Circulating Nitric Oxide Is Suppressed in Obstructive Sleep Apnea and Is Reversed by Nasal Continuous Positive Airway Pressure

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Epidemiological studies have implicated obstructive sleep apnea (OSA) as an independent comorbid factor in cardiovascular and cerebrovascular diseases. The recurrent episodes of occlusion of upper airways during sleep result in pathophysiological changes that may predispose to vascular diseases, and we postulate that nitric oxide may be one of the mediators involved. This study investigates the levels of circulating nitric oxide (NO), measured as serum nitrites and nitrates, in the early morning in OSA subjects compared with control subjects, and the effect of overnight nasal continuous positive airway pressure (nCPAP) in OSA subjects. Thirty men with moderate to severe OSA (age = 41.9 ± 9.0; apnea-hypopnea index, AHI = 48.0 ± 18.1) were compared with 40 healthy men (age = 40.6 ± 5.4; AHI = 1.4 ± 1.2). Serum nitrite/nitrate levels were significantly lower in OSA subjects (OSA = 38.9 ± 22.9 μM, control subjects = 63.1 ± 47.5 μM, p = 0.015). There was significant negative correlation between serum nitrites/nitrates and the following parameters: AHI (r = −0.389, p = 0.001), oxygen desaturation time (r = −0.346, p = 0.004), and systolic blood pressure (BP) (r = −0.335, p = 0.005). Stepwise multiple linear regression with systolic or diastolic BP as the dependent variable identified serum nitrites/nitrates as the only significant correlate. Twenty-two OSA subjects had measurements of serum NO at baseline and after an overnight application nCPAP. There was significant increase in serum NO after nCPAP (baseline = 30.5 ± 14.4 μM, after nCPAP = 81.0 ± 82.1 μM, p = 0.01). This study demonstrates, for the first time, that circulating NO is suppressed in OSA, and this is promptly reversible with the use of nCPAP. The findings offer support for nitric oxide being one of the mediators involved in the acute hemodynamic regulation and long-term vascular remodeling in OSA.

Nitric oxide (NO) is an almost ubiquitous signaling molecule in the human body, and it is involved in numerous biological functions, including regulation of vascular tone, inflammation, and neurotransmission (1–3). NO is the most potent vascular relaxing factor known in the human body, and it is formed constitutively by endothelial NO synthase (eNOS) together with the substrate L-arginine, cosubstrate nicotinamide adenine dinucleotide phosphate (NADPH), and other cofactors (4). eNOS and NO play a key role in regulation of vascular tone, and their disturbance is postulated to be essential in the development of atherosclerosis associated with various conditions such as hypertension, hypercholesterolemia, and diabetes mellitus (5–8).

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Obstructive sleep apnea (OSA) is known to be associated with increased prevalence of cardiovascular and cerebrovascular morbidity (9–14). It has been well documented that there are surges of arterial blood pressure at the end of each obstructive episode (15), and there is now ample evidence to show that OSA may be an independent causative risk factor for systemic hypertension (13, 14). Many mechanisms have been postulated to be involved in the regulation of blood pressure both acutely at the time of sleep-disordered breathing and in the long term in OSA (16–18). Because the expression of eNOS and subsequent endothelial NO release may be affected by some of the prevailing conditions, notably hypoxemia, hypoxia–reoxygenation, shear stress, and ischemia–reperfusion of the vascular wall (16, 18–22), we hypothesize that endothelial NO may play an important role in the regulation of blood pressure in OSA. In this study, we measured and analyzed the total levels of serum nitrites plus nitrates as metabolites of NO in subjects with OSA and their relationship with sleep apneic activity and blood pressure.

METHODS

Subjects

All subjects were recruited from the Sleep Laboratory at the University Department of Medicine, Queen Mary Hospital. They had been referred for sleep studies either as part of a community-based study for prevalence of sleep apnea (23) or for clinically suspected sleep apnea. Inclusion criteria for the present study were apnea hypopnea index (AHI) > 20 for OSA subjects and AHI < 5 for control subjects. Presence of other diseases and the use of medications were documented. Anthropometric measurements of body mass index (BMI), neck, waist, and hip circumference were obtained. Fasting venous blood was obtained and blood pressure (BP) was measured the next morning after polysomnogram (PSG) recording.

