Decreased plasma levels of nitric oxide derivatives in obstructive sleep apnoea: response to CPAP therapy

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Abstract

Background—Reduced endothelium dependent vasodilation has been reported in patients with obstructive sleep apnoea (OSA) but direct measurements of the most potent naturally occurring vasodilator, nitric oxide (NO) or its derivatives (nitrate and nitrite, NO_x), have not yet been performed in these patients.

Methods—In 21 patients with OSA of mean (SE) age 54 (2) years, body mass index (BMI) 30.9 (1.1) kg/m², and apnoea-hypopnoea index (AHI) 37 (4)/h, NO_x levels were measured in peripheral venous blood samples by chemiluminescence. Blood samples were obtained before and after two nights of continuous positive airway pressure (CPAP) and after 5.5 (1.5) months of follow up. Thirteen age matched, healthy volunteers and 18 patients without OSA but with a similar spectrum of comorbidity served as controls (control groups 1 and 2).

Results—Before CPAP NO_x levels were 21.7 (1.5) µM in patients with OSA compared with 42.6 (2.2) µM and 36.7 (1.7) µM in control groups 1 and 2, respectively ($p < 0.01$ for each comparison). NO_x concentrations increased to 32.1 (2.7) µM after two nights of CPAP and remained constant at 32.9 (2.3) µM at follow up ($p < 0.01$ compared with levels before CPAP).

Conclusions—Plasma NO_x levels are reduced in OSA and can be increased by short and long term CPAP therapy. Although the precise mechanism underlying this observation remains to be clarified, it may have important implications for the development of cardiovascular disease in patients with OSA and for the life saving effect of CPAP.

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Keywords: obstructive sleep apnoea; nitric oxide; CPAP therapy

Obstructive sleep apnoea (OSA) is associated with cardiovascular disorders such as arterial hypertension, coronary artery disease, and cerebrovascular disease.¹ It is thought that this accounts for the increased mortality observed in patients with OSA,^{2,3} but the causal relationship between OSA and cardiovascular disease remains to be fully established.⁴

One hypothesis to explain this association is based on the concept of disturbed vascular control in OSA.⁵ Nitric oxide (NO) is the main vasodilating substance released from the endothelium. Deficiency of NO has been implicated in the pathogenesis of cardiovascular disease.^{6,7} Two studies have already suggested reduced endothelium dependent vasodilation in OSA. Carlson and colleagues administered intra-arterial acetylcholine and sodium nitroprusside infusions to 16 awake patients with OSA and measured forearm blood flow using venous occlusion plethysmography.⁸ They found that endothelium dependent vascular relaxation was impaired independently of hypertension. Duchna *et al* recently demonstrated reduced vascular responsiveness to bradykinin and nitroglycerin after intravenous infusion in 10 of 12 patients with OSA using the dorsal hand vein technique.⁹ After one month of treatment with continuous positive airway pressure (CPAP) vascular reactivity was reversed to normal in all subjects with an abnormal initial response.

Direct measurements of NO or its derivatives have, however, not yet been performed in patients with OSA. Because NO is a gas with a very short plasma half life of only a few seconds, it is difficult to measure NO itself. More feasible is the measurement of the more stable derivatives of NO (nitrate and nitrite (NO_x)) in biological fluids. NO_x concentrations not only depend on endothelial NO formation but also on other aspects of NO metabolism such as the distribution in various body compartments and the rate of elimination by the kidneys. Nevertheless, NO_x levels have been suggested as the most appropriate measure of overall NO production.¹⁰ The aim of the present study was therefore to determine plasma levels of NO_x in a group of patients with OSA before and after CPAP therapy.

Methods

STUDY POPULATION

Twenty one consecutive patients with OSA were investigated. All gave written informed consent and the study protocol was approved by the local ethics committee.

