Delayed Neutrophil Apoptosis in Patients with Sleep Apnea

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***Rationale*: Obstructive sleep apnea (OSA), characterized by intermittent hypoxia/reoxygenation (IHR), is associated with atherosclerosis. Polymorphonuclear leukocytes (PMNs) are implicated in atherogenesis by producing oxidizing radicals and proteolytic enzymes during PMN–endothelium interactions. PMN apoptosis is a fundamental, injury-limiting mechanism, which prevents their destructive potential.**

***Objectives*: To determine whether PMN apoptosis and expression of adhesion molecules are affected by OSA and IHR *in vitro*.**

***Methods*: Apoptosis and expression of adhesion molecules were assessed in whole blood PMNs by flow cytometry, verified by various culture conditions, and morphology. These were complemented by exposing whole blood and purified PMNs to IHR and to sustained hypoxia *in vitro*.**

***Measurements and Main Results*: This study demonstrates for the first time that, in patients with moderate to severe OSA, PMN apoptosis is delayed. Apoptosis was attenuated in patients with an apnea–hypopnea index (AHI) of more than 15, determined by decreased expression of low-CD16/annexin-V–positive PMNs, by lowered caspase-3 activity and nuclear condensation. Concomitantly, selectin-CD15 expression was increased in a severity-dependent manner in patients with moderate to severe OSA having an AHI greater than 15. The percentage of apoptotic PMNs was negatively correlated with OSA severity, determined by AHI, and positively with CD15 expression. In nasal continuous positive airway pressure–treated patients, CD15 expression was attenuated and low CD16 was increased, whereas omitting nasal continuous positive airway pressure for a single night increased CD15 expression and decreased the percentage of low CD16. IHR *in vitro* delayed PMN apoptosis as well.**

***Conclusions*: Decreased apoptosis and increased expression of adhesion molecules were noted in OSA PMNs. Although adhesion molecules may facilitate increased PMN–endothelium interactions, decreased apoptosis may further augment these interactions and facilitate free radical and proteolytic enzyme release.**

Keywords: obstructive sleep apnea; polymorphonuclear leukocytes; apoptosis; adhesion molecules; atherosclerosis

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Sleep apnea is associated with increased vascular risk. Inflammatory cells participate in vascular morbidity and are also activated by sleep apnea. Yet, the mechanisms by which neutrophils mediate atherogenesis in this syndrome are currently unknown.

What This Study Adds to the Field

Neutrophil apoptosis decreases and expression of selectins increases in sleep apnea. Treatment with positive pressure reverses both measures, whereas neutrophils of healthy subjects exposed to intermittent hypoxia *in vitro* show delayed apoptosis.

Obstructive sleep apnea (OSA) has emerged as an important risk factor for atherosclerosis. Intermittent hypoxia/reoxygenation (IHR) accompanied by oxidative stress and inflammation, are fundamental processes initiating cardiovascular morbidity (1). Polymorphonuclear leukocytes (PMNs) possess the ability to produce large quantities of reactive oxygen species (ROS), which can cause DNA protein and lipid peroxidation (2, 3). In addition, PMNs release inflammatory leukotrienes and proteolytic enzymes, which may directly induce vascular damage (2). Thus, intensive PMN activation and infiltration were found in lesions of acute coronary syndromes (4, 5), signifying increased risk of subsequent cardiovascular events (6). Moreover, PMNs were implicated in the pathogenesis of lethal myocardial reperfusion injury (7), whereas their depletion reduced myocardial infarct size (8) and protected the myocardium (9).

Adhesion of PMNs to endothelial cells is largely attributed to the CD15 carbohydrate subgroup on the L-selectin adhesion molecule (10). CD15 also mediates PMNs and monocytes binding to platelets (11) and contributes to cell–cell interactions leading to vascular dysfunction (12).

PMNs are bone marrow–derived ($1–2 \times 10^{11}$/d), short-lived (half-life < 1 d), terminally differentiated cells that are released to the circulation and die by programmed cell death termed "apoptosis" (13). This is a highly regulated process to maintain PMN homeostasis. *In vivo*, apoptosis is a fundamental injury-limiting mechanism, which restricts PMN function by lowering adhesion, migration, phagocytosis, degranulation, and ROS formation. Conversely, its delay may exacerbate these functions (13–15). Importantly, CD16 receptors participate in PMN activation and apoptosis. Thus, PMNs undergoing apoptosis lose their high expression but retain "low CD16" expression. In parallel, degranulation and phagocytic abilities are decreased. Therefore, low CD16 is considered a well-established apoptotic marker (16–19). Additional apoptotic markers include phosphatidylserine, detected by annexin-V binding; morphologic changes, such as chromatin structure and nuclear condensation; and caspase-3 activity. Using these markers, delayed PMN apoptosis was observed in acute coronary syndromes (20) and during exacerbation of chronic obstructive pulmonary disease (21). In OSA, PMN activation was documented via increased ROS formation and nuclear factor (NF)-κB up-regulation (22, 23).

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Generally, hypoxia promotes apoptosis. However, in PMNs, unlike in other cells, hypoxia profoundly inhibits apoptosis (15, 24, 25). Given that IHR is a prominent feature of OSA, we hypothesized that OSA PMNs would undergo activation, decreased apoptosis, and increased expression of adhesion molecules. Therefore, PMN phenotype and function were rigorously investigated: namely, apoptotic markers, adhesion molecules (CD15-selectin, CD11c-integrin), and systemic inflammatory markers. To determine whether the impaired PMN apoptosis and adhesion molecule changes could be reversed with treatment for OSA, PMNs were examined after nasal continuous positive airway pressure (nCPAP). Finally, to determine if hypoxia/reoxygenation impairs PMN apoptosis, PMNs from healthy individuals were examined for changes in apoptosis in response to IHR and sustained hypoxia *in vitro*.

Some of the results of these studies have been previously reported in abstract form (26, 27).

METHODS

Subjects

All subjects investigated underwent full-night polysomnography at the Technion Sleep Medicine Center (Haifa, Israel) using a computerized recording system (Embla; Flaga Medical, Reykjavik, Iceland) with the following channels: electroencephalogram, electrooculogram, chin electromyogram, arterial oxygen saturation (finger oxymetry), electrocardiogram, chest and abdominal wall motion (piezo electrodes), nasal airflow (pressure cannula), tibialis electromyogram, and body position. The polysomnography recordings were scored manually for sleep stages and respiratory events. An apnea or hypopnea event was defined as an airflow amplitude reduction of more than 50% from the baseline lasting at least 10 seconds, or a less significant reduction in the airflow amplitude accompanied by the presence of arousal or oxygen desaturation of at least 3%. A diagnosis of OSA was based on apnea–hypopnea index (AHI), calculated as the total number of apneas plus hypopneas divided by hours of sleep, and characteristic complaints. Arousals were determined according to the American Academy of Sleep Medicine guidelines (28). Blood samples were withdrawn under fasting conditions at 6:00–6:30 A.M. after polysomnography. Exclusion criteria included known hypertension or blood pressure greater than 140/90 mm Hg, glucose levels above 126 mg/dl or treated diabetes, any known cardio/cerebrovascular or other comorbidities, and sickness during the 2 weeks before the study, or regularly using medications. Sixty-eight consecutive patients referred to undergo sleep recordings because of suspected sleep apnea were recruited for experiment 1. On the basis of the results of the first experiment, participants in experiment 2 were newly recruited in pairs comprising a patient with sleep apnea (AHI $>$ 15) meeting the exclusion/inclusion criteria as before, and a sex-, age- and body mass index (BMI)–matched control subject (AHI $<$ 15). In some cases, two patients and a matched control or vice versa were selected. The protocol was approved by the local human rights committee, and all participants signed an informed consent form.

Flow Cytometry

The PMN phenotype was determined by FACSSorter (Becton Dickinson, Lincoln Park, NJ). PMNs were gated using forward and side light scatter. Data are presented as percentage of positive cells and as mean fluorescence intensity (MFI), corrected for background fluorescence. Annexin-V binding to phosphatidylserine (18, 21, 29, 30) and low-CD16 expression (16–20, 30) were used as standard markers of PMN apoptosis. An expanded version is presented in the online supplement.

