Intermittent and sustained hypoxia induce a similar gene expression profile in human aortic endothelial cells

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Submitted 27 May 2009; accepted in final form 24 February 2010

OBSTRUCTIVE SLEEP APNEA (OSA) is associated with high cardiovascular mortality and morbidity (38, 49, 71). High cardiovascular risk in OSA can be attributed to accelerated atherosclerosis (10). Treatment with continuous positive airway pressure (CPAP) for 3 mo inhibited the progression of atherosclerosis in OSA or IH are not clear, but multiple factors are likely to contribute, including dyslipidemia (39, 53, 58), hypertension (46), and systemic oxidative stress (29). OSA is also characterized by systemic inflammation with increased circulating levels of the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-8, and IL-6 (56, 57, 69), which can accelerate atherosclerosis (7), and decreased levels of anti-inflammatory and antiatherogenic IL-10 (15, 65). IH may lead to atherosclerosis by inducing direct vascular injury (8, 32). Interestingly, IH upregulated redox-sensitive proinflammatory nuclear factor-κB (NF-κB) in HeLa cells and bovine aortic endothelial cells (55, 56), whereas SH activated the transcription factor hypoxia-inducible factor 1 (HIF-1) but not NF-κB, suggesting differential cellular responses to IH and SH (56). The in vitro findings are clinically relevant, because NF-κB is tightly linked to oxidative stress and hypercytokinemia observed in OSA (7, 57). Despite the fact that endothelial cells are likely to be a major site of injury during the development of atherosclerosis, the expression profiles in human endothelial cells exposed to IH and SH have not been compared previously. We chose human aortic endothelial cells (HAEC) for our study because of their relevance for atherosclerosis and commercial availability. We hypothesized that IH will induce genes of inflammation and oxidative stress in HAEC, which will not occur after SH. We exposed HAEC to IH or SH for 8 h and 1) performed gene arrays with total RNA isolated from HAEC with subsequent verification of differentially expressed genes of inflammation and oxidative stress in real-time PCR and 2) measured protein levels of the proinflammatory and proatherogenic cytokines TNF-α, IL-8, and IL-6 (56, 57, 69) and anti-inflammatory, antiatherogenic IL-10 (15, 65) in media.
METHODS

Cell culture. HAEC from three different donors were obtained commercially from Cambrex (East Rutherford, NJ). Cells at passage 9 were grown to 100% confluence in T-75 tissue culture flasks in EGM-2 complete medium (Clonetics) and placed in basal medium 24 h before exposures began. Immediately before exposure, 40 ml of fresh medium was added to each flask and small holes for gas inlets and outlets were created in the tops of the flasks with a heated awl. Flasks were placed in a heated water bath, which maintained medium temperature at 37°C, and were gassed with 1) 16% O₂, 5% CO₂, balance N₂ (control); 2) 5 min of 16% O₂, 5% CO₂, balance N₂ alternating with 5 min of 0% O₂, 5% CO₂, balance N₂ (IH); or 3) 4% O₂, 5% CO₂, balance N₂ (SH-4%) as shown in Fig. 1. Preliminary experiments had shown that six cycles per hour was the highest frequency of IH that could reach the desired zenith and nadir of O₂ in the medium (Fig. 2). Initial experiments were performed to record temperature and % O₂ in the medium simultaneously during the protocol with an OM-4 oxygen meter (Microelectrodes, Bedford, NH) and an electronic temperature probe (4600; YSI, Dayton, OH), and data were captured with a Powerlab 8Sp Data Acquisition system (AD Instruments, Colorado Springs, CO). The % O₂ in the medium cycled according to the desired profile, and the temperature remained stable throughout the 8-h exposure. After our model was validated, HAEC were exposed to IH, SH, or control conditions for 8 h. The exposure was repeated five times (n = 5, including 3 times with HAEC from donor 1 and once each with HAEC from donors 2 and 3). Upon cessation of the exposure, flasks were immediately placed on ice, and cells and medium were collected, snap-frozen in liquid nitrogen, and stored at −80°C.

