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## Alveolar-Derived Exhaled Nitric Oxide Is Reduced in Obstructive Sleep Apnea Syndrome\*

Antonio Foresi, MD, FCCP; Clementina Leone, PhD; Dario Olivieri, MD, FCCP; and George Cremona, MD

**Background:** Obstructive sleep apnea syndrome (OSAS) is associated with cardiovascular diseases, in particular systemic arterial hypertension. We postulated that intermittent nocturnal hypoxia in OSAS may be associated to decreased fractional exhaled nitric oxide (FENO) levels from distal airspaces.

**Methods:** Multiple flow rate measurements have been used to fractionate nitric oxide (NO) from alveolar and bronchial sources in 34 patients with OSAS, in 29 healthy control subjects, and in 8 hypertensive non-OSAS patients. The effect of 2 days of treatment with nasal continuous positive airway pressure (nCPAP) on FENO was examined in 18 patients with severe OSAS.

**Results:** We found that the mean [ $\pm$  SE] concentrations of exhaled NO at a rate of 50 mL/s was  $21.8 \pm 1.9$  parts per billion (ppb) in patients with OSAS,  $25.1 \pm 3.3$  ppb in healthy control subjects, and  $15.4 \pm 1.7$  ppb in hypertensive control patients. The mean fractional alveolar NO concentration (CANO) in OSAS patients was significantly lower than that in control subjects ( $2.96 \pm 0.48$  vs  $5.35 \pm 0.83$  ppb, respectively;  $p < 0.05$ ). In addition, CANO values were significantly lower in OSAS patients with systemic hypertension compared to those in normotensive OSAS patients and hypertensive patients without OSAS. The mean values of CANO significantly improved after nCPAP therapy ( $2.67 \pm 0.41$  to  $4.69 \pm 0.74$  nL/L, respectively;  $p = 0.01$ ).

**Conclusions:** These findings suggest that alveolar FENO, and not bronchial FENO, is impaired in patients with OSAS and that this impairment is associated with an increased risk of hypertension. NO production within the alveolar space is modified by treatment with nCPAP.

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**Key words:** exhaled nitric oxide; hypertension; nasal continuous positive airway pressure; obstructive sleep apnea syndrome

**Abbreviations:** AHI = apnea-hypopnea index; BMI = body mass index; CANO = alveolar nitric oxide concentration; DLCO = diffusing capacity of the lung for carbon monoxide; FENO = fractional exhaled nitric oxide; nCPAP = nasal continuous positive airway pressure; NO = nitric oxide; NOS = nitric oxide synthase; OSAS = obstructive sleep apnea syndrome; ppb = parts per billion

Obstructive sleep apnea syndrome (OSAS) is associated with cardiovascular disorders such as systemic arterial hypertension, ischemic heart disease, and stroke.<sup>1,2</sup> Systemic vascular endothelial dysfunction, as evidenced by reduced levels of circulating metabolites of nitric oxide (NO),<sup>3</sup> which is a potent vasodilator and inhibitor of platelet aggregation and messengers, as well as by the impaired responsiveness of systemic resistance vessels in OSAS patients,<sup>4</sup> have been postulated as a possible cause of these cardiovascular disorders. In the lung, NO regulates pulmonary vascular

tone,<sup>5</sup> and reduced NO levels may contribute to pulmonary vascular smooth muscle proliferation and remodeling associated with the development of pulmonary hypertension.<sup>6</sup> Hypoxia may impair NO release, both by reducing substrate availability or by inhibiting the NO synthase (NOS), or perhaps, by increasing the levels of circulating NOS inhibitors. Evidence of upper airway inflammation<sup>7</sup> and systemic inflammation<sup>8</sup> have been described in patients with OSAS,<sup>9</sup> which in turn may be linked to the development of cardiovascular disorders.<sup>10</sup>

Fractional exhaled NO (FENO) is a simple non-invasive measurement of pulmonary NO production and has been used mainly as a measure of airway inflammation as well as a way of monitoring pulmonary and systemic NO production. FENO levels appear to be decreased in patients with primary pulmonary hypertension and chronic heart failure, supporting the hypothesis of reduced vascular NO production. However, measurements of FENO levels in OSAS patients have been reported as unchanged<sup>11</sup> or even increased.<sup>12</sup> Despite the complexity of NO exchange dynamics, the partitioning of exhaled NO from proximal airways, as opposed to distal airspaces, is possible using measurements obtained at different flow rates and has been successfully used in distinguishing inflammation due to alveolitis from that of the bronchial compartment.<sup>13</sup>