In all patients serum creatinine (normal <1.2 mg/dl), total cholesterol (normal <200 mg/dl), and fasting blood glucose levels (normal <110 mg/dl) were measured. The patients were asked about their regular medications and smoking habits. The medical history of the patients was evaluated with special refer-

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Table 1 Characteristics of subjects in the OSA group and control group 2

	OSA (n=21)	Control group 2 (n=18)	p value
Sex (M/F)	17/4	12/6	
Age (years)	54 (2)	61 (2)	<0.05
BMI (kg/m ²)	30.9 (1.1)	25.5 (1.1)	<0.05
AHI (n/hour)	37 (4)	—	
Sao ₂ <90% (% of TST)	23.9 (5.4)	1.2 (2.0)	
Mean Sao ₂ (%)	91.8 (0.7)	94.5 (1.4)	
Minimum Sao ₂ (%)	66.1 (2.9)	84.3 (2.5)	
Arterial hypertension	12 (57%)	7 (39%)	<0.05
Coronary artery disease	1 (5%)	2 (11%)	NS
Cerebrovascular disease	2 (10%)	3 (17%)	NS
Diabetes mellitus	1 (5%)	3 (17%)	NS
Chronic renal failure	—	—	
Hypercholesterolaemia	7 (33%)	5 (28%)	NS
Smoking	8 (38%)	8 (44%)	NS
ACE inhibitors	4 (19%)	5 (28%)	NS

Data are given as mean (SE) or as total numbers.

BMI = body mass index; AHI = apnoea-hypopnoea index; Sao₂ = nocturnal oxygen saturation; TST = total sleep time; NS = not significant.

ence to cardiovascular disease (arterial hypertension, coronary artery disease, and cerebrovascular disease). Blood pressure at rest was measured at fixed time intervals during their stay in the sleep laboratory at 06.00, 12.00, 16.00, and 20.00 hours. Arterial hypertension was diagnosed if blood pressure values exceeded 140/90 mm Hg during at least two different measurements or if there was known and medically treated hypertension. Patients taking oral nitrate preparations were excluded from the study.

Thirteen healthy non-smoking volunteers (nine men) of mean (SE) age 53 (2) years and body mass index (BMI) 27.9 (0.4) kg/m² comprised control group 1 and 18 patients without OSA admitted to hospital with other diagnoses served as control group 2. The main diagnosis in these patients was lung cancer, followed by other bronchopulmonary diseases such as pneumonia and chronic bronchitis.

In the control subjects OSA was excluded by a negative history of sleep related symptoms (snoring, witnessed apnoeas, excessive daytime sleepiness) and overnight pulse oximetry recordings. The characteristics of the OSA group and control group 2 are shown in table 1.

POLYSOMNOGRAPHY

Polysomnography was performed on three consecutive nights. The electroencephalogram (electrodes at positions C3-A2 and C4-A1 of the international 10–20 system), electrooculogram and electromyogram of the submandibular and pretibial muscles were simultaneously recorded. Ventilatory airflow at the nose and mouth was registered with thermistors, and the breathing movements of the chest and abdomen were monitored by inductive plethysmography. Oxygen saturation (Sao₂) was measured transcutaneously by pulse oximetry at the finger tip of the patient. Finally, an electrocardiogram was obtained. All data were stored on a computerised polysomnograph with capability for analogue registration (Sidas GS, IfM GmbH, Wettenberg, Germany). Analysis of sleep stages was performed manually at 30 second intervals according to the criteria of Rechtschaffen and Kales.¹¹

An obstructive apnoea was defined as complete cessation of oronasal flow in the presence of thoracoabdominal breathing move-

ments. If neither oronasal flow nor breathing efforts of the chest and abdomen could be detected, this was defined as a central apnoea. Hypopnoea was defined as a reduction in the respiratory amplitude of more than 50% with regard to the preceding effort signals. All apnoeas and hypopnoeas were required to have a duration of at least 10 seconds. The apnoea-hypopnoea index (AHI) was obtained by dividing the total number of apnoeas and hypopnoeas by the total sleep time (TST). An AHI of more than 10 per hour of sleep was considered diagnostic of OSA.

