

# Evidence for activation of nuclear factor kappaB in obstructive sleep apnea

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**Abstract** Obstructive sleep apnea (OSA) is a risk factor for atherosclerosis, and atherosclerosis evolves from activation of the inflammatory cascade. We propose that activation of the nuclear factor kappaB (NF-kappaB), a key transcription factor in the inflammatory cascade, occurs in OSA. Nine age-matched, nonsmoking, and non-hypertensive men with OSA symptoms and seven similar healthy subjects were recruited for standard polysomnography followed by the collection of blood samples for monocyte nuclear p65 concentrations (OSA and healthy groups). In the OSA group, p65 and of monocyte production of tumor necrosis factor alpha (TNF- $\alpha$ ) were measured at the same time and after the next night of continuous positive airway pressure (CPAP). p65 Concentrations in the OSA group were significantly higher than in the control group [median, 0.037 ng/ $\mu$ l (interquartile range, 0.034 to 0.051) vs 0.019 ng/ $\mu$ l (interquartile range, 0.013 to 0.032);  $p=0.008$ ], and in the OSA group were significantly correlated with apnea-hypopnea index and time spent below an oxygen saturation of 90% ( $r=0.77$  and 0.88, respectively) after adjustment for age and BMI. One night of CPAP resulted in a reduction in p65 [to 0.020 ng/ $\mu$ l (interquartile range, 0.010 to 0.036),  $p=0.04$ ] and levels of TNF- $\alpha$  production in cultured monocytes [16.26 (interquartile range, 7.75 to 24.85) to 7.59 ng/ml (interquartile range, 5.19 to 12.95),  $p=0.01$ ]. NF-kappaB activation occurs with sleep-disordered breathing. Such activation of NF-kappaB may contribute to the pathogenesis of atherosclerosis in OSA patients.

**Keywords** Sleep apnea · Nuclear factor kappaB · Tumor necrosis factor alpha · Atherosclerosis · Cardiovascular disease

## Introduction

Obstructive sleep apnea (OSA) is a condition characterized by sleep disruption and cycles of intermittent hypoxemia, and it is a known risk factor for hypertension and cardiovascular disease [1–6]. Previous studies have demonstrated an independent relationship between the severity of OSA and the carotid artery intima-media thickness [7, 8], a marker for atherosclerosis, but the mechanisms underlying this association are not entirely understood. Immune response alterations in atherosclerosis have led it to be redefined as an inflammatory condition, a pathway operating in other chronic diseases [9, 10].

Activated nuclear factor kappaB (NF-kappaB) is a key transcription factor in chronic inflammatory diseases [11, 12]. Recently, exposure of transformed cervical cells (HeLa cells) to intermittent hypoxia was demonstrated to result in the activation of NF-kappaB [13]. This observation seemed potentially relevant to reports in OSA populations of increased circulating cytokines and proteins in the downstream pathways from NF-kappaB activation, including plasma levels of tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin (IL)-6 [14, 15], adhesion molecule-1 and vascular cell adhesion molecule-1 [16], and increased matrix metalloproteinases [17].

Our hypothesis was that circulating blood monocytes, a key player in the inflammatory theories for atherosclerosis, would exhibit NF-kappaB activation in OSA patients. We report nuclear fraction p65 levels, one marker of NF-kappaB activation, comparing OSA patients to healthy

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controls, and both p65 levels and TNF- $\alpha$  production in peripheral blood monocytes before and after one night of treatment of OSA with nasal continuous positive airway pressure (CPAP) to test this hypothesis.

## Materials and methods

**Subjects** Over a 12-month period of time, nine nonsmoking men, age <50 years, with OSA symptoms but without known hypertension or presence of a morning blood pressure elevation (systolic >140 and diastolic >90 mmHg) were recruited from approximately 100 patients referred to the sleep clinic for suspected sleep apnea. Other exclusion criteria were a known or suspected diagnosis of asthma, chronic obstructive pulmonary disease, arthritis, autoimmune diseases, stroke, rhinitis, and coronary artery disease. The primary symptoms in the OSA group were excessive daytime sleepiness and/or snoring and disturbed sleep, and OSA was confirmed by full standard polysomnography (PSG; see below). Seven male nonsmoking healthy volunteers with similar recruitment and exclusion criteria were recruited as control subjects from the greater hospital community and entered the study on the basis of absence of symptoms of snoring, disturbed sleep, or observed apneas. No patient or healthy subject was on prescribed medications. Before this study, we explained its purpose and the procedures involved to the subjects, and we obtained written consent, as approved by the Human Subjects Committee of Nara Medical University.

