Effects of Exposure to Intermittent Hypoxia on Oxidative Stress and Acute Hypoxic Ventilatory Response in Humans

Vincent Pialoux[1,2], Patrick J. Hanly[2,3,4], Glen E. Foster[1,2], Julien V. Brugniaux[1,2], Andrew E. Beaudin[1,2], Sara E. Hartmann[1,2], Matiram Pun[1,2], Cailean T. Duggan[1,2], and Marc J. Poulin[1,2,4,5,6,7]

[1]Departments of Physiology and Pharmacology, [2]Faculty of Medicine, [3]Department of Medicine, [4]Hotchkiss Brain Institute, [5]Department of Clinical Neurosciences, [6]Department of Kinesiology, and [7]Libin Cardiovascular Institute of Alberta, University of Calgary, Calgary, Alberta, Canada

Rationale: **Periodic occlusion of the upper airway in patients with obstructive sleep apnea leads to chronic intermittent hypoxia, which increases the acute hypoxic ventilatory response (AHVR). Animal studies suggest that oxidative stress may modulate AHVR by increasing carotid body sensitivity to hypoxia. This has not been shown in humans.**

Objectives: **To determine whether 4 days of exposure to chronic intermittent hypoxia increases AHVR and oxidative stress and to determine the strength of the association between oxidative stress and AHVR.**

Methods: **After two normoxic control days (Day −4 and Day 0), 10 young healthy men were exposed awake to 4 days (Days 1–4) of intermittent hypoxia for 6 hours per day.**

Measurements and Main Results: **AHVR, assessed using an isocapnic hypoxia protocol, was determined as the slope of the linear regression between ventilation and oxygen desaturation. Oxidative stress was evaluated by measuring plasma DNA, lipid and protein oxidation, uric acid and antioxidant status by measuring α-tocopherol, total vitamin C, and antioxidant enzymatic activities. Between baseline and Day 4, there were significant increases in AHVR, DNA oxidation, uric acid, and vitamin C, whereas antioxidant enzymatic activities and α-tocopherol were unchanged. There were strong correlations between the changes in AHVR and DNA oxidation ($r = 0.88$; $P = 0.002$).**

Conclusions: **Chronic intermittent hypoxia increases oxidative stress by increasing production of reactive oxygen species without a compensatory increase in antioxidant activity. This human study shows that reactive oxygen species overproduction modulates increased AHVR. These mechanisms may be responsible for increased AHVR in patients with obstructive sleep apnea.**

Keywords: OSA; intermittent hypoxia; ventilation; oxidative stress

Obstructive sleep apnea (OSA) occurs in at least 4% of women and 9% of men in a middle-aged community-based population (1) and is found more frequently in patients with hypertension, heart failure, and stroke (1). Patients with OSA experience periodic occlusion of the upper airway during sleep, which is associated with chronic intermittent hypoxia (CIH) and reduced cellular oxygen supply. It was recently suggested that exposure to CIH associated with recurrent apneas (2) is responsible for the increased cardiovascular and cerebrovascular morbidity associated with OSA (3).

Studies in patients with OSA (4) and in some (5) but not all (6, 7) animal models of OSA indicate that this disorder may contribute to an increase in the acute hypoxic ventilatory response (AHVR). Although some studies have shown that the peripheral chemoreflex sensitivity to hypoxia is increased in patients with OSA (8), Foster and colleagues (9) did not find a difference in AHVR between patients with moderate to severe OSA free from overt cardiovascular disease and healthy control subjects, suggesting that the duration of the disease and cardiovascular health might mediate changes in the chemoreflex sensitivity to hypoxia and to alterations in AHVR. In this context, recent studies report that CIH leads to increased carotid body sensitivity to hypoxia in animals (2, 8, 10) and increased AHVR in humans (11–13). The mechanisms responsible for this association between AHVR and carotid body sensitivity are not well understood, but the generation of reactive oxygen species (ROS) is thought to play a major role by increasing carotid body sensitivity to hypoxia (14). More specifically, CIH generates ROS during reoxygenation similar to ischemia-reperfusion by the activation of the xanthine oxidase and NADPH oxidase pathways (15, 16). ROS overproduction during CIH could also be facilitated by down-regulation of the mitochondrial Complex I, leading to an increase in electrons escaping from the electron transport chain (17).

Some clinical studies have shown that OSA is associated with an increase in oxidative stress, which is defined as an imbalance between ROS production and antioxidant status (18–24). However, other studies (25–28) have failed to show an increase in oxidative stress in patients with OSA. Comorbid

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

In patients with obstructive sleep apnea, evaluation of the relationship between intermittent hypoxia and oxidative stress has been hindered by the potential confounding effects of medical comorbidities and obesity. Furthermore, data from animal models of obstructive sleep apnea have suggested that oxidative stress increases the ventilatory sensitivity to acute hypoxia.

What This Study Adds to the Field

In an experimental human model of intermittent hypoxia, we found that oxidative stress is increased. The changes in oxidative stress are associated with increased ventilatory sensitivity to hypoxia.

(*Received in original form May 5, 2009; accepted in final form August 24, 2009*)

Supported by the Alberta Heritage Foundation for Medical Research (AHFMR) (G.E.F., V.P.); the Heart and Stroke Foundation of Alberta, NWT (G.E.F., V.P.); The Canadian Foundation for Innovation; Canadian Institutes of Health Research (CIHR) Focus-on-Stroke Program (C.T.D., V.P.); the Hotchkiss Brain Institute (J.V.B.); and TORCH (Tomorrow's Research Cardiovascular Health Professionals) (G.E.F. and C.T.D.).

Correspondence and requests for reprints should be addressed to Marc J. Poulin, Ph.D., D.Phil., HMRB-2120, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. E-mail: poulin@ucalgary.ca

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 180. pp 1002–1009, 2009
Originally Published in Press as DOI: 10.1164/rccm.200905-0671OC on August 27, 2009
Internet address: www.atsjournals.org

medical disorders that are often associated with OSA, such as obesity (29), diabetes (30) and renal disease (31), increase the generation of ROS independently of CIH, which may explain these conflicting reports. Moreover, animal models of sleep fragmentation have reported increases in oxidative stress in specific areas of the brain (32). The use of an experimental human model of intermittent hypoxia provides one approach to study the effects of intermittent hypoxia on oxidative stress and AHVR without the potential confounding issues associated with comorbid disease. Furthermore, exposure to intermittent hypoxia during wakefulness excludes the potential confounding impact of sleep disruption that characteristically accompanies OSA. To our knowledge, these questions have not been addressed in such a model.