The regimen of hypoxia was selected on the basis of the following considerations: 1) control conditions corresponded to partial pressure of O₂ (P₀₂) ~114 mmHg slightly exceeding the PO₂ of arterial blood in a healthy human and ensuring that neither hypoxia nor hyperoxia was present; 2) SH corresponded to a PO₂ of 28.5 mmHg, which was consistent with severe hypoxia in vivo; 3) IH was comparable in severity to SH, given that although the mean O₂ concentration during IH was ~7.5%, P₀₂ of 53.5 mmHg, the nadir of O₂ was 1%, P₀₂ of 7 mmHg. Nevertheless, given that the mean O₂ during IH was higher than during SH-4%, we performed a second set of experiments, which included identical IH and control exposure but SH induced with 8% O₂, 5% CO₂, balance N₂ (SH-8%) corresponding to PO₂ of 57 mmHg.

Immunocytochemistry. HAEC at passage 9 were fixed with 10% buffered formalin and then stained with 1) mouse monoclonal anti-human thrombomodulin antibody (R&D Systems, Minneapolis, MN) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) or 2) mouse monoclonal anti-smooth muscle actin from Sigma and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and counterstained with DAPI. Staining was detected with filters U-N31000 [exciter 360 nm/emitter 460 nm (DAPI)], U-N31001 [exciter 480 nm/emitter 535 nm (actin/Alexa Fluor 488)], and U-N31002 [exciter 540 nm/emitter 605 nm (thrombomodulin/Alexa Fluor 594)] from Chroma Technology (Rockingham, VT) on an Olympus microscope.

Illumina array studies. Microarray studies were performed in the experiments with IH and SH-4% with standard protocols. Total RNA was isolated from HAEC with the TRIZol reagent method (Invitrogen; catalog no. 15596-026) and subsequent RNEasy clean up (Qiagen, Valencia, CA; catalog no. 74104). Total RNA (0.5 μg from each sample) was labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX; catalog no. IL1791). mRNA was converted into double-stranded cDNA with an oligo(dT) primer containing the T7 RNA polymerase promoter. Single-stranded RNA (cRNA) was synthesized from double-stranded cDNA in an in vitro transcription reaction. cRNA was labeled by incorporating biotin-16-UTP. Biotin-labeled cRNA (0.85 μg) was hybridized (16 h) to Illumina’s Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA; catalog no. 511972) on an Illumina Bead Array Reader. Total RNA from HAEC was labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX; catalog no. IL1791). mRNA was converted into double-stranded cDNA with an oligo(dT) primer containing the T7 RNA polymerase promoter. Single-stranded RNA (cRNA) was synthesized from double-stranded cDNA in an in vitro transcription reaction. cRNA was labeled by incorporating biotin-16-UTP. Biotin-labeled cRNA (0.85 μg) was hybridized (16 h) to Illumina’s Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA; catalog no. 511972) on an Illumina Bead Array Reader.
log 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 with Illumina BeadStudio software. The resulting digitized matrix was processed by a platform approach modified for Illumina described previously (16).

**Real-time PCR.** cDNA was synthesized from total RNA with the Advantage RT for PCR kit from Clontech (Palo Alto, CA). Real-time reverse transcriptase-PCR (RT-PCR) was performed with primers from Invitrogen and Taqman probes from Applied Biosystems (Foster City, CA). The sequences of primers and probes for 18S were designed previously (33–35, 58, 59). The sequences of primers and probes for heat shock protein 90 kDa [HSP90-B1] were designed based on GenBank sequence NM_003299: forward primer 5’-CCAGTGTGGTGCGTTTCTATTCT-3’; reverse primer 5’-TCTGGGTACCAATTACAGAAA-3’, and probe 5’-AGGTTATTGTCACTTCAAAAAC-3’. The sequences of primers for thrombospondin 1 (THBS1) were designed based on GenBank sequence NM_003246:2; forward primer 5’-CGTTAGAAGCTCTCTTGCAAAC-3’ and reverse primer 5’-GCCCTGACTCTCGCAGGAAAC-3’. The sequences of primers for tumor necrosis factor superfamily member 4 (TNFSF4) were designed based on GenBank sequence NM_002133.1: forward primer 5’-CAATAAAGTCATCCAGGGAGGTATTG-3’ and reverse primer 5’-CAAG-3’.