During the first night a diagnostic study was performed. After confirming the diagnosis of OSA, CPAP therapy was given to all patients during the second night (Aria LX, Respiration Inc, Germany). On the third night all patients slept with the finally titrated pressure to assure adequate elimination of all apnoeas, hypopnoeas, and snoring.

After using CPAP at home for a variable length of time (range 92–265 days) the patients were re-examined in our sleep laboratory. Compliance with CPAP therapy was evaluated by the readings on the time counter built into the CPAP machine. Acceptable compliance was defined as CPAP use of at least five hours per night for five days of the week. On the control night the adequacy of the initially chosen CPAP pressure was checked and increased if snoring or apnoeas were again detected, otherwise it was kept constant or decreased if possible.

DETERMINATION OF NO_x PLASMA LEVELS

At 07.00 hours, immediately after polysomnography, a peripheral venous blood sample was withdrawn into a tube prepared with EDTA and centrifuged at 2000 rpm for 10 minutes. The supernatant plasma was stored in a refrigerator at –20°C. Blood samples were obtained before and after initiating CPAP treatment (after the first two nights of CPAP therapy and at follow up). NO_x levels were determined using a chemiluminescence technique as previously described.¹²

Briefly, the plasma samples were deproteinized and double volumes of 0.5 M NaOH and zinc sulphate (30%) were added to the samples. The mixture was shaken continuously for 15 minutes at 600 rpm and thereafter centrifuged for 10 minutes at 13 000 rpm. The protein free supernatant was put into Eppendorf cups, two portions each of 600 µl. The samples were injected into a heated reaction chamber at a constant flow (98°C; 600 µl/min) and the chamber was filled with a solution of vanadium (III) chloride 0.1 M in 2 M HCl which quantitatively reduced the NO decomposition products (nitrate and nitrite) back to NO. Oxygen free nitrogen at a flow rate of 160 ml/min was used to carry the NO through a cooling tower to a chemiluminescence NO analyser (UPK 3100; UPK, Bad Nauheim, Germany) where NO reacted with ozone to produce NO₂. The energy of the NO₂ emitted photons was filtered, enhanced by a photomultiplier, and converted into a voltage signal. The NO concentrations (in ppb) were calculated

from the intensity of this signal after prior calibration with defined gases (NO in oxygen free nitrogen; Messer Griesheim, Herborn, Germany). The NO levels were continuously monitored on a personal computer with the help of a program developed by one of the authors (KM). The maximal increases in the concentrations of the two different probes were averaged and converted to $\mu\text{M NO}_x$ in the original plasma samples using a calibration curve obtained from prior measurements of calibration samples containing known concentrations of nitrate and nitrite.

The average NO_x concentration in control group 1 was interpreted as the normal reference value for NO_x . An increase in NO_x after CPAP was defined as a change in NO_x concentrations of more than $2 \mu\text{M}$. This cut off value was chosen because the day to day variability of NO_x between individuals had been found to lie within this range in earlier experiments.

STATISTICAL ANALYSIS

All data are given as mean (SE) values. The plasma levels of NO_x in the two control groups and in the OSA group were compared using the Wilcoxon-Mann-Whitney test. Differences in NO_x values in individuals before and after CPAP therapy were evaluated by the two tailed paired Wilcoxon test in the whole OSA group as well as in normotensive and hypertensive patients with OSA. Differences in plasma concentrations of NO_x between the OSA group and the controls and between patients with OSA before and after CPAP were further quantified by calculating the Hodges-Lehmann estimator including 95% confidence intervals (CI). While performing calculations, the family wise error rate was taken into consideration. A p value of <0.05 was regarded as significant.

Results

PATIENT CHARACTERISTICS

In the OSA group 12 subjects suffered from concomitant cardiovascular disease (mainly systemic arterial hypertension). All patients had normal renal function and only one patient suffered from diabetes mellitus. Seven patients had mild hypercholesterolaemia (200–220 mg/dl). Four patients were being treated with an ACE inhibitor and eight were habitual smokers.