The primary objective of this study was to examine whether 4 days of CIH, which replicates the cyclical pattern of hypoxemia and reoxygenation that occurs in patients with moderately severe OSA, alters oxidative stress and antioxidant status in healthy subjects. Secondary objectives of our study were to determine the strength of the association between the changes in oxidative stress and AHVR induced by CIH. Therefore, we hypothesized that 4 days of CIH would increase oxidative stress and AHVR and that the changes in these parameters over the 4 days of CIH would be correlated. Some of the results of these studies have been previously reported in the form of an abstract (33).

METHODS

Subjects

Ten healthy male subjects participated in the study. Women were not studied because fluctuations in estrogen throughout the menstrual cycle may affect the oxidative stress response to intermittent hypoxia (34). All subjects were nonsmokers who were not taking medication and had no history of cardiovascular, cerebrovascular, or respiratory disease. All participants were residents of Calgary, Alberta, Canada. The research study was approved by the Conjoint Health Research Ethics Board at the University of Calgary, and written informed consent was obtained from each subject prior to participating in the study.

Protocol

The experiments were conducted in the Laboratory of Human Cerebrovascular Physiology at the University of Calgary. The laboratory is located 1,100 m above sea level, and the average barometric pressure during the study was 663 ± 5 mm Hg (mean ± SD). After two baseline measurements (Day −4 and Day 0) taken after subjects breathed room air for 6 consecutive hours, all subjects were exposed to 4 days (Day 1 to Day 4) of intermittent hypoxia (IH) while awake (cycling between 2 minutes at end-tidal P_{O_2}[$P_{ET_{O_2}}$] = 45 mm Hg and 2 minutes at $P_{ET_{O_2}}$ = 88 mm Hg) 6 hours per day followed by 4 days of recovery (Day 5 to Day 8). End-tidal P_{CO_2} ($P_{ET_{CO_2}}$) was not controlled during episodes of intermittent hypoxia. Heart rate and Sa_{O_2} were measured continuously by electrocardiogram (Micromon, 7142B monitor; Kontron Medical, Plaisir, France) and by pulse oximetry (3900; Datex-Ohmeda, Louisville, CO), respectively. Oxygen desaturation index was calculated as the average number of oxygen desaturations to below 90% per hour. A detailed description of this methodology can be found in the online supplement.

Home Cardiopulmonary Monitoring

Subjects were screened for OSA by continuous, overnight cardiopulmonary monitoring at home (Remmers Sleep Recorder Model 4.2; Saga Tech Electronic, Calgary, AB, Canada). The respiratory disturbance index was calculated as the number of episodes of oxyhemoglobin desaturation greater than 4% divided by the duration of the recording. A detailed description of this methodology can be found in the online supplement.

Determination of Acute Hypoxic and Hypercapnic Ventilatory Responses

The AHVR and the acute hypercapnic ventilatory response (AHCVR) were determined using a slightly modified protocol previously described by our group (35) on Days −4, 0, 1, 2, 4, and 8 within 30 minutes after the end of exposure to intermittent hypoxia or room air. A detailed description of this methodology can be found in the online supplement.

Resting Mean Arterial Blood Pressure

Resting mean arterial blood pressure (MABP) was assessed on the right arm as the mean of three values taken every 3 minutes from an automated blood pressure monitoring device (Dinamap; Johnson and Johnson Medical, Inc., New Brunswick, NJ) provided that the three values were within 5% of each other. MABP was taken on Days −4, 0, 1, 2, 4, and 8 within 30 minutes after the end of exposure to intermittent hypoxia or room air.

Biochemical Analyses

Blood glucose status was determined from finger capillary samples (ABL700; Radiometer Canada, London, ON, Canada). Blood was collected from the antecubital vein in two 7-ml ethylenediaminetetraacetic acid tubes for biochemical analysis. The plasma was obtained by centrifugation of the samples at 1,000 × *g* for 10 minutes at 4°C. Plasma was separated into aliquots and frozen at −80°C until assays could be performed.

Plasma levels of cholesterol, triglycerides, oxidative stress (i.e., advanced oxidation protein products [AOPP], 8-hydroxy-2′-deoxyguanosine [8-OHdG], malondiadehide [MDA]), uric acid (as one of the end-products of the xanthine oxidase pathway inducing superoxide generation during hypoxia-reoxygenation), and nonenzymatic antioxidant (i.e., total vitamin C and α-tocopherol) and antioxidant enzymatic activities (i.e., plasma glutathione peroxidase [GPX] and catalase activities) were measured on Day −4, Day 0, Day 1, Day 2, Day 4 and Day 8 within 30 minutes after the end of exposure to intermittent hypoxia or room air. A detailed description and relevance of these assays can be found in the online supplement.

Statistics

The results are expressed as mean ± SD. The effect of the 4 days of IH exposure (time effect: Day −4, Day 0, Day 1, Day 2, Day 4, and Day 8) on the physiological variables (8-OHdG, MDA, AOPP, uric acid, total vitamin C, α-tocopherol, catalase, and GPX activities, ventilation when end-tidal gases are controlled near-resting values [isocapnic and eucapnia], $P_{ET_{O_2}}$ and $P_{ET_{CO_2}}$, AHVR, and AHCVR) was evaluated by one-way repeated measures ANOVA followed by Sidak *post hoc* test. Pearson's coefficient correlation was also used to determine the strength of the association between AHVR and oxidative stress parameters (8-OHdG, MDA, and AOPP). Statistical analyses were performed with analytical software (Version 15.0; SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Ten healthy subjects (29.3 ± 1.7 years of age; body mass index, 25.6 ± 0.4 kg/m^2) completed the protocol, which involved 24 hours of intermittent poikilocapnic hypoxia over 4 days (6 h/d; 8 AM to 2 PM daily) in a purpose-built chamber. The concentrations of blood cholesterol, triglycerides, and glucose were within the normal range at baseline (3.65 ± 0.41 μmol/L, 0.74 ± 0.29 μmol/L, and 5.86 ± 0.55 mmol/L, respectively) and did not change after 4 days of CIH (3.36 ± 0.39 μmol/L, 0.71 ± 0.56 μmol/L, and 5.50 ± 0.50 mmol/L, respectively). Home cardiopulmonary monitoring was completed in seven subjects; no subject had evidence of sleep apnea (mean respiratory disturbance index, 2.5 ± 1.8) or nocturnal hypoxemia (mean arterial oxyhemoglobin saturation, 94.2 ± 1.1%). The remaining three subjects had no clinical indication of sleep apnea or intermittent hypoxemia. Over 4 days of exposure to intermittent

