Selective Activation of Inflammatory Pathways by Intermittent Hypoxia in Obstructive Sleep Apnea Syndrome

Silke Ryan, MD; Cormac T. Taylor, PhD*; Walter T. McNicholas, MD*

**Background**—Obstructive sleep apnea syndrome (OSAS), characterized by intermittent hypoxia/reoxygenation (IHR), is an independent risk factor for cardiovascular disease. We investigated the underlying molecular mechanisms of this association in a translational study.

**Methods and Results**—In a novel in vitro model of IHR, we used HeLa cells transfected with reporter constructs and DNA binding assays for the master transcriptional regulators of the inflammatory and adaptive pathways (NFκB and HIF-1, respectively) to investigate underlying transcriptional events initiated by repeated cell exposure to IHR. Furthermore, we prospectively studied 19 male OSAS patients (median apnea-hypopnea frequency, 48.5 episodes per hour; interquartile range [IQR], 28.5 to 72.9) and 17 matched normal control subjects. Circulating levels of the proinflammatory cytokine tumor necrosis factor-α and the adaptive factor erythropoietin were assayed in all subjects at baseline and again after 6 weeks of continuous positive airway pressure therapy in patients. Full blood count was measured as part of a detailed baseline evaluation. HeLa cells exposed to IHR demonstrated selective activation of the proinflammatory transcription factor NFκB ($P<0.001$ by ANOVA), whereas the adaptive regulator HIF-1 was not activated, as demonstrated by luciferase reporter assays and DNA binding studies. Circulating tumor necrosis factor-α levels were higher in OSAS patients (2.56 pg/mL; IQR, 2.01 to 3.42 pg/mL) than in control subjects (1.25 pg/mL; IQR, 0.94 to 1.87; $P<0.001$) but normalized with continuous positive airway pressure therapy (1.24 pg/mL; IQR, 0.78 to 2.35 pg/mL; $P=0.002$). In contrast, erythropoietin levels were similar throughout. Furthermore, circulating neutrophil levels were higher in OSAS patients than in control subjects, whereas the hematocrit was unaltered.

**Conclusions**—These data demonstrate selective activation of inflammatory over adaptive pathways in IHR and OSAS, which may be an important molecular mechanism of cardiovascular disease. (**Circulation. 2005;112:2660-2667.**)

**Key Words:** cardiovascular diseases ■ inflammation ■ intermittent hypoxia ■ sleep apnea, obstructive

Obstructive sleep apnea syndrome (OSAS) is a highly prevalent disorder, affecting $\approx4\%$ of adults,1 and is associated with repetitive episodes of transient oxygen desaturation during sleep. The predominant physical morbidity of the disorder is cardiovascular, and OSAS is an independent risk factor for a number of cardiovascular diseases, particularly systemic arterial hypertension2-5 but also coronary artery disease, congestive cardiac failure, and cerebrovascular events.6,7 Furthermore, therapy with nasal continuous positive airway pressure (CPAP), which ameliorates oxygen desaturations, decreases cardiovascular morbidity5 and mortality.6,7 Although the pathophysiological basis of cardiovascular complications in OSAS is likely multifactorial, including sympathetic excitation, endothelial dysfunction, inflammation, and insulin resistance,8 it is likely that the intermittent episodes of hypoxia, particularly the associated episodes of intermittent reoxygenation, are important mediators. However, the molecular mechanisms underlying such events remain unclear.

A greater understanding of the cellular response to intermittent hypoxia and reoxygenation (IHR) should provide insight into pathophysiological pathways in OSAS. Sustained cellular hypoxia is associated with the activation of a ubiquitous transcriptionally initiated response mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is activated in sustained hypoxia (SH) through a well-defined mechanism,9 resulting in increased expression of a number of genes encoding proteins such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and inducible nitric oxide synthase. Such factors mediate an adaptive response to hypoxia directed toward increasing tissue perfusion and oxygenation and hence overcoming the initial hypoxic insult.10 However, the molecular response to IHR is not well defined. Reoxygenation after a brief period of hypoxia as experienced repetitively and systemically by OSAS patients may predispose to cell stress, possibly because of mitochon-

Received April 22, 2005; revision received June 2, 2005; accepted June 27, 2005.