The mRNA expression levels of all RT-PCRs was 85–110%. The mRNA expression levels were normalized to 18S rRNA concentrations with the following formula:

\[
\frac{C_{t} \text{(gene of interest)}}{C_{t} \text{(18S)}} = \text{2}^{-\Delta C_{t}} = \text{2}^{-\left(C_{t} \text{(gene of interest)} - C_{t} \text{(18S)}\right)}.
\]

The sequences of primers for nuclear factor (erythroid-derived 2)-like 2 (NRF2) were designed based on GenBank sequence NM_003326.2: forward primer 5’-GCCCTGTCTTCCTGCACAAAC-3’ and reverse primer 5’-AGGTTATTGTCACTTCAAAAAC-3’. The sequences of primers for thrombospondin 1 (THBS1) were designed based on GenBank sequence NM_003246:2; forward primer 5’-CGTTAGAAGCTCTCTTGCAAAC-3’ and reverse primer 5’-GCCCTGACTCTCGCAGGAAAC-3’. The sequences of primers for tumor necrosis factor superfamily member 4 (TNFSF4) were designed based on GenBank sequence NM_002133.1: forward primer 5’-CAATAAAGTCATCCAGGGAGGTATTG-3’ and reverse primer 5’-CAAG-3’.

The sequences of primers and probes for 18S were described previously (67). The mRNA expression levels were normalized to 18S rRNA concentrations with the following formula: gene of interest/18S normalized to 18S rRNA concentrations with the following formula:

\[
\frac{C_{t} \text{(gene of interest)}}{C_{t} \text{(18S)}} = \text{2}^{-\Delta C_{t}} = \text{2}^{-\left(C_{t} \text{(gene of interest)} - C_{t} \text{(18S)}\right)}.
\]

The Medline analysis of the differentially expressed genes revealed that IH and SH-4% activated a number of the proinflammatory genes with 1,000 permutations of 5 control and 5 treated HAEC samples without application of arbitrary restrictions. Genes with $p<0.05$ were considered significantly affected by IH or SH. Finally, the Medline database (www.pubmed.com; National Library of Medicine, Bethesda, MD) was searched for all differentially expressed genes through December 2009. The search strategy was as follows: [gene of interest(MeSH)] OR [inflammation(MeSH)] OR [atherosclerosis(MeSH)] OR [oxidative stress(MeSH)] OR [mitochondrial electron transport(MeSH)] OR [hypoxia (MeSH)].

**RESULTS**

HAEC at passage 9 preserved the endothelial cell phenotype, which was evident from positive thrombomodulin staining and negative smooth muscle actin staining (Fig. 3).

In HAECs, IH upregulated 407 genes and downregulated 151 genes, whereas SH-4% upregulated 213 genes and downregulated 221 genes (Supplemental Tables S1 and S2). The Medline analysis of the differentially expressed genes revealed that IH and SH-4% activated a number of the proinflammatory genes.