The healthy control group 1 was age matched with the OSA group, but the average BMI was somewhat lower than in the patients with OSA. Compared with the OSA group, patients in control group 2 were slightly older but were not overweight. In this group the percentage of hypertensive patients was lower (39% vs 57%, $p<0.05$). The other characteristics did not differ significantly between the OSA group and control group 2.

POLYSOMNOGRAPHIC DATA

The 21 patients who participated in the study had moderate to severe OSA with markedly disturbed sleep architecture (data not shown). After starting CPAP therapy the AHI de-

Figure 1 Plasma levels of nitric oxide derivatives (NO_x) in untreated OSA patients and control groups 1 (healthy volunteers) and 2 (patients without OSA). All data are given as mean (SE) and as individual values. The SE is not depicted if it is so small that it would be obscured by the symbol of the mean value. * $p<0.05$, ** $p<0.01$.

creased to $<10/\text{h}$ in all patients and nocturnal oxygen saturation was markedly improved or even normalised. The mean CPAP pressure was set at 10.0 (2.4) cm H_2O .

After a mean follow up period of 5.5 (1.5) months 15 patients were re-evaluated by polysomnography. Thirteen patients were compliant with CPAP (average use 7.1 (1.8) h per night) and reported improvement in their daytime sleepiness. One patient had been unable to sleep with her CPAP machine for two weeks before admission because of upper respiratory tract infection. One patient was non-compliant with CPAP (average use 3.5 h per night). These two patients were excluded from statistical analysis. During the control night the CPAP pressure was kept constant in nine patients, had to be increased in four patients, and was reduced in two patients. Six patients were lost to follow up either because of non-compliance (four patients), intervening severe illness (one patient), or long distance to their place of residence (one patient).

NO_x PLASMA LEVELS BEFORE CPAP

Mean plasma levels of NO_x were 42.6 (2.2) μM in the healthy control group 1 compared with 36.7 (1.7) μM in control group 2 ($p<0.05$, fig 1). In the OSA group the mean NO_x level was further reduced to 21.7 (1.5) μM , which was highly significant compared with the two control groups ($p<0.01$ for each comparison; Hodges-Lehmann estimators $-19.7 \mu\text{M}$ (95% CI -12.7 to -28.0) and $-15.1 \mu\text{M}$ (95% CI -8.7 to -22.0) respectively, fig 1).

When looking at individual data, all patients with OSA had lower NO_x levels than the mean values of both control groups although there was some overlap between NO_x levels in patients with OSA and controls (fig 1). The presence of hypertension had no effect on NO_x levels in the patients with OSA (21.4 (2.3) μM in hypertensive vs 22.2 (1.7) μM in normotensive patients; fig 2).

NO_x PLASMA LEVELS AFTER CPAP

After two nights of CPAP therapy NO_x levels increased to a mean of 32.1 (2.7) μM ($p<0.01$ compared with before CPAP; Hodges-

Figure 2 Plasma levels of nitric oxide derivatives (NO_x) in (A) hypertensive and (B) normotensive patients with OSA before CPAP, after two nights of CPAP, and at follow up. All data are given as mean (SE) and as individual values. * $p < 0.05$; ** $p < 0.01$; NS = non-significant.

Lehmann estimator $10.1 \mu\text{M}$ (95% CI 2.6 to 16.9)). The NO_x levels remained unchanged in eight patients (38%) but increased in 13 patients (62%). The increase in NO_x concentrations was more pronounced in hypertensive patients than in normotensive patients ($33.6 (4.5) \mu\text{M}$ vs $30.2 (2.2) \mu\text{M}$, $p < 0.05$, fig 2).