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*Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.556746

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drial dysfunction.\textsuperscript{11} We have hypothesized that such events favor the activation of a proinflammatory response as mediated through the transcription factor nuclear factor-\textkappa B (NF\textkappa B), a master regulator of inflammatory gene expression.\textsuperscript{12} The downstream effects of this activation include increased expression of proatherogenic factors such as tumor necrosis factor-\alpha (TNF\alpha), which may contribute to endothelial dysfunction and subsequently cardiovascular complications.\textsuperscript{13,14} We suggest that the selective activation of such an inflammatory pathway by IHR may be an important underlying factor in the cardiovascular pathophysiology of OSAS.

We developed an in vitro model of IHR that involved exposing cells to increasing numbers of cycles of hypoxia/reoxygenation and investigating the activation profiles of NF\textkappa B and HIF-1 compared with SH. To translate our findings of the cell culture model into the clinical setting, we performed a prospective controlled study in a tightly selected group of OSAS patients who experienced frequent intermittent hypoxia but always resaturated to normoxia between apneas and thus did not experience SH. We compared circulating levels of HIF-1 and NF\textkappa B-dependent genes before and after CPAP therapy and compared levels with those from a matched group of normal control subjects.

\textbf{Methods}

\textbf{Cell Culture}

HeLa cells were grown in minimum essential medium as described before.\textsuperscript{15} Normoxic cells were maintained at atmospheric oxygen concentrations for 24 hours (21\% \textsubscript{O2}, 5\% \textsubscript{CO2}; 37\°C). Cells exposed to SH were maintained in a hypoxia chamber (1\% \textsubscript{O2}, 5\% \textsubscript{CO2}; 37\°C, Optronix). The actual PO2 values at the cell monolayer were determined from the cellular perception of hypoxia or reoxygenation was instantaneous. The actual \textsubscript{PO2} values at the cell monolayer were determined by fluorescence quenching oximetry (Oxylite 2000, Oxford Optronix).

\textbf{HIF-1 and NF\textkappa B-Dependent Reporter Gene Assay}

HeLa cells were grown to 50\% to 70\% confluence on Petri dishes and transfected with a reporter plasmid consisting of a luciferase reporter gene under the control of concatomers of basic promoter element consensus binding sites for HIF-1 or NF\textkappa B (HIF construct was a gift from Dr K. Williams; NF\textkappa B construct, Stratagene). Transfections were performed overnight with Effectene transfection reagent (Qiagen).\textsuperscript{16} After exposure to normoxia, SH, or the indicated number of cycles of preconditioned hypoxic (5 minutes) and normoxic (10 minutes) medium.

The use of preconditioned medium was necessary to ensure that the cellular perception of hypoxia or reoxygenation was instantaneous. The actual \textsubscript{PO2} values at the cell monolayer were determined by fluorescence quenching oximetry (Oxylite 2000, Oxford Optronix).

\textbf{HIF-1- and p65-DNA Binding}

HeLa cells were grown to 90\% confluence and exposed to normoxia, SH, or IHR as above. Nuclear extracts were obtained immediately after treatment with a commercial kit (Active Motif). For the DNA binding assays, either the TransAM p65 activation assay kit or the HIF-1 kit (Active Motif) was used. Nuclear extracts were plated on 96-well plates coated with DNA oligonucleotides containing the relevant consensus binding elements. After binding and washing, an antibody to HIF-1 or NF\textkappa B was added. A horseradish peroxidase antibody was used for signal detection and quantification.

\textbf{Subject Details}

Consecutive men with suspected OSAS and no other medical disorder were considered for the study, which was approved by the St Vincent’s Hospital Ethics Committee. Age- and body mass index–matched normal men were also recruited, and all gave written consent. Each subject underwent clinical assessment, testing for full blood count, liver and kidney function, and cardiac enzymes, and was assessed for cardiovascular risk factors. Overnight polysomnography (PSG) studies were recorded on an automated system (SleepLab, Viaysis) using standard techniques and manually analyzed according to the criteria of Rechtschaffen and Kales.\textsuperscript{17} Two leads of electroencephalogram (C4/A1, C3/A2), bilateral electrooculogram, and submental electromyogram were recorded continuously from surface electrodes, and a thermistor recorded oronasal flow. Thoracic and abdominal movements were monitored by inductive plethysmography, and events were classified as obstructive or central on the basis of the presence or absence, respectively, of respiratory effort during apnea or hypopnea. Apneas and hypopneas were defined as a reduction in thoracoabdominal motion of at least 80\% or 50\%, respectively, for at least 10 seconds, together with an oxygen desaturation of \textgeq 4\%. Arterial oxyhemoglobin saturation (\textsubscript{SaO2}) was measured with a pulse oximeter (BMI, Viaysis). Snoring was detected by a surface microphone attached above the sternal notch. All sleep studies were performed in the sleep laboratory and supervised throughout by an experienced sleep technician.