PMN Isolation and Culture

PMNs were isolated using a two-layer Ficoll-Histopaque density gradient centrifugation (Histopaque 1.077 and 1.119; Sigma Diagnostics, St. Louis, MO) (31). PMN purity was greater than 96%, and viability was greater than 99%, as determined by Trypan blue exclusion. PMN suspensions were cultured for 2, 8, or 24 hours at 37°C in RPMI 1640 medium, supplemented with 10% fetal calf serum in a humidified incubator. Whole blood was cultured under the same conditions.

Microscopic Assessment of PMN Apoptosis

Purified PMNs were cytospun, stained with May-Grunwald-Giemsa (MGG) and read blindly using an Axiovert 25 microscope (Carl Zeiss Microscopy, Zeppelinstrasse, Germany). Apoptotic PMNs showed a single nucleus with dense chromatin condensation or nuclear fragments not connected by strands (16, 21, 30, 32, 33).

Morphologic counting of pyknotic nuclei and the fluorescence properties of the DNA-binding dye Hoechst 33342 (Sigma) in combination with or without propidium iodide (PI) was used to further confirm apoptosis (29). Slides were observed by fluorescence microscopy under ultraviolet light (365/380 nm).

Measurement of Caspase-3 Activity

Caspase-3 activity was determined in $CD16^+$ PMNs by flow cytometry. Cells were stained with anti–active caspase-3 (Clone C92–605; BD Pharmingen, San Jose, CA) for 1 hour in the presence of 0.1% saponin.

In Vitro IHR Protocol

Whole blood or purified PMNs were exposed to three or six cycles of intermittent hypoxia. Air-phase set-point consisted of a 35-minute hypoxic period, followed by 25 minutes of reoxygenation (21% O_2 and 5% CO_2), using the BioSpherix OxyCycler C42 system (BioSpherix, Redfield, NY). Actual blood O_2 saturation was kept at 2 or 6% for 6.6 $\pm$ 3.6 minutes or 6.55 $\pm$ 2.75 minute durations, respectively, at each 1-hour cycle (*see* Figure E2 of the online supplement). Sustained hypoxia (SH) was used for the same duration (at 2% actual oxygen in the blood). Control-treated blood was maintained in normoxic conditions for the same durations. An oxygen electrode immersed in the blood monitored dissolved oxygen concentration continuously (details provided in the online supplement).

Biochemical Plasma and Serum Measures

Determinations of lipid profile, C-reactive protein (CRP), adiponectin, soluble P-selectin (sP-selectin), and cortisol levels are specified in the online supplement.

Statistical Analysis

Data are expressed as mean $\pm$ SD. Differences between groups as a function of sleep apnea severity were evaluated by Kruskal-Wallis one-way analysis of variance and by analysis of the covariance followed by Bonferroni pairwise comparisons. Differences in frequencies between groups were compared by χ^2 test. A Mann-Whitney test was used for two-group comparisons. Pearson correlation was used to determine the relationship between selected variables if normally distributed; otherwise, Spearman correlation was used. Stepwise logistic regression was used to determine the independent predictors of low CD16 and CD15. The effects of nCPAP and *in vitro* experiments were analyzed using a paired *t* test. Two-tailed tests were used and $P < 0.05$ was considered significant. The NCSS 2004 statistical package (NCSS, Kaysville, UT) was used.

RESULTS

Experiment 1

Phenotype of fresh peripheral blood PMNs. PMN phenotype was determined in fresh whole blood. A total of 68 subjects were classified into four groups according to AHI as follows: non-obstructive sleep apnea (NOSA) (AHI $\leqslant$ 5, n = 17), mild OSA (5 $<$ AHI $\leqslant$ 15, n = 14), moderate OSA (15 $<$ AHI $\leqslant$ 30, n = 23), and severe OSA (AHI $>$ 30, n = 14). Table 1 presents subjects' demographic, blood chemistry, sleep apnea, circulating inflammatory marker, and flow cytometry data. There were no significant differences between the groups in age, but BMI was significantly different ($P < 0.02$). Post hoc comparisons revealed that patients with NOSA had lower BMI as compared with mild- and severe-OSA groups, but there were no significant differences between the sleep apnea groups. There were no significant differences in male/female ratio and in the rate of

TABLE 1. DEMOGRAPHIC, SLEEP, BLOOD CHEMISTRY, AND FLOW CYTOMETRY DATA FOR SUBJECTS WITH NONOBSTRUCTIVE SLEEP APNEA AND SUBJECTS WITH OBSTRUCTIVE SLEEP APNEA

	NOSA	Mild OSA	Moderate OSA	Severe OSA
Demographics				
No. of subjects	17	14	23	14
Age, yr	40 ± 11	45 ± 11	46 ± 10	47 ± 13
Sex, female/male	8/9	3/11	4/19	3/11
BMI, kg/m^2	25.2 ± 3.5	28.8 ± 6.2*	26.8 ± 3.3	29.2 ± 3.4*
AHI, events/h	3.9 ± 1.5	10.9 ± 2.8*†‡	21.7 ± 4.8*†§	41.4 ± 10.1*‡§
ODI 3%	2.5 ± 1.4	5.5 ± 4.5	9.3 ± 8.6	28.9 ± 12.3*‡§
Min Sa_{O_2}	93.2 ± 2.4	90.5 ± 3.6	88.4 ± 7.1	81.1 ± 11.4*‡§
% Time < 90% Sat	0.21 ± 0.59	0.84 ± 1.31	3.33 ± 5.9	20.9 ± 26.3*‡§
Arousal index	15.2 ± 4.0	21.6 ± 6.9	28.9 ± 9.5	45.5 ± 8.5*§
Current smokers, n (%)	1/17 (5.9)	4/14 (28.6)	9/23 (39.1)	2/14 (14.3)
Blood chemistry				
Cholesterol, mg/dl	175.7 ± 26.6	188.8 ± 33.3	192.9 ± 37.3	204.6 ± 39.3
Triglycerides, mg/dl	102.9 ± 40.7	155.6 ± 55.0	152.3 ± 73.4	212.9 ± 223.4*
HDL, mg/dl	47.3 ± 14.9	38.0 ± 8.9	44.1 ± 9.3	45.1 ± 16.3
LDL, mg/dl	108.1 ± 26.7	119.7 ± 26.5	118.5 ± 31.0	125.0 ± 38.3
Glucose, mg/dl	91.4 ± 9.4	92.2 ± 7.9	95.9 ± 11.8	99.6 ± 11.8
CRP, mg/L	3.4 ± 3.3	3.03 ± 2.65	2.89 ± 3.45	2.93 ± 2.55
Adiponectin, μg/ml	7.6 ± 2.8	3.5 ± 1.6	5.3 ± 2.7	5.1 ± 3.0
sP-selectin, ng/ml	46.7 ± 22.6	35.9 ± 12.0	54.1 ± 28.3	67.7 ± 40.0‡
Flow cytometry markers				
PMNs from total leukocytes, %	56.9 ± 8.3	56.4 ± 6.7	60.6 ± 5.6	56.9 ± 7.4
CD11c, %	29.3 ± 26.8	34.3 ± 20.9	31.3 ± 25.2	27.0 ± 22.4
CD11c, MFI	16.7 ± 9.3	14.0 ± 7.4	17.1 ± 8.7	16.4 ± 4.8
CD15, %	97.4 ± 2.4	96.4 ± 4.0	97.5 ± 2.3	97.3 ± 3.5
CD15, MFI	1,048.1 ± 370	963.9 ± 453.7	1,482.9 ± 457.9*†	1,484.8 ± 470.1‡
CD15, MFI, nonsmokers subgroup	1,071 ± 373	968 ± 386	1,693 ± 492*†	1,613 ± 435*‡
Low CD16, %	2.75 ± 0.90	2.69 ± 1.17	0.99 ± 0.54*†	1.0 ± 0.59*‡
Low CD16, %, nonsmokers subgroup	2.80 ± 0.92	2.9 ± 1.30	0.84 ± 0.47*†	1.0 ± 0.60*‡

Definition of abbreviations: AHI = apnea–hypopnea index; BMI = body mass index; CRP = C-reactive protein; HDL = high density lipoproteins; LDL = low density lipoproteins; MFI = mean fluorescence intensity; Min Sa_{O_2} = minimum oxygen saturation; ODI 3% = oxygen desaturation index (number of desaturation events of at least 3% divided by hours of sleep); NOSA = nonobstructive sleep apnea; OSA = obstructive sleep apnea; PMNs = polymorphonuclear leukocytes; % time < 90% Sat = percentage of time with arterial oxygen saturation < 90%.

A Kruskal-Wallis one-way analysis of variance with Bonferroni correction was used.