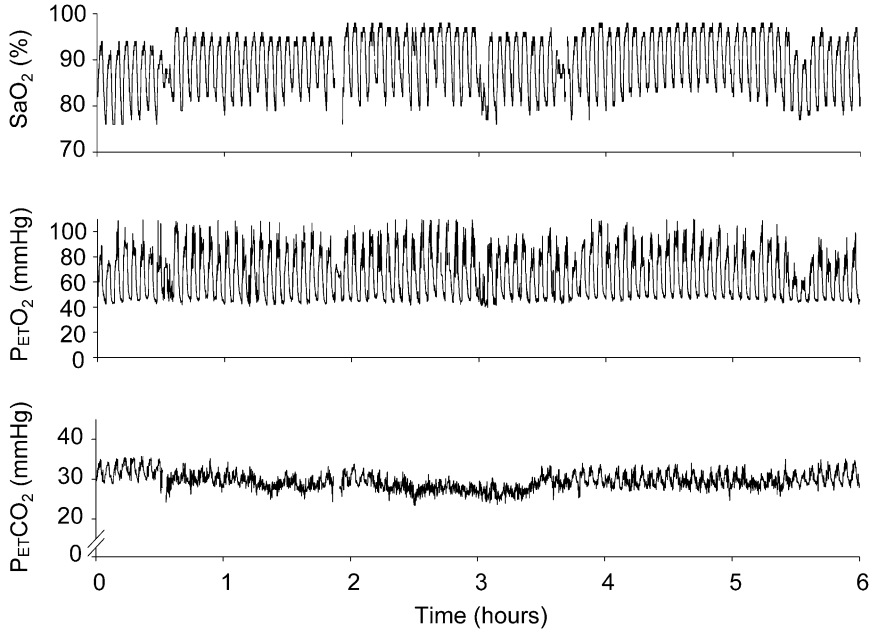

Figure 1. Sa_{O_2}, partial pressure of end-tidal O_2 ($P_{ET_{O_2}}$), and partial pressure of end-tidal CO_2 ($P_{ET_{CO_2}}$) profile of a subject during 6 hours of intermittent hypoxia (2 minutes at $P_{ET_{O_2}}$ = 45 mm Hg, and 2 minutes at $P_{ET_{O_2}}$ = 88 mm Hg).

hypoxia, the mean oxygen desaturation index was 14.8 ± 0.1 events per hour (Figure 1). Because values of oxidative stress (8-OHdG, MDA, AOPP, and uric acid), total vitamin C, α-tocopherol, and antioxidant enzyme activities (catalase and GPX), resting isocapnic eucapnia ventilation ($\dot{V}_E$, $P_{ET_{O_2}}$, and $P_{ET_{CO_2}}$), AHVR, and AHCVR were not significantly different between the two baseline measurements (Day −4 and Day 0), baseline values are expressed as the mean of values on Day −4 and Day 0.

Resting Ventilation

Mean results of ventilation when the end-tidal gases are controlled near-resting values (isocapnic and eucapnia) at baseline, Day 1, Day 2, Day 4, and Day 8 are listed in Table 1. Isocapnic and eucapnia ventilation did not change significantly throughout the study despite a trend for higher values on Day 4 (P = 0.07). Resting isocapnic and eucapnic $P_{ET_{CO_2}}$ progressively decreased from baseline to Day 4 (−5.8%; P = 0.03), whereas $P_{ET_{O_2}}$ did not change significantly. Air-breathing values for $P_{ET_{O_2}}$ did not change significantly throughout the study, but air-breathing values for $P_{ET_{CO_2}}$ decreased progressively from baseline to Day 4 (−7.8%; P = 0.015).

AHVR

AHVR increased progressively on Day 1, Day 2, and Day 4 (Figure 2) compared with baseline. On Day 4, AHVR was 83% higher than baseline (P = 0.016). After 4 days of recovery (Day 8), AHVR was significantly lower compared with Day 4 and had returned to baseline values.

AHCVR

Mean results for AHCVR at baseline, Day 1, Day 2, Day 4, and Day 8 are outlined in Table 1. AHCVR did not change significantly throughout the study (Table 2), although there was a trend for a higher AHCVR on Day 4 (P = 0.06) compared with baseline.

MABP

Resting MABP increased after exposure to IH on Day 1 (+1.1 ± 3.6 mm Hg), Day 2 (+3.1 ± 3.7 mm Hg), and Day 4 (+3.9 ± 4.4 mm Hg) and returned to baseline values on Day 8 (Table 1).

Plasma Oxidative Stress

Mean results of AOPP, MDA, and uric acid at baseline, Day 1, Day 2, Day 4, and Day 8 are outlined in Table 2. Analysis of

TABLE 1. RESTING MEAN ARTERIAL BLOOD PRESSURE, ACUTE HYPERCAPNIC VENTILATORY RESPONSE, RESTING END-TIDAL O_2 AND CO_2, ISOCAPNIC EUCAPNIA VENTILATION, END-TIDAL P_{O_2}, AND P_{CO_2} AT BASELINE (MEAN DAY −4, DAY 0) DURING 4 DAYS OF INTERMITTENT HYPOXIA (DAY 1, DAY 2, AND DAY 4) AND AFTER 4 DAYS OF RECOVERY (DAY 8)

	Baseline	Day 1	Day 2	Day 4	Day 8
$P_{ET_{O_{2\text{-REST}}}}$, mm Hg	86.5 ± 2.1	88.0 ± 2.5	89.1 ± 4.0	88.3 ± 2.1	87.0 ± 3.8
$P_{ET_{CO_{2\text{-REST}}}}$, mm Hg	36.4 ± 1.5	35.1 ± 1.4*	34.0 ± 1.9*	34.3 ± 1.8*	35.9 ± 2.0
$P_{ET_{O_{2\text{-IC-EUC}}}}$, mm Hg	87.5 ± 1.0	88.0 ± 1.0	87.4 ± 1.0	87.9 ± 1.0	87.4 ± 1.7
$P_{ET_{CO_{2\text{-IC-EUC}}}}$, mm Hg	37.8 ± 1.2	36.4 ± 1.3†	35.8 ± 1.6*	35.6 ± 1.8*	37.3 ± 1.3
$V_{E_{IC\text{-}EUC}}$, L/min	10.6 ± 2.1	11.1 ± 2.4	11.2 ± 3.1	12.3 ± 3.0	11.4 ± 2.0
AHCVR, L/min/mm Hg	3.52 ± 1.22	3.54 ± 1.31	3.38 ± 1.68	4.01 ± 1.32	3.22 ± 1.50
MABP, mm Hg	79.5 ± 6.3	80.6 ± 6.4*	82.6 ± 6.6*	83.4 ± 6.5*	77.9 ± 6.1

Definition of abbreviations: AHCVR = acute hypercapnic ventilatory response; MABP = mean arterial blood pressure; $P_{ET_{CO_{2\text{-IC-EUC}}}}$ = end-tidal P_{CO_2}; $P_{ET_{CO_{2\text{-REST}}}}$ = resting end-tidal CO_2; $P_{ET_{O_{2\text{-IC-EUC}}}}$ = end-tidal P_{O_2}; $P_{ET_{O_{2\text{-REST}}}}$ = resting end-tidal O_2; $V_{E_{IC\text{-}EUC}}$ = isocapnic eucapnia ventilation.