We postulated that, consistent with the concept of reduced vascular NO production, FENO levels from distal airspaces are decreased in OSAS patients while airway inflammation would increase NO levels in the bronchial compartment. In this study, we measured exhaled NO levels at different flow rates in order to distinguish between alveolar and proximal airway levels of NO, in subjects with OSAS before and after treatment with nasal continuous positive airway pressure (nCPAP).

## MATERIALS AND METHODS

### Population

Thirty-four consecutive patients, who were referred to our institution for clinical suspicion of sleep-disordered breathing, were recruited into the study. All patients had newly diagnosed OSAS.<sup>14</sup> A detailed clinical history, including the presence or absence of hypertension, was obtained and a complete physical examination was performed in each patient. Hypertension was judged to be present in patients receiving antihypertensive drug therapy on the basis of a diagnosis of hypertension, a systolic BP of > 140 mm Hg, or a diastolic BP of > 90 mm Hg at rest.

The control group consisted of twenty-nine healthy volunteers

who were recruited from among hospital staff. All were nonsmokers (*ie*, never-smokers or ex-smokers), normotensive, were receiving no medication, and had no evidence of cardiovascular, respiratory, and allergic disease at the time of the study. In addition, a group of eight hypertensive patients were recruited from the outpatient clinic. All were nonsmokers (*ie*, never-smokers or ex-smokers), had no history of airway disease, and were receiving appropriate treatment to control their hypertension. Before performing the physical examinations, all subjects gave informed consent according to the Helsinki declaration.

### Study Design

All of the patients and volunteers underwent full polysomnography in the sleep laboratory. On a separate study day (48 h later), FENO was measured followed by spirometry and diffusing capacity of the lung for carbon monoxide (DLCO). A group of 18 patients with OSAS, who had a baseline apnea-hypopnea index (AHI) of > 20 events per hour of sleep and accepted nCPAP treatment, were asked to perform FENO measurements again following 2 nights of nCPAP treatment.

### Polysomnography

The diagnosis of OSAS and nCPAP treatment requirements were ascertained using standard polysomnography (Somnostar 4100; SensorMedics; Yorba Linda, CA), which included EEG, electrooculogram, submental and anterior tibial electromyogram, measurements of oronasal airflow with pressure transducer, chest wall and abdominal excursions, oxygen saturation, single-lead ECG, body position, and snoring sensor. The evaluation started at approximately 10:00 PM and ended at 6:00 AM. An apnea was defined as the complete absence of airflow for > 10 s with or without oxyhemoglobin desaturation. Hypopnea was defined as a reduction of airflow of > 50% associated with  $\geq$  4% oxygen desaturation. Apneas were classified as obstructive, mixed, or central according to the standard criteria of the American Academy of Sleep Medicine.<sup>14</sup> The number of apneas and hypopneas per hour of sleep were computed, and the AHI value was calculated manually by a sleep laboratory expert.<sup>14</sup> The Epworth sleepiness scale was used to investigate subjective daytime sleepiness.

### Measurements of Exhaled NO

Exhaled NO measurements were performed following international recommendations<sup>15</sup> using a chemiluminescent NO analyzer (NOA 280; Sievers Instruments; Boulder, CO), which was designed for the online recording of exhaled NO concentration. The sensitivity of the analyzer for the measurement of gas-phase NO is < 1 part per billion (ppb) by volume. The analyzer was calibrated at 0 and 50 ppm in accordance with the manufacturer's recommendations. Subjects inspired "NO-free" air (*ie*, < 1 ppb) during measurements. After inhaling to total lung capacity, the subjects exhaled through a mouthpiece attached to a one-way valve containing two sampling ports. NO was sampled directly in the analyzer through a plastic (Teflon; DuPont; Wilmington, DE) side-arm tube attached to one of the sampling ports. Exhalation mouth pressure was measured by a pressure transducer in the analyzer via the second sampling port. Data were stored and analyzed on the computer, using NO analysis software. The flow rates were achieved by the placement of expiratory resistances in the exhalation circuit and by asking the subject to exhale at a constant mouth pressure, which was displayed and readily visualized on a computer screen. The pressure bar remained red until the target pressure was obtained, at which time it changed to