* Statistically significant as compared with NOSA.

† Statistically significant, mild OSA versus moderate OSA.

‡ Statistically significant, mild OSA versus severe OSA.

§ Statistically significant, moderate OSA versus severe OSA.

"current" smokers. Analysis of blood chemistry data revealed a significant difference in triglyceride levels ($P < 0.03$), but post hoc comparisons revealed a significant difference only between the severe- and NOSA groups. There were no significant differences in CRP and adiponectin. The levels of sP-selectin were significantly higher in patients with severe OSA than in those with mild OSA, but this became nonsignificant after adjustment to BMI. Also, there were no significant differences between groups in the percentage of PMNs among blood leukocytes, as detected by flow cytometry (Table 1).

Selectin and integrin adhesion molecule expression was assessed using CD15 and CD11c markers, respectively. Normally, CD15 selectin receptors are constitutively expressed on most PMNs (95–99%; Table 1). However, analysis of the variance revealed significant between-group differences in CD15 MFI ($P < 0.005$). Post hoc comparisons across the four groups of subjects identified differences only for the groups with moderate and severe OSA versus those with NOSA and mild OSA (Table 1). Analysis of the covariance, using as covariates BMI and triglycerides, which were significantly different between the groups, revealed identical results. A separate analysis performed on only nonsmokers revealed identical results (Table 1). Pearson correlation between CD15 MFI and AHI was statistically significant (Figure 1; $r = 0.43$, $P < 0.001$). In addition, CD15 MFI positively correlated with arousal index ($r = 0.38$, $P < 0.02$). Stepwise regression analysis was used to determine the independent contribution of each of the sleep apnea severity indices (AHI, ODI 3% [oxygen desaturation index (number of desaturation events of at least 3% divided by hours of sleep)], % time < 90% Sat [percentage of time with arterial oxygen saturation

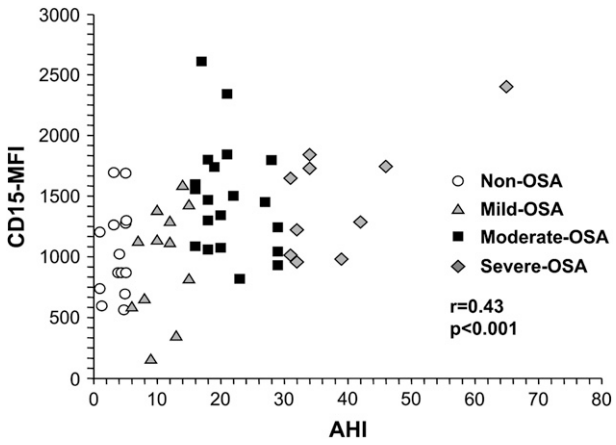

Figure 1. Positive correlation between the intensity of CD15 expression (mean fluorescence intensity [MFI]) in polymorphonuclear leukocytes and apnea severity (apnea–hypopnea index [AHI]). OSA = obstructive sleep apnea.

$< 90\%$], minimum Sa_{O_2}). This revealed that only AHI was a significant predictor ($P < 0.0002$), accounting for 36.6% of the total variance. Adding arousal index did not change these results. No significant differences between groups were found for integrin-CD11c expression (Table 1).

Delayed apoptosis in fresh whole blood PMNs in patients with OSA. CD16 is constitutively expressed on PMNs (92–96%) (*see also* the online supplement). Yet, as PMNs undergo apoptosis, their expression on the cell surface declines and a new PMN population expressing fewer CD16 receptors termed "low CD16," is defined (16–20). This low-CD16 PMN population, which denotes apoptotic PMNs, is illustrated in Figure 2A. Although 4.9% of PMNs from subjects with NOSA express low CD16, only 1.02% of the OSA PMNs express this population. Figure 2B represents the average values of apoptotic low CD16 obtained for all groups investigated by flow cytometry in fresh whole blood. There were significant between-group differences in the levels of low CD16 ($P < 0.000001$); post hoc comparisons revealed that patients with moderate and severe OSA had lower low-CD16 expression than patients with mild or NOSA, with no differences between mild and NOSA or between moderate and severe OSA, indicating that apoptosis of moderate and severe OSA PMNs was delayed. Identical results were obtained after adjusting the data for BMI and triglycerides by analysis of the covariance. Of note, the percentage of apoptotic low-CD16 PMNs was negatively correlated with AHI ($r = -0.55$, $P < 0.00001$), ODI 3% ($r = -0.33$, $P < 0.01$), arousal index ($r = -0.50$, $P < 0.001$), and % time $< 90\%$ Sat ($r = -0.29$, $P < 0.02$), and positively correlated with minimum Sa_{O_2} ($r = 0.34$, $P < 0.006$) (*see* Table 2). The low CD16 was also negatively correlated with CD15 expression ($r = -0.37$, $P < 0.006$). Stepwise regression analysis was used to determine the significant predictors of low CD16. This revealed that AHI ($P < 0.000001$) was a significant predictor and ODI 3% ($P < 0.07$) was nearly significant, accounting together for 39.3% of the total variance. Once arousal index was added to the analysis, only AHI remained a significant predictor ($P < 0.004$), accounting for 17.5% of the variance. Correlating the soluble inflammatory markers adiponectin, sP-selectin, and CRP with low CD16 revealed a significant negative correlation only with sP-selectin (Table 2).

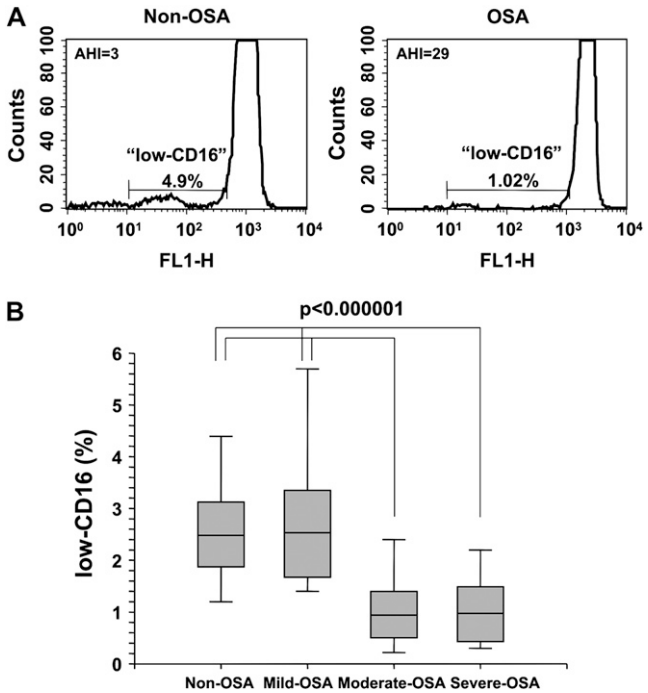

Figure 2. Expression of low CD16 in polymorphonuclear leukocytes (PMNs) by flow cytometry analysis. (*A*) Original recordings of fluorescence histograms of blood PMNs of two representative subjects. The percentage of apoptotic PMNs expressing low CD16 is indicated. (*B*) The percentage of low-CD16 PMNs in fresh whole blood of nonobstructive sleep apnea patients (n = 14) and patients with mild (n = 14), moderate (n = 23), and severe OSA (n = 14). *Boxes* represent values within the interquartile range and *whiskers* show the data range; *lines* across the boxes represent the median values. OSA = obstructive sleep apnea.

Experiment 2

Kinetics of PMN apoptosis in cultured blood. The current experiment was performed on a newly recruited group of subjects. Apoptosis was redetermined by low-CD16 expression but also verified by annexin-V binding, caspase-3 activity, and characteristic morphology. On the basis of the results of the first experiment demonstrating no differences between subjects with mild OSA and NOSA or between those with moderate and severe OSA, in this experiment subjects were divided into two groups based on an AHI of less than 15 (n = 19) and an AHI of greater than 15 (n = 22). The two groups were of similar age, BMI, male/female ratio, and smoking status, and had similar blood chemistry values. Their demographic and sleep apnea data are presented in Table 3. Because not all flow cytometry and morphologic analyses in experiment 2 could be performed simultaneously on all subjects investigated, the number of participants in each determination and their average age and BMI are indicated in each legend. In addition, in view of the fact that cortisol was reported to delay apoptosis (34), cortisol levels were determined in this experiment (Table 3). There were no significant differences in cortisol levels between the two groups, thus not affecting the apoptotic parameters measured (additional data are provided in the online supplement).