**Sleep study** PSG was performed using a computerized polysomnography system (Alice 4; Respironics; Pittsburgh, PA, USA). Data acquisition started from 9:00 p.m. and continued until 6:00 a.m. on the following morning. Apnea was defined as cessation of airflow for 10 s or more, while hypopnea was defined as a decrease in airflow by at least 50% for a minimum of 10 s or a clear decrease in airflow (>20%) followed by either oxygen desaturation of more than 3% or signs of physiological arousal. The apnea–hypopnea index (AHI) was calculated as the number of apnea–hypopnea events per hour of total sleeping time. To assess the severity of hypoxia induced by apnea–hypopnea events, we also measured the duration for which the oxygen saturation was below 90% and expressed this as a percentage of the total sleeping time (T90). We also calculated the oxygen desaturation index (ODI), defined as the number of dips in oxygen saturation by >3% per hour of sleep.

**CPAP** On the night after the diagnosis of OSA by PSG, patients underwent a second PSG during which apneas

were largely reduced or eliminated with nasal CPAP (REMstar Auto; Respironics).

**Isolation of peripheral blood monocytes for nuclear p65 levels and TNF- $\alpha$  production** Heparinized whole blood (50 ml) was collected from the OSA patients in the early morning after the diagnostic PSG and also in the early morning after the CPAP treatment PSG. In the control subjects, blood was collected once in the early morning after the PSG night. From the collected blood, peripheral blood monocytes were isolated using standard methods [18]. Greater than 90% of the cells thus obtained expressed CD14, which was detected by the fluorescent antibody method with a fluorescein isothiocyanate-labeled anti-CD14 antibody. In the OSA group, the remainder was placed in culture for TNF- $\alpha$  production. For measurement of NF-kappaB activation, nuclear fractions were prepared from  $0.5 \times 10^6$  monocytes using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Levels of nuclear p65 concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Active Motif). Measurement of TNF- $\alpha$  production was determined from short-term culture of isolated monocytes [18]. Two hundred microliters of the suspension ( $2.5 \times 10^6$  monocytes/ml) was poured into each well of a 96-well TC plate (3799 Corning, Corning). Lipopolysaccharide (*E. coli* 055:B5, Calbiochem, San Diego, CA, USA) was added to the cell suspension at a final concentration of 10  $\mu$ g/ml. After the plate was cultured for 24 h at 37°C under a 5% CO<sub>2</sub> atmosphere, the culture supernatant was collected and stored at –80°C until analysis. The TNF- $\alpha$  concentration was determined with a Human TNF- $\alpha$  ELISA kit (R&D System, Minneapolis, MN, USA).

## Statistical analysis

The power of the study was projected using previous studies of TNF- $\alpha$  measurement in OSA populations, with consideration of the number of subjects that could be recruited given the inclusion and exclusion criteria. Data are presented as medians and interquartile range (IQR). Comparison of continuous variables between OSA patients and control subjects was performed by the Mann–Whitney test. To investigate correlations between NF-kappaB and AHI, ODI and T90, we performed Spearman's correlation analysis. For statistical evaluation of changes in the variables in the morning before and after CPAP, the Wilcoxon signed rank test was used. A probability value of less than 0.05 was considered to indicate significance. Statistical analysis was done with SPSS software for Windows (version 10, SPSS, Chicago, IL).

## Results

Values indicative of OSA (AHI, ODI, T90, and the Epworth Sleepiness Scale) and BMI were significantly different between groups. Age was not statistically different (Table 1).

Morning p65 concentration after the diagnostic PSG was significantly higher in the OSA group than in the control group [median, 0.037 ng/ $\mu$ l (IQR, 0.034 to 0.051) vs 0.019 ng/ $\mu$ l (IQR, 0.013 to 0.032);  $p=0.008$ ] and was not affected by adjustment for BMI and age ( $p<0.001$ ; Fig. 1). Using values from the OSA group alone, p65 concentration adjusted for BMI and age were found to be significantly correlated with the AHI ( $r=0.77$ ), ODI ( $r=0.80$ ), and T90 ( $r=0.88$ ) during the preceding PSG (Fig. 2).