1 The online version of this article contains supplemental material.
and anti-inflammatory genes (Tables 1 and 2). Gene microarrays showed that both stimuli upregulated the following proinflammatory genes: heat shock proteins 70 kDa and 90 kDa (HSP70, HSP90-A1, and HSP90-B1); histone 2, H2be; histone 1, H3d; and histone deacetylase 5; and proatherogenic chemokine (C-X-C motif) receptor 4 (CXCR4) (40). Robust induction of HSP90-B1 by both IH and SH-4% was verified by real-time PCR (Fig. 4). Gene microarrays also showed a 1.5- to 1.9-fold increase in expression of several proinflammatory genes by IH but not by SH-4% (Table 1). These genes included THBS1 (64), TNFSF4 (also known as OX40 ligand) (68), ZMPSTE24 (55), and apoptotic protein caspase 3 (27). How- ever, real-time PCR revealed that expression of THBS1 and TNFSF4 was increased by both IH and SH-4% (Fig. 4). Gene microarrays also showed that both stimuli upregulated a number of antioxidant genes, including HO-1, selenoproteins, thioredoxin, and an important transcription factor of antioxidant defense, NRF2 (24). Induction of HO-1 and NRF2 by IH and SH-4% was confirmed by real-time PCR (Fig. 4). Both conditions resulted in upregulation of genes encoding such critical proteins of the mitochondrial electron transport chain as cytochrome-c oxidases, NAD(P)H quinone oxidoreductases (NQO), and ATP synthase (11) (Table 3). IH, but not SH-4%, induced expression of antioxidant thioredoxin reductase 1, and von Hippel-Lindau (VHL) binding protein 1. In contrast, SH-4%, but not IH, upregulated VEGF.

Only five genes demonstrated statistical differences in the levels of expression between IH and SH-4% (Table 4). Compared with SH-4%, IH resulted in higher levels of expression of proinflammatory IL-8, anti-inflammatory annexin A1, and

### Table 1. Proinflammatory genes differently regulated by intermittent hypoxia and sustained hypoxia (4% O2) compared with normoxia

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>IH (Fold Change)</th>
<th>q, %</th>
<th>SH-4% (Fold Change)</th>
<th>q, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI_4507676-S</td>
<td>Heat shock protein 90 kDa β1 (HSP90-B1)</td>
<td>3.70</td>
<td>&lt;0.10</td>
<td>2.28</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_40254815-S</td>
<td>Heat shock protein 90 kDa, α1 (HSP90-A1)</td>
<td>3.40</td>
<td>&lt;0.10</td>
<td>1.71</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_22027639-S</td>
<td>Histone 2, H2be (HIST2H2BE)</td>
<td>1.89</td>
<td>&lt;0.10</td>
<td>2.07</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_4504522-S</td>
<td>H2A histone family, member X (H2AFX)</td>
<td>1.81</td>
<td>0.289</td>
<td>1.84</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_39812261-S</td>
<td>Histone 1, H3d (HIST1H3D)</td>
<td>1.80</td>
<td>&lt;0.10</td>
<td>1.76</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_4503174-S</td>
<td>Chemokine (C-X-C motif) receptor 4 (CXCR4)</td>
<td>1.70</td>
<td>&lt;0.10</td>
<td>1.94</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_21237796-A</td>
<td>Histone deacetylase 5 (HDAC5), transcript variant 1</td>
<td>1.69</td>
<td>0.162</td>
<td>1.64</td>
<td>0.178</td>
</tr>
<tr>
<td>GI_23510446-S</td>
<td>Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4)</td>
<td>1.78</td>
<td>&lt;0.10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_19743812-I</td>
<td>Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)</td>
<td>1.66</td>
<td>1.656</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_4505204-S</td>
<td>Matrix metalloproteinase 10 (stromelysin 2) (MMP10)</td>
<td>1.66</td>
<td>0.386</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_22027623-A</td>
<td>TNF receptor-associated factor 4 (TRAF4), transcript variant 2</td>
<td>1.65</td>
<td>&lt;0.10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_14790114-A</td>
<td>Caspase 3, apoptosis-related cysteine protease (CASP3), transcript variant β</td>
<td>1.64</td>
<td>0.162</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_21361242-S</td>
<td>Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (HSPA5)</td>
<td>2.08</td>
<td>&lt;0.10</td>
<td>1.83</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_19743818-A</td>
<td>Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)</td>
<td>1.60</td>
<td>2.136</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GI_28610153-S</td>
<td>Interleukin 8 (IL8)</td>
<td>1.57</td>
<td>2.931</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

IH, intermittent hypoxia; SH-4%, sustained hypoxia (4% O2); q, false discovery rate; NS, not significant.