Kinetics of low-CD16 expression and annexin-V binding. As in experiment 1, the percentage of apoptotic PMNs expressing low CD16 was lower in patients with an AHI greater than 15 (n = 13) than in patients with an AHI less than 15 (n = 14) at all time points investigated ($P < 0.05$) (Figure 3A). Similarly to the low-CD16 expression, the percentage of annexin-V–positive PMNs was significantly lower in patients with an AHI greater than 15 (n = 10) than in patients with an AHI less than 15 (n = 10) at all time points investigated ($P < 0.01$) (Figure 3B).

TABLE 2. LINEAR REGRESSION ANALYSIS (*r*, *P*) OF THE RELATIONSHIP BETWEEN LOW CD16 AND CD15 MEAN FLUORESCENCE INTENSITY, AND VARIOUS INDEPENDENT VARIABLES

Variable	Low CD16 (*r*, *P value*)	CD15 MFI (*r*, *P value*)
AHI	−0.55, <0.00001	0.42, <0.001
ODI 3%	−0.33, <0.01	0.24, NS
Min Sa_{O_2}	0.34, <0.02	−0.09, NS
% Time < 90%Sat	−0.29, <0.02	0.07, NS
Arousal index	−0.50, <0.001	0.38, <0.02
BMI	0.10, NS	−0.10, NS
CRP	0.04, NS	0.16, NS
Adiponectin	0.01, NS	0.02, NS
sP-selectin	−0.30, <0.03	0.15, NS
Triglycerides	−0.16, NS	0.04, NS

Definition of abbreviations: AHI = apnea–hypopnea index; BMI = body mass index; CRP = C-reactive protein; MFI = mean fluorescence intensity; Min Sa_{O_2} = minimum oxygen saturation; ODI 3% = oxygen desaturation index (number of desaturation events of at least 3% divided by hours of sleep); % time < 90% Sat = percentage of time with arterial oxygen saturation < 90%.

TABLE 3. DEMOGRAPHIC, SLEEP, AND BLOOD CHEMISTRY DATA FOR SUBJECTS PARTICIPATING IN EXPERIMENT 2

Demographics	Subjects with AHI < 15	Subjects with AHI > 15
No. of subjects	22	19
Age, yr	45.5 ± 10.4	48.7 ± 10.8
Sex, female/male	5/17	2/17
BMI, kg/m^2	26.3 ± 3.5	28.0 ± 3.9
AHI, events/h	7.4 ± 4.0	38.9 ± 16.8*
% Time < 90% Sat	1.13 ± 1.96	14.59 ± 19.97*
Current smoking, n (%)	10 (45.5)	4 (21.1)
Cholesterol, mg/dl	187 ± 33	205 ± 45
Triglycerides, mg/dl	126.8 ± 38.9	203.4 ± 140.1
HDL, mg/dl	47.8 ± 9.6	43.3 ± 15.3
LDL, mg/dl	113.9 ± 31.2	126.8 ± 42
Glucose, mg/dl	94.0 ± 12.0	99.3 ± 21.4
Cortisol, μg/dl	21.96 ± 6.0	17.83 ± 6.7

Definition of abbreviations: AHI = apnea–hypopnea index; BMI = body mass index; HDL = high density lipoproteins; LDL = low density lipoproteins; % time < 90% Sat = percentage of time with arterial oxygen saturation < 90%.

A Kruskal-Wallis one-way analysis of variance with Bonferroni correction was used.

* Statistically significant ($P < 0.001$) between the groups.

Moreover, similarly to earlier studies (18), PMNs capable of binding annexin-V also expressed low CD16 (Figure 3C).

Morphologic markers of apoptosis. Morphologic measures of apoptosis were also purified PMNs. Figure 4A is a representative photomicrograph of PMN cytospin preparations. PMNs that have reached the terminal stage of apoptosis, in which chromatin condensation occurs, show a bright staining with Hoechst 33342 and chromatin condensation with MGG staining. These PMNs were predominant in the AHI < 15 group (n = 8) as compared with the AHI > 15 group (n = 10) (26.4 ± 2.1% vs. 12.2 ± 2.6%, $P < 0.01$, and 46.4 ± 11.4% vs. 30.6 ± 8.7%, $P < 0.01$ after 8 and 24 h in culture, respectively). In parallel, flow cytometry histograms of low-CD16 expression of the same subjects are also depicted in Figure 4B, showing PMNs of one subject with low and one with high AHI after 8 and 24 hours in culture.

Viability was assessed using flow cytometry by PI staining. After 24-hour incubation, the proportion of necrotic cells was determined. Less then 10% of PMNs in all subjects were positive for PI, indicating that PMNs had not yet started to show significant evidence of necrosis, as shown previously (29, 30, 32).

Caspase-3 activation. Increased caspase-3 activation has been widely demonstrated to be one of the main events in PMN apoptosis (13, 33, 35). Therefore, its activity was determined as well. The most notable differences between the groups were detected after 24 hours of culture. As depicted in Figure 5A, caspase-3 activity was markedly attenuated in patients with an AHI greater than 15 (n = 12), compared with subjects with an AHI less than 15 (n = 10) ($P < 0.0001$). Moreover, as illustrated in Figure 5B, most of the caspase-3–positive cells were detected among the PMNs with decreased CD16 expression.

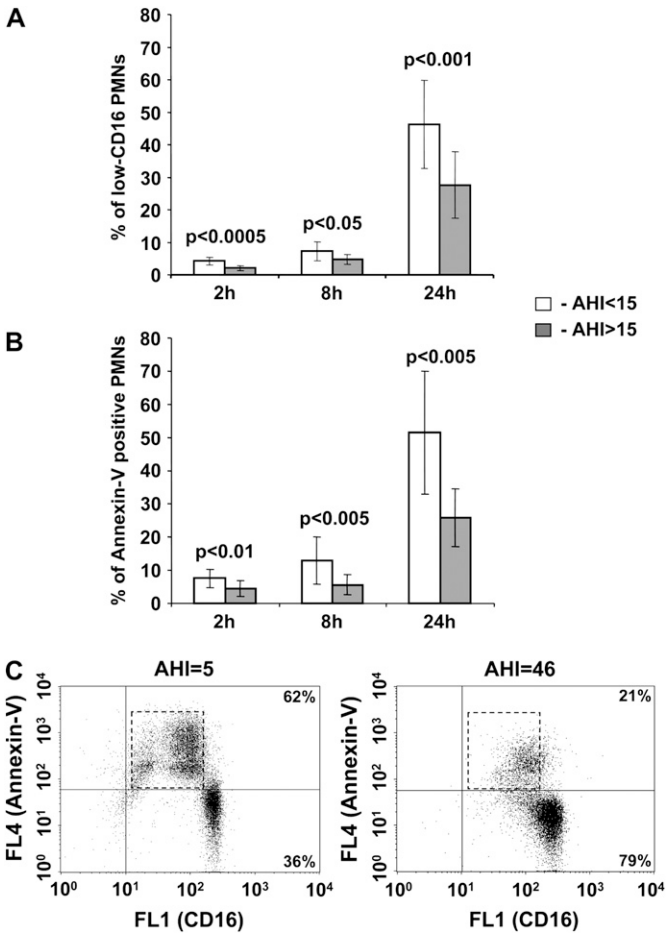

Figure 3. Kinetics of polymorphonuclear leukocyte (PMN) apoptosis in cultured whole blood, analyzed by flow cytometry. (*A*) The percentage of low-CD16 cells detected in 14 subjects with an apnea–hypopnea index (AHI) < 15 (age, 46.6 ± 10.4 yr; body mass index [BMI], 26.3 ± 3.4; AHI, 7.3 ± 4.3) and 13 patients with moderate to severe OSA with an AHI > 15 (age, 48.4 ± 10.2 yr; BMI, 27.4 ± 3.3; AHI, 36.9 ± 16.3) at 2, 8, and 24 hours in culture. (*B*) The percentage of annexin-V–positive PMNs in 10 subjects with an AHI < 15 (age, 46.3 ± 10.9 yr; BMI, 26.8 ± 2.9; AHI, 7.4 ± 4) and in 10 patients with OSA with an AHI > 15 (age, 49.6 ± 10.5 yr; BMI, 27.0 ± 3.1; AHI, 39.3 ± 18.6) at 2, 8, and 24 hours in culture. (*C*) Representative dot plots of surface expression of annexin-V on low-CD16 PMNs (*dashed rectangles*) of two representative subjects (AHI, 5; BMI, 23.5; and AHI, 46; BMI, 24.5) maintained for 24 hours in culture.