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green. If the flow dropped below or increased to above the desired range, the green light would again change to red. FENO values were obtained at five different flow rates (*ie*, 50, 120, 190, 250, and 300 mL/s). The exhalation proceeded until a stable plateau was reached. Plateau levels of NO were determined and expressed in ppb. Three successive recordings were made at 3-min intervals, and the mean value was used in the analysis. All measurements were recorded between 9:00 and 10:00 AM. The background NO level was measured and was always < 100 ppb.

### Statistical Analysis

The two-compartment model, first described by George et al<sup>16</sup> and Tsoukias and George,<sup>17</sup> was used to partition exhaled NO into two important subdivisions of the lungs (the airways and the alveolar region). This model of pulmonary NO dynamics can be used to predict FENO (in ppb) at any constant exhalation flow by using an exponential expression. Since the exponential function approaches the first-order linear approximation when exhalation flow is larger than conductance for the radial mass transfer of NO from the airway wall to the gas stream, using measurements of the NO elimination rate (in picoliters per second) at multiple constant exhalation flows (in milliliters per second), this equation provides a reliable and simple means to assess the NO output of the bronchial airways (in picoliters per second) [the intercept] and the alveolar NO concentration (CANO) [in ppb or nanoliters per liter; *ie*, the slope].

Analysis of variance was used to compare the different groups, and the correlation coefficient was used to measure the associations among variables. Multiple regression analysis was used to examine the relationships between CANO and variables relating to lung function, nocturnal hypoventilation, and nocturnal hypoxia. The results are presented as the mean  $\pm$  SE. A *p* value of 0.05 was considered to be statistically significant in all data analyses.

## RESULTS

The main characteristics of the study populations are presented in Table 1. The study group consisted

of 34 individuals and was largely male, middle-aged (mean age, 56 years), and overweight (mean body mass index [BMI], 31 kg/m<sup>2</sup>). Approximately half of the participants were ex-smokers, and 18 had previously been diagnosed with arterial hypertension and were receiving treatment with at least one drug. The control group consisted of 29 healthy individuals, of whom 7 were women. They were younger (mean age, 46 years; *p* < 0.01 [unpaired *t* test]) and had normal weight (mean BMI, 25 kg/m<sup>2</sup>; *p* < 0.05 [unpaired *t* test]). Lung function was normal in all individuals in both groups.

In a subgroup of 18 patients, multiple-flow exhaled NO was measured again after 2 nights of nCPAP treatment. Their breathing during sleep was normalized as shown by changes in mean AHI (47.3  $\pm$  13.1 to 3.9  $\pm$  2.3 events per hour, respectively; *p* < 0.001) and by the percentage of time spent with arterial oxygen saturation of < 90% (22.1  $\pm$  20.0 to 2.1  $\pm$  0.5% of time, respectively; *p* < 0.001).

### Exhaled NO

Representative exhaled NO curves measured at 50 and 300 mL/s in an OSAS patient and a healthy subject are shown in Figure 1. At a standard expiratory flow rate of 50 mL/s, the mean FENO was similar in patients with OSAS compared with values in healthy and hypertensive control subjects (21.8  $\pm$  1.9 vs 25.1  $\pm$  3.3 vs 15.4  $\pm$  1.7 ppb, respectively).

Linear fitting of the relationship between measurements of NO elimination rate and multiple constant exhalation flows, using least squares analysis