Subjects who had a diagnosis of OSA with AHI > 20 based on PSG were readmitted for nasal continuous positive airway pressure (nCPAP) application for one night within 1 wk of the diagnostic (baseline) PSG. They have not been started on nocturnal nCPAP at home during this week. Fasting venous blood was obtained and blood pressure was measured the next morning after nCPAP.

For the baseline comparison of OSA and control subjects, all those with known hypertension on medications were excluded. For the comparison of OSA subjects at baseline and after nCPAP application, subjects with known hypertension were not excluded as long as the medications were constant in the 1-wk interval between the two tests.

Informed consent for blood taking was obtained from all subjects. The protocol was approved by the Institutional Ethics Committee.

Polysomnogram

All subjects underwent overnight polysomnogram (Alice 3 Diagnostics System; Healthdyne, Atlanta, GA) with documentation of sleep stages by electroencephalogram, respiratory movement by impedance plethysmography, airflow by thermistors, arterial oxygen saturation by pulse oximetry, snoring by tracheal microphone, and sleep position by position sensor. All polysomnograms were manually scored according to standard criteria (24). Apnea was defined as an absence of

TABLE 1

CLINICAL FEATURES OF OSA SUBJECTS AND CONTROL SUBJECTS

	All Subjects			BMI-Matched Subjects		
	OSA	Control	p Value	OSA	Control	p Value
No. of subjects	30	40		18	29	
Age, yr	42 ± 9	41 ± 5	NS	42 ± 10	41 ± 5	NS
Body mass index	30.2 ± 4.6	24.7 ± 2.5	< 0.001	27.4 ± 3.1	25.7 ± 1.9	NS
Systolic BP, mm Hg	130 ± 14	127 ± 13	NS	129 ± 15	126 ± 14	NS
Diastolic BP, mm Hg	80 ± 9	76 ± 11	NS	79 ± 7	76 ± 11	NS
Apnea–hypopnea index	48.0 ± 18.1	1.4 ± 1.2	< 0.001	47.6 ± 16.7	1.6 ± 1.3	< 0.001
O_2 saturation < 90%, min	114.7 ± 92.9	1.8 ± 4.0	< 0.001	95.3 ± 79.6	2.0 ± 4.6	< 0.001

Definition of abbreviations: BMI = body mass index; NS = not significant; OSA = obstructive sleep apnea.

airflow for > 10 s, and hypopnea was defined as a reduction of airflow associated with a reduction of oxygen saturation by 4% from baseline. The apnea–hypopnea index (AHI) was defined as the average number of apneic and hypopneic events per sleep hour.

Subjects with OSA were admitted to the sleep laboratory within 1 wk of the baseline study for overnight nCPAP therapy, which was delivered by application of the Sullivan Autoset Flow Generator II (Resmed, Sydney, Australia).

Blood Sample Collection and Measurement of NO Concentrations in Serum of Patients

After overnight polysomnogram (PSG) or nCPAP treatment, fasting blood was collected by venesection into EDTA vials and centrifuged at 1,000 *g* for 15 min. After centrifugation, serum was aliquoted and stored at −70° C until batch analysis.

Measurement of serum concentrations of total nitrites and nitrates was performed by one technician blinded to patient status of the serum samples, according to previously described methodology (25), using an NO analyzer (NOA) (Sievers 280, USA). Because there is no evidence that the blood should contain any significant amounts of nitrites and nitrates other than that contributed by NO, we can assume that the NO concentration deduced from the chemiluminescence experiment does represent the true NO concentration in the blood. The procedure is described briefly as follows. Serum samples were deproteinized by zinc sulfate and the supernatants after deproteinization were collected for further analysis (25). Assay of serum nitrites and nitrates was then carried out according to the NOA manufacturer's instructions. Vanadium (III) chloride was used as the reducing agent in the system. A sodium nitrate (100 mM) solution ($NaNO_3$) was prepared and diluted to various concentrations for the calibration test. Ten microliters of a standard concentration of $NaNO_3$ was injected into the Radical Purger, which was linked to the NOA, to obtain the calibration curve and the peak area for each standard concentration was measured. Deproteinized serum samples were then injected and the NO concentrations measured after correction for background noise.