Experiment 3

Effects of nCPAP treatment. To further demonstrate that PMN apoptosis and expression of adhesion molecules were directly affected by sleep apnea rather then associated conditions, a third experiment was conducted on nCPAP-treated patients. The expression of CD15 and low-CD16 receptors was determined in nine patients with OSA after 8.5 ± 4.1 months of initiation of the nCPAP treatment (eight males/one female, age = 48.0 ± 7.6 yr, BMI = 33.7 ± 7.1 kg/m^2). These patients were investigated on two consecutive nights, the first with and the second without nCPAP. AHI and arousal index were lower in the nCPAP treatment night (8.8 ± 8.4 and 11.2 ± 4.2 events/h, respectively) and were increased after omitting nCPAP (47.1 ± 18.4, $P < 0.0005$, and 51.3 ± 21.9 events/h, $P < 0.0005$, respectively). After omitting nCPAP, CD15 expression increased from 1,083 ± 101 MFI to 1,262 ± 140 MFI ($P < 0.0005$), and low-CD16 expression decreased from 6.1 ± 2.9% to 2.3 ± 1.1% ($P < 0.005$). The individual data are illustrated in Figures 6A and 6B. The average cortisol levels with and without nCPAP were similar (18.2 ± 3.1 vs. 18.1 ± 3.0 μg/dl, respectively) and thus did not affect apoptosis in patients with treated OSA. Individual patients' demographic, biochemistry, and sleep apnea data are presented in the online supplement.

Experiment 4

***Effects of IHR and SH on PMN apoptosis* in vitro.** To determine whether intermittent hypoxia *per se* can affect PMN apoptosis,

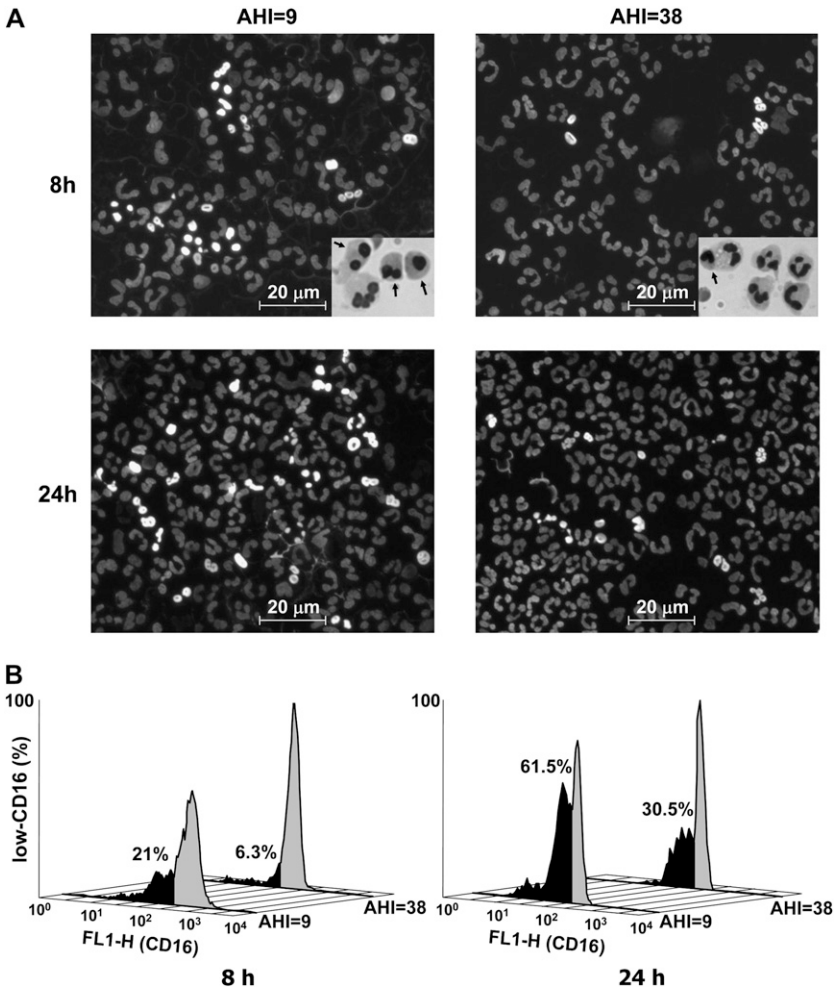

Figure 4. Assessment of apoptosis by morphology in purified cultured polymorphonuclear leukocytes (PMNs). (*A*) Typical photomicrographs of cytospin preparations of PMNs cultured for 8 and 20 hours. Two representative subjects of each apnea–hypopnea index (AHI) group out of n = 8 (AHI < 15) and n = 10 (AHI > 15) are depicted. After 8 hours in culture, chromatin condensation and apoptotic bodies, determined by bright fluorescence of Hoechst 33342 staining, are predominant in the subject with an AHI of 9 (body mass index [BMI], 23.5), whereas fewer apoptotic cells are seen in the subject with an AHI of 38 (BMI, 25.2). These were confirmed by May-Grunwald-Giemsa stain (inset photomicrograph on *top right panel*). Increased apoptosis was noted after 24 hours in culture, particularly in the control subject. PMNs were considered apoptotic if they showed dense condensation of chromatin, either as a single nucleus or nuclear fragments not connected by strands (*arrows*). (*B*) Representative histograms of low-CD16 expression on cultured purified PMNs from the same subjects after 8 and 24 hours, respectively.

whole blood PMNs from eight healthy subjects (five males/three females, BMI = 25.1 ± 3.8, and age = 35.6 ± 10.2 yr) without polysomnographic evidence of sleep apnea (AHI = 2.6 ± 2.4) were exposed *in vitro* to IHR and compared with SH and with normoxia.

Treatment of PMNs with six cycles of IHR *in vitro* resulted in a significant decrease in the percentage of apoptotic low-CD16 PMNs as compared with normoxic or SH conditions (Figure 7A). Importantly, the differences between all three conditions—normoxia, SH, and IHR—were statistically significant. Moreover, PMN apoptosis, detected by low CD16, was already significantly decreased after three cycles of IHR at 6% actual oxygen concentrations in the blood as compared with normoxia (5.8 ± 1.9 vs. 7.0 ± 1.4%, $P < 0.02$), indicative of a relatively fast PMN activation over a period of 3 hours.

Increased PMN apoptosis due to IHR was also confirmed by morphology and flow cytometry in purified PMN cultures in four of the subjects. By using the DNA-binding dye Hoechst 33342, the percentage of apoptotic cells was 19.6 ± 2.3 in normoxia, 12.3 ± 2.2 in SH ($P < 0.01$ vs. normoxia), and 5.2 ± 0.9 in IHR ($P < 0.0002$ vs. normoxia; $P < 0.01$ SH vs. IHR). Staining with MGG also showed similar results; the percentage of apoptotic cells was 17.3 ± 3.3 in normoxia, 10.9 ± 3.7 in SH ($P < 0.005$ vs. normoxia), and 7.5 ± 2.9 in IHR ($P < 0.002$ vs. normoxia; $P < 0.02$ SH vs. IH). Typical photomicrograph cytospin preparations with Hoechst 33342 and MGG staining are presented in Figures 7B and 7C, respectively. These values were also confirmed by determination of percentage of low-CD16 expression using flow cytometry of the purified PMNs (normoxia, 19.3 ± 4.1; SH, 13.5 ± 2.8, $P < 0.02$, vs. normoxia; IHR, 6.5 ± 1.7, $P < 0.005$, vs. normoxia, $P < 0.005$, SH vs. IHR). Collectively, these data further confirm delayed PMN apoptosis *in vitro* in response to IHR as compared with normoxia or SH, as observed in whole blood. This trend was seen in each subject individually, but values were slightly higher in purified PMNs compared with whole blood (Figure 7A), due to PMN purification.

DISCUSSION

Atherosclerosis, one of the major complications of sleep apnea, is associated with exaggerated inflammation. PMNs are among the first circulating leukocytes involved in acute inflammatory responses that induce vessel and tissue injury (4–9, 36). Moreover, endothelial cell damage induced by primed PMNs constitutes a risk factor for atherosclerosis (37). Therefore, in the present study, we sought to investigate OSA-associated PMN phenotypic and functional changes. Our major findings are summarized as follows:

1. Expression of CD15-selectin adhesion molecules was increased in patients with moderate to severe OSA with an AHI greater than 15 and was also OSA severity dependent, whereas CD11c-integrin was unaffected.
2. PMN apoptosis was decreased in patients with an AHI greater than 15, as determined by the reduced expression of low CD16.