In OSA patients on CPAP the next night, the AHI was significantly lower on CPAP [62.3 (IQR, 21.7 to 72.5) to 4.0 (IQR, 3.0 to 4.8);  $p<0.001$ ], as was ODI [57.3 (IQR, 19.7 to 62.7) to 3.9 (IQR, 3.0 to 4.5);  $p<0.001$ ]. T90 was also significantly lower [19.8% (IQR, 0.3 to 25.3) to 0% (IQR, 0 to 0);  $p<0.001$ ]. In this group, p65 concentrations upon awakening before CPAP were significantly higher in the morning compared to those after one night of CPAP morning values  $\sim 24$  h later [0.037 ng/ $\mu$ l (IQR, 0.034 to 0.051) to 0.020 ng/ $\mu$ l (IQR, 0.010 to 0.036);  $p=0.03$ ]. The post-CPAP level of p65 concentration was no different from that in control subjects the morning after the PSG. In OSA patients, TNF- $\alpha$  production was lower after one night of CPAP [16.26 ng/ $\mu$ l (IQR, 7.75 to 24.85) to 7.59 ng/ $\mu$ l (IQR, 5.19 to 12.95);  $p=0.01$ ; Fig. 3).

**Table 1** Subject's characteristics

	OSA	Control	<i>p</i>
No. of subjects	9	7	
Age (year)	41.0 (34.5–46.5)	35.0 (32.8–36.8)	0.17
BMI (kg/m <sup>2</sup> )	29.6 (26.7–30.3)	23.3 (23.1–24.6)	0.003
ESS	11.0 (6.0–12.3)	5.0 (3.0–7.8)	0.03
AHI	62.3 (21.7–72.5)	4.0 (3.0–4.8)	<0.001
ODI	57.3 (19.7–62.7)	3.9 (3.0–4.5)	<0.001
T90%	19.8 (0.3–25.3)	0 (0–0)	<0.001

Data are presented as median (interquartile range).

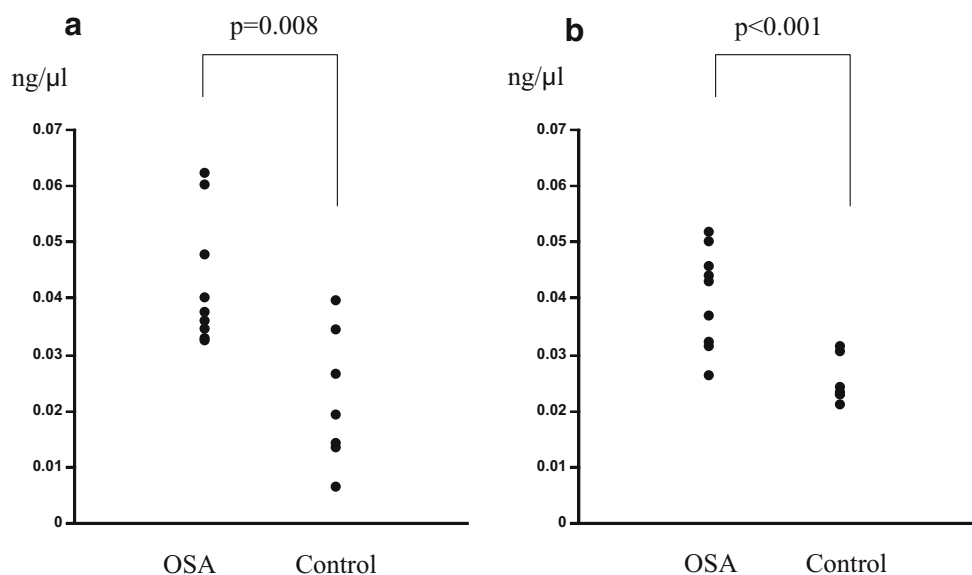
*BMI* Body mass index, *ESS* Epworth Sleepiness Scale, *AHI* apnea-hypopnea index, *ODI* oxygen desaturation index, *T90* percentage of time spent with SpO<sub>2</sub><90%.

