particularly in relation to the future risk of cardiovascular diseases. Although the basic mechanisms underlying the inflammatory process in OSA remain unclear, recent studies suggest that not only repeated episodes of intermittent hypoxia but also increased fat tissue due to comorbid obesity seem to play significant roles [15, 16]. In particular, visceral adipose tissue accumulation may contribute to systemic inflammation by releasing numerous cytokines [17, 18].

As compared to systemic inflammation, less attention has been paid to airway inflammation in OSA. However, recent studies indicate that OSA causes respiratory symptoms such as cough [19] and increases proximal airway resistance [20], airway wall thickness and possibly airway hyperreactivity [21], or worsens comorbid airway diseases [22, 23], all of which might be related to airway inflammation. Airway inflammation in OSA seems to be directly and locally affected through mechanical stress [24], snoring-induced airway vibration [25, 26], or local oxidative stress [27].

The relationship between systemic and airway inflammation in OSA has not been investigated so far, but on the basis of differences in the speculated underlying mechanisms, we hypothesized that inflammatory processes in the two compartments would be differently related to OSA and comorbid obesity. Therefore, in the present study, by simultaneously measuring various biomarkers both in serum and induced sputum, we investigated their interrelationships between systemic and local compartments and also compared their relationships with polysomnographic data and obesity measurements.

Methods

Subjects

We recruited 43 consecutive patients who visited the Sleep Unit of Kyoto University Hospital. None had been previously diagnosed with or treated for OSA. Exclusion criteria included the following: (1) respiratory tract infection within the previous 4 weeks; (2) smoking history of more than 5 pack-years or during the past 6 months; (3) presence of other respiratory diseases such as asthma or chronic obstructive pulmonary disease (COPD) based on clinical history, chest radiograph, and spirometry; (4) treatments with corticosteroids or other immunosuppressive drugs; (5) comorbidities that may affect systemic inflammation such as collagen vascular disease or cancer; and (6) central sleep apnea. This study was approved by the Ethics Committee of Kyoto University (E558). Written informed consent was obtained from all patients.

Polysomnography

The diagnosis of OSA was confirmed by polysomnography (PSG) [28]. Apnea was defined as the complete cessation of airflow and hypopnea as a clear decrease in airflow of 50 % or more lasting for 10 s or more accompanied by a decrease in SpO_2 of at least 3 % [29]. All apnea/hypopnea index (AHI) values were expressed as the number of episodes of apnea and hypopnea per hour over the total sleep time (TST). Respiratory effort-related arousal (RERA) was defined as a sequence of breaths lasting at least 10 s characterized by increasing respiratory effort or flattening of the nasal pressure waveform leading to an arousal from sleep but not meeting criteria for apnea or hypopnea. The respiratory disturbance index (RDI) includes the AHI and the number of episodes of RERA per hour of sleep. Nocturnal oxygen desaturation was assessed by the lowest SpO_2 during sleep and $SpO_2<90$ % time per TST (%TST<90 %).

Blood sample collection and sputum induction and processing

Following overnight PSG, peripheral venous blood samples were collected in the morning after an overnight fast. Sputum induction and processing were performed shortly after the blood collection, as previously described [30–32]. Briefly, after premedication with 200 μg of inhaled salbutamol, subjects inhaled hypertonic (3 %) saline solution delivered with an ultrasonic nebulizer for 15 min. Each collected sample was immediately separated from contaminating saliva by visual examination, then mixed with 0.1 % dithiothreitol (Suptasol; Oxoid Ltd., Hampshire, UK) and diluted with Dulbecco's phosphate-buffered saline. After centrifugation, cell differentiation was determined by counting at least 400 non-squamous cells stained using the Diff-Quik method. The supernatants were collected and stored at −80 °C.

Biomarkers

Leptin concentrations were measured using a radioimmunoassay kit (Human Leptin RIA kit; Linco Research, St. Charles, MO, USA). IL-6, IL-8, TNF-α, and VEGF concentrations were determined using the Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions [33]. Cytokine-specific antibody-coated beads were used for these experiments. Beads were read on the Bio-Plex 200 suspension array system, and cytokine concentrations were automatically calculated with Bio-Plex Manager Software by a standard curve derived from a recombinant cytokine standard. Albumin concentrations were measured by turbidimetric immunoassay (Superior-Microalbumin kit; Mitsubishi Chemical Medience, Tokyo, Japan), after which, we calculated the airway vascular permeability index (ratio of albumin concentrations in induced sputum and serum) [34]. It has been shown to be reliable and useful in assessing extravasation in airway diseases [34, 35].

Body fat distribution

Amounts of abdominal subcutaneous and visceral fat deposition were assessed by computed tomography (CT) [17]. The area of visceral fat was measured in a single cross-sectional scan at the level of the umbilicus. An image histogram was computed for the subcutaneous fat layers in order to determine the range of CT numbers for the fat tissue. The total fat area was then calculated by counting the pixels that had intensities within the selected range of CT numbers. The intraperitoneal space was defined by tracing its contour on the scan image. The total area with the same CT numbers was considered to represent the visceral fat area (VFA). Subtraction of the visceral fat area from the total fat area was defined as the subcutaneous fat area (SFA).