Values are mean ± SD.

* $P < 0.05$ compared with baseline.

† $P < 0.01$ compared with baseline.

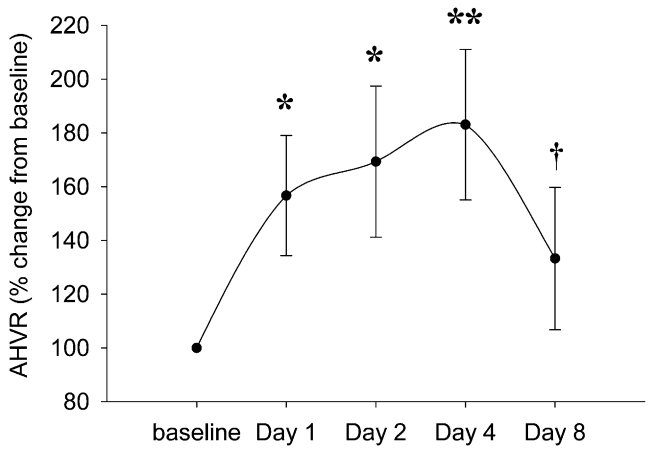

Figure 2. Acute hypoxic ventilatory response (AHVR) at baseline (mean Day −4, Day 0), during 4 days of intermittent hypoxia (Day 1, Day 2, and Day 4), and after 4 days of recovery (Day 8). Values are mean ± SD. *$P < 0.05$ and **$P < 0.01$ compared with baseline. †$P < 0.05$ compared with Day 4.

oxidative stress showed an increase in three blood oxidation markers during the 4 days of IH: (*1*) 8-OHdG increased on Day 1 (+46%; $P = 0.04$) and remained significantly elevated on Day 2 (+39%; $P = 0.05$) and Day 4 (+40%; $P = 0.02$) (Figure 3). (*2*) MDA increased significantly on Day 1 (+35%; $P = 0.003$) and returned toward baseline values on Day 2 and Day 4 despite a trend toward higher values in Day 2 and Day 4 (baseline vs. Day 2, +16% and $P = 0.09$; baseline vs. Day 4, +20% and $P = 0.09$). (*3*) Uric acid increased significantly on Day 1 (+14%; $P = 0.04$) and Day 2 (+11%; $P = 0.04$) and returned toward baseline values on Day 4 (+10%; $P = 0.08$). Uric acid, 8-OHdG, and MDA values on Day 8 were not significantly different from baseline levels.

Antioxidant Status

Mean results of vitamin C, α-tocopherol, and enzymatic GPX and catalase activities at baseline, Day 1, Day 2, Day 4, and Day 8 are outlined in Table 2. Total vitamin C (i.e., reduced form: ascorbic acid; oxidized form: dehydroascorbic acid) increased on Day 1 (+15%; $P < 0.001$) and remained significantly elevated on Day 2 (+13%; $P = 0.02$) and Day 4 (+14%; $P = 0.03$). The plasma antioxidant enzymatic activities GPX and catalase and α-tocopherol concentration did not change significantly throughout the protocol. The increase in DNA and lipid oxidation markers without a change in antioxidant enzymatic activity suggests that the oxidative stress observed in response to exposure to intermittent hypoxia is the result of ROS overgeneration.

Association between Oxidative Stress, AHVR, and HCVR

We performed correlation analyses to determine the strength of the association between plasma oxidative stress levels and AHVR. Throughout the protocol (10 subjects, five time points per subject), AHVR was positively correlated with 8-OHdG ($r = 0.39$; $P = 0.003$) and MDA ($r = 0.30$; $P = 0.02$). Analysis of the changes between baseline and Day 4 showed a significant correlation between the percentage changes in AHVR and the changes in 8-OHdG ($r = 0.88$; $P = 0.002$) (Figure 4), suggesting a moderately strong association between the increases in AHVR and ROS overproduction. Between baseline and Day 4, the percentage changes in AHVR were significantly correlated with the changes in total vitamin C ($r = 0.68$; $P = 0.02$). Changes in total vitamin C and 8-OHdG were significantly correlated between baseline and Day 4 ($r = 0.64$; $P = 0.03$). AHCVR was not significantly correlated with oxidative stress markers (8-OHdG and MDA) or with AHVR.

DISCUSSION

We report a strong association between the generation of ROS and the increase in AHVR after exposure to CIH in healthy humans. In support of the hypotheses being tested, 4 days of CIH (6 h/d) induced an increase in oxidative stress without changes in antioxidant enzyme activities, suggesting that ROS were overproduced during the CIH phase of the protocol. During the 4 days of CIH, these changes in oxidative stress were correlated with individual changes in AHVR. To our knowledge, this is the first human study in which the effects of CIH on oxidative stress have been assessed and correlated with changes in AHVR. Our data strengthen the notion that the reported increase in oxidative stress in OSA is associated with CIH and corroborate previous animal studies demonstrating that ROS up-regulates respiratory responses after exposure to CIH.

Effects of CIH on Oxidative Stress

Oxidative stress is caused by an imbalance between the production of ROS and antioxidant activity. Therefore, the lack of change in antioxidant enzyme efficiency indicates that the oxidative stress observed over 4 days of CIH is caused by an overproduction of ROS.