Statistical Analysis

For comparison between OSA and control subjects, the Mann–Whitney U test was used; for comparison of pre- and post-CPAP samples of OSA subjects, the Wilcoxon rank sum test was used. To evaluate the relationship between baseline serum nitrite/nitrate levels and anthropometric indices, sleep apneic activity, and blood pressure, and between the changes in serum nitrite/nitrate levels and changes in sleep apneic activity and blood pressure after nCPAP, Spearman's rank correlation test was applied. To further evaluate the relationship of blood pressure and serum nitrites/nitrates, stepwise multiple linear regression was performed with systolic blood pressure (SBP) and diastolic blood pressure (DBP) as the dependent variables, respectively. Parameters included in the analysis were serum nitrite/nitrates, AHI (categorized as < 5 and ⩾ 5), time with oxygen saturation < 90%, arousal index, BMI, and waist:hip ratio. All values were expressed as mean ± SD and two-sided p values of < 0.05 were considered to be significant. Data processing was performed with the SPSS program (26).

RESULTS

Thirty subjects with OSA and 40 healthy control subjects fulfilling set criteria were included in the analysis. They were all men and their clinical data are shown in Table 1.

The serum levels of total nitrite/nitrate levels are significantly different between OSA and control subjects (OSA subjects 38.9 ± 22.9 μM, control subjects 63.1 ± 47.5 μM, p = 0.015) (Figure 1a). There was significant negative correlation between nitrite/nitrate levels and indices of sleep apnea, and systolic blood pressure (Table 2), but no correlation between

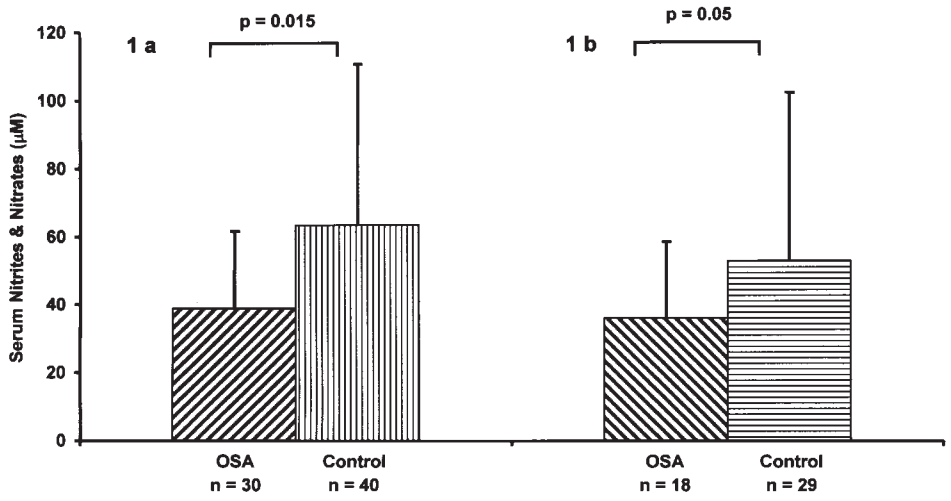

Figure 1. (*a*) Serum nitrite/nitrate levels in OSA subjects (n = 30) and control subjects (n = 40). (*b*) Serum nitrite/nitrate levels in BMI-matched OSA subjects (n = 18) and control subjects (n = 29).

TABLE 2

SIGNIFICANT CORRELATION OF SERUM NITRITE/ NITRATE LEVELS WITH OTHER PARAMETERS

	For All Subjects ($n = 70$)		For BMI-Matched Subjects ($n = 47$)	
	r	p Value	r	p Value
Apnea–hypopnea index	−0.389	0.001	−0.420	0.003
Time with $Sa_{O_2} < 90\%$	−0.346	0.004	−0.406	0.005
Systolic BP	−0.335	0.005	−0.334	0.022

Definition of abbreviations: BMI = body mass index; BP = blood pressure.

nitrite/nitrate levels and any anthropometric indices. Stepwise multiple linear regression analysis identified nitrite/nitrate levels as the only significant correlate of both systolic and diastolic blood pressure (Table 3).

To exclude any potential confounding effect of body mass on circulating NO levels, statistical analysis was repeated after exclusion of the heaviest subjects in the OSA group and the lightest subjects in the control group until the mean BMI of the two groups was similar with the maximal preservation of sample size (OSA 18, control 29). Their clinical data are shown in Table 1 and their serum nitrite/nitrate levels are shown in Figure 1b. The absolute values of most parameters except BMI were very similar to the previous data set, although the difference in serum nitrite/nitrate levels was of lesser significance due to the decrease in sample size (Figure 1b). Correlation analysis was performed as for the original data set, and the results were similar (Table 2).

