In vivo Platelet Activation Is Increased during Sleep in Patients with Obstructive Sleep Apnea Syndrome

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Cardiovascular events · Obstructive sleep apnea syndrome · Platelet activation · Platelet-derived microparticles · Stroke

Abstract
Background: Patients with obstructive sleep apnea syndrome (OSAS) have an increased risk of cardiovascular events including myocardial infarction and stroke. Objective: To determine whether in vivo platelet activation and the generation of procoagulant platelet-derived microparticles (PMP) are increased during sleep in patients with OSAS. Methods: In vivo platelet activation and PMP formation was determined using flow cytometry in 12 patients with untreated OSAS during and after sleep (4 and 7 a.m.). To study the effect of treatment with continuous positive airway pressure (CPAP), the measurements were repeated at the same time points after initiation of CPAP therapy. Healthy volunteers served as controls (n = 6). Results: Patients with OSAS had an increased percentage of platelets positive for the activation-dependent epitopes CD63 and CD62P during sleep (4 a.m.) compared to controls (4.8 ± 0.8% vs. 1.9 ± 0.4% for CD63, p < 0.01, and 2.0 ± 0.5 vs. 1.1 ± 0.3% for CD62P, p < 0.05). In OSAS patients, the amount of CD63- and CD62P-positive platelets was significantly elevated at 4 compared to 7 a.m. (4.8 ± 0.8 vs. 2.6 ± 0.4% for CD63 and 2.0 ± 0.5 vs. 1.1 ± 0.2% for CD62P, p < 0.05), but not in the control group. The levels of PMP were similar in patients with OSAS and controls at 4 and 7 a.m. After 1 night of CPAP therapy, there was a trend to reduced levels of CD63- and CD62P-positive platelets at 4 a.m. Conclusions: Patients with OSAS have increased in vivo platelet activation during sleep, which may contribute to the increased incidence of cardiovascular events in patients with OSAS.

Introduction
Patients with an obstructive sleep apnea syndrome (OSAS) have an increased mortality [1] and are at an increased risk of cardiovascular events such as myocardial infarction [2] and stroke [3]. Both events frequently occur during sleep and in the early morning hours [4–6]. Snoring and OSAS have been suggested as possible risk factors for myocardial infarction and stroke [7, 8].
Increased platelet activation was suggested as a potential cause for the increased incidence of cardiovascular events in patients with OSAS. Spontaneous platelet activation was increased in patients with OSAS [9], and an increased frequency of myocardial infarction and sudden cardiac death in the early morning hours was shown to be associated with increased platelet aggregability [10]. Platelet-derived microparticles (PMP) are generated during platelet activation and have a potent procoagulant activity facilitating the assembly of the prothrombinase complex and accelerating the coagulation cascade and thrombin formation several thousand times [11]. Previous studies have shown that PMP formation might be clinically relevant in cerebrovascular disease [12, 13]. However, the possible role of PMP in OSAS is not known.

We hypothesized that in patients with OSAS, in vivo platelet activation and/or PMP formation are increased during sleep compared to healthy controls. We therefore measured in vivo platelet activation and PMP formation using flow cytometry with activation-specific antibodies during and after sleep (at 4 and 7 a.m.) in patients with OSAS and healthy controls. To elucidate a possible effect of nasal continuous positive airway pressure (nCPAP) therapy on in vivo platelet activation and PMP formation, the measurements were done without and with CPAP therapy during 2 consecutive nights.

**Patients and Methods**

**Patients and Controls**

Twelve consecutive patients hospitalized on a routine basis for CPAP training were included in the study after having given informed consent. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bern (Switzerland). The diagnosis of OSAS was made prior to hospitalization by clinical history and polysomnography [apnea-hypopnea index (AHI) >10/h] and before CPAP therapy. Patients treated with anticoagulants, aspirin or glucocorticoids were excluded from the study. A detailed history was taken before examinations, focusing on symptoms and cardiovascular risk factors (arterial hypertension, smoking history, hypercholesterolemia or diabetes). Moreover, lung function studies including spirometry and arterial blood gases were performed.

In all patients, a polysomnography was performed before study entry, in order to confirm and quantify the OSAS and to rule out any other sleep disturbances. For standard polysomnography, two electroencephalograms, two electro-oculograms, three electromyograms (submental, left and right titial), oronasal flow (thermistors), thoracoabdominal movements (respiratory inductive plethysmography, Respiritrace), electrocardiography, and position were recorded.

Six healthy volunteers served as controls according to the protocol. In all controls, the presence of OSAS was excluded by measuring the AHI with the diagnostic AutoSet [14] during the study night. The AHI was below 10/h in each control (5.0 ± 2.9/h). The 6 controls were non-smokers without any risk factor for cardiovascular disease and did not take any medication 2 weeks prior to the examination. The body mass index of the control group was 23.2 ± 0.7 compared to 28.5 ± 4.8 kg/m² in the patient group (p < 0.01).

