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Lipid peroxidation and osmotic fragility of red blood cells in sleep-apnea patients☆

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Abstract

Background: Obstructive sleep apnea (OSA) refers to the occurrence of episodes of complete or partial pharyngeal obstruction with oxyhemoglobin desaturation during sleep. These hypoxia/reoxygenation episodes may cause generation of reactive oxygen species. Reactive oxygen species are toxic to biomembranes and may lead to the peroxidation of lipids. We tested the hypothesis that obstructive sleep apnea is linked to increased oxidative stress and lipid peroxidation. In order to identify target tissue/cell damage, we studied the osmotic fragility of red blood cells. *Methods*: Six subjects polysomnographically diagnosed as obstructive sleep apnea syndrome and 10 controls were included. After all subjects gave written informed consent, blood samples were collected in the morning between 08:00 and 09:00 a.m. following polysomnography. Blood samples were immediately transferred to the laboratory. Glutathione, lipid peroxidation and osmotic fragility of red blood cells were measured manually. *Results*: Mean glutathione and lipid peroxidation concentrations of patients were not different than those of control subjects (105.6 ± 38.6 U/g Hb and 3.1 ± 2.3 nmol MDA/l vs. 100.6 ± 62.1 U/g Hb and 3.2 ± 2.8 nmol MDA/l, respectively). In both groups, osmotic fragility of red blood cells was not changed. *Conclusion*: The present study failed to support the hypothesis that obstructive sleep apnea is linked with increased oxidative stress and lipid peroxidation.

Keywords: Sleep apnea; Lipid peroxidation; Osmotic fragility; Glutathione

1. Introduction

Obstructive sleep apnea (OSA) refers to the occurrence of episodes of complete or partial pharyngeal obstruction during sleep. It is characterized by cyclical alterations of arterial oxygen concentration with oxy-

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hemoglobin desaturation developing in response to apneas followed by resumption of oxygen saturation during hyperventilation [1]. These hypoxia/reoxygenation episodes, which may, to some extent, be compared with ischemia/reperfusion [2], may cause an imbalance between reactive oxygen species and the antioxidant reserve that is important for detoxification of these molecules.

Reactive oxygen species are generated as by-products of oxidative metabolism particularly in mitochondria of aerobic cells as well as in red blood cells (RBC) concomitant to spontaneous oxidation of hemoglobin to methemoglobin [3]. Oxygen free radicals are highly reactive and may cause cell and tissue damage by interacting with cell membranes and organelles. Extensive lipid peroxidation in biological membranes causes loss of fluidity, decrease in membrane potential, increased permeability to ions and eventual rupture leading to release of cell and organelle contents [4]. Red blood cell membrane is also prone to lipid peroxidation owing to its high content of polyunsaturated lipids [5] and it has been extensively used to investigate the role of oxidative membrane damage in various pathological conditions [6,7]. Abnormal susceptibility of RBC lipids to peroxidation is known to reflect similar abnormalities in other organs and tissues [6].

Recent studies accumulated evidence against or in support of increased oxidative stress within the vascular system of patients with OSA. Schulz et al. [2] have shown that the release of free radicals from circulating neutrophils is markedly enhanced in OSA patients. Increased superoxide radical generation, which might have a major impact on the development of cardiovascular disorders, was fully reversed by effective continuous positive airway pressure therapy. Wali et al. [8] failed to find any difference in low-density lipoprotein (LDL) peroxidation and antioxidant enzyme activities between OSA and control subjects. However, data referring to existence and consequences of lipid peroxidation under apneic conditions are not abundant, and thus it is not very clear which enzymes, metabolites and tissues are more sensitive to oxidative stress and hence lipid peroxidation. In this study, we tested the hypothesis that obstructive sleep apnea is linked to increased oxidative stress and lipid peroxidation. We also studied osmotic fragility of RBCs in an attempt to identify target tissue/cell damage.