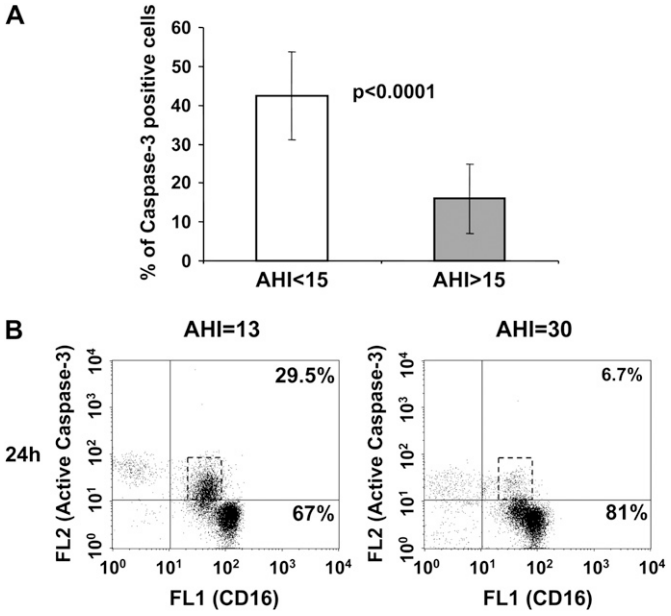

Figure 5. The percentage of caspase-3–positive cells. (*A*) Caspase-3 was detected by flow cytometry in polymorphonuclear leukocytes (PMNs) cultured for 24 hours in 10 subjects with an apnea–hypopnea index (AHI) < 15 (age, 47.3 $\pm$ 8.3 yr; body mass index [BMI], 25.9 $\pm$ 3.5; AHI, 8.5 $\pm$ 4.3) and 12 patients with an AHI > 15 (age, 49.8 $\pm$ 9.1 yr; BMI, 26.5 $\pm$ 2.5; AHI, 36.2 $\pm$ 16). (*B*) Representative dot plots of intracellular expression of active caspase-3 on low-CD16 PMNs (*dashed rectangles*) are depicted for a subject with an AHI of 13 and a BMI of 30.2, and a subject with an AHI of 30 and a BMI of 30.5.

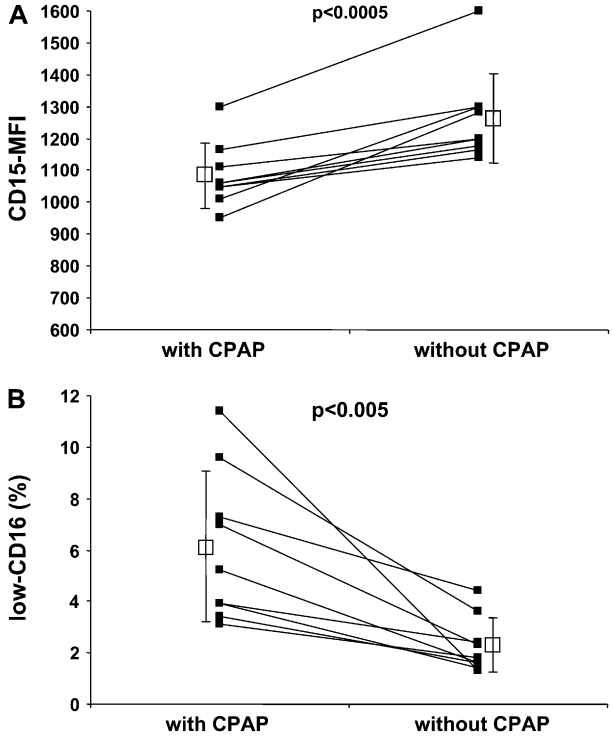

Figure 6. The effects of omitting nasal continuous positive airway pressure (CPAP) treatment for 1 night on the expression of adhesion molecules and apoptosis. (*A*) The intensity of CD15 expression, detected by mean fluorescence intensity (MFI), and (*B*) the percentage of low-CD16 apoptotic polymorphonuclear leukocytes. Nine nasal CPAP–treated patients were investigated for 2 consecutive nights. *Open squares* represent the average values $\pm$ SD.

3. Treatment of PMNs in culture for up to 24 hours further confirmed these findings at all time points investigated. The percentage of low-CD16/annexin-V–positive cells was lower in patients with an AHI greater than 15. This was further verified by morphologic features of apoptosis as nuclear and chromatin condensation and a significant reduction in caspase-3 activity.
4. AHI, but not arousal index, was an independent predictor of the percentage of apoptotic PMNs. The contribution of ODI 3% was of borderline significance. Also, the expression of sP-selectin and CD15-adhesion molecules was negatively correlated with the percentage of apoptotic PMNs.
5. In nCPAP-treated patients, CD15 expression and the percentage of apoptotic low-CD16 PMNs were comparable to values obtained in subjects with an AHI less than 15. However, omitting nCPAP for a single night increased the expression of CD15 and decreased the percentage of low-CD16 PMNs.
6. No differences were found between NOSA (AHI < 5) and mild OSA ($5 <$ AHI ≤ 15) with regard to CD15 and low-CD16 expression.
7. IHR *in vitro* alone profoundly inhibited apoptosis in whole blood as well as in purified cultured PMNs of healthy subjects and was more effective than SH.

PMNs are vital to the body's defense against infections. However, uncontrolled release of their formidable array of toxic substances may inflict damage to surrounding tissues and propagate inflammatory responses, leading to tissue scarring and destruction. Normally, PMNs are removed by apoptosis, to limit their activation (13–15). When PMNs die by apoptosis, they retain their granular contents but lose chemotactic and secretory responsiveness. These PMNs are recognized and phagocytosed by macrophages (14, 38). Thus, by down-regulating potentially harmful PMN functions and triggering their clearance by phagocytes, apoptosis provides a mechanism for safe removal of inflammatory cells. Moreover, phagocytosis of apoptotic PMNs triggers various powerful antiinflammatory signals that attenuate inflammation and regulate PMN homeostasis in the circulation (38).

PMNs undergo spontaneous apoptosis *in vivo* as well as *in vitro*. Under *in vitro* conditions, significant numbers of PMNs become apoptotic within 18 to 24 hours of culture, which is coupled with down-regulation of PMN functions and altered expression of surface molecules. Specifically, the low-affinity receptor for immunoglobulin G CD16 (FcγRIII) is a functional neutrophil receptor, which participates in degranulation, microfilament reorganization, and phagocytosis (39). In aged PMNs, apoptosis was shown to be directly modulated by signaling through CD16 (30). This receptor is constitutively expressed on most PMNs, but as PMNs undergo apoptosis, its expression is drastically attenuated (14, 16–20). Therefore, the loss of functionally active CD16 receptors results in the appearance of low-CD16 PMNs. This PMN subset expressing low CD16, which is used as a standard marker of apoptosis, is therefore also indicative of a decreased functional activity of PMNs. Thus, the delayed PMN apoptosis observed in patients with OSA, as indicated by the decreased percentage of low-CD16 expression, denotes a remarkable shift toward a highly up-regulated inflammatory response. Moreover, PMN apoptosis was further confirmed in culture