### Table 2. Anti-inflammatory genes differently regulated by intermittent hypoxia and sustained hypoxia (4% O2) compared with normoxia

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>IH (Fold Change)</th>
<th>q, %</th>
<th>SH-4% (Fold Change)</th>
<th>q, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI_4502100-S</td>
<td>Annexin A1 (ANXA1)</td>
<td>2.97</td>
<td>&lt;0.10</td>
<td>1.76</td>
<td>0.178</td>
</tr>
<tr>
<td>GI_17136146-A</td>
<td>Adenosine A2a receptor (ADORA2A)</td>
<td>2.63</td>
<td>&lt;0.10</td>
<td>2.93</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_21361242-S</td>
<td>Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (HSPA5)</td>
<td>2.08</td>
<td>&lt;0.10</td>
<td>1.83</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_29743323-S</td>
<td>Similar to Heat shock 27 kDa protein (HSP 27) (Stress-responsive protein 27) (SRP27)</td>
<td>1.89</td>
<td>&lt;0.10</td>
<td>1.94</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_10092618-S</td>
<td>Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (NFKBIA)</td>
<td>1.87</td>
<td>&lt;0.10</td>
<td>2.01</td>
<td>&lt;0.10</td>
</tr>
</tbody>
</table>

The Medline analysis of the differentially expressed genes revealed that both IH and SH-4% upregulated a number of antioxidant genes, including HO-1, selenoproteins, thioredoxin, and an important transcription factor of antioxidant defense, NRF2 (24). Induction of HO-1 and NRF2 by IH and SH-4% was confirmed by real-time PCR (Fig. 4). Both conditions resulted in upregulation of genes encoding such critical proteins of the mitochondrial electron transport chain as cytochrome-c oxidases, NAD(P)H quinone oxidoreductases (NQO), and ATP synthase (11) (Table 3). IH, but not SH-4%, induced expression of antioxidant thioredoxin reductase 1, and von Hippel-Lindau (VHL) binding protein 1. In contrast, SH-4%, but not IH, upregulated VEGF.

Only five genes demonstrated statistical differences in the levels of expression between IH and SH-4% (Table 4). Compared with SH-4%, IH resulted in higher levels of expression of proinflammatory IL-8, anti-inflammatory annexin A1, and
In a separate series of experiments, we explored whether similarities between IH and SH persist when the same regimen of IH is compared with SH-8%. Compared with control conditions, IH induced a 2.61 ± 0.84-fold increase in HSP90-B1 mRNA (P < 0.05), whereas SH-8% induced a 2.12 ± 0.91-fold increase (P = 0.05); there was no difference in HSP90-B1 mRNA between IH and SH-8% (P = 0.67). IH induced a 2.84 ± 0.62-fold increase in THBS1 mRNA (P < 0.05), and SH-8% induced a 3.36 ± 0.73-fold increase (P < 0.05); there was no difference in THBS1 mRNA between IH and SH-8% (P = 0.71). IH induced a 2.41 ± 0.38-fold increase in TNFSF4 mRNA (P < 0.05), and SH-8% induced a 3.19 ± 0.48-fold increase (P < 0.05); there was no difference in TNFSF4 mRNA between IH and SH-8% (P = 0.46). IH induced a 3.25 ± 0.62-fold increase in NRF2 mRNA (P < 0.01), and SH-8% induced a 3.17 ± 0.82-fold increase (P < 0.05); there was no difference in NRF2 mRNA between IH and SH-8% (P = 0.72). Levels of IL-8 in the medium were identical after IH and SH-8%, 1,148 ± 141 and 1,145 ± 88 pg/ml, respectively, which was significantly higher than under control conditions (429 ± 48 pg/ml, P < 0.001 with IH and SH-8%). Levels of IL-6 in the medium were also similar in both hypoxic conditions. Thus IH and SH-8% comparisons yielded the same results as IH and SH-4% comparisons: IH and SH-8% induce similar changes in expression of proinflammatory and antioxidant genes and secretion of proinflammatory cytokines.