Of the 30 OSA subjects in the baseline study, 19 had serum nitrite/nitrate levels measured after overnight use of nCPAP. The other 11 were excluded from post-nCPAP analysis because overnight nCPAP was not delivered within 1 wk of baseline study, lack of a second blood sample due to patient refusal or technical problems, or intolerance to nCPAP. Another three OSA subjects on antihypertensive medications (who were not included in the baseline comparison) were included in this pre- and post-nCPAP analysis. All three were taking angiotensin-converting enzyme inhibitor and beta-blockers, and one was also taking calcium antagonist and indapamide. Their medications were kept constant in the interval (less than 1 wk) between baseline and post-nCPAP measurements. These 22 subjects had moderate to severe OSA (age = 42.2 ± 8.6 yr, AHI = 52.9 ± 17.8). They received overnight nCPAP for 8.6 ± 1.1 h and the 95th percentile pressure was 11.9 ± 2.1 cm H_2O. The AHI recorded on the Autoset machine was 8.6 ± 6.4 (apneas and hypopneas were defined by airflow limitation and not by the previously set criteria for PSG). There was a significant increase in serum nitrite/nitrate after nCPAP (baseline 30.5 ± 14.4 μM, after nCPAP = 81.0 ± 82.1 μM, $p = 0.01$) (Figure 2a). The nitrite/nitrate levels after nCPAP was not significantly different from that of the 40 control subjects. There was no significant correlation between the change in serum nitrite/nitrate levels and the baseline AHI or desaturation time (which is taken to reflect the change in sleep apneic activity assuming that auto-nCPAP could abolish most of the obstructive apneas and hypopneas) or the change in morning blood pressure (baseline: SBP 134 ± 12, DBP 83 ± 9; after nCPAP: SBP 130 ± 17, DBP 74 ± 12; mean ± SD in mm Hg). When the three subjects on antihypertensive medications were excluded, all analysis results were very similar (Figure 2b).

DISCUSSION

The increased association with vascular morbidity, including hypertension, myocardial infarction, and stroke, is a major concern in OSA (8–14). Epidemiological studies suggest that OSA may independently contribute to these diseases, therefore attention has been drawn to the potential vascular pathogenetic mechanisms caused by OSA.

Patients with OSA demonstrate both acute and chronic hemodynamic changes attributable to the sleep-disordered breathing (15, 17, 27–29). The recurrent obstructive events have been shown to lead to repetitive oscillations in arterial blood pressure, heart rate, stroke volume, and consequently cardiac output. There are several mechanisms by which obstructive apnea can lead to these acute hemodyanmic changes. The possible initiating culprits would include hypoxemia, hypoxia–reoxygenation, pronounced intrathoracic pressure swings and cerebral excitation, which may all lead to metabolic and autonomic nervous activation and ultimately hemodynamic changes (17, 18). Apart from the acute hemodynamic effects, the physiological disturbances may also predispose to atherosclerosis (16, 18), a common pathogenic factor to the vascular diseases known to be associated with OSA. Many of these mechanisms may involve NO as a mediator (16, 18–22). Notably, hypoxia may affect the vasomotor tone by direct action or via tissue-derived or blood vessel-derived vasoactive substances, including adenosine, prostanoids, endothelin, and nitric oxide (19, 20).

Nitric oxide was initially called the endothelium-derived relaxing factor (EDRF) due to its potent relaxing action on blood vessels. In addition to its potent effect on regulation of basal vasomotor tone and hence systemic blood pressure in healthy subjects, nitric oxide may also play an important role in vascular homeostasis through its ability to inhibit proinflammatory events such as platelet activation and aggregation (30, 31) and leukocyte adhesion (32), or regulation of endothelial macrophage-mediated low-density lipoprotein oxidation (18), all of which may contribute to atherosclerosis (33, 34). Prolonged hypobaric hypoxia has been shown to induce systemic hypertension, which could be mitigated by L-arginine supplementation (20). Attenuation of endothelium-dependent vascular relaxation, a process mediated by NO (5), has been demonstrated in OSA (35, 36), supporting a role of NO in the regulation of vascular resistance in OSA.

In a recent report (37), serum nitrites and nitrates, measured by both high-performance liquid chromatography analy-

TABLE 3

STEPWISE MULTIPLE LINEAR REGRESSION MODELS FOR BLOOD PRESSURE*

	Systolic BP Model			Diastolic BP Model		
Independent Variable	β	SE (β)	p Value	β	SE (β)	p Value
Constant	4.893	0.019	< 0.001	4.412	0.025	< 0.001
Nitrite and nitrate	-9.13×10^{-4}	0.000	0.002	-1.31×10^{-3}	0.000	0.001

Definition of abbreviations: BP = blood pressure; OSA = obstructive sleep apnea.
* Number of subjects: OSA = 30, control = 40.