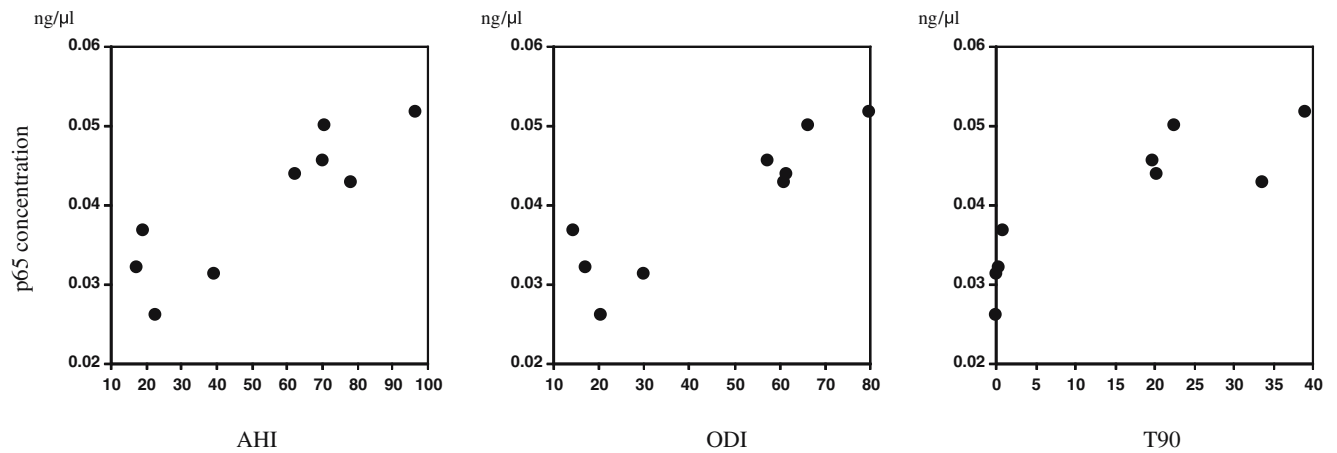
## Discussion

The present study shows that there is an activation of NF-kappaB in peripheral blood monocytes, as a representative transcription factor involved in regulating inflammation, and there is evidence showing that this activation is functionally reversed by one night of CPAP titration.

Of the potential mechanisms for this activation of NF-kappaB, our results implicate that events (AHI, ODI, and T90) in sleep are different from healthy subjects and reversed by one night of CPAP. Recent studies have proposed the importance of the oxidative stress in OSA in the development of cardiovascular diseases [19–25]. Reactive oxygen species (ROS) are produced during reoxygenation [26, 27], which occurs after episodes of sleep apnea, and ROS were implicated in the cell culture demonstration

**Fig. 1** Monocyte NF-kappaB activity as measured by p65 concentration of OSA patients and control subjects in the morning. **a** Unadjusted p65 concentration, **b** p65 concentration adjusted for BMI and age





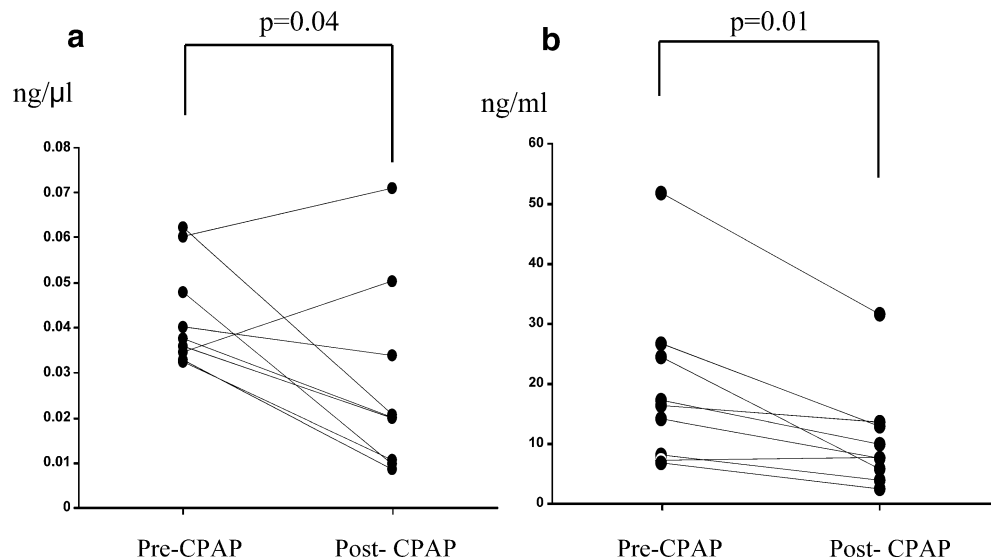
**Fig. 2** Scatterplots of p65 concentration adjusted for BMI and age vs apnea–hypopnea index (AHI; left), oxygen desaturation index (ODI; middle), and percentage of time spent with SpO<sub>2</sub><90% (T90; right). p65 Concentration were significantly correlated with AHI ( $r=0.77$ ), ODI ( $r=0.80$ ), and T90 ( $r=0.88$ ) by Spearman’s correlation analysis