OSAS patients initiated CPAP therapy 1 week after diagnosis, and PSG was repeated 6 weeks later. Serum samples were obtained from all subjects after initial PSG and again from patients after 6 weeks of therapy and stored at \textminus 80\°C for further analysis. TNF\alpha and EPO levels were assayed with commercially available ELISA kits (TNF\alpha, BioSource; EPO, R&D).

\textbf{Statistical Analysis}

Subject baseline characteristics and serum ELISA results are expressed as medians and interquartile range (IQRs) and compared by use of the Wilcoxon rank-sum test for independent samples and the Wilcoxon signed-rank test for paired samples. Data generated in luciferase reporter assay and DNA binding assay experiments were analyzed by 1-way ANOVA. In vitro data are expressed as mean\pm SEM for independent experiments. Statistical analysis was performed with a commercial software package (SPSS version 11).

\textbf{Results}

\textbf{HIF-1– and NF\textkappa B-Dependent Reporter Gene Assay}

Using a novel model of IHR, we exposed cells to 0, 2, 4, 8, and 16 cycles of IHR by treating with preconditioned hypoxic medium in a hypoxia chamber, followed by oxygenated medium at ambient oxygen concentrations. This model results in cyclic fluctuations of cellular \textsubscript{PO2} values (between \textapprox 14 and 0.5 kPa) as measured by fluorescence quenching-based oximetry (Figure 1). As shown in Figure 2a, IHR activated NF\textkappa B in a dose-dependent manner, whereas SH had only moderate effects on activation. In contrast, IHR did not activate the classic hypoxia-dependent transcriptional pathway mediated via HIF-1, but SH activated a robust response (Figure 2b). To rule out shear stress related to media change as a mechanism of selective NF\textkappa B activation we demonstrated that a medium change alone for the same number of cycles did not mimic this effect (data not shown).

\textbf{HIF-1 and p65-DNA Binding Activity}

To confirm the activation and specifically the DNA binding activity of NF\textkappa B, we used a DNA binding assay (TransAM, ActiveMotif). Nuclear extracts were prepared immediately.

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after exposure to normoxia, SH, or 2 to 16 cycles of IHR as detailed above. In separate experiments, we quantified specific DNA binding activity in the nuclear extracts to consensus NFκB and HIF-1 response elements. In a manner exactly reflecting the reporter experiments, we demonstrated a significant dose-dependent NFκB activation in IHR as measured by increased nuclear p65 abundance, with a maximum of 213±37% increase after 16 cycles compared with normoxia (Figure 3a). Again, SH had only moderate effects on NFκB activity. In contrast, HIF-1 was not significantly activated by IHR, whereas it did show robust 4.6-fold activation in SH (Figure 3b).

Patient and Control Populations
The cell culture data indicate a selective activation of inflammatory pathways over adaptive pathways in IHR. To determine the importance of these findings for the cardiovascular pathophysiology in OSAS, we performed a controlled prospective clinical study.

Baseline details in OSAS patients and control subjects are given in the Table. Patients and control subjects were middle-aged and moderately obese. No subject had clinical evidence of any other medical disorder, and a detailed biochemical profile, including liver and renal function and cardiac enzyme, was within normal limits in all subjects, consistent with the inclusion criteria. OSAS patients demonstrated significantly higher neutrophil counts compared with control subjects (the Table), suggesting an increased basal systemic inflammatory state. In contrast, hemoglobin and hematocrit levels were the same in both groups, consistent with the lack of activation of an adaptive response. Patients and control subjects had normal fasting lipid and glucose levels and thyroid function. All subjects had normal blood pressure levels based on the average of 3 resting supine recordings. Because the principal objective of the present study was to study the effects of IHR, we included only those patients with OSAS who consistently resaturated to oxygen saturation (Sao2) levels >90% between apneas and thus did not experience SH. All subjects completed the Epworth Sleepiness Score.18

OSAS patients, on average, had moderate to severe disease (apnea/hypopnea index, 48.5; IQR, 28.5 to 72.9) and reported marked daytime sleepiness using the Epworth Sleepiness Score (17; IQR, 14 to 19; the Table). Control subjects were all below the upper limit of normal for an apnea/hypopnea index of 5 episodes per hour, and none reported significant daytime sleepiness.