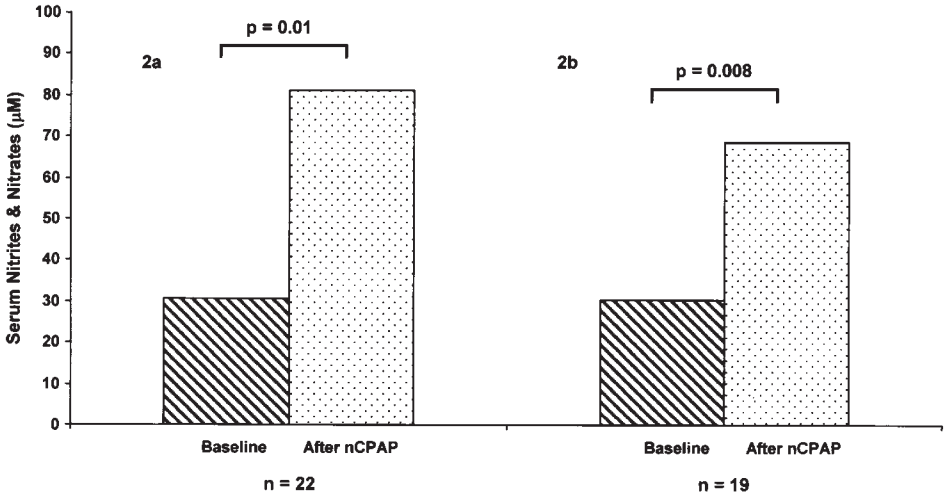

Figure 2. (*a*) Serum nitrite/nitrate levels in OSA subjects at baseline and after nCPAP (n = 22). (*b*) Serum nitrite/nitrate levels in OSA subjects, excluding three subjects on antihypertensive mediations, at baseline and after nCPAP (n = 19).

sis and ultraviolet detection, of blood samples taken from the deep antecubital vein of human subjects were compared and analyzed with results representing changes of forearm blood flow via endothelium-dependent and endothelium-independent mechanisms, with intravenous injections of eNOS inhibitor (NMMA) and NO biosynthesis substrate (L-arginine). The relevant analysis indicated that serum nitrite concentrations reflected sensitively changes in endothelial NO formation in forearm circulation, whereas nitrate concentration remained about constant, and the total NO concentration was reflected by the sum of nitrite and nitrate concentrations. Thus the nitrite concentration in blood taken from the forearm can be taken as a marker of NO concentration generated. For subjects not suffering from major inflammation in internal organs, nitrite/nitrate concentration in the circulation measures NO generated mainly by eNOS, rather than iNOS, from the blood vessels. In fact, it has been recognized that at baseline situation, NO is formed continuously by eNOS (2). With the previous background analysis, we postulate that nitric oxide may play a role in vascular homeostasis in obstructive sleep apnea. We therefore measured serum nitrite and nitrate levels in subjects with and without OSA, and also similar levels before and after nCPAP treatment, which would remove the recurrent apneic events. Because it is likely that any effect of sleep apnea would be greatest in the early morning, just after a night of repetitive apneic events, a fasting blood sample in the morning on waking up was taken for measurement. We recruited only subjects with moderately elevated AHI (> 20) as there has been no previously published literature on this aspect to guide us on the magnitude of the changes, if any, in serum NO, and choosing those with moderately severe OSA would help to avoid missing small changes caused by sleep apnea.

We demonstrated that early morning circulating NO levels, reflected by total nitrite/nitrate levels in serum, are lower in subjects with OSA compared with control subjects without OSA. The negative correlation between NO levels and sleep apneic activity indicated by apnea–hypopnea indices and duration of sleep time with hypoxemia suggests that the more severe the sleep apnea, the greater the NO suppression. The negative correlation of serum NO with systolic blood pressure suggests that NO may be involved in the regulation of blood pressure in these subjects.

