in IL-8 and its receptor gene expression during control exposure (Table 2) are consistent with circadian variations of IL-8 levels peaking in the early morning hours.\textsuperscript{[32]} This circadian decline was no longer present during intermittent hypoxia suggesting that IL-8 may have been up-regulated. Of note, short-term exposure of human endothelial cells to intermittent hypoxia is known to up-regulate IL-8 gene expression.\textsuperscript{[8]} Studies in animal models of intermittent hypoxia showed that chronic intermittent hypoxia up-regulates NF-\(\kappa\)B and downstream pro-inflammatory cytokines in multiple tissues.\textsuperscript{[33–36]} Moreover, it has also been demonstrated that chronic intermittent hypoxia causes atherosclerosis in C57BL/6J mice that are fed a high fat diet.\textsuperscript{[20;36]} In vitro studies have demonstrated that hypoxia can lead to a robust induction of TLR2 in various cell types acting via a master regulator of hypoxic responses, hypoxia inducible factor 1 (HIF-1).\textsuperscript{[37]} It is conceivable that induction of TLR-2 by intermittent nocturnal hypoxemia contributes to the progression of systemic inflammation and atherosclerosis in patients with OSA. Indeed, nocturnal oxyhemoglobin desaturation in patients with OSA has been independently associated with atherosclerosis as assessed by increased carotid artery intima-media thickness \textsuperscript{[38;39]} that can be reversed with continuous positive airway pressure therapy.\textsuperscript{[40]} Given that exposure to intermittent hypoxia in healthy subjects, as done in the current study, can induce insulin resistance,\textsuperscript{[16]} it is certainly possible that systemic induction of TLR2 may also lead to the development of insulin resistance in OSA.

\textbf{Fig 2. Intermittent hypoxia increased expression of toll-like receptor 2 in peripheral blood mononuclear cells.} Expression of toll-like receptor two (TLR2) in peripheral blood mononuclear cells of healthy volunteers was measured during daytime exposure to intermittent hypoxia or control conditions for 5 hours and compared to baseline by real time PCR. The results are expressed as ratios to 18s. * denotes \(p < 0.05\) for the difference between baseline and 5 hours data points.

doi:10.1371/journal.pone.0144725.g002
The current study has several caveats. First, TLR2 protein levels were not measured and which cell type increases TLR2 expression was not assessed. We were unable to collect sufficient amount of blood from volunteers to perform these additional measurements due to ethical limitations of the human study. Nevertheless, transcriptional regulation is an important element of TLR functioning and up-regulation of TLR2 may lead to inflammatory response regardless whether it occurs in lymphocytes or monocytes.[41;42] Second, expression of pro-inflammatory cytokines downstream of TLR2 was not characterized with exception of IL-8, plasma levels of which remained undetectable throughout hypoxic and control exposures. Plasma IL-8 levels in healthy young adults is frequently below the sensitivity threshold of the ELISA assay.[43;44] The 5 hour exposure to intermittent hypoxia may not be long enough to induce measurable changes in cytokine protein levels.

In conclusion, we have demonstrated that TLR2 was one of 20 genes induced by exposure to intermittent hypoxia in healthy volunteers and were able to validate our data by real time PCR in a larger cohort. Thus, the current study suggests that hypoxic induction of TLR2 could be one of the mechanisms leading to systemic inflammation, atherosclerosis and insulin resistance in OSA.

Acknowledgments
This work has been supported by the National Institutes of Health grants HL075078 and HL080105.

Author Contributions
Conceived and designed the experiments: VYP NMP. Performed the experiments: VYP SBF DNG NMP. Analyzed the data: VYP SBF DNG NMP. Contributed reagents/materials/analysis tools: VYP SBF DNG NMP. Wrote the paper: VYP DNG NMP.