2. Methods

2.1. Subjects

Sixteen non-smoking patients (52 ± 11 years) with suspected sleep apnea were participated in the study. Six subjects were diagnosed as obstructive sleep apnea syndrome. The remaining 10 subjects without sleep apnea served as controls. The study was approved by the local Ethics Committee and written informed consent was signed by the patients before recruitment. All participants underwent venous blood sampling between 08:00 and 09:00 a.m. after an overnight fast following the polysomnographic recording. Blood samples were immediately transferred to the laboratory for glutathione (GSH) and lipid peroxidation measurements. Osmotic fragility of RBCs was studied in fresh blood samples during the sampling day.

2.2. Sleep study

Polygraphic sleep study was performed by using a computerized polysomnography system (Alice 3, Respironics, Pittsburgh, PA). To determine the stages of sleep, two channels electroencephalogram (C4-A1, C3-A2), chin electromyogram, and left and right electrooculograms were obtained. Thoracoabdominal movements were monitored by thoracic and abdominal strain gauges. Airflow was monitored by oronasal thermistor. Arterial oxyhemoglobin saturation was recorded by using a pulse oxymeter. Electrocardiogram, snoring and body position were also recorded. Recordings were manually scored according to standard criteria [9]. An episode of obstructive apnea was defined as the absence of airflow for at least 10 s in the presence of ribcage and abdominal excursions. Hypopnea was defined as 50% reduction in airflow with respect to baseline lasting 10 s or more and associated with at least a 4% decrease in arterial oxyhemoglobin saturation, an electroencephalographic arousal or both. The number of episodes of apnea and hypopnea per hour is referred to as the apnea–hypopnea index.

2.3. RBC lipid peroxidation and GSH measurements

RBC lipid peroxidation was measured as described by Stocks and Dormandy [10]. Hydrogen-peroxide

Table 1
Characteristics of OSA patients and control subjects[a]

	OSA patients ($n=6$)	Control subjects ($n=10$)
Age (y)	45 ± 8	51 ± 10
Sex (M/F)	5:1	9:1
Height (cm)	175 ± 11	171 ± 9
Body mass index[b]	31 ± 3	29 ± 2
Hemoglobin (g/dl)	14.7 ± 0.9	13.9 ± 1.8
Hematocrit (%)	43 ± 2	41 ± 4
RBC Count ($\times 10^3$/mm^3)	4973 ± 421	4626 ± 408

[a] Values represent mean ± S.D. There were no significant differences between the two groups.
[b] Body mass index is the weight in kg/height^2 in meters.

induced lipid peroxidation was determined after incubation with H_2O_2 for 2 h at 37 °C. The final incubation mixture contained 5 mmol/l H_2O_2, 1.65 mmol/l NaN_3 and erythrocyte suspension in phosphate-buffered saline (14 mg hemoglobin/ml of incubation mixture). Lipid peroxidation was assayed by measurement of malondialdehyde (MDA) production using 1,1,3,3-tetraethoxypropane as standard. Results were expressed as nmol MDA/g hemoglobin.

GSH concentrations were determined according to Beutler [11]. RBC hemolysates were incubated in a solution containing glacial metaphosphoric acid (1.67%, w/v), disodium EDTA (0.2%, w/v) and sodium chloride (30.0%, w/v). After centrifugation the supernatants were mixed with 0.3 mmol/l Na_2HPO_4 and Ellman's reagent (0.4% dithio-*bis*-nitrobenzoic acid). Absorbances were measured at 412 nm and data were expressed as U/g hemoglobin.

2.4. Osmotic fragility

Osmotic fragility of RBCs was determined by the method of Dacie and Lewis [12]. Briefly, heparinized venous blood (0.01 ml) was added into tubes with increasing concentration of buffered salt solution (pH 7.4; NaCl (%) 0, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.70, 0.90). The tubes were gently mixed and incubated at 25 °C for 30 min. Then, the samples were centrifuged at 1500 × *g* for 10 min, absorbances were measured at 540 nm.