**Table 1—Characteristics of the Patients With OSAS, Healthy Control Subjects, and Hypertensive Patients\***

Characteristics	OSAS Patients (n = 34)	Healthy Control Subjects (n = 29)	Hypertensive Patients (n = 8)
Age, yr	56.6 $\pm$ 8.1 (41–75)	46.6 $\pm$ 15.2† (24–76)	54 $\pm$ 7.4 (38–59)
Sex			
Male	27	22	5
Female	7	7	3
Ex-smokers (yes)	15	3	3
BMI, kg/m <sup>2</sup>	31.6 $\pm$ 5.5 (24–48)	25.3 $\pm$ 4.2‡ (16–30)	26.3 $\pm$ 1.7‡ (24–29)
FEV <sub>1</sub> , % predicted	97.4 $\pm$ 17.4 (74–127)	100.7 $\pm$ 10.7 (81–112)	98.2 $\pm$ 4.2 (91–101)
Total lung capacity, % predicted	101.3 $\pm$ 16.6 (76–133)	100.1 $\pm$ 9.0 (80–119)	97.3 $\pm$ 5.1 (95–108)
DLCO/VA adjusted, % of predicted	110.7 $\pm$ 18.3 (83–156)	109.8 $\pm$ 13.3 (82–146)	98.2 $\pm$ 4.8 (89–105)
AHI, events/h of sleep	31.3 $\pm$ 17.4 (11–74)	1.4 $\pm$ 0.3† (0.4–4.5)	ND
Nadir SaO <sub>2</sub> , %	79.2 $\pm$ 8.0 (56–89)	90.2 $\pm$ 1.9† (87–94)	ND
SaO <sub>2</sub> at < 90%, % time	5.6 $\pm$ 6.9 (0–34)	0.5 $\pm$ 0.9† (0–2.6)	ND
Oxygen desaturation index, No. of dips $\geq$ 4%/h	22.8 $\pm$ 16.5 (4–72)	0.9 $\pm$ 0.7† (0.1–2.6)	ND
Epworth sleepiness scale score	10 $\pm$ 4 (3–20)		

\*Values are given as the mean  $\pm$  SD (range) or No. ND = not done; VA = alveolar volume.

†*p* < 0.001 vs OSAS and hypertensive patients, if applicable.

‡*p* < 0.05 vs OSAS patients.

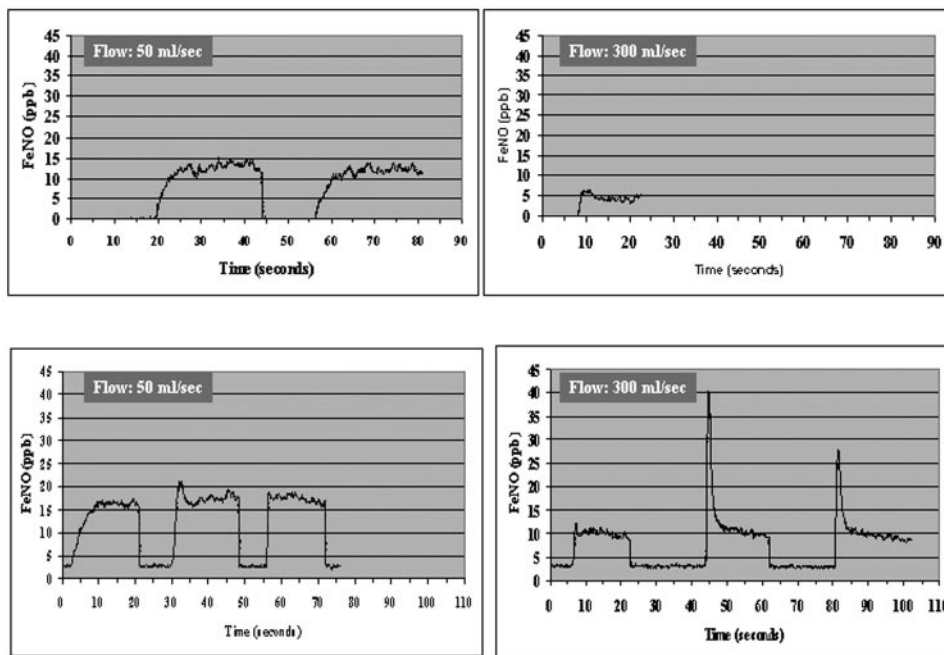


FIGURE 1. Representative traces of exhaled NO at 50 and 300 mL/s in an OSAS patients (*top panels*) and in a healthy control subjects (*bottom panels*).

did not show any gross departure from linearity (Fig 2). The mean  $R^2$  values were  $0.91 \pm 0.01$  for the OSAS patients and  $0.94 \pm 0.02$  for the control subjects. The slopes of the graph were shallower in the OSAS patients compared with control subjects, as reflected by lower mean values of CANO ( $2.96 \pm 0.48$  and  $5.35 \pm 0.38$  ppb or nL/L, respectively;  $p < 0.05$ ) [Fig 2], suggesting a lower alveolar