Oxidative stress is a well-established mechanism of cellular injury. Because DNA is known to be very sensitive to ROS,

TABLE 2. PLASMA MALONDIALDEHYDES, 8-HYDROXY-2′-DEOXYGUANOSINE, ADVANCED OXIDATION PROTEIN PRODUCTS, URIC ACID, TOTAL VITAMIN C, α-TOCOPHEROL, CATALASE, AND GLUTATHIONE PEROXIDASE ACTIVITIES, CHOLESTEROL, TRIGLYCERIDES, AND BLOOD GLUCOSE AT BASELINE (MEAN DAY −4, DAY 0), DURING 4 DAYS OF INTERMITTENT HYPOXIA (DAY 1, DAY 2, AND DAY 4), AND AFTER 4 DAYS OF RECOVERY (DAY 8)

	Baseline	Day 1	Day 2	Day 4	Day 8
AOPP, μmol/L	130.4 ± 42.4	141.5 ± 48.5	115.0 ± 52.0	98.3 ± 35.0	164.7 ± 70.5
MDA, μmol/L	3.59 ± 0.55	4.85 ± 0.30†	4.15 ± 0.45	4.31 ± 0.48	4.35 ± 0.44
Uric acid, mg/dL	5.06 ± 1.23	5.77 ± 2.04†	5.63 ± 1.54†	5.57 ± 1.68	5.09 ± 1.57
Total vitamin C, μmol/L	37.0 ± 5.6	42.5 ± 4.7†	41.9 ± 6.9*	42.3 ± 10.7*	38.5 ± 10.9
α-tocopherol, μmol/L	19.0 ± 2.5	18.9 ± 4.8	18.7 ± 2.6	18.7 ± 2.0	18.4 ± 1.4
GPX, μmol/L/min	65.1 ± 20.9	66.1 ± 14.3	62.8 ± 18.2	64.6 ± 26.4	68.3 ± 27.3
Catalase, μmol/L/min	7.31 ± 0.83	7.63 ± 0.98	8.68 ± 1.74	8.10 ± 1.68	8.01 ± 1.50

Definition of abbreviations: AOPP = advanced oxidation protein products; GPX = glutathione peroxidase; MDA = malondialdehydes.

Values are mean ± SD.

* $P < 0.05$ compared with baseline.

† $P < 0.01$ compared with baseline.

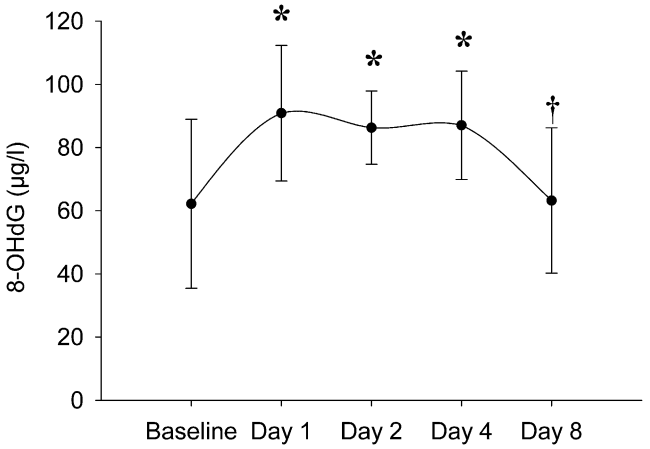

Figure 3. DNA oxidation (8-OHdG) at baseline (mean Day −4, Day 0), during 4 days of intermittent hypoxia (Day 1, Day 2, and Day 4), and after 4 days of recovery (Day 8). Values are mean ± SD. *$P < 0.05$ compared with baseline. †$P < 0.05$ compared with Day 4.

8-OHdG, an end-product of DNA oxidation, is one of the most reliable markers for oxidative stress in diseases processes (36) and was selected as the primary marker of oxidative stress in this study. Two secondary markers of oxidative stress were also investigated: (*1*) uric acid, which is produced by purine metabolism during reoxygenation and reflects ROS production through activation of the xanthine oxidase pathway, and (*2*) MDA, which is produced by oxidation of polyunsaturated fatty acids and reflects lipid peroxidation (37). The phospholipids of cell membranes contain a large amount of polyunsaturated fatty acids, which are highly susceptible to oxidative stress. Although correlated with clinical outcomes such as stroke (38), the measurement of MDA presents some methodological challenges (37) that may result in underestimation of the oxidative stress.

Three hypotheses have been proposed to explain ROS production during CIH. (*1*) CIH may trigger a partial inhibition of the mitochondrial electron transport chain activity by inducing a leak of electrons from Complex I, thereby leading to ROS generation (39). (*2*) Enzymatic generation of ROS and uric acid from the xanthine oxidase pathway may occur during the cycle of hypoxia/reoxygenation, similar to what occurs during ischemia/reperfusion (40, 41). (*3*) The involvement of the NADPH oxidase complex may be a third pathway for ROS production during the cycle of hypoxia-oxygenation (42). Assuming that long-term CIH enhances oxidative stress in animals (40), a progressive increase in lipid and DNA oxidation markers would be expected. However, in our study, MDA increased on the first day of CIH and diminished slightly during the next 3 days of exposure. The lack of a statistically significant increase in MDA on Days 2 and 4 compared with baseline values may reflect our small sample size. Indeed, power calculations reveal relatively high power (0.67 and 0.72 for Days 2 and 4, respectively) and a calculated sample size of 15 subjects to achieve a power of 80% and a P value of 0.05. Conversely, 8-OHdG increased on Day 1 and remained elevated for the duration of the CIH protocol. Finally, the increase in uric acid during the 4 days of CIH suggests that ROS are mainly produced during the reoxygenation process through the xanthine oxidase pathway. Because antioxidant enzyme activities and α-tocopherol remained at constant levels during the CIH, we propose that healthy subjects respond differently from patients with OSA who exhibit lower global antioxidant capacity compared with control subjects (18, 22). Although antioxidant enzymes have not been measured in studies of patients with OSA, the possibility of impaired enzymatic antioxidant systems in this patient population warrants further investigation. It is also possible that 4 days of CIH is not sufficiently long to induce adaptive antioxidant responses in humans, as reported after intermittent hypoxia preconditioning in rats cardiomyocytes (43). The reported increase in circulating vitamin C suggests an increased mobilization of ascorbic acid from tissues to blood to fight against the likely ROS overproduction. This is supported by the positive relationship between the changes in vitamin C and 8-OHdG between baseline and Day 4. Additionally, our results are consistent with studies reporting higher levels of oxidative stress in patients with OSA (18–24) and in animals exposed to intermittent hypoxia (44). By demonstrating that CIH increases oxidative stress in healthy humans, our data suggest that CIH may contribute to ROS overproduction in OSA.