**Study Protocol**

Hospital admission was on a routine basis for 2 nights to introduce and adjust nCPAP treatment in patients with previously diagnosed OSAS. During the first night, a diagnostic reassessment with the AutoSet device (AutoSet, RespMed, Australia) [14] was performed without CPAP. During the 2nd night, CPAP titration with the AutoSet device was performed with automatic adjustment of the CPAP pressure. During both nights, transcutaneous oxygen saturation was continuously measured. Blood sampling was done during sleep at 4 a.m. and after awakening at 7 a.m. on both nights (with and without CPAP treatment).

**Blood Sampling**

Blood sampling was standardized and always performed by the same person (F.B.). At 4 a.m. the patients and controls were woken up, kept in the supine position and blood was immediately drawn. At 7 a.m. most of the patients were awake for 30–60 min, but still in supine position and under CPAP therapy (after the 2nd night) until blood was drawn. No permanent intravenous lines were used because of possible platelet activation and activation of the coagulation system. Instead, the procedure was performed by a clean venipuncture from an antecubital vein (Monovette system, Sarstedt, Nuembrecht, Germany) under controlled venous stasis at 60 Torr for less than 45 s. Previous studies have shown normal values for fibrinopeptide A and thrombin-antithrombin III complexes, indicating that no fibrin or thrombin generation has taken place using this technique [15].

**Flow Cytometry**

The procedures for ex vivo detection of activated platelets and PMP have been described previously [13, 16]. Briefly, whole blood anticoagulated with acid citrate dextrose was immediately fixed after blood sampling by adding 1 ml 1% paraformaldehyde (ratio 1:1) in phosphate-buffered saline (20 mM, containing 0.33 g/l NaH2PO4·H2O, 3.06 g/l Na2HPO4·2 H2O, and 8.18 g/l NaCl, adjusted to pH 7.4), followed by incubation with the primary antibodies for 30 min at a final concentration of 10 μg/ml. Two different, independent antibodies specific for activated platelets were used: CD62P (P-selectin, Becton-Dickinson, San Jose, Calif., USA) that is released on the platelet membrane after platelet granule secretion and CD63 (Immunotech) that appears on the platelet membrane after lysosome degranulation. Platelets and PMP were identified in whole blood using mAb 7H2 directed against GPIIbα and mAb 6D1 directed against GPIbα, respectively (kindly provided by Dr. B.S. Coller, Mount Sinai Hospital, New York, N.Y., USA). Non-specific mouse IgG1 (Dako, Denmark) and phosphate-buffered saline instead of the primary antibody were used as negative controls. Fluorescein-isothiocyanate-labeled goat anti-mouse antibody F(ab') fragment (final concentration 1.5 μg/ml; Dako) was then added without washing (in order not to lose any PMP) and incubated for another 30 min. All steps were performed at room temperature. The samples were analyzed on a Becton-Dickinson FACSScan equipped with a 15-mW air-cooled argon laser (Becton-Dickinson) and 10,000 gated events per sample were analyzed using the Cell Quest software (Becton-Dickinson). Standard beads of different sizes (2, 0.5, 0.1,
Table 1. Platelet activation and PMP formation in patients with OSAS

<table>
<thead>
<tr>
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<th>Patients (n = 12)</th>
<th>Controls (n = 6)</th>
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<tr>
<td></td>
<td>without CPAP</td>
<td>with CPAP</td>
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<tr>
<td></td>
<td>4 a.m. 7 a.m.</td>
<td>4 a.m. 7 a.m.</td>
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<tr>
<td>Platelet activation, %</td>
<td></td>
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<tr>
<td>Anti-CD 63</td>
<td>4.8 ± 0.8</td>
<td>2.6 ± 0.4</td>
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<td></td>
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<td>2.5 ± 0.5</td>
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<tr>
<td>Anti-CD 62P</td>
<td>2.0 ± 0.5</td>
<td>1.1 ± 0.2</td>
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<td></td>
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<td>1.3 ± 0.4</td>
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<tr>
<td>PMP formation, %</td>
<td></td>
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<tr>
<td>Anti-GPIb</td>
<td>11.5 ± 1.0</td>
<td>9.7 ± 0.7</td>
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<td></td>
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<td>11.0 ± 0.9</td>
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<tr>
<td>Anti-GPIIIa</td>
<td>12.0 ± 0.8</td>
<td>10.8 ± 0.8</td>
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<td>10.0 ± 0.7</td>
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* p < 0.01, b p < 0.05 (night vs. morning levels), c p < 0.01, d p < 0.05 (patients vs. controls).