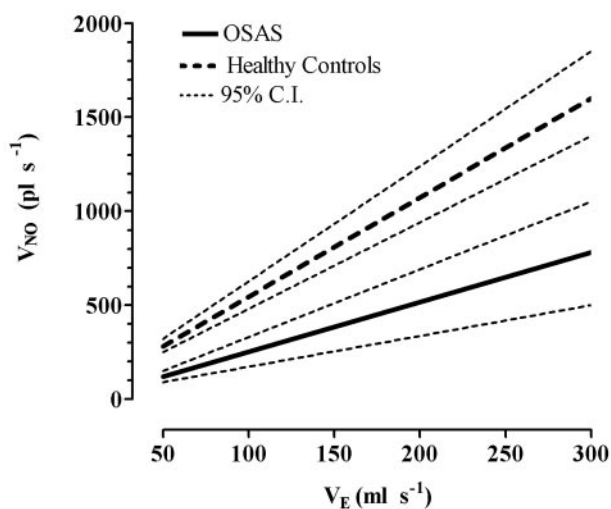


FIGURE 2. Mean linear regression lines for exhaled NO elimination rate (VNO) at different constant expiratory flows (VE) in patients with OSA ( $n = 34$ ; continuous line) and in healthy control subjects ( $n = 29$ ; dashed lines). Dotted lines show 95% confidence intervals (C.I.).

production of NO. No difference was observed in the mean intercept values between the two groups ( $771 \pm 98$  vs  $596 \pm 84$  pL/s, respectively).

CANO did not correlate with BMI in the healthy and OSAS groups. There was no relationship between bronchial and alveolar NO and any lung function parameter and polysomnographic data in the OSAS group. However, values of CANO were significantly lower in patients with systemic hypertension ( $p < 0.01$ ) [Fig 3]. In addition, multiple linear regression analysis showed that the oxygen

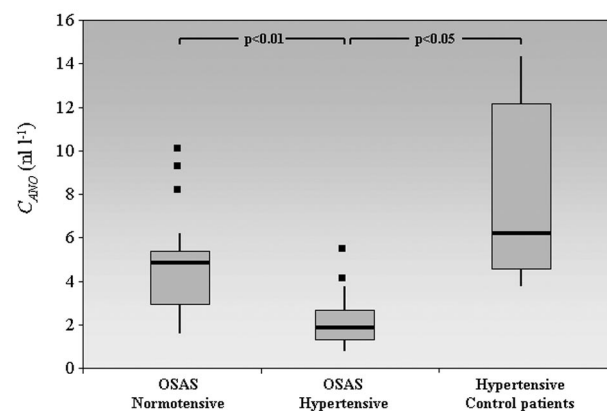


FIGURE 3. Box-and-whisker plot of CANO in normotensive patients ( $n = 16$ ) and hypertensive patients ( $n = 18$ ) with OSAS and in hypertensive control subjects ( $n = 8$ ). Each box shows the median (horizontal bar), quartiles (box), and extreme values (whiskers) within a category.



desaturation index (*ie*, the number of dips of  $\geq 4\%$  in oxygen saturation per hour of sleep), DLCO, total lung capacity, and hypertension were the most important variables affecting the slope ( $R^2 = 0.5$ ;  $p = 0.002$ ).

Linear fitting of the relationship between measurements of NO elimination rate and multiple constant exhalation flows using least squares analysis varied after nCPAP therapy. Following 2 nights of nCPAP treatment, CANO increased in 15 of 18 patients ( $2.67 \pm 0.41$  to  $4.69 \pm 0.74$  ppb or nL/L, respectively;  $p = 0.01$ ) [Fig 4], while no effect was observed on FENO at a rate of 50 mL/s.