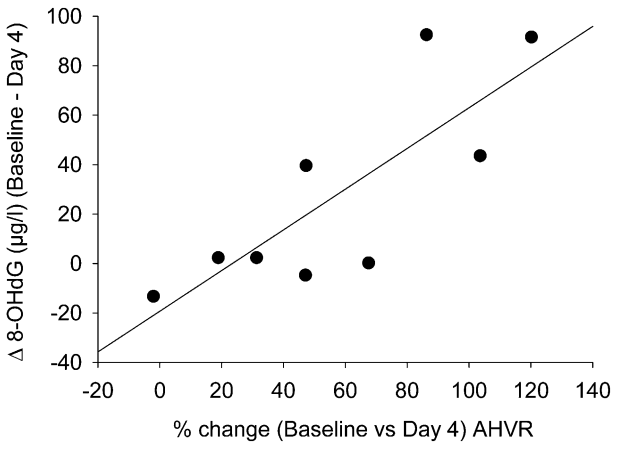

Figure 4. Relationship between the changes in the AHVR and DNA oxidation (8-OHdG) from baseline (mean of Day −4 and Day 0) to Day 4.

Effects of CIH on AHVR and AHCVR

The isocapnic AHVR was significantly increased after 4 days of CIH. After conclusion of the intermittent hypoxic protocol, AHVR returned to baseline within 4 days. Similar results have been found in other human studies (13, 45). Foster and colleagues (13) reported a 37% increase in AHVR in subjects exposed to short durations of intermittent hypoxia over 12 days (10 1-hour sessions of IH with 5 minutes at 12% of O_2 and 5 minutes in normoxia). By doubling the daily exposure to CIH, Koehle and colleagues (45) found a 68% increase in AHVR in 1 week.

The higher increase in AHVR observed in the present study (82 vs. 37 and 67% for the studies by Foster and colleagues [11] and Koehle and colleagues [43], respectively) suggests that the total time spent in hypoxia and the frequency of hypoxia/reoxygenation periods may alter the magnitude of the increase in AHVR. In this context, Tun and colleagues (4) reported a 25% decrease in AHVR after 2 weeks of continuous positive airway pressure (CPAP) therapy in a patient with severe OSA (AHI = 58). In a similar group of patients with OSA (AHI, 55), 1 month of CPAP decreased AHVR by 51%, whereas a single night of CPAP therapy did not affect the chemosensitivity to hypoxia (46).

Effect of Oxidative Stress Induced by CIH on AHVR

A growing body of evidence suggests that the increase in AHVR induced by CIH arises, at least partly, from activation

TABLE 3. MEAN VALUES AND COEFFICIENTS OF VARIATION FOR ACUTE HYPOXIC VENTILATORY RESPONSES, PLASMA MALONDIALDEHYDES, URIC ACID, 8-HYDROXY-2′-DEOXYGUANOSINE, AND TOTAL VITAMIN C DURING BASELINE (MEAN OF DAY −4, DAY 0) AND SHAM-TREATMENT GROUPS

	Sham-treatment Group		Experimental Group, Baseline (Day −4 and Day 0)		*P* Value,* Sham-treatment vs. Experimental Groups	
Variables	Mean	CV	Mean	CV	Mean	CV
AHVR, L/min (%)	1.18 ± 0.40	0.22 ± 0.06	0.95 ± 0.15	0.20 ± 0.04	0.51	0.79
MDA, μmol/L	5.86 ± 0.86	0.12 ± 0.09	3.59 ± 0.55	0.15 ± 0.14	0.71	0.71
Uric acid, mg/dl	5.68 ± 0.71	0.14 ± 0.08	5.06 ± 1.23	0.09 ± 0.07	0.65	0.33
8-OHdG, μg/L	79.2 ± 1.2	0.02 ± 0.02	62.2 ± 25.8	0.12 ± 0.12	0.51	0.11
Vitamin C, μmol/L	32.0 ± 2.8	0.11 ± 0.09	37.0 ± 5.6	0.10 ± 0.09	0.49	0.73

Definition of abbreviations: AHVR = acute hypoxic ventilatory responses; CV = coefficient of variation; MDA = malondialdehydes; 8-OHdG = 8-hydroxy-2′-deoxyguanosine.

Values are means ± SD for sham-treatment group (n = 4) and experimental group (n = 10).

* Unpaired *t* tests.

of oxidative stress and molecular signaling cascades. The strong relationships that were found between the changes in DNA oxidation markers and AHVR between baseline and Day 4 (Figure 4) strengthen the hypothesis that ROS is involved in the respiratory plasticity observed during CIH (2, 10). In support of this notion, the carotid body sensitization induced by CIH was suppressed in rats after 10 days of antioxidant treatment (17). Up-regulation of the HIF-1α gene by ROS during CIH also seems to play an important role in the sensitization of carotid bodies to hypoxia (14).

The effects of an increased ventilatory sensitivity to hypoxia in OSA are controversial. An augmented AHVR should help maintain oxygenation and thus reduce the deleterious effects of hypoxia/reoxygenation. However, some investigators (47) have suggested that increased ventilatory sensitivity to hypoxia promotes unstable breathing and thereby perpetuates OSA. Finally, sensory long-term facilitation (i.e., higher baseline sensitivity) of the carotid bodies, resulting in increased sympathetic activity, may contribute to the development of hypertension in patients with OSA (2) and likely contributes to the increase in resting MABP in the present study.

We have addressed whether factors such as cumulative stress associated with the protocol and/or habituation to the experimental procedures altered our outcome measurements of oxidative stress and ventilatory sensitivity to hypoxia. In an ancillary study, four healthy male volunteers were exposed to 6 hours of sham-intermittent hypoxia (i.e., room air) over four consecutive days. The sham-treatment protocol did not change the ventilatory (AHVR, $P = 0.51$) or oxidative stress (MDA, $P = 0.71$; uric acid, $P = 0.65$; 8-OHdG, $P = 0.51$; vitamin C, $P = 0.49$) parameters. Moreover, the mean values and the coefficients of variation for the sham-treatment group (see protocol description, online supplement) were not significantly different from the coefficients of variation calculated during the baseline period (mean of Day −4 and Day 0) for the original experimental group (Table 3). Thus, results from the sham-treatment group support our conclusion that the changes we found in the ventilatory and biochemical parameters are due to the IH stimulus and not to other aspects of the experimental protocol.

Perspectives and Limitations

Studies on animals suggest that CIH-induced increases in oxidative stress, HIF-1α expression (14), carotid body activity (48, 49), and ventilation (17) are prevented by antioxidant treatment with a potent superoxide scavenger. Although the causal relationship between oxidative pathways and perpetuation of apnea in patients with OSA remains to be completely elucidated, the available data suggest that reductions in oxidative stress via antioxidant medications (50) and supplementation (51) may offer potential therapeutic benefits in patients with OSA. Future studies should investigate whether antioxidant supplementation modulates AHVR in an experimental human model of CIH and in patients with OSA.