**DISCUSSION**

There is a growing body of literature suggesting that IH of OSA induces systemic and vascular inflammation leading to atherosclerosis that is uniquely different from SH. However, genomic responses in human endothelial cells exposed to IH and SH have not been compared. The purpose of this work was to examine changes in gene expression in HAEC induced by IH and SH. Although we hypothesized that IH and SH would induce distinct genetic profiles, the main finding of our study was that IH and SH exhibited similar expression profiles, upregulating proinflammatory and antioxidant genes. The only general transcription factor IIIF, which is important for RNA polymerization (44). Compared with IH, SH-4% induced higher levels of expression of proinflammatory HSP40 (51), whereas antiapoptotic BCL2-associated athanogene 3 (BAG3) (4) was decreased. Thus IH and SH-4% induced similar genomic profiles in HAEC.

Secretion of proinflammatory cytokines into supernatant was examined by ELISA. In contrast to the microarray data, both IH and SH-4% resulted in 3.5- to 4.5-fold increases in IL-8 protein levels (Fig. 5A). Moreover, IH and SH-4% induced striking 6- to 8-fold increases in IL-6 protein levels, despite unchanged mRNA levels (Fig. 5B). TNF-α and IL-10 protein levels were undetectable in both control and hypoxic conditions.

**Table 3. Genes of oxidative stress, mitochondrial electron transport and hypoxia differentially regulated by intermittent hypoxia and sustained hypoxia (4% O_2) compared with normoxia**

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>IH Fold Change</th>
<th>IH q, %</th>
<th>SH-4% Fold Change</th>
<th>SH-4% q, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes upregulated by intermittent hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI_4504436-S</td>
<td>Heme oxygenase (decycling) 1 (H01)</td>
<td>3.18</td>
<td>&lt;0.10</td>
<td>2.76</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_27754195-S</td>
<td>NAD(P)H quinone oxidoreductase 3 (NQO3)</td>
<td>3.16</td>
<td>&lt;0.10</td>
<td>3.27</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_27754205-S</td>
<td>Cytochrome-c oxidase II (MTCO2)</td>
<td>3.01</td>
<td>&lt;0.10</td>
<td>3.00</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_27754207-S</td>
<td>ATP synthase 6 (MTATP6)</td>
<td>2.55</td>
<td>&lt;0.10</td>
<td>2.49</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_20149575-S</td>
<td>Nuclear factor (erythroid-derived) 2-like 2 (NRF2)</td>
<td>2.31</td>
<td>&lt;0.10</td>
<td>1.65</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_27754203-S</td>
<td>Cytochrome-c oxidase 1 (MTCO1)</td>
<td>2.20</td>
<td>0.289</td>
<td>2.61</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_25014098-S</td>
<td>Selenoprotein K (SELK)</td>
<td>2.16</td>
<td>&lt;0.10</td>
<td>2.24</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>GI_27754201-S</td>
<td>NAD(P)H quinone oxidoreductase 2 (NQO2)</td>
<td>2.02</td>
<td>0.693</td>
<td>2.7</td>
<td>0.178</td>
</tr>
<tr>
<td>GI_27754199-S</td>
<td>NAD(P)H quinone oxidoreductase 1 (NQO1)</td>
<td>1.98</td>
<td>0.162</td>
<td>2.11</td>
<td>&lt;0.10</td>
</tr>
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**Genes upregulated by intermittent hypoxia only**

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>q, %</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GI_33519429-A</td>
<td>Thioredoxin reductase 1 (TXNRD1), transcript variant 4</td>
<td>1.96</td>
<td>1.656</td>
<td>NS</td>
</tr>
<tr>
<td>GI_42741647-S</td>
<td>15 kDa selenoprotein (SEP15), transcript variant 1</td>
<td>1.77</td>
<td>&lt;0.10</td>
<td>NS</td>
</tr>
<tr>
<td>GI_34222132-S</td>
<td>Thioredoxin domain containing (TXNDC)</td>
<td>1.76</td>
<td>0.693</td>
<td>NS</td>
</tr>
<tr>
<td>GI_21614497-S</td>
<td>von Hippel-Lindau binding protein 1 (VPB1)</td>
<td>1.61</td>
<td>1.656</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Genes upregulated by sustained hypoxia only**