References
1. Gastaut H, Tassinari CA, Duron B. 1966. Polygraphic study of the episodic diurnal and nocturnal (hypnic and respiratory) manifestations of the Pickwick syndrome. Brain Res. 1:167–186. PMID:5923125
2. Young T, Finn L, Peppard PE, Szklo-Coxe M, Austin D, Nieto FJ, et al. 2008. Sleep disordered breathing and mortality: eighteen-year follow-up of the Wisconsin sleep cohort. Sleep 31:1071–1078. PMID:18714778
3. Marshall NS, Wong KK, Liu PY, Cullen SR, Knuiman MW, Grunstein RR. 2008. Sleep apnea as an independent risk factor for all-cause mortality: the Busselton Health Study. Sleep 31:1079–1085. PMID:18714779
4. Punjabi NM, Cafo BS, Goodwin JL, Gottlieb DJ, Newman AB, O'Connor GT, et al. 2009. Sleep-disordered breathing and mortality: a prospective cohort study. PLoS Med 6:e1000132. doi: 10.1371/journal.pmed.1000132 PMID:19689045
5. Ryan S, Taylor CT, McNicholas WT. 2005. Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. Circulation 112:2660–2667. PMID:16246965
6. Vgontzas AN, Papanicolaou DA, Bixler EO, Hopper K, Lotsikas A, Lin HM, et al. 2000. Sleep apnea and daytime sleepiness and fatigue: relation to visceral obesity, insulin resistance, and hypercytokinemia. J Clin.Endocrinol.Metab 85:1151–1158. PMID:10720054
7. Vgontzas AN, Zourakis E, Lin HM, Bixler EO, Trakada G, Chrousos GP. 2004. Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor-alpha antagonist. J Clin.Endocrinol.Metab 89:4409–4413. PMID:15356039
8. Polotsky VY, Savransky V, Bevans-Fonti S, Reinke C, Li J, Grigoryev DN, et al. 2010. Intermittent and sustained hypoxia induce a similar gene expression profile in human aortic endothelial cells. Physiol Genomics 41:306–314. doi:10.1152/physiolgenomics.00091.2009 PMID:20197421
9. Poulain L, Richard V, Levy P, Dematteis M, Arnaud C. 2015. Toll-like receptor-4 mediated inflammation is involved in the cardiometabolic alterations induced by intermittent hypoxia. Mediators.Inflamm. 2015:20258. doi:10.1155/2015/20258 PMID:25873766
10. Khalyfa AO, Capdevila S, Buazza MO, Serpero LD, Kheirandish-Gozal L, Gozal D. 2009. Genome-wide gene expression profiling in children with non-obese obstructive sleep apnea. Sleep Med 10:75–86. doi: 10.1016/j.sleep.2007.11.006 PMID: 18261956
11. Hoffmann MS, Singh P, Wolk R, Romero-Corral A, Raghavakaimal S, Somers VK. 2007. Microarray studies of genomic oxidative stress and cell cycle responses in obstructive sleep apnea. Antioxid. Redox.Signal. 9:661–669. PMID: 17511582
12. Amardottir ES, Mackiewicz M, Gislason T, Teff KL, Pack AI. 2009. Molecular signatures of obstructive sleep apnea in adults: a review and perspective. Sleep 32:447–470. PMID: 19413140
13. Fan C, Iacobas DA, Zhou D, Chen Q, Lai JK, Gavrialov O, et al. 2005. Gene expression and phenotypic characterization of mouse heart after chronic constant or intermittent hypoxia. Physiol Genomics 22:292–307. PMID: 15928208
14. Li J, Grigoryev DN, Ye SQ, Thorne L, Schwartz AR, Smith PL, et al. 2005. Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice. J Appl.Physiol 99:1643–1648. PMID: 16037401
15. Ryan S, McNicholas WT, Taylor CT. 2007. A critical role for p38 map kinase in NF-kappaB signaling during intermittent hypoxia/reoxygenation. Biochem.Biophys.Res.Commun. 355:728–733. PMID: 17316568
16. Louis M, Punjabi NM. 2009. Effects of acute intermittent hypoxia on glucose metabolism in awake healthy volunteers. J.Appl.Physiol 106:1538–1544. doi: 10.1152/japplphysiol.91523.2008 PMID: 19265062
17. 1998. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: The evidence report. Public Health Service, Department of Health & Human Services, Washington DC.
18. Ogden CL, Carroll MD, Kit BK, Flegal KM. 2014. Prevalence of childhood and adult obesity in the United States, 2011–2012. JAMA 311:806–814. doi: 10.1001/jama.2014.732 PMID: 24570244
19. Grigoryev DN, Mathai SC, Fisher MR, Girgis RE, Zaiman AL, Houston-Harris T, et al. 2008. Identification of candidate genes in scleroderma-related pulmonary arterial hypertension. Transl. Res. 151:197–207. doi: 10.1016/j.trsl.2007.12.010 PMID: 18355767
20. Savransky V, Jun J, Li J, Nanayakkara A, Fonti S, Moser AB, et al. 2008. Dyslipidemia and atherosclerosis induced by chronic intermittent hypoxia are attenuated by deficiency of stearoyl coenzyme A desaturase. Circ. Res. 103:1173–1180. doi: 10.1161/CIRCRESAHA.108.178533 PMID: 18832746
21. Schober A. 2008. Chemokines in vascular dysfunction and remodeling. Arterioscler.Thromb.Vasc.Biol. 28:1950–1959. doi: 10.1161/ATVBAHA.107.161224 PMID: 18814241
22. Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30:207–210. PMID: 11752295
23. Barrett T, Suzek TO, Troup DB, Wilhite SE, Ngau WC, Ledoux P et al. 2005. NCBI GEO: mining millions of expression profiles—database and tools. Nucleic Acids Res. 33:D562–D566. PMID: 15608262
24. Iwaski A., Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. Nat.Immunol. 5:987–995. PMID:15608262
25. Moghimpour BF, Valiejo JG, Rezaei N. 2012. Toll-like receptor signaling pathways in cardiovascular diseases: challenges and opportunities. Int.Rev.Immunol. 31:379–395. doi: 10.3109/08830185.2012.706761 PMID: 23085347
26. Erridge C. 2011. Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis, type 2 diabetes and non-alcoholic fatty liver disease. Atherosclerosis 216:1–6. doi: 10.1016/j.atherosclerosis.2011.02.043 PMID: 21439367
27. Apostolakis S, Vogiatzi K, Amanatidou V, Spandidos DA. 2009. Interleukin 8 and cardiovascular disease. Cardiovasc.Res. 84:353–360. doi: 10.1093/cvr/cvp241 PMID: 19617600
28. Madan M, Amar S. 2008. Toll-like receptor-2 mediates diet and/or pathogen associated atherosclerosis: proteomic findings. PLoS.ONE. 3:e3204. doi: 10.1371/journal.pone.0003204 PMID: 18787704
29. Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. 2009. The c-Jun N-Terminal Kinase Mediates doi: 10.1677/JOE-08-0354 PMID: 18787058
30. Caricelli AM, Nascimento PH, Pauli JR, Tsukumo DM, Velloso LA, Carvalheira JB, et al. 2008. Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet. J Endocrinol. 199:399–406. doi: 10.1677/JOE-08-0354 PMID: 18787058
31. Ryan S, Taylor CT, McNicholas WT. 2006. Predictors of elevated nuclear factor-kappaB-dependent genes in obstructive sleep apnea syndrome. Am.J.Respir.Crit Care Med. 174:824–830. PMID: 16840748
32. Rahman SA, Castanon-Cervantes O, Scheer FA, Shea SA, Czeisler CA, Davidson AJ, et al. 2015. Endogenous circadian regulation of pro-inflammatory cytokines and chemokines in the presence of bacterial lipopolysaccharide in humans. Brain Behav. Immun. 47:4–13. doi: 10.1016/j.bbi.2014.11.003 PMID: 25452149