At follow up NO_x levels remained constant (mean $32.9 (2.3) \mu\text{M}$; $p < 0.01$ compared with pre-CPAP values; Hodges-Lehmann estimator $12.4 \mu\text{M}$ (95% CI 3.6 to 21.0)). Compared with levels before CPAP therapy, individual NO_x levels were still reduced in three patients (23%) but had increased in the remaining 10 patients (77%). In the OSA patients with hypertension NO_x levels did not change significantly during the follow up period ($32.2 (3.5) \mu\text{M}$, fig 2A). The normotensive patients with OSA had a small but significant further increase in NO_x levels ($33.8 (3.1) \mu\text{M}$, $p < 0.05$ compared with the level after two nights of CPAP, fig 2B).

Discussion

In this study plasma levels of NO_x were found to be markedly reduced in a group of 21 untreated patients with OSA. After two nights of CPAP therapy NO_x levels increased in more than half of the patients. After a mean follow up period of 5.5 months an even higher percentage of those patients regularly using their CPAP device had increased NO_x levels.

Blood samples for the measurement of NO_x were taken in the morning shortly after waking. Since nitrate and nitrite have a plasma half life of 4–7 hours,¹⁰ the NO_x concentrations achieved reflect NO generation during the night while the patients were asleep. It is also crucial to the understanding of our results that a recent study has found a significant correlation between changes in forearm blood flow as measured by venous occlusion plethysmography and the serum nitrite concentration.¹³ The current observation of reduced plasma levels of NO_x in patients with OSA thus suggests reduced endothelium dependent vascular relaxation, as previously assumed for this disease.^{8,9}

The healthy control group had the highest mean NO_x level, subjects in control group 2

had intermediate NO_x levels, and the OSA patients had the lowest mean NO_x levels of all groups. There are many variables that can influence NO_x levels which have to be considered when discussing these observations. Arterial hypertension, diabetes mellitus, chronic renal failure, hypercholesterolaemia, smoking, and aging have all been shown to be associated with decreased plasma levels of NO_x .^{14–19} On the other hand, drugs such as nitrates and ACE inhibitors directly or indirectly increase the generation of NO in the vasculature.^{20,21}

One might argue that the reduction in NO_x was the result of the higher percentage of arterial hypertension in the OSA group compared with control group 2. However, the NO_x concentrations were also low in those OSA patients without concomitant hypertension. This observation is in agreement with the study by Carlson *et al* who reported that endothelium dependent vascular relaxation was reduced in OSA independently of hypertension.⁸

It is very unlikely that metabolic disturbances had a major impact on the levels of NO_x in our study. Only a few patients suffered from diabetes mellitus and all had normal serum creatinine concentrations. Total cholesterol levels were not in the range known to be linked with decreased NO_x concentrations.¹⁷

Smoking might negatively affect plasma levels of NO_x ⁸ but it has to be kept in mind that blood samples were withdrawn at 07.00 hours—that is, after some hours without inhalation of cigarette smoke. It is known that NO production decreases with advancing age.¹⁹ The OSA group was age matched with the healthy control group, whereas patients in control group 2 were older. Thus, the difference between the OSA group and control group 2 might be regarded as even more significant. Finally, the influence of drugs on NO_x levels has to be considered. Nitrates were not taken by any of the subjects studied, but a similar number of patients in the OSA group and in control group 2 were receiving ACE inhibitors. These drugs may actually lead to an increase in NO_x concentrations,²¹ thereby possibly attenuating the true difference in NO_x levels between the OSA group and the healthy control group.

The patients in control group 2 all suffered from bronchopulmonary disease. In the presence of airway inflammation, bronchopulmonary disorders might be associated with high levels of exhaled NO.²² However, these changes do not usually affect the intravascular compartment—that is, plasma levels of NO_x.^{23, 24} Furthermore, most of our patients were free from infection at the time of the investigation.