of NF-kappaB activation by cycles of hypoxia and reoxygenation [11, 13]. Therefore, our findings indicate this effect. CPAP acts to improve oxygenation but could have other conceivable but less likely effects, for instance effects on sleep continuity or sleep architecture.

With regard to the relevance of the association of cardiovascular disease and OSA, various pro-inflammatory cytokines, including TNF- $\alpha$ , IL-6, and adhesion molecules, induce the monocyte migration into the endothelial cells, after which the monocytes become foam cells and contribute to the progression of atherosclerosis [9]. NF-kappaB regulates the genes of pro-inflammatory cytokines, chemokines that attract inflammatory cells to sites of inflammation. This suggests that higher monocyte and circulating levels of inflammatory cytokines and adhesion molecules would contribute, if not initiate, atherosclerosis in those with OSA.

There are potential limitations of our study. First, the BMI of the OSA patients was higher than that of the control subjects. Although we used statistical adjustments in an attempt to eliminate the effect of BMI, we cannot completely exclude its influence. One of the issues effecting this was that we had a relatively small sample size, which handicapped our ability to control for the effect of BMI. However, we also found a decline of NF-kappaB activation and a decrease in the production of TNF- $\alpha$  by monocytes after only one session of CPAP, suggesting that obesity, per se, has only a small effect in this population. Unfortunately, it remains unknown what the effect of CPAP on NF-kappaB would be in clinically normal controls. It may be the case that CPAP lowers the levels of NF-kappaB in both those with OSA and those without. Second, p65 is only one marker of NF-kappaB activity and TNF- $\alpha$  production by monocytes is not solely dependent on NF-

**Fig. 3 a** Level of p65 concentration (nuclear fraction) in monocytes in the morning before and in the morning after CPAP. **b** Level of TNF- $\alpha$  production by cultured monocytes from the morning before and in the morning after CPAP. Symbols connected by lines indicate results from an individual patient



kappaB. Values of p65 and the impact of CPAP varied even in this highly selected population, suggesting that variations on this relationship may exist in clinical populations, including OSA in women, children, or the elderly. Third, it is known that TNF- $\alpha$  itself activates NF-kappaB [11]. Thus, it is still unclear what the causal relationship between a decline of NF-kappaB activation and a decrease in the production of TNF- $\alpha$  by monocytes after CPAP is.

Unlike better characterized diseases like asthma where atopy is a known trigger, in OSA the triggers are related to recurrent apneas during sleep, and monocyte NF-kappaB activity is modified by a CPAP treatment. These results suggest that the modification of inflammation is a specific target for treatments of OSA. The presence or development of obesity, hypertension, or hyperlipidemia may amplify the effects of sleep apnea, and, hence, the process of atherosclerosis.