Oximetry recordings during PSG studies demonstrated frequent transient oxygen desaturations with resaturation to normal levels in OSAS patients, with normal Sao2 levels throughout sleep in control subjects. Interapnea Sao2 levels averaged >92% in OSAS patients (the Table).

After 6 weeks of nasal CPAP therapy in OSAS patients, the apnea/hypopnea index fell to 7 (IQR, 3 to 15) (P<0.001 versus pre-CPAP levels), and all showed Sao2 levels >90% during sleep (total sleep time <90%; median, 0%; IQR, 0 to 0; minimum Sao2, 93%; IQR, 91 to 94). Objective recordings from CPAP machines revealed a nightly compliance of 4.4±0.9 hours (mean±SD).

TNFα and EPO Measurements
Serum TNFα levels were higher in OSAS patients compared with control subjects (P<0.001; Figure 4a) and reverted to control levels after 6 weeks of CPAP therapy (P=0.002). In contrast, there was no significant difference in serum EPO levels between patients and control subjects or between OSAS patients before and after CPAP therapy (Figure 4b).

These data support the selective activation of NFκB over HIF-1 in IHR compared with SH, an event that favors the production of inflammatory cytokines such as TNFα over adaptive genes such as EPO.
Discussion

These data indicate a selective and dose-dependent activation of inflammatory pathways by IHR and support a specific role for this event in the pathophysiology of cardiovascular complications in OSAS. OSAS is widely recognized as an independent risk factor for cardiovascular diseases, particularly systemic arterial hypertension, even after adjustment for potential confounding factors.\textsuperscript{2,3} For example, a recent study has found occult OSAS in 83% of patients with drug-resistant hypertension.\textsuperscript{19} Moreover, the US Sleep Heart Health Study of 6000 patients identified OSAS as an independent risk factor for cardiovascular diseases, including coronary artery disease, congestive cardiac failure, and stroke.\textsuperscript{2} CPAP therapy prevents apneas and associated oxygen desaturations, and there is growing evidence of long-term benefit from CPAP therapy to cardiovascular morbidity and mortality. Peker et al\textsuperscript{5} reported an increased incidence of cardiovascular disease among incompletely treated OSAS patients compared with those efficiently treated over a 7-year follow-up period. Furthermore, we have recently demonstrated a reduction in deaths from cardiovascular causes in OSAS patients by comparing those treated with CPAP with untreated patients over an average follow-up of 7.5 years.\textsuperscript{6} Similarly, in a large cardiovascular outcome study with a 10-year period of follow-up, severe untreated OSAS significantly increased the risk of fatal and nonfatal cardiovascular events.\textsuperscript{7}
The precise mechanisms of cardiovascular complications in OSAS have not yet been elucidated but are almost certainly of multiple origins. Potential mechanisms include sympathetic overactivity secondary to recurrent hypoxias and arousals from sleep, endothelial dysfunction, increased coagulation, and metabolic dysregulation.8,20–22 There has been considerable interest during recent years in the possibility that intermittent hypoxia in OSAS may activate molecular pathways that predispose to vascular disease. In particular, the intermittent reoxygenation that distinguishes intermittent hypoxia from SH resembles reperfusion injury and may result in the activation of inflammatory pathways such as those mediated by NFκB.12 Although the detailed molecular mechanisms underlying the cellular response to intermittent hypoxia remain unknown, the cellular response to SH is well described. Under conditions of physiological normoxia, ≈90% of oxygen is consumed by the mitochondria,23 leaving 10% for nonmitochondrial functions such as the degradation of HIF-1α, an event signaled by oxygen-dependent hydroxylation of specific residues.9 In hypoxia, the high affinity of the mitochondrial cytochrome C oxidase makes this the site of consumption of virtually all available oxygen, allowing the rapid stabilization of HIF-1. We have shown that this event may be reversed by inhibition of mitochondrial respiration by the endogenous gas nitric oxide, causing a redistribution of cellular oxygen.24 We believe that in intermittent hypoxia,