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>q, %</th>
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<tbody>
<tr>
<td>GI_19924300-S</td>
<td>Vascular endothelial growth factor C (VEGFC)</td>
<td>1.57</td>
<td>0.384</td>
</tr>
</tbody>
</table>
proinflammatory atherogenic cytokine differentially expressed in the two conditions was IL-8, but the differences between IH and SH did not persist at the level of secreted protein. Finally, similarities between gene expression and proinflammatory cytokine secretion were present when IH was compared with two levels of SH, 4% and 8% O2. In the discussion below, we explore a potential role of IH and SH in atherosclerosis and putative pathways involved in cellular responses to IH and SH in the human endothelium.

Overall, our data suggest that IH and SH induce similar inflammatory changes in the human endothelium at the transcriptional and posttranscriptional levels. There are several possible interpretations of our data in relationship to atherosclerosis in humans. First, SH may be as atherogenic as IH, as could also be inferred from previous work in animal models of SH and atherosclerosis (28, 43). Human observational studies in denizens of high altitude lack appropriate controls (12) and cannot dispel this hypothesis. In addition, these subjects can be genetically adapted to residence at high altitude. Second, we have identified early changes in the expression profile during IH and SH; it is conceivable that proinflammatory changes will persist in IH because of the lack of adaptation, which will occur at in SH conditions. Late changes in gene expression may differ from acute changes, and our findings may not be relevant for a chronic disease such as atherosclerosis. Third, IH may have a more profound proinflammatory effect on other cellular populations important for the development of atherosclerosis, such as macrophages and lymphocytes (1, 31), which were not examined in our study. Finally, proatherogenic effects of IH of OSA may be systemic rather than local and related to dyslipidemia (58, 59), insulin resistance (47, 48, 50), and hypertension (46), which do not develop in SH.

We were surprised by the finding that both IH and SH induced proinflammatory genes regulated by NF-κB. Our results stand in stark contrast to those of Ryan et al. (56), where IH, but not SH, induced NF-κB-regulated genes and increased NF-κB DNA binding activity in HeLa cells. In our study, both stimuli induced the NF-κB pathway, including NF-κB-regulated HSP90 (3), TNFSF4 (22), and CXCR4 (25) and upregulated NF-κB activator histone deacetylase (36). IL-8 was the only NF-κB-regulated gene, which was differentially expressed in the two conditions, but the difference was no longer present at the protein level. The dissimilarities between our study and that of Ryan et al. could be attributed to differences in cell types and exposure regimens. Ryan et al. (56) maintained HeLa cells at constant levels of O2 in the SH and control protocols and alternated preconditioned hypoxic and normoxic media in the IH protocol, whereas we used HAEC and perfused the media with gas in all types of exposure. In addition, levels of O2 at control and SH conditions differ in our study and that of Ryan et al., which could also contribute to the differences in outcomes. Our study has also shown that, while IH led to a modest 1.57-fold increase in IL-8 mRNA, both IH and SH induced a robust 2.5- to 4-fold increase in IL-8 protein in the medium. These findings suggest that both hypoxic stimuli acted posttranscriptionally, at the level of either protein synthesis or secretion. A similar phenomenon occurred with IL-6, which was unchanged by hypoxia at the mRNA level but exhibited a striking 6- to 8-fold increase in the protein level in both IH and SH conditions. An increase in secretion of IL-6 and IL-8 by human endothelium in response to hypoxia has been reported previously and was attributed to the effect of