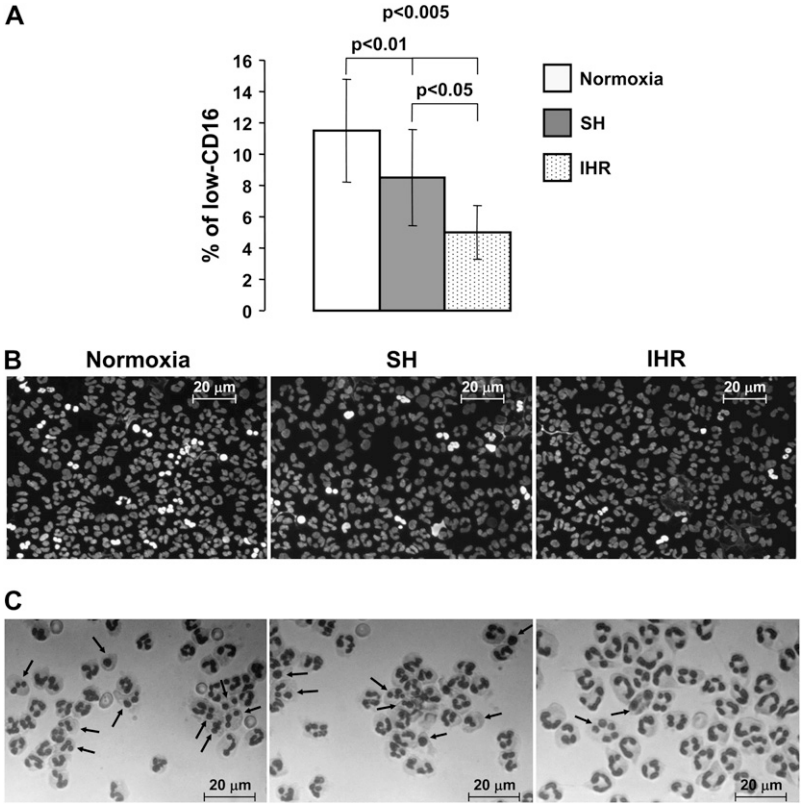

Figure 7. *In vitro* effects of intermittent hypoxia/reoxygenation (IHR) and sustained hypoxia (SH) as compared with normoxia on polymorphonuclear leukocyte (PMN) apoptosis measures of healthy subjects. (*A*) Expression of low CD16 in whole blood PMNs determined by flow cytometry. (*B*) Representative photomicrographs of cytospin preparations of purified PMNs stained by the fluorescent DNA binding dye Hoechst 33342, showing bright fluorescence of chromatin condensation and apoptotic bodies. Fluorescence is lower in SH- and is lowest in IHR-treated PMNs, compared with normoxia. (*C*) Typical photomicrographs of cytospin preparations of purified PMNs stained by May-Grunwald-Giemsa. Apoptotic PMNs were identified by dense chromatin condensation, either as a single nucleus or nuclear fragments not connected by strands (*arrows*). Apoptosis is highest in normoxia and lower under IHR. In IHR treatment, blood samples (in *A*) or purified PMNs (in *B, C*) were exposed to six cycles. Each cycle lasted an hour. In the hypoxic period, the actual blood O_2 concentration was 2% for a duration of 6.6 ± 3.6 minutes. During the normoxic period, blood O_2 was restored above 15% for 14.0 ± 1.5 minutes. In the SH treatment, actual blood or medium O_2 concentration was kept at 2% for the entire period. Controls were maintained under normoxic conditions (21% O_2, 5% CO_2 balanced with N_2) for the same duration. Carbon dioxide was held constant (5%) at all treatments.

conditions. It was detected by appearance of low-CD16/annexin-V–binding PMNs and verified by apoptotic morphology, as determined by Hoechst 33342 and MGG staining. In agreement with this finding, the involvement of another widely accepted pathway of apoptotic signaling that involves a caspase activation cascade was demonstrated in OSA. Caspases are a family of cysteine proteases existing in normal cells as inactive enzymes. They are activated by sequential proteolytic events that lead to degradation or functional alteration of cellular proteins, which contribute to cell death, characterized by the typical apoptotic morphology described above (35). Therefore, caspase-3 activation is one of the main requirements for the execution phase of apoptosis (13, 33, 35). Indeed, our data show that delayed PMN apoptosis in patients with moderate to severe OSA was accompanied by a significantly lower caspase-3 activity as well. Moreover, high levels of ROS produced by activated PMNs were shown to inhibit caspase function (35). Therefore, the increased ROS production by OSA PMNs (22) (*see also* the online supplement) could be possibly involved in caspase-induced delayed PMN apoptosis. Collectively, these complementary *in vitro* data on annexin-V binding, nuclear morphology, and caspase-3 activity further strengthen our results.

Because delayed apoptosis results in functional longevity of PMNs, it can also facilitate increased procoagulant activity, leukocyte plugging in the capillaries, and release of proinflammatory cytokines and ROS, all promoting endothelial cell damage and dysfunction and possibly exacerbating systemic and myocardial damage (40). Accordingly, such delayed PMN apoptosis was implicated in the pathogenesis of inflammatory diseases (41, 42), including cardiovascular pathology and chronic obstructive pulmonary disease (20, 21). Thus, it is likely that the intermittent hypoxia experienced by patients with OSA could possibly activate oxidative/inflammatory pathways in PMNs, leading to cardiovascular morbidities similar to those caused by ischemia/reperfusion injury, which were shown to be mediated by various mechanisms including up-regulation of selectins and delayed apoptosis (7, 9, 20, 43–45).

Together with delayed PMN apoptosis in OSA, increased expression of the selectin-CD15 adhesion molecules was observed. Notably, most PMN effector functions are performed in an adherent state, and selectin receptors were suggested to trigger these PMN functions (46). Thus, the up-regulation of selectin-CD15 noted in OSA PMNs may facilitate increased PMN rolling and interactions with the endothelium, and subsequently may promote endothelial cell damage via exacerbated PMN activity that is further exacerbated by PMN delayed apoptosis. This was previously shown for acute coronary syndromes (4) and myocardial infarction (5). Integrins (CD11c), unlike the selectins, were not up-regulated in OSA PMNs. Such data were previously reported using another clone of CD11c monoclonal antibodies (B-ly6; BD Pharmingen) (22), suggesting that the observed expression did not occur as a result of a loss of a single epitope. Importantly, adhesion of PMNs is preceded by rolling on the endothelium of the postcapillary venules via selectin receptors. Rolling is followed by either detachment from the venules and their return to the circulation, or by firm adhesion in preparation for migration out of the vasculature, using integrin adhesion receptors. However, the selectin-mediated rolling is more pronounced on inflamed venules (12, 47).

Treatment with nCPAP as compared with no nCPAP decreased the expression of CD15 adhesion molecules and increased PMN apoptosis, as determined by low-CD16 expression. The fact that notable changes were eminent immediately after omitting nCPAP for a single night clearly attests to the significance of eliminating the recurrent apneic events and the associated intermittent hypoxia. This is further indicated by exposing circulating PMNs to IHR *in vitro* where there was no involvement of sleep fragmentation. Although hypercapnia, arousals, and sympathetic activation cannot be excluded as possible contributors, the impact of catecholamines *in vitro* on the