<table>
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<th>Baseline Characteristics in OSAS Patients and Control Subjects</th>
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<td><strong>OSAS Patients</strong></td>
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<td><strong>Age, y</strong></td>
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<td><strong>BMI, kg/m²</strong></td>
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<td><strong>Fasting lipids, mmol/L</strong></td>
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<td>Fasting glucose, mmol/L</td>
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<td>TSH, mIU/L</td>
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<td>Epworth Sleepiness Score</td>
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BMI indicates body mass index; WBC, white blood cell count; HB, hemoglobin; RCC, red cell count; Hct, hematocrit; PLT, platelet count; TSH, thyroid-stimulating hormone; TST, total sleep time; AHI, apnea/hypopnea index; and DI, desaturation index. Values in parentheses are IQR.

*Bold values are statistically significant.
there is insufficient SH to allow HIF-1α stabilization; however, some signaling pathway, possibly because of mitochondrial stress, results in the activation of NFkB. Such events would account for the selective activation of an inflammatory phenotype over an adaptive phenotype in intermittent hypoxia compared with SH (Figure 5).

The role of oxidative stress in cellular responses to SH remains a controversial issue. It is more likely that increased production of reactive oxygen species is a feature of intermittent hypoxia, possibly as a consequence of repetitive reoxygenation. Oxidative stress can profoundly regulate the cellular transcriptome through activation of transcription factors, including (but not limited to) specificity protein 1 (Sp1), c-jun, and possibly NFkB. The possible role of oxidative stress as a signaling mechanism in the selective activation of NFkB in intermittent hypoxia is currently being investigated in our laboratory.

A number of previous reports have selectively examined the expression of either adaptive or inflammatory factors in OSAS patients before and after CPAP therapy. Downstream products of NFkB activation, including TNFα, interleukin-6, interleukin-8, and intercellular adhesion molecule-1, have been reported to be elevated in OSAS patients and to fall with CPAP therapy.25–27 These genes have been implicated in the pathogenesis of endothelial dysfunction and subsequent cardiovascular disease.13,28 However, other reports have indicated activation of the HIF-1 pathway in OSAS. EPO levels have been reported as increased in OSAS, but this increase was observed only in patients with severe disease and higher body mass index.29 A possible explanation for these different findings is that patients with severe OSAS may be exposed to sufficient cumulative periods of SH during sleep to activate the HIF-1–dependent pathway. Support for this possibility comes from the recent report by Yuan and coworkers30 demonstrating that more severe IHR results in the activation of HIF-1 in a manner dependent on activation of Ca2+/calmodulin kinase. However, these authors found that moderate IHR (which is sufficient to activate NFkB in our model) did not activate HIF-1. Thus, we hypothesize that NFkB is more sensitive to activation by intermittent hypoxia than SH and conversely that HIF-1 is more sensitive to activation by SH than intermittent hypoxia.

An important feature of both the OSAS patient cohort and our in vitro model of intermittent hypoxia is that full resaturation occurred after each hypoxic period. Thus, the impact of such events on cell function was due solely to intermittent hypoxia without the development of SH. Patients with severe OSAS, particularly if severely obese, may remain mildly hypoxemic between apneas, resulting in a degree of SH in addition to intermittent hypoxia. Such SH would have resulted in HIF-1 activation according to our proposed model. We excluded patients who demonstrated interapnea hypoxemia from our patient cohort, and all showed interapnea SaO2 levels >90% during sleep. The mean interapnea SaO2 level in the overall patient cohort before CPAP was 92%. Thus, like our cell culture model, our patient population allowed the study of pure IHR without the confounding effects of SH.