### Table 4. Genes differentially regulated by intermittent hypoxia compared with sustained hypoxia (4% O2)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>q, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI_28610153-S</td>
<td>Interleukin 8 (IL8)</td>
<td>1.78</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GI_4502100-S</td>
<td>Annexin A1 (ANXA1)</td>
<td>1.69</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GI_4758487-S</td>
<td>General transcription factor II, polypeptide 2 (30 kDa subunit) (GTF2F2)</td>
<td>1.66</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GI_33354248-S</td>
<td>DnaJ (Hsp40) homolog, subfamily A, member 4 (DNAJA4)</td>
<td>−1.56</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GI_14043023-S</td>
<td>BCL2-associated athanogene 3 (BAG3)</td>
<td>−1.58</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Fig. 5. Levels of proinflammatory cytokines interleukin (IL)-8 (A) and IL-6 (B) in the medium of HAEC after exposure to normoxia, IH, or SH (4% O2) for 8 h measured by ELISA (n = 5 for each exposure). *P < 0.05, †P < 0.01, ‡P < 0.001 for difference between hypoxia and control.
reactive oxygen species, but molecular mechanisms remain obscure (2). Thus our work demonstrates that IH and SH induce similar proinflammatory changes in HAEC at transcriptional and posttranscriptional levels.

In our study, neither IH nor SH upregulated classical HIF-1-dependent genes, such as endothelin and glucose transporter GLUT1 (20, 61). Several potential HIF-1 targets, HO-1, cytochrome-c oxidase, and adenosine A2a receptor (13, 37, 45), were induced by IH and SH, while VEGFC was induced only by SH. However, these genes are also NF-κB targets (6, 41, 42) and therefore, could be upregulated by NF-κB. In addition, HO-1 is regulated by NRF2, as discussed further below. Our findings differ from previous reports showing HIF-1 induction in cell culture by SH (20, 56, 62). Such a discrepancy may be a result of differences in cell type or more severe sustained hypoxia used by other investigators (20, 56, 62). A lack of HIF-1 induction by IH was previously reported by Ryan et al. (56), who exposed HeLa cells to 16 cycles of IH with inspired O2 fraction (FIO2) alternating between 12% and 1%. We have observed several limitations. First, proatherogenic and proinflammatory changes in HAEC at transcriptional level. In unstressed basal conditions NRF2 is bound to Keap1 and localized in the cytoplasm. Upon the addition of electrophiles or oxidants, NRF2 is released from Keap1 and translocates into nucleus to bind to an antioxidant/electrophile response element (ARE) and activate transcription of antioxidant genes and cytoprotective enzymes. We found that both SH and IH increased expression of NRF2 mRNA. Although this finding does not necessarily suggest that NRF2 is activated, increased expression of such NRF2-regulated genes as NQO1, HO-1, and thioredoxin reductase indicates that both hypoxic stimuli may indeed induce NRF2 (14, 23, 24, 66, 73).

In conclusion, we demonstrated that short exposures to IH and SH upregulate proinflammatory and antioxidant genes in HAEC and increase secretion of proinflammatory cytokines IL-6 and IL-8 into the medium in similar fashions. Our study had several limitations. First, proatherogenic and proinflammatory effects of IH may occur systematically as a result of IH-induced hypertension, dyslipidemia, and lipid peroxidation, and therefore in vitro models may have inherent limitations for studying IH. Second, our protocol of exposure to IH and SH-4% could be considered too severe compared with OSA or IH in vivo. Other investigators used comparable or even more severe regimens of in vitro hypoxia (56, 72). In addition, moderate levels of sustained hypoxia (SH-8%) alter gene expression and cytokine secretion in a similar manner, suggesting that our findings in IH and SH-4% may have clinical relevance. Third, our study describes the gene expression profile but does not dissect mechanisms of endothelial cell response to hypoxic stimuli. Nevertheless, an important implication of the study is that perturbation of NF-κB and NRF2 pathways and excessive secretion of proinflammatory cytokines by endothelial cells may contribute to the pathogenesis of atherosclerosis in hypoxic conditions, including OSA.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-68715, HL-80105, and PO1-HL-084945, American Heart Association Grant 0756293U, American Heart Association Mid-Atlantic Affiliate Postdoctoral Fellowship 0625514U, and Research Fellowship Grant RE 2842/1-1 from the German Research Foundation (DFG).

DISCLOSURES

The authors have no conflicts of interest to report.

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