0.01 μm) were used to calibrate the system (Polysciences, Warrington, Pa., USA). Antibody binding was expressed as the percentage of platelets positive for antibody.

PMP were defined by their size and their specific positive fluorescence for platelet glycoprotein and quantified as described by Warkentin et al. [16]. Briefly, the number of GPIb- or GPIIIa-positive particles of less than 87 relative fluorescence units was expressed as percentage of the total number of GPIb- or GPIIIa-positive particles gated. This cutoff value was derived from the analysis of 25 normal donors, in accord with the findings of Warkentin et al. [16]. This threshold (87 fluorescence units) optimally separates the well-defined platelet population from PMP, while non-specific binding of the control mouse mAb sets a much lower fluorescence margin.

Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was done by Mann-Whitney U test, unpaired Student’s t test, paired t test, or ANOVA, where appropriate. Intergroup differences were further analyzed by a post-hoc Student-Newman-Keuls analysis. The results were considered significant if p < 0.05.

Results

Patient Characteristics

The mean age of the 12 patients enrolled in the study (all men) was 59.8 ± 9.5 years. All patients reported three or more of the typical signs of OSAS (excessive daytime sleepiness, chocking or gasping during sleep, recurrent awakenings during sleep, unrefreshing sleep, daytime fatigue, or impaired concentration [17]). Three patients had arterial hypertension (systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg), 2 patients were current and 3 patients were former smokers. One patient had hypercholesterolemia; none of the patients had diabetes. One patient reported a transient ischemic attack 2 years ago, otherwise there were no thromboembolic complications such as myocardial infarction, stroke or pulmonary emboli. Medication consisted of ACE inhibitors (2 patients), calcium antagonists (3 patients), β-blockers (1 patient), diuretics (3 patients), uricoscutasis (1 patient) and tyroxin substitution (1 patient). Three patients had mild obstructive lung disease with an FEV1 >80% predicted. None of the patients had chronic hypoxia or hypercapnia.

Six patients had a mild-to-moderate OSAS with an AHI below 25/h (mean AHI 20.3 ± 34.5/h), 5 patients had a moderate-to-severe OSAS with an AHI between 25 and 50/h (33.4 ± 6.4/h), and 1 patient had a severe OSAS with an AHI of >50/h (54.5/h). Apneic episodes were accompanied by minimal oxygen saturation of 73.6 ± 8.9%.

Platelet Activation in Patients with OSAS Compared to Controls

The amount of CD63-positive platelets in patients with OSAS during sleep (at 4 a.m.) was significantly increased compared to controls using antibodies towards the activation-dependent CD63 (fig. 1) and CD62P (table 1). At 7 a.m., patients with OSAS had an increased level of CD63-positive platelets compared to controls. However, this difference could not be confirmed using anti-CD62P (table 1). The total number of platelets was not significantly different in patients compared to controls, neither at 4 nor at 7 a.m. (232 ± 17.1 vs. 242 ± 14.6 × 10⁹ at 7 a.m.). These data indicate that increased platelet activation is present in patients with OSAS during sleep compared to normal controls. We did not find significant correlations between the levels of platelet activation (CD63- and CD62P-positive platelets) and the sever-
Platelet Activation in Patients with OSAS at 4 Compared to 7 a.m.

Patients had higher levels of CD63-positive platelets during sleep at 4 a.m. compared to the morning levels at 7 a.m. (fig. 2). This result was confirmed using anti-CD62P. However, there was no statistically significant difference in the levels of CD63-positive and CD62P-positive platelets at 4 compared to 7 a.m. in controls (table 1).

Platelet Microparticles in Patients with OSAS

PMP were measured in patients with OSAS and controls at 4 and 7 a.m. The levels of PMP were similar in patients with OSAS and controls. There were also no statistically significant differences in PMP levels in blood samples obtained at 4 a.m. compared to those obtained at 7 a.m. (table 1).

Effect of CPAP Therapy

In 11/12 patients, the AHI decreased significantly during the 2nd night with CPAP therapy (27.2 ± 12.4/h before CPAP compared to 9.6 ± 6.1 with CPAP, p < 0.01), indicating that CPAP therapy was effective.

The level of CD63-positive platelets was slightly decreased during sleep at 4 a.m. in OSAS patients under CPAP therapy compared to the levels before CPAP therapy, however without reaching a statistically significant difference (fig. 3). A similar trend was obtained using anti-CD62P. Interestingly, at 7 a.m. the levels of CD63-positive platelets were similar with and without CPAP therapy (table 1).