33. Greenberg H, Ye X, Wilson D, Htoo AK, Hendersen T, Liu SF. 2006. Chronic intermittent hypoxia activates nuclear factor-kappaB in cardiovascular tissues in vivo. Biochem. Biophys. Res. Commun. 343:591–596. PMID: 16554025

34. Savransky V, Bevans S, Nanayakkara A, Li J, Smith PL, Torbenson MS, et al. 2007. Chronic intermittent hypoxia causes hepatitis in a mouse model of diet-induced fatty liver. Am. J. Physiol. Gastrointest. Liver Physiol. 293:G871–G877. PMID: 17690174

35. Savransky V, Nanayakkara A, Li J, Bevans S, Smith PL, Rodriguez A, et al. 2007. Chronic intermittent hypoxia induces atherosclerosis. Am. J. Respir. Crit Care Med. 175:1290–1297. PMID: 17332479

36. Savransky V, Nanayakkara A, Vivero A, Li J, Bevans S, Smith PL, et al. 2007. Chronic intermittent hypoxia predisposes to liver injury. Hepatology 45:1007–1013. PMID: 17393512

37. Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK. 2007. Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. PLoS ONE. 2:e1364. PMID: 18159247

38. Drager LF, Bortolotto LA, Lorenzi MC, Figueiredo AC, Krieger EM, Lorenzi-Filho G. 2005. Early signs of atherosclerosis in obstructive sleep apnea. Am. J. Respir. Crit Care Med. 172:613–618. PMID: 15901608

39. Minoguchi K., Yokoe T, Tazaki T, Minoguchi H, Tanaka A, Oda N, et al. 2005. Increased carotid intima-media thickness and serum inflammatory markers in obstructive sleep apnea. Am. J. Respir. Crit Care Med. 172:625–630. PMID: 16120716

40. Drager LF, Bortolotto LA, Figueiredo AC, Krieger EM, Lorenzi-Filho G. 2007. Effects of continuous positive airway pressure on early signs of atherosclerosis in obstructive sleep apnea. Am. J. Respir. Crit Care Med. 176:706–712. PMID: 17556718

41. Palm NW, Medzhitov R. 2009. Pattern recognition receptors and control of adaptive immunity. Immuno. Rev. 227:221–233. doi: 10.1111/j.1600-065x.2008.00731.x PMID: 19120487

42. Foster SL, Hargreaves DC, Medzhitov R. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature 447:972–978. PMID: 17538624

43. Hill DB, Marsano LS, McClain CJ. 1993. Increased plasma interleukin-8 concentrations in alcoholic hepatitis. Hepatology 18:576–580. PMID: 8359798

44. https://www.mdsystems.com/products/human-cxcl8-il-8-quantikine-elisa-kit_d8000c. 2015.