## DISCUSSION

The main findings of this study are as follows in patients with OSAS: (1) alveolar NO concentrations are decreased while bronchial NO concentration is unchanged; (2) nCPAP treatment for 2 nights is sufficient to restore alveolar NO concentration; and (3) alveolar NO concentration appears to be more reduced in those OSAS patients with hypertension. Previous work on FENO in OSAS patients has provided inconsistent results. Olopade et al<sup>12</sup> found an increase in mixed expired NO levels in patients with OSAS after sleep compared to presleep values. However, presleep levels were low and similar to those in healthy control subjects. An offline measurement of mixed exhaled NO was used in this study, with patients wearing nose-clips, and a slow vital capacity maneuver was performed without an expiratory resistance into a plastic (Tedlar; DuPont;

Wilmington, DE) sample bag.<sup>12</sup> Thus, this study does not guarantee against some mixing of upper and lower airway NO. In another study<sup>11</sup> using online exhaled NO measurements, mixed exhaled NO values in OSAS patients with or without cardiovascular disease were similar to those in healthy control subjects. In the present study, there was also no difference in FENO at a standard low flow rate of 50 or 100 mL/s between subjects with OSAS and healthy control subjects. This suggests that airway NO output is not as high in OSAS patients, as confirmed by the estimation of bronchial NO output in both groups. However, we demonstrated that CANO levels were significantly lower in OSAS patients. It is unlikely that the lower CANO values in OSAS patients are related to differences in BMI and age. We found that BMI is not related to CANO both in OSAS patients and in healthy control subjects. Thus, although our study groups were not well balanced as regards BMI, it is unlikely that differences in CANO values were related to differences in BMI. Indeed, FENO at 50 mL/s is age-dependent in children,<sup>18</sup> but not in adults,<sup>19</sup> whereas whether CANO values are also age-dependent is unknown.

The source of alveolar NO reflects the balance between the local production of NO from distal parts of the lung and diffusion across the alveolar capillary wall. Increased alveolar levels of NO have been found in patients with alveolitis,<sup>13</sup> asthma,<sup>20</sup> hepatic cirrhosis,<sup>21</sup> and COPD,<sup>22</sup> ostensibly due either to increased NO production by inflammatory cells, epithelium, or endothelial cells.

Intermittent nocturnal hypoxemia in OSAS patients may be associated with systemic inflammation, increased oxidative stress, and endothelial dysfunction. A systemic inflammatory response has been shown in patients with sleep apnea with reported increases in circulating levels of C-reactive protein,<sup>8</sup> interleukin-6,<sup>23</sup> tumor necrosis factor- $\alpha$ ,<sup>24,25</sup> interleukin-8,<sup>26</sup> intercellular adhesion molecule-1, and monocyte chemoattractant protein-1.<sup>27,28</sup> Potential sites of inflammation in patients with OSAS are nasal,<sup>29</sup> oropharyngeal,<sup>30</sup> and tonsillar tissue.<sup>31</sup> A reduction in the circulating levels of tumor necrosis factor- $\alpha$  has been found following tonsillectomy,<sup>32</sup> and prolonged treatment with nCPAP decreases the levels of C-reactive protein and interleukin-6.<sup>23</sup> The presence of systemic inflammation may link OSAS causally with the metabolic syndrome.<sup>33</sup> However, the increase in circulating levels of inflammatory markers may be, at least in part, a consequence of visceral obesity and of strenuous breathing of respiratory muscles.<sup>34</sup> In the lungs, evidence suggesting local inflammation and oxidative stress in patients with OSAS derives from the findings of increased neutrophils in induced sputum<sup>35</sup> along with a de-

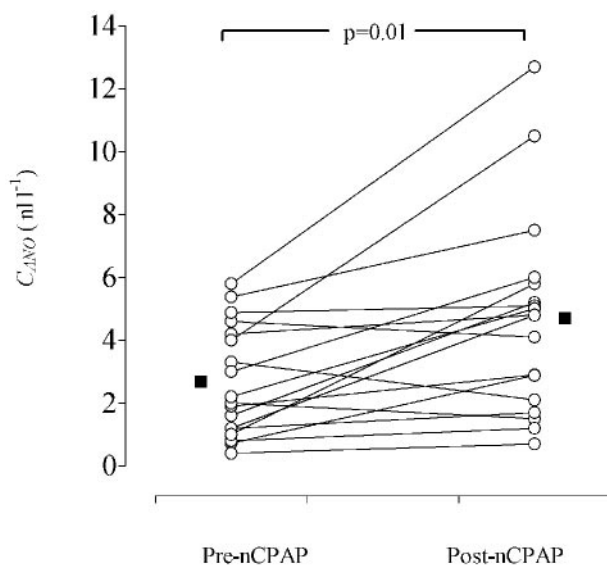


FIGURE 4. CANO values before and after 2 nights of nCPAP treatment in 18 patients with OSAS.