There are no previous data on the effect of body mass on circulating NO levels, although there were reports of endothelial dysfunction in obesity (38) and endothelium-dependent vasodilation is known to be NO dependent (5). To adjust for any potential confounding effect of obesity on circulating NO levels, we further analyzed the data after matching for BMI in the two groups of subjects, and serum nitrites/nitrates levels remained negatively correlated to AHI, desaturation time, and systolic blood pressure. Moreover, stepwise multiple linear regression analysis for blood pressure in all 70 subjects indicated that serum nitrates/nitrates were the only parameter significantly related to blood pressure, in the presence of BMI and waist–hip ratio as confounding variables. Results of these two analyses would suggest that serum nitrites/nitrates are related to OSA and blood pressure independent of obesity.

In this study, we delivered overnight nCPAP in the sleep laboratory to the OSA subjects using the Autoset System (Resmed). This device operates on the principles of automatic adjustment of CPAP in response to airflow limitation of the subject as measured by a pneumotachograph located between the mask and the exhaust port. The device has been shown to be capable of abolishing sleep apneic–hypopneic episodes and arousals with efficacy equivalent to the application of a constant predetermined pressure (39). This device generates data on apneas and hypopneas defined by criteria of flow limitation rather than respiratory effort and oximetry. The use of overnight nCPAP to abolish sleep apnea resulted in an increase of the circulating serum NO levels to levels that were comparable with control subjects. This would suggest that whatever caused the suppression of NO synthesis or release, this effect was acute and promptly reversible with reversal of sleep apnea despite a long history of disease that was almost universally seen in subjects presenting for evaluation. However, reduced NO levels every night over a long period of time may have caused other vascular modification that may not be as reversible, such as increased atherosclerosis through decreased inhibition of platelet adhesion and leukocyte adhesion or other processes of vascular wall remodeling. This postulate is highly tenable in view of the lack of consistent effect of nCPAP on established systemic hypertension in subjects with OSA (40), and the lack of correlation between changes in early morning blood pressure and the changes in NO levels after overnight nCPAP in this group of subjects.

We postulate that circulating nitrites/nitrates reflect NO of mainly endothelial origin, based on the argument previously presented, but we cannot exclude other sources of variation of serum NO. It is well established that cerebral excitation and elevation of intracranial pressure occur in OSA (41), and this may lead to changes in neuronal NO (nNO) synthesis, which

theoretically may be represented as circulating NO, and these changes are also subject to modification by nCPAP treatment, which also decreases the cerebral arousals. So far, however, we are not aware of significant evidence to relate nNOS and OSA. Another potential source of variation of circulating NO is the production from circulating neutrophils (42). There is preliminary evidence to suggest that neutrophils may be activated in OSA (43), but under such circumstances, NO production should be increased rather than suppressed. Levels of exhaled NO have also been reported to be elevated in OSA (44), and this is not unexpected as the paranasal sinuses are the major sources of exhaled NO and any inflammation of the upper airways induced by the repeated airflow turbulence in sleep apnea would cause generation of NO at the luminal surface, and be brought out by respiration and measured as exhaled NO. This increased production would have masked any suppressed production of NO by airway epithelial or endothelial cells from recurrent hypoxia and hypoxemia. Hence, we believe that suppression of systemic vascular NO production generated by eNOS is the major source of the changes of serum nitrite/nitrate levels detected in these subjects.

A previous study reported that acute hypoxia produced systemic vasodilation mediated by an increased production of endothelial NO (19), which apparently contradict our finding of decreased circulating NO in OSA in which hypoxia is one obvious pathogenetic phenomenon. However, in moderate to severe sleep apnea, hypoxia is recurrent throughout the night, and prolonged hypoxia has been shown to result in hypertension associated with depressed NO production in rats (20) and decreased NO release in cultured endothelial cells *in vitro* (47). Furthermore, in moderate to severe sleep apnea, apart from hypoxia, there are other pathophysiological changes. Notably, the arterial vessel wall is subjected to increased shear stress and together with the cyclical nature of apneic–hypopneic episodes resulting in recurrent hypoxia–reoxygenation and ischemia–reperfusion of the vascular endothelium, there may be generation of superoxides, free radicals, and other changes in the endothelial mileau (18), all of which may result in decreased eNO release or activity (45, 46).

In summary, our findings demonstrate that circulating NO levels may be suppressed by obstructive sleep apneic events, and suggest that NO is one of the mediators involved in the regulation of vascular homeostasis in obstructive sleep apnea. This novel finding is a strong documentation of the potential adverse vascular sequelae caused by sleep apnea and further basic and clinical research into the cascade of hemodynamic and vascular events along this line would be indicated.

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