Hemolysis in each tube was expressed as percentage of the absorbance in distilled water.

2.5. Statistical analysis

Distribution of variables was tested by Kolmogorov–Smirnof test. Comparisons of means between the two groups were performed by Mann–Whitney *U*-test. A $p<0.05$ was considered as statistically significant.

3. Results

There were no significant differences between the patients who met the criteria for obstructive sleep apnea and those who did not with respect to demographic characteristics and body mass index (Table 1). None of the patients were on medications and/or taking alcohol or vitamin supplements. Among patients with obstructive sleep apnea, the mean apnea–hypopnea index was 37 ± 16 episodes/h. Eventually these patients had arterial oxyhemoglobin desaturation and a high number of arousals from sleep (Table 2). The differences in AHI and the severity of oxyhemoglobin desaturation were highly significant in patient and control groups ($p<0.001$ and $p<0.01$, respectively). Table 2 lists the characteristics of sleep

Table 2
Characteristics of OAS patients and control subjects during sleep[a]

	OSA patients ($n=6$)	Control subjects ($n=10$)
Total dark time (min)	464.5 ± 75.9	464.4 ± 26.5
Total sleeping time (min)	411.8 ± 56.3	403.7 ± 43.3
Sleep efficiency (%)[b]	88 ± 5	86 ± 6
Wakefulness after sleep onset (min)	38 ± 25	50 ± 35
Sleep stage (% of total sleeping time)		
1	6.8 ± 2.3	5.7 ± 3.1
2	180.6 ± 62.9	228.7 ± 38.3
3–4	143.5 ± 50.4	101.4 ± 44.9
Rapid Eye Movement	75.5 ± 9.9	62.9 ± 42.8
Apnea–hypopnea index (episodes/hr)	37 ± 16[‡]	2 ± 1
Arterial oxyhemoglobin saturation		
Baseline value (%)	93	94
Lowest value (%)	77[§]	88
<90% (min)	58.2[‡]	3.8

[a] Values represent mean ± S.D.
[b] Sleep efficiency was calculated as the ratio of total sleeping time to total dark time.
[‡] $p<0.001$ when compared with control subjects.
[§] $p<0.01$ when compared with control subjects.

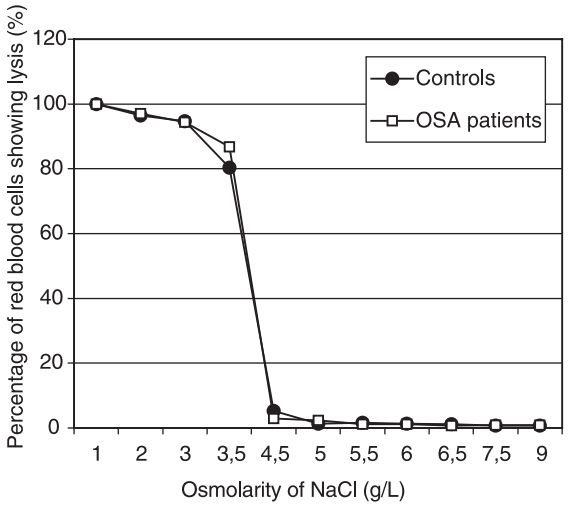

Fig. 1. Osmotic fragility curves of OSA patients and control subjects. Heparinized venous blood (0.01 ml) was added into tubes with increasing concentration of buffered salt solution (pH 7.4). The tubes were gently mixed and incubated at 25 °C for 30 min. Then, the samples were centrifuged at 1500 × *g* for 10 min, absorbances of the supernatants were measured at 540 nm. Hemolysis in each tube was expressed as percentage of the absorbance in distilled water.

and disordered breathing events and oxyhemoglobin saturation parameters during sleep.