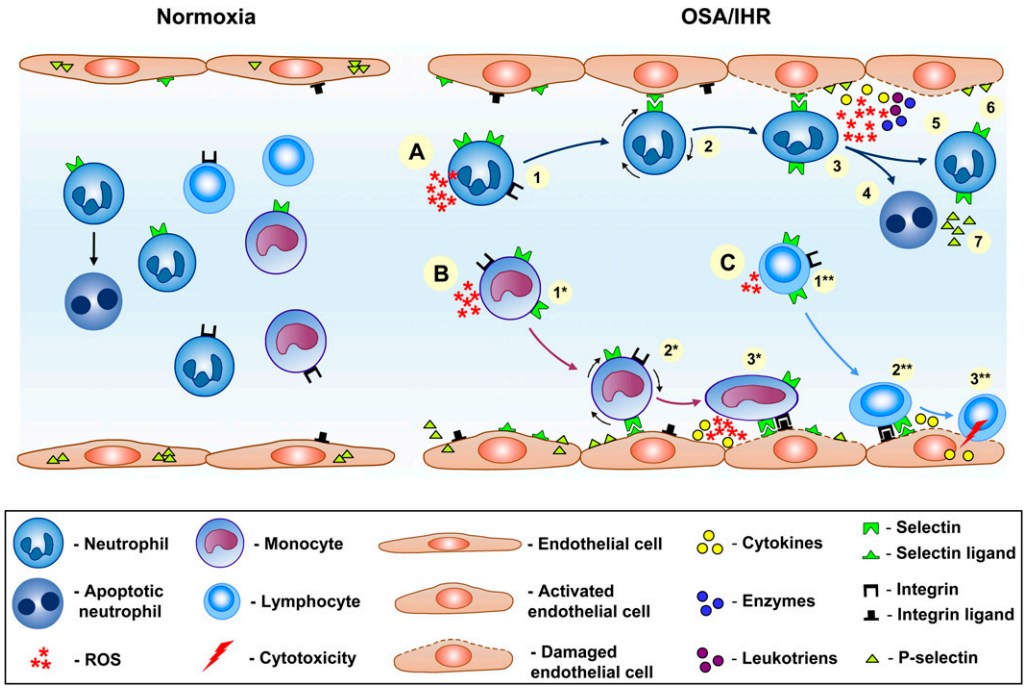

Figure 8. A schematic representation of the roles of leukocytes in endothelial cell injury in sleep apnea. In normoxia, leukocytes are freely flowing in the bloodstream and endothelial cells are in a non-activated state. Yet, basal levels of adhesion molecules are expressed on the surface of leukocytes and endothelial cells at low levels. P-selectin is stored intracellularly in Weibel-Palade bodies of endothelial cells (and in α-granules in platelets). Polymorphonuclear leukocytes (PMNs) are continuously undergoing apoptosis, identified by low-CD16/annexin-V–positive, decreased caspase-3 activity and characteristic nuclear morphology. However, in response to intermittent hypoxia, endothelial cells undergo activation that promotes increased surface expression of adhesion molecules as selectins and integrins. It is also likely that the intracellular P-selectin undergoes translocation from intracellular granules to the cell surface, or shedding to the circulation. In parallel, PMNs, monocytes, and lymphocytes undergo activation as well. *A*: In activated PMNs, reactive oxygen species (ROS) production is increased and as is the expression of selectins (*1*). The increased expression of selectins on PMNs and endothelial cells promotes rolling and capture of the PMNs (*2*). After attachment, PMNs release inflammatory cytokines, proteolytic enzymes, leukotrienes, and additional ROS molecules in the vicinity of the endothelial cells inducing endothelial cell injury (*3*). Delayed PMN apoptosis may further exacerbate this injury. The captured PMNs may then either undergo apoptosis (*4*), or if apoptosis is delayed, PMNs could possibly detach from the endothelium and return to the circulation (*5*). This may facilitate their recapture to continue endothelial cell injury. The intracellular P-selectin, which is rapidly translocated from activated endothelial cells (or platelets) to the cell surface (*6*), could also be shed to the circulation (*7*), and may possibly contribute to delay PMN apoptosis. *B*: Similarly, activated monocytes (*1**) release ROS molecules and express increased amounts of selectins and integrins in response to intermittent hypoxia while rolling onto the endothelium via selectins (*2**) and firmly adhering to the endothelial cells via integrins (*3**), further releasing ROS molecules and inflammatory cytokines, which can damage the endothelium (described in Reference 22). C: Activated lymphocytes (*1***) release low amounts of ROS and express adhesion molecules which facilitate rolling and firm adhesion to endothelial cells (*2***), promoting endothelial cell damage by using various mechanisms of cytotoxicity (*3***), as described previously (52–54).

expression of adhesion molecules by human PMNs was shown to be limited (48). Moreover, using stepwise regression analysis, we found that AHI was the only independent sleep apnea severity measure that predicted the levels of low CD16. Although arousal index significantly correlated with the markers of apoptosis, when both AHI and arousal index were simultaneously tested as possible predictors of low CD16 by multivariate analysis only AHI was a significant predictor. When all three variables—AHI, ODI 3%, and arousal index—were used simultaneously, AHI remained a significant predictor, ODI 3% bordered on statistical significance, and arousal index was not significant.

The intrinsic apoptotic threshold of a cell is modifiable by an array of extracellular proinflammatory cytokines and various stress-inducing stimuli (13, 15). Generally, regulation of apoptosis by various hypoxic conditions, including intermittent hypoxia, is a cell-specific phenomenon that may reflect different abilities of cells to adapt to anaerobic metabolism (49). Interestingly, in PMNs, unlike in other cells investigated, hypoxia inhibits apoptosis (15, 24, 25). Moreover, hypoxia-inducible factor (HIF)-1α–dependent NF-κB activity was implicated in this hypoxia-dependent PMN function (25). In the current study, IHR *in vitro* was more effective then sustained hypoxia in inhibiting apoptosis of PMNs in whole blood of healthy subjects. However, the specific molecular pathways that delay PMN apoptosis under IHR conditions and OSA remain to be elucidated.

Additional soluble markers of systemic inflammation and cortisol, which could possibly affect apoptosis, were measured as well. We did not find significant differences between patients with OSA and control subjects in the systemic inflammatory markers CRP, adiponectin, or sP-selectin. However, the observation that sP-selectin was negatively correlated with low CD16 of PMNs may implicate this soluble adhesion molecule, which is released from activated endothelial cells and platelets, in delayed apoptosis of PMNs. This relationship should be further explored. Also, cortisol levels, previously reported to influence apoptosis (34), were similar in control subjects and patients with OSA, as was also reported recently (50), and did not significantly change after omitting nCPAP. Collectively, the circulating inflammatory markers investigated and cortisol did not play a significant role in the delayed PMN apoptosis observed in patients with OSA.

The possible limitations of our study should be acknowledged. In experiment 1, the four groups were not closely matched with respect to BMI and patients with severe and mild OSA had higher BMIs than the control subjects. Also, there was a significant difference in triglyceride levels between the severe-OSA group and nonapneic control subjects, as shown in earlier studies (51). We should note, however, that despite their higher BMI, there were no significant differences in the levels of low CD16 and CD15 MFI between NOSA and mild-OSA groups, and that both groups had lower CD15 and higher low-CD16 levels than patients with moderate and severe OSA. Moreover,

adjusting for both BMI and triglycerides by analysis of the covariance confirmed these results for both low CD16 and CD15 MFI. To overcome this limitation, these data were verified by experiment 2 using closely matched groups and additional markers of apoptosis, and were further supported by experiments 3 and 4 using nCPAP treatment and exposure of PMNs from healthy individuals to IHR and to SH *in vitro*. Collectively, these data strongly support our conclusion that delayed apoptosis of PMNs is related to the intermittent hypoxia itself and not to any associated conditions of sleep apnea. Another possible limitation could be that the intervals used in the experimental conditions for the IHR *in vitro,* using whole blood or purified PMNs, do not mimic the IHR of patients with OSA, because this is difficult to replicate *in vitro*. Yet, PMN apoptosis was delayed in response to IHR without the involvement of sleep fragmentation, and in parallel, IHR was shown to be a stronger stimulus for delayed apoptosis than SH.

It should also be noted that we did not use a conventional paradigm to test the effect of nCPAP on delayed apoptosis, in which previously untreated patients are tested before and after nCPAP treatment. The purpose of experiment 3, however, was not to test the effects of initiating nCPAP treatment on delayed PMN apoptosis but to minimize as much as possible any differences in confounding factors and other potential differences between the treated and untreated conditions. On the basis of our clinical experience, patients on nCPAP may show post-treatment decrease in BMI, and no less important, changes in lifestyle, such as increased physical activity and change in diet, which could potentially affect the results. Thus, testing patients after using nCPAP for several months, with and without treatment, allowed the investigation of the role of apneas with minimal effect of any other potential confounding variables.

In summary, decreased apoptosis and increased selectin adhesion molecule expression were noted in PMNs of patients with OSA. These were OSA severity dependent and could be normalized by nCPAP treatment. IHR and SH experiments *in vitro* further corroborated these findings. Importantly, the measures investigated were detectable only at an AHI greater than 15. These data confirm earlier observations on the effects of OSA pathology on inflammatory and immunologic changes in blood leukocytes (52–54). Importantly, thus far, various leukocyte subpopulations of patients with OSA, such as monocytes, $CD4^+$, $CD8^+$, and γδ T cells, also demonstrated increased activation and cytotoxicity against endothelial cells accompanied by increased expression of adhesion molecules and proinflammatory cytokines and various cytotoxic mechanisms. A schematic representation suggestive of this course of events, based on the current data and previous publications (22, 52–54), is illustrated in Figure 8. Jointly with delayed PMN apoptosis, leukocytes pose an atherosclerotic threat to the endothelium in OSA. This can be down-regulated by treatment with nCPAP.

Conflict of Interest Statement: L.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.L. has been reimbursed by MedCare for attending a scientific conference in 2005. He is a board member and scientific consultant of Itamar Medical, a company that produces ambulatory devices for sleep monitoring and cardiology, and he owns options in this company. He received $40,000 in 2006 and 2005 for consulting activities to Itamar Medical. He is a scientific advisor to SLP, which produces sensors for diagnosis of sleep disorders, and he owns options in this company. He is a board member of the Sleep Medicine Center Israel and Sleep Health Centers US, which provide diagnosis and treatment of sleep disorders. He has five patents relevant to diagnosis of sleep disorders. L.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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