Patient compliance with CPAP averaged 4.4 hours per night, indicating that patients spent part of each night without effective therapy. However, there is evidence of a residual benefit from CPAP during periods of sleep when the device is not used.31 Thus, the level of OSAS should be substantially less during these periods compared with pretreatment levels. On average, our subjects slept 7 hours, and the average nightly compliance represents ~65% of each night’s sleep. However, the reported compliance represents the average over the 6-week period of CPAP. In fact, compliance was better in the latter part of the treatment period as the patients adapted to the device. Previous reports that have evaluated

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**Figure 4.** Cytokine levels in OSAS. Serum level of TNFα (A) and EPO (B) were assayed in control subjects, OSAS patients before CPAP, and OSAS patients after 6 weeks of CPAP therapy by ELISA. Probability values between control subjects and OSAS patients by the Wilcoxon rank-sum test and between OSAS patients before and after CPAP therapy were determined by the Wilcoxon signed-rank test. Boxes represent values within the interquartile range; whiskers, the data range; and lines across the boxes, median values.
compliance with CPAP in OSAS have found average nightly compliance to range from 4 to 6 hours per night.32–34 Another HIF-1–dependent gene, VEGF, has been reported to be increased in serum35,36 and plasma37 of OSAS patients. We chose not to measure VEGF in the present study as a marker of HIF-1 activation because VEGF activation is complex and can be regulated by a wide range of transcription factors other than HIF-1, including Sp1, Sp3, or activating protein 2.38 Sp1 has been demonstrated to regulate VEGF transcription after oxidative stress, which could account for the increased VEGF levels in OSAS patients.39 EPO is activated solely in response to hypoxia and therefore represents a better marker for HIF-1 activation.40

We recognize that our cell culture model of IHR differs from the pattern of intermittent hypoxia that occurs in OSAS patients in terms of both duration and frequency of episodes. However, the selective activation of NFxB over HIF-1 by IHR in this model is all the more striking given the relatively few numbers of episodes involved and thus, we believe, adds support to our central hypothesis of increased sensitivity of inflammatory pathways. Furthermore, it is very difficult to mimic exactly the intermittent hypoxia of OSAS patients, particularly because the level of cellular oxygen tension during apneic episodes is unknown.

Although vascular endothelial cells are likely the primary target cells for cytokine-mediated damage in the cardiovascular complications of OSAS, the identity of the source cells that sense intermittent hypoxia and produce cytokines remains unknown and could be of endothelial, epithelial, immune, or other origin. In fact, multiple cell types are probably involved in this response to various degrees because whole tissues will experience intermittent hypoxia in OSAS. We selected HeLa cells, a tumor cell line of epithelial origin, to investigate differential activation of NFkB and HIF-1 in response to intermittent hypoxia for 3 main reasons. First, HeLa cells have a relatively high mitochondrial mass and demonstrate a significant degree of basal aerobic metabolic activity as attested to by significant ATP depletion in hypoxia (unpublished data). Thus, these cells are metabolically well poised to allow measurement of the hypoxic response. Second, HeLa cells have a robust and remarkably well-defined hypoxic response as measured by activation of both HIF-1 and NFkB in response to various stimuli. Finally, a physically robust cell line was necessary to withstand the shear stress associated with multiple media changes that is characteristic of our model. Thus, HeLa cells represent an excellent model to compare and contrast the effects of intermittent hypoxia and SH on HIF-1– and NFkB-dependent transcriptional activity.

Although the present report specifically relates to OSAS, the data have implications for other disorders associated with intermittent hypoxia. Patients with Cheyne-Stokes breathing also experience intermittent hypoxia and reoxygenation in association with central apneas, and there is evidence that patients with congestive heart failure who have associated Cheyne-Stokes breathing have a higher mortality rate than congestive heart failure patients without, even after adjustment for severity of the underlying cardiac disease.41 Furthermore, patients with chronic respiratory disorders such as chronic obstructive pulmonary disease also experience periods of intermittent hypoxia, particularly during exercise and sleep.42 Thus, it is possible that IHR in these disorders may result in activation of inflammatory pathways and contribute to adverse outcomes.

The present data provide evidence for a selective activation of inflammatory pathways in response to IHR that differs substantially from the response to SH and provides a mechanism to explain the high prevalence of cardiovascular disease in OSAS patients.

Acknowledgments

This work was supported by grants from the Science Foundation of Ireland, Wellcome Trust, and Health Research Board. We are grateful to Mairead Ryan for analyzing the PSGs, Anne-Marie Griffin for her expert technical assistance with the cell culture, all staff members of the Sleep Laboratory at St Vincent’s University Hospital for their help and support, and all patients and control subjects for participating in this study.

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Circulation. 2005;112:2660-2667
doi: 10.1161/CIRCULATIONAHA.105.556746
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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