Although our model provides the opportunity to improve our understanding of the interaction between OSA, oxidative stress, and the control of breathing, it has limitations in this regard. First, intermittent hypoxia was induced by alteration of the environmental gas mixtures rather than by recurrent apnea. The associated reduction in arterial P_{CO_2} is different from what happens during an obstructive apnea when arterial P_{CO_2} typically rises. Furthermore, intrathoracic pressure changes considerably during obstructive apnea, compared with the minor changes in our model. Hypercapnia and negative intrathoracic pressure increase sympathetic activation (52) and reduce vagal activity (32). Second, the study was performed in awake subjects, but OSA occurs during sleep. The arousals resulting from apnea and associated sleep fragmentation (44) are also known to increase sympathetic activation. However, our model was designed to study the direct effects of intermittent hypoxia *per se* in the absence of potential confounding parameters such as arousals and sleep fragmentation. Future studies using an experimental human model of CIH should be conducted during sleep to address these issues.

Conclusion

This study is the first to demonstrate that exposure of healthy humans to CIH increases oxidative stress by overproduction of ROS. Our findings corroborate previous animal studies suggesting that overproduction of ROS leads to increased ventilatory sensitivity to hypoxia. Future studies should determine the importance of this physiologic phenomenon in patients with OSA and whether it can be altered by antioxidant therapy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank the subjects for their participation in this study.

References

1. Somers VK, White DP, Amin R, Abraham WT, Costa F, Culebras A, Daniels S, Floras JS, Hunt CE, Olson LJ, *et al.* Sleep apnea and cardiovascular disease. An American Heart Association/American College of Cardiology Foundation Scientific Statement from the American Heart Association Council for High Blood Pressure Research Professional Education Committee, Council on Clinical Cardiology, Stroke Council, and Council on Cardiovascular Nursing Council. *Circulation* 2008;118:1080–1111.