A possible limitation of our study is that the patients did not adhere to a low nitrate/nitrite diet which has been recommended for the measurement of NO_x.¹⁰ Unfortunately, it was not feasible to submit all the patients in the sleep laboratory to such a diet so we are not able to exclude a confounding influence of the individual diet on the level of NO_x. A further potential limitation to our study is that the control subjects were not followed up to have repeated measurements of NO_x. However, we think it unlikely that NO_x levels would have risen in all or most of these subjects to an extent comparable to the OSA patients after CPAP. Finally, methodological factors such as nitrite contamination of the blood tubes containing EDTA²⁵ or the influence of the tourniquet pressure applied during bleeding²⁶ might have biased our results. Unfortunately, it was impossible to control for these confounding factors but they were presumably of equal importance in all subjects studied.

Taken together, the difference in NO_x levels between the two control groups might be attributed to the lack of cardiovascular disease in the healthy volunteers while the further reduction in NO_x levels in the OSA group is assumed to be caused by the sleep related breathing disorder itself. A final argument for the validity of our results is that effective CPAP therapy led to a rapid and long lasting increase in NO_x concentrations in patients with OSA.

From the present study it is not possible to explain the mechanism of NO_x reduction in OSA. However, it might be speculated that intermittent nocturnal oxygen desaturation and/or apnoea related haemodynamic changes are of major importance.

In patients with OSA nocturnal hypoxaemia might adversely affect the metabolic balance between production and breakdown of endothelium derived NO. NO is synthesised from the amino acid L-arginine by NO synthases. It is rapidly metabolised to its oxidative products nitrate, nitrite, and peroxynitrite. Both the synthesis and breakdown of NO are critically dependent on the presence of oxygen, either in its free or haemoglobin bound form. The formation of peroxynitrite occurs primarily under pathological conditions by the interaction of NO with superoxide anions.

There is some evidence that hypoxaemia associated with apnoea could lead to decreased production of NO. Firstly, oxygen is a co-substrate of NO synthase. Secondly, recent data, as yet only published in abstract form, suggest that NO synthase inhibitors are significantly increased in patients with OSA.²⁷ Thirdly, and most important, hypoxia suppresses both the transcriptional rate of the

eNOS gene and the stability of its mRNA.^{28, 29} Finally, free oxygen radicals which have been shown to accumulate in untreated OSA patients³⁰ might cause an exaggerated destruction of NO.

During each apnoea the blood pressure gradually rises, thereby imposing increased shear stress to the vessel wall. Shear stress primarily enhances NO release from the endothelium causing vasodilation.³¹ Chronic exposure of the vascular bed to high shear and pressure forces in OSA might, however, ultimately lead to endothelial damage and dysfunction with reduced release of NO and consecutive paradoxical vasoconstriction.³² The increase in NO_x after the initiation of CPAP would then reflect the amelioration of OSA related blood pressure changes induced by this form of treatment.

It is important to point out that Duchna *et al* did not find restoration of vascular responsiveness in patients with OSA after the infusion of nitroglycerin.⁹ This suggests that defects in the second messenger systems activated by NO might contribute to the loss of endothelium dependent vascular relaxation in OSA. Such disturbances might thus add to the currently observed reduction in NO generation in patients with OSA.

The findings of the present study may have clinically important implications. The deficiency in the NO system might contribute to the development of hypertension and atherosclerosis, and thereby to the emergence of cardiovascular disease in OSA. CPAP treatment is believed to normalise life expectancy in patients with OSA by its favourable influence on the progression of cardiovascular disorders.^{2, 3} However, the mechanisms responsible for this beneficial effect of CPAP therapy are not well understood, and hitherto the evidence that CPAP reduces cardiovascular morbidity and mortality has come primarily from epidemiological observations.³³ Since NO deficiency has an important role in the pathogenesis of cardiovascular disease, we hypothesise that the increase in NO_x levels which occurs after starting CPAP therapy might constitute one mechanism by which CPAP therapy saves life in patients with OSA.

In conclusion, plasma levels of nitric oxide derivatives are significantly reduced in OSA and may be increased by both short and long term CPAP treatment. Although the precise mechanism underlying this observation remains to be fully elucidated, it may have important implications for the development of cardiovascular disease in patients with OSA and for the life saving effect of CPAP.

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