The mean GSH and lipid peroxidation concentrations in OSA patients (105.6 ± 38.6 U/g Hb and 3.1 ± 2.3 nmol MDA/l, respectively) were at the same concentration with those of the control subjects (100.6 ± 62.1 U/g Hb and 3.2 ± 2.8 nmol MDA/l). Similarly, RBC osmotic fragility of both groups failed to differ from each other. Fig. 1 shows the mean of osmotic fragility curves of OSA patients and control subjects.

4. Discussion

The prevalance of cardiovascular disease is increased in OSA [13]. It is thought that this association may be the cause of increased mortality seen in untreated OSA patients [1]. On the other hand, considerable data suggest that LDL oxidation triggers a number of events that can promote both the establishment and progression of atherosclerosis [14] which is a hallmark of cardiovascular disease. Thus, interest in the effects of lipid peroxidation is both clinically relevant to discerning the pathophysiological mechanisms of cardiovascular disease in OSA patients and physiologically relevant to understanding the adaptive changes that occur in response to hypoxia/reoxygenation episodes. In the present study, we failed to find increased RBC lipid peroxidation in OSA patients when compared to control subjects. To our knowledge, there are only two studies in the literature which have investigated lipid peroxidation in OSA. One of these studies [8] failed to identify abnormal or elevated lipid peroxidation in OSA in accordance with our results. The other study reported abnormal lipid peroxidation which was improved by chronic use of continuous positive airway pressure [15].

GSH plays a major role in cellular defense against oxidative stress and is the most abundant antioxidant in cells [16]. GSH can directly scavenge free radicals [17] or act as a substrate for glutathione peroxidase and glutathione-*S*-transferase in detoxification of lipid peroxides [18]. Various oxidants have been reported to cause either a direct increase [19] or first an initial acute decrease and then an overproduction of GSH [20]. Oxidized LDL has also been shown to cause an initial decrease followed by an adaptive increase of GSH in different cell types [21]. Since OSA is a chronic long-term disease, one can expect increased GSH concentrations in response to suspected abnormal lipid peroxidation. Furthermore, intracellular GSH concentrations are mainly regulated by the activity of rate-limiting enzyme γ-glutamylcysteine synthetase [22] which has been shown to increase under oxidative stress [23]. In this study, an adaptive increase was not found in RBC GSH concentrations of OSA patients. This finding is consistent with the absence of increase in lipid peroxidation concentrations of the same patient group.

Lipid peroxidation is a ubiquitous phenomenon in the body under the influence of oxidative stress. Thus, it cannot be restricted to one organ or tissue type. We therefore studied RBCs to determine the end organ effect of oxidative stress. We found that there was no alteration in osmotic fragility of RBCs in OSA patients. To our knowledge, this is the first study that has evaluated osmotic fragility in OSA. Since Stocks and Dormandy [10] performed first exhaustive experiments in human RBCs showing the ability of oxidative stress to induce lipid peroxidation and haemolysis in 1971, many in vitro and in vivo studies showed that

RBC function and integrity are negatively affected by increased oxidative stress. The challenge of RBCs with different oxygen radical-generating systems led to increase in lipid peroxidation [24], oxidation of protein groups [25] and energy metabolism changes including glycolytic enzymes and AMP-deaminase activity [26]. On the basis of the above-mentioned findings, we hypothesized that if there is an increase in lipid peroxidation in OSA patients, then there should be increased osmotic fragility as well. In fact, absence of osmotic fragility changes has suggested the absence of abnormal lipid peroxidation.

In this study, only six patients and 10 control subjects were studied. Although the amount of hypoxia and the severity of disease were distinctive between the two groups, it can be questioned whether these data are applicable to large member of OSA patients. We acknowledge that the present study did not assess all parameters of oxidative stress and antioxidant system. These include superoxide radical, superoxide dismutase, catalase and glutathione peroxidase. Adding these parameters would increase the validity of our study. In conclusion, the results of this preliminary study did not support the hypothesis that OSA may be linked to abnormal lipid peroxidation and suggested that RBCs of OSA patients were not prone to haemolysis as evidenced by osmotic fragility test.

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