2. Prabhakar NR, Peng YJ, Kumar GK, Pawar A. Altered carotid body function by intermittent hypoxia in neonates and adults: relevance to recurrent apneas. *Respir Physiol Neurobiol* 2007;157:148–153.
3. Punjabi NM, Newman AB, Young TB, Resnick HE, Sanders MH. Sleep-disordered breathing and cardiovascular disease: an outcome-based definition of hypopneas. *Am J Respir Crit Care Med* 2008;177: 1150–1155.
4. Tun Y, Hida W, Okabe S, Kikuchi Y, Kurosawa H, Tabata M, Shirato K. Effects of nasal continuous positive airway pressure on awake ventilatory responses to hypoxia and hypercapnia in patients with obstructive sleep apnea. *Tohoku J Exp Med* 2000;190:157–168.
5. Kimoff RJ, Brooks D, Horner RL, Kozar LF, Render-Teixeira CL, Champagne V, Mayer P, Phillipson EA. Ventilatory and arousal responses to hypoxia and hypercapnia in a canine model of obstructive sleep apnea. *Am J Respir Crit Care Med* 1997;156:886–894.
6. Edge D, Skelly JR, Bradford A, O'Halloran KD. Ventilatory drive is enhanced in male and female rats following chronic intermittent hypoxia. *Adv Exp Med Biol* 2009;648:337–344.
7. Katayama K, Smith CA, Henderson KS, Dempsey JA. Chronic intermittent hypoxia increases the CO_2 reserve in sleeping dogs. *J Appl Physiol* 2007;103:1942–1949.
8. Narkiewicz K, Somers VK. Sympathetic nerve activity in obstructive sleep apnoea. *Acta Physiol Scand* 2003;177:385–390.
9. Foster GE, Hanly PJ, Ostrowski M, Poulin MJ. Ventilatory and blood pressure responses to isocapnic hypoxia in OSA patients. *Adv Exp Med Biol* 2008;605:463–468.
10. Macfarlane PM, Wilkerson JE, Lovett-Barr MR, Mitchell GS. Reactive oxygen species and respiratory plasticity following intermittent hypoxia. *Respir Physiol Neurobiol* 2008;164:263–271.
11. Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ, Olson EB Jr. Invited review: intermittent hypoxia and respiratory plasticity. *J Appl Physiol* 2001;90:2466–2475.
12. Katayama K, Sato K, Matsuo H, Hotta N, Sun ZH, Ishida K, Iwasaki K, Miyamura M. Changes in ventilatory responses to hypercapnia and hypoxia after intermittent hypoxia in humans. *Respir Physiol Neurobiol* 2005;146:55–65.
13. Foster GE, McKenzie DC, Milsom WK, Sheel AW. Effects of two protocols of intermittent hypoxia on human ventilatory, cardiovascular and cerebral responses to hypoxia. *J Physiol* 2005;567:689–699.
14. Peng YJ, Yuan G, Ramakrishnan D, Sharma SD, Bosch-Marce M, Kumar GK, Semenza GL, Prabhakar NR. Heterozygous HIF-1alpha deficiency impairs carotid body-mediated systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia. *J Physiol* 2006;577:705–716.
15. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am J Physiol Cell Physiol* 2002;282:C227–C241.
16. Zhan G, Serrano F, Fenik P, Hsu R, Kong L, Pratico D, Klann E, Veasey SC. NADPH oxidase mediates hypersomnolence and brain oxidative injury in a murine model of sleep apnea. *Am J Respir Crit Care Med* 2005;172:921–929.
17. Peng YJ, Overholt JL, Kline D, Kumar GK, Prabhakar NR. Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. *Proc Natl Acad Sci USA* 2003;100:10073–10078.
18. Barcelo A, Barbe F, de la Pena M, Vila M, Perez G, Pierola J, Duran J, Agusti AG. Antioxidant status in patients with sleep apnoea and impact of continuous positive airway pressure treatment. *Eur Respir J* 2006;27:756–760.
19. Dyugovskaya L, Lavie P, Lavie L. Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* 2002;165:934–939.
20. Yamauchi M, Nakano H, Maekawa J, Okamoto Y, Ohnishi Y, Suzuki T, Kimura H. Oxidative stress in obstructive sleep apnea. *Chest* 2005; 127:1674–1679.
21. Carpagnano GE, Kharitonov SA, Resta O, Foschino-Barbaro MP, Gramiccioni E, Barnes PJ. 8-Isoprostane, a marker of oxidative stress, is increased in exhaled breath condensate of patients with obstructive sleep apnea after night and is reduced by continuous positive airway pressure therapy. *Chest* 2003;124:1386–1392.
22. Christou K, Markoulis N, Moulas AN, Pastaka C, Gourgoulianis KI. Reactive oxygen metabolites (ROMs) as an index of oxidative stress in obstructive sleep apnea patients. *Sleep Breath* 2003;7:105–110.
23. Lavie L, Vishnevsky A, Lavie P. Evidence for lipid peroxidation in obstructive sleep apnea. *Sleep* 2004;27:123–128.
24. Barcelo A, Miralles C, Barbe F, Vila M, Pons S, Agusti AG. Abnormal lipid peroxidation in patients with sleep apnoea. *Eur Respir J* 2000;16:644–647.
25. Alzoghaibi MA, Bahammam AS. Lipid peroxides, superoxide dismutase and circulating IL-8 and GCP-2 in patients with severe obstructive sleep apnea: a pilot study. *Sleep Breath* 2005;9:119–126.
26. Svatikova A, Wolk R, Lerman LO, Juncos LA, Greene EL, McConnell JP, Sommers VK. Oxidative stress in obstructive sleep apnoea. *Eur Heart J* 2005;26:2435–2439.
27. Ozturk L, Mansour B, Yuksel M, Yalcin AS, Celikoglu F, Gokhan N. Lipid peroxidation and osmotic fragility of red blood cells in sleep-apnea patients. *Clin Chim Acta* 2003;332:83–88.
28. Wali SO, Bahammam AS, Massaeli H, Pierce GN, Iliskovic N, Singal PK, Kryger MH. Susceptibility of LDL to oxidative stress in obstructive sleep apnea. *Sleep* 1998;21:290–296.
29. Sharma SK, Kumpawat S, Goel A, Banga A, Ramakrishnan L, Chaturvedi P. Obesity, and not obstructive sleep apnea, is responsible for metabolic abnormalities in a cohort with sleep-disordered breathing. *Sleep Med* 2007;8:12–17.
30. Ip MS, Lam B, Ng MM, Lam WK, Tsang KW, Lam KS. Obstructive sleep apnea is independently associated with insulin resistance. *Am J Respir Crit Care Med* 2002;165:670–676.
31. Hanly P. Sleep apnea and daytime sleepiness in end-stage renal disease. *Semin Dial* 2004;17:109–114.
32. D'Almeida V, Lobo LL, Hipolide DC, de Oliveira AC, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. *Neuroreport* 1998;9:2853–2856.
33. Pialoux V, Foster GE, Brugniaux JV, Duggan TC, Hanly PJ, Poulin MJ. Effect of 4 days of intermittent hypoxia on oxidative stress in healthy men [Abstract]. *FASEB J* 2008;22:960.3.
34. Castelao JE, Gago-Dominguez M. Risk factors for cardiovascular disease in women: relationship to lipid peroxidation and oxidative stress. *Med Hypotheses* 2008;71:39–44.
35. Kolb JC, Ainslie PN, Ide K, Poulin MJ. Protocol to measure acute cerebrovascular and ventilatory responses to isocapnic hypoxia in humans. *Respir Physiol Neurobiol* 2004;141:191–199.
36. Loft S, Moller P, Cooke MS, Rozalski R, Olinski R. Antioxidant vitamins and cancer risk: is oxidative damage to DNA a relevant biomarker? *Eur J Nutr* 2008;47:19–28.
37. Lefevre G, Beljean-Leymarie M, Beyerle F, Bonnefont-Rousselot D, Cristol JP, Therond P, Torreilles J. [Evaluation of lipid peroxidation by measuring thiobarbituric acid reactive substances.] *Ann Biol Clin (Paris)* 1998;56:305–319.
38. Cherubini A, Ruggiero C, Polidori MC, Mecocci P. Potential markers of oxidative stress in stroke. *Free Radic Biol Med* 2005;39:841–852.
39. Prabhakar NR, Kumar GK, Nanduri J, Semenza GL. ROS signaling in systemic and cellular responses to chronic intermittent hypoxia. *Antioxid Redox Signal* 2007;9:1397–1403.
40. Xu W, Chi L, Row BW, Xu R, Ke Y, Xu B, Luo C, Kheirandish L, Gozal D, Liu R. Increased oxidative stress is associated with chronic intermittent hypoxia-mediated brain cortical neuronal cell apoptosis in a mouse model of sleep apnea. *Neuroscience* 2004;126:313–323.
41. Yuan G, Adhikary G, McCormick AA, Holcroft JJ, Kumar GK, Prabhakar NR. Role of oxidative stress in intermittent hypoxia-induced immediate early gene activation in rat PC12 cells. *J Physiol* 2004;557:773–783.
42. Abramov AY, Scorziello A, Duchen MR. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J Neurosci* 2007;27: 1129–1138.
43. Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca2+ overload. *Am J Physiol Heart Circ Physiol* 2005;288:H1900–H1908.
44. Yamauchi M, Kimura H. Oxidative stress in obstructive sleep apnea: putative pathways to the cardiovascular complications. *Antioxid Redox Signal* 2008;10:755–768.
45. Koehle MS, Sheel AW, Milsom WK, McKenzie DC. Two patterns of daily hypoxic exposure and their effects on measures of chemosensitivity in humans. *J Appl Physiol* 2007;103:1973–1978.
46. Spicuzza L, Bernardi L, Balsamo R, Ciancio N, Polosa R, Di MG. Effect of treatment with nasal continuous positive airway pressure on ventilatory response to hypoxia and hypercapnia in patients with sleep apnea syndrome. *Chest* 2006;130:774–779.
47. Younes M. Role of respiratory control mechanisms in the pathogenesis of obstructive sleep disorders. *J Appl Physiol* 2008;105:1389–1405.
48. Peng YJ, Prabhakar NR. Effect of two paradigms of chronic intermittent hypoxia on carotid body sensory activity. *J Appl Physiol* 2004;96:1236–1242.

49. Mitchell GS, Johnson SM. Neuroplasticity in respiratory motor control. *J Appl Physiol* 2003;94:358–374.
50. El Solh AA, Saliba R, Bosinski T, Grant BJ, Berbary E, Miller N. Allopurinol improves endothelial function in sleep apnoea: a randomised controlled study. *Eur Respir J* 2006;27:997–1002.
51. Grebe M, Eisele HJ, Weissmann N, Schaefer C, Tillmanns H, Seeger W, Schulz R. Antioxidant vitamin C improves endothelial function in obstructive sleep apnea. *Am J Respir Crit Care Med* 2006;173:897–901.
52. Kara T, Narkiewicz K, Somers VK. Chemoreflexes–physiology and clinical implications. *Acta Physiol Scand* 2003;177:377–384.