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## References

- Lavie P, Herer P, Hoffstein V (2000) Obstructive sleep apnoea syndrome as a risk factor for hypertension: population study. *BMJ* 320:479–482
- Peppard PE, Young T, Palta M, Skatrud J (2000) Prospective study of the association between sleep-disordered breathing and hypertension. *N Engl J Med* 342:1378–1384
- Peker Y, Kraiczi H, Hedner J, Loth S, Johansson A, Bende M (1999) An independent association between obstructive sleep apnoea and coronary artery disease. *Eur Respir J* 14:179–184
- Dyken ME, Somers VK, Yamada T, Ren ZY, Zimmerman MB (1996) Investigating the relationship between stroke and obstructive sleep apnea. *Stroke* 27:401–407
- Schafer H, Koehler U, Ewig S, Hasper E, Tasci S, Luderitz B (1999) Obstructive sleep apnea as a risk marker in coronary artery disease. *Cardiology* 92:79–84
- Minoguchi K, Yokoe T, Tazaki T, Minoguchi H, Tanaka A, Oda N, Okada S, Ohta S, Naito H, Adachi M (2005) Increased carotid intima-media thickness and serum inflammatory markers in obstructive sleep apnea. *Am J Respir Crit Care Med* 172:625–630
- Drager LF, Bortolotto LA, Lorenzi MC, Figueiredo AC, Krieger EM, Lorenzi-Filho G (2005) Early signs of atherosclerosis in obstructive sleep apnea. *Am J Respir Crit Care Med* 172:613–618
- Suzuki T, Nakano H, Maekawa J, Okamoto Y, Ohnishi Y, Yamauchi M, Kimura H (2004) Obstructive sleep apnea and carotid-artery intima-media thickness. *Sleep* 27:129–133
- Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. *Circulation* 105:1135–1143
- Glass CK, Witztum JL (2001) Atherosclerosis: the road ahead. *Cell* 104:503–516
- Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 336:1066–1071
- Monaco C, Paleolog E (2004) Nuclear factor kappaB: a potential therapeutic target in atherosclerosis and thrombosis. *Cardiovasc Res* 61:671–682
- Ryan S, Taylor CT, McNicholas WT (2005) Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. *Circulation* 112:2660–2667
- Entzian P, Linnemann K, Schlaak M, Zabel P (1996) Obstructive sleep apnea syndrome and circadian rhythms of hormones and cytokines. *Am J Respir Crit Care Med* 153:1080–1086
- Yokoe T, Minoguchi K, Matsuo H, Oda N, Minoguchi H, Yoshino G, Hirano T, Adachi M (2003) Elevated levels of C-reactive protein and interleukin-6 in patients with obstructive sleep apnea syndrome are decreased by nasal continuous positive airway pressure. *Circulation* 107:1129–1134
- Chin K, Nakamura T, Shimizu K, Mishima M, Nakamura T, Miyasaka M, Ohi M (2000) Effects of nasal continuous positive airway pressure on soluble cell adhesion molecules in patients with obstructive sleep apnea syndrome. *Am J Med* 109:562–567
- Tazaki T, Minoguchi K, Yokoe T, Samson KT, Minoguchi H, Tanaka A, Watanabe Y, Adachi M (2004) Increased levels and activity of matrix metalloproteinase-9 in obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 170:1354–1359
- Havlic DV, Ellner JJ, Chervenak KA, Boom WH (1991) Selective expansion of human gamma delta T cells by monocytes infected with live *Mycobacterium tuberculosis*. *J Clin Invest* 87:729–733
- Schulz R, Mahmoudi S, Hattar K, Sibelius U, Olschewski H, Mayer K, Seeger W, Grimminger F (2000) Enhanced release of superoxide from polymorphonuclear neutrophils in obstructive sleep apnea. Impact of continuous positive airway pressure therapy. *Am J Respir Crit Care Med* 162:566–570
- Dyugovskaya L, Lavie P, Lavie L (2002) Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* 165:934–939
- Barcelo A, Miralles C, Barbe F, Vila M, Pons S, Agusti AG (2000) Abnormal lipid peroxidation in patients with sleep apnoea. *Eur Respir J* 16:644–647
- Christou K, Moulas AN, Pastaka C, Gourgoulisanis KI (2003) Antioxidant capacity in obstructive sleep apnea patients. *Sleep Med* 4:225–228
- Carpagnano GE, Kharitonov SA, Resta O, Foschino-Barbaro MP, Gramiccioni E, Barnes PJ (2003) 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* 124:1386–1392
- Lavie L, Vishnevsky A, Lavie P (2004) Evidence for lipid peroxidation in obstructive sleep apnea. *Sleep* 1(27):123–128
- Yamauchi M, Nakano H, Maekawa J, Okamoto Y, Ohnishi Y, Suzuki T, Kimura H (2005) Oxidative stress in obstructive sleep apnea. *Chest* 127:1674–1679
- McCord JM (2000) The evolution of free radicals and oxidative stress. *Am J Med* 108:652–659
- McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 312:159–163