Discussion

This study shows a significant increase in CD63- and CD62P-positive platelets during sleep in patients with OSAS compared to healthy controls. In patients with OSAS, platelet activation was enhanced during sleep at 4 compared to 7 a.m., in contrast to the controls. There was a trend for reduced platelet activation under CPAP therapy, although the difference did not reach statistical significance. The levels of PMP were similar in patients with OSAS and controls. These data indicate that patients with OSAS have an increased platelet activation during sleep, suggesting that activated platelets may contribute to the increased incidence of cerebrovascular events in patients with OSAS.
The immediate physiological consequences of OSAS are nocturnal hypoxia, hemodynamic changes and sleep disruption, which can lead to an increased sympathetic activity shown by increased epinephrine levels in patients with OSAS [18, 19]. It was therefore hypothesized that the increased sympathetic activity can induce an increase in platelet activation, especially during sleep [19]. Our data support this hypothesis showing increased levels of platelet activation during sleep in patients with OSAS compared to healthy controls.

Our results are in accordance with several other studies reporting enhanced platelet activation and aggregation in patients with OSAS [9, 20, 21], although there are methodological differences that need to be emphasized. First, the time point of blood sampling seems to be important, and secondly, the determination of in vivo platelet activation by flow cytometry using activation-specific antibodies may be superior to methods studying platelet activation in vitro, since the aim of our study focuses on the role of in vivo platelet activation in patients with OSAS [22]. Based on our results and the data from recently published studies there is increasing evidence that platelet activation is enhanced during sleep when the pathophysiological changes in patients with OSAS are maximal. We therefore decided to draw the blood samples during sleep in order to get representative data for the status of platelet activation during sleep, similar to the study published by Bokinsky et al. [9]. This study showed highest levels of platelet activation 6 h after sleep onset, which corresponds to the time point we chose for blood sampling (4 a.m.). In contrast to this study, direct venipunctures were repeated since a profound alteration in platelet function cannot be excluded using a continuously placed peripheral intravenous catheter.

Whole blood flow cytometry is a well-validated method for the evaluation of platelet function in disease states [23]. Platelet activation testing may be improved by directly studying platelet glycoprotein alterations during in vivo platelet activation. This approach is therefore superior to conventional in vitro platelet activation assays since it directly reflects the level of platelet activation occurring in vivo. The two independent antibodies used to determine in vivo platelet activation are specific for activated platelets. CD62P (P-selectin) is released on the platelet membrane after platelet secretion, whereas CD63 is exposed on the platelet membrane after lysosome degranulation. The fact that similar results were obtained using different activation-dependent antibodies further supports our findings that patients with OSAS have increased platelet activation during sleep.

There is increasing evidence in the literature that PMP may be clinically relevant in several pathological conditions including cardiovascular disease [13, 16], since it was shown that PMP are highly procoagulant [13]. To our knowledge, there are no reports on the role of PMP in patients with OSAS in the literature. Using flow cytometry, we did not find a significant difference in PMP formation in patients with OSAS compared to controls, neither at 4 nor at 7 a.m. Since there was a considerable variability in PMP in patients with OSAS, the patient number may have been small to obtain statistically significant differences between patients with OSAS and controls. However, since relatively small amounts of PMP may contribute to a clinically relevant procoagulant state, further studies elucidating the role of PMP in patients with OSAS are needed.

Although the body mass index was much lower in our patient group compared to other study populations, we cannot rule out that obesity contributed to the different levels of platelet activation in our study. Arterial hypertension could be another factor which may affect platelet activation. However, platelet activation did not significantly differ between the 3 patients with a history of arterial hypertension and the other patients with OSAS in our patient group.

The AHI of the consecutive patients studied was rather low, indicating that the OSAS was mild to moderate in these patients. Although patients with more severe OSAS may have demonstrated a more significant increase in in vivo platelet activation, we conclude from our data that the increase in in vivo platelet activation must be relevant, since we obtained a significant increase in a patient group with relatively mild OSAS.

CPAP therapy reduced the AHI in most patients, indicating that CPAP therapy was effective. Platelet activation was lower during sleep on CPAP (fig. 3), although this difference did not reach statistical significance. Interestingly, the morning levels were similar with and without CPAP therapy. These results suggest that CPAP therapy may in part reduce platelet activation during sleep, possibly by reducing the number and severity of episodes with apnea/hypopneas. The short duration of CPAP therapy may be responsible for the fact that the effect of the CPAP therapy on platelet activation did not reach statistical significance. Moreover, CPAP titration may have been suboptimal during the study night, since the patients had to be awakened for blood sampling.

In summary, in vivo platelet activation is increased in patients with OSAS during sleep, whereas no significant difference could be detected in PMP generation. These
data support the hypothesis that increased in vivo platelet activation may contribute to the increased risk of cardiovascular events including myocardial infarction and stroke in patients with OSAS.

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