crease in macrophages,<sup>35</sup> and of increased interleukin-6 and isoprostane levels in breath condensate.<sup>27</sup>

The effects of inflammation on NO production in OSAS patients are complex. Intermittent hypoxia in wild-type mice caused increased cerebral inducible NOS activity and a proinflammatory gene response.<sup>36</sup> These changes were not seen in transgenic inducible NOS-deficient mice, suggesting that oxidative injury and proinflammatory gene responses are inducible NOS dependent.<sup>36</sup> However, circulating nitrotyrosine, a biomarker of NO-induced oxidative/nitrosative stress, was unchanged in subjects with OSAS,<sup>37</sup> arguing against an increase in inducible NOS-induced NO levels.

The decrease in alveolar NO levels observed in the OSAS patients in this study could be due to increased free radical production in leukocytes in peripheral airspaces,<sup>38</sup> although this would be unlikely given the unchanged bronchial output of NO and the immediate increase in alveolar NO levels after only 2 nights of nCPAP therapy. The failure to find elevated levels of exhaled NO in OSAS does not preclude the presence of an inflammatory component in these patients but merely that it may not involve the inducible NOS pathway in the lung.

There is considerable evidence of alterations in NO production in OSAS patients. Impaired endothelium-dependent relaxation of systemic arteries in patients with OSA has been reported.<sup>4,39</sup> This impairment is independent of hypertension and is related to the severity of apnea-related hypoxemia.<sup>39</sup> Moreover, circulating plasma nitrite/nitrate levels are decreased in OSAS patients and increase with prolonged nCPAP.<sup>40–42</sup> Treatment with nCPAP also appears to restore endothelial function,<sup>43</sup> and is associated with significant increases in plasma NO levels and decreases in plasma endothelin levels.<sup>44</sup> Intriguingly, oxygen administration improves the serum level of NO metabolites in patients with OSA,<sup>45</sup> implicating decreased substrate availability. In a rat model, intermittent hypoxia causes a widespread increase of vascular endothelial growth factor in neurones and glial cells.<sup>46</sup> Endothelial NOS appears to blunt the cerebrovascular inflammatory response to intermittent hypoxia and exerts neuroprotective effects in mice,<sup>47</sup> suggesting that it has a key role in the pathogenesis of hypoxia-induced vascular damage.

Similar to the reductions in systemic NO production, the evidence for endothelial dysfunction, such as systemic hypertension, supports the concept of reduced pulmonary distal endothelial NOS activity in OSAS patients. Systemic hypertension is associated with lower FENO values that fail to rise following treatment with angiotensin-converting

enzyme inhibitors than those in normotensive control subjects, suggesting a generalized impairment of endothelial function.<sup>48</sup> The rapid increase in alveolar NO levels observed after only 2 nights of nCPAP treatment suggests that the lack of molecular oxygen, a substrate of NO, may be a direct cause of the low levels of alveolar NO. Acute hypoxia causes a reduction in exhaled NO both in isolated lungs<sup>49,50</sup> and in humans susceptible to high-altitude pulmonary edema.<sup>51</sup> This reduction has been suggested to be one of the factors contributing to pulmonary hypertension in these subjects. Intermittent hypoxia has been associated with vascular inflammation, which is blunted by endothelial NOS.<sup>47</sup> The greater decrease in alveolar NO observed in hypertensive OSAS patients likely reflects a greater impairment in endothelial NOS function, resulting in enhanced endothelial cell damage and dysfunction. It is unlikely that hypertension *per se* is the cause of the lower alveolar NO concentrations because, first, the hypertensive patients in our study (those with and without OSA) were all receiving treatment and had normal arterial BP levels; second, in the hypertensive group of patients without OSAS alveolar NO concentrations were similar to those found in healthy subjects.

In conclusion, our results suggest that alveolar NO levels are decreased in subjects with OSAS, and nocturnal nCPAP rapidly improved alveolar NO levels, suggesting that hypoxia is a direct cause. The reduction in alveolar NO levels was found to be greater in hypertensive OSAS patients, which may be linked to a possible defect in endothelial function in these patients.

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