Spirometry and impulse oscillometry

Spirometry and impulse oscillometry (IOS) (Masterscreen IOS-J, Jaeger, Wurzburg, Germany) for measuring respiratory impedance were performed as previously reported [20, 36, 37]. As increased proximal airway resistance was reported as a clinical feature of OSA using IOS [20, 38, 39], we used respiratory resistance at 20 Hz (R20) in the supine position for the analyses.

Statistics

All statistical analyses were performed using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA). Results are expressed as mean ± SD. Comparisons of variables between two groups were made by Fisher's exact tests or unpaired *t* tests. Relationships between two variables were analyzed by Pearson's correlation coefficient tests. Stepwise multiple regression analyses were performed to identify variables that could best explain inflammatory biomarker levels in serum and sputum using measurements that were significantly related to each biomarker level as explanatory variables. When assessing the relationships of proximal airway resistance with sputum biomarker levels, we adjusted for body mass index (BMI) by multiple regression analyses. A p value less than 0.05 was considered to indicate statistical significance.

Results

Clinical characteristics and polysomnographic data

Patient characteristics and polysomnographic data are shown in Table 1. Although the study included 43 patients at entry, samples from five patients could not be examined simultaneously due to scheduling considerations, and those patients were therefore excluded. Thereafter, the further analysis included the following 38 patients: 6 non-OSA (AHI<5), 9 mild OSA (5≤AHI<15), 13 moderate OSA (15≤AHI<30), and 10 severe OSA patients (AHI≥30). Sputum was successfully induced in 28 patients [sputum (+) group] but could not be induced in ten patients [sputum (−) group]. CT analysis of body fat distribution was not performed in 3 of the 38 patients. There were no significant differences in patients' background, smoking history, body fat distribution, and polysomnographic data between sputum (+) and sputum (−) groups (Table 1).

Table 1 Clinical characteristics and polysomnographic data

	Total (n=38)	Sputum (+) (n=28)	Sputum (−) (n=10)	p value[a]
Sex, male/female	21:17	15:13	6:4	0.99
Age, years	53.4±16.0	51.2±15.9	59.5±15.5	0.16
Smoking history, ex/never	5:33	4:24	1:9	0.99
Smoking, pack-years	0.4±1.3	0.5±1.5	0.02±0.05	0.28
Respiratory resistance at 20 Hz, kPa/l/s	0.34±0.14	0.34±0.14	0.32±0.15	0.68
BMI, kg/m^2	26.7±6.6	27.0±5.9	25.7±8.4	0.58
Waist circumference, cm	92.5±15.6	93.2±15.8	90.5±15.9	0.65
SFA, cm^2	182.7±147.2	185.7±131.3	173.8±198.0	0.85
VFA, cm^2	94.7±67.1	98.7±65.1	82.7±76.0	0.57
3 % ODI	24.0±25.7	21.4±22.0	31.3±34.4	0.30
4 % ODI	19.6±24.6	17.2±20.8	26.1±33.5	0.34
AHI, events/h	24.7±24.7	22.1±21.0	32.2±33.2	0.27
RDI, events/h	26.7±24.2	24.2±20.5	33.7±32.7	0.29
SpO_2<90 % time/TST, %	12.1±22.5	11.0±20.5	15.3±27.9	0.61
Lowest SpO_2, %	79.4±13.4	79.9±14.5	78.1±10.0	0.73

Data are presented as number or mean ± SD

BMI body mass index, *SFA* subcutaneous fat area, *VFA* visceral fat area, *ODI* oxygen desaturation index, *AHI* apnea/hypopnea index, *RDI* respiratory disturbance index, *TST* total sleep time

[a]Comparison between sputum (+) and sputum (−) groups

Table 2 Concentrations of albumin and biomarkers in serum and induced sputum

	Serum[a]		Induced sputum
	Sputum (+) (n=28)	Sputum (−) (n=10)	Sputum (+) (n=28)
Albumin, g/dl	4.2±0.3	4.1±0.3	0.03±0.03
Leptin, ng/ml	10.1±7.4	10.7±17.4	2.5±1.3
IL-6, pg/ml	0.9±0.6	1.2±0.7	42.8±53.1
IL-8, pg/ml	3.9±2.3	4.6±1.6	5,272.4±8,730.8
TNF-α, pg/ml	1.0±0.9	1.1±1.6	16.8±33.4
VEGF, pg/ml	61.8±60.8	40.2±39.0	978.0±967.2

Data are presented as mean ± SD

IL-6 interleukin-6, *IL-8* interleukin-8, *TNF-α* tumor necrosis factor-α, *VEGF* vascular endothelial growth factor

[a] Significant differences were not found in biomarker levels between sputum (+) and sputum (−) groups

Concentrations of albumin and biomarkers in serum and induced sputum

Concentrations of albumin and biomarker levels in serum and induced sputum samples are shown in Table 2. Significant differences were not found in serum biomarker levels between sputum (+) and sputum (−) groups (p=0.17 to 0.88).

Relationships of systemic inflammation markers with obesity measurements and polysomnographic data

Table E1 shows the relationships of serum biomarker levels with obesity measurements and polysomnographic data. Obesity measurements according to BMI, waist circumference, SFA, and VFA were significantly related to serum leptin, IL-6, IL-8, TNF-α, and VEGF [|r (correlation coefficients)|=0.37 to 0.78, p<0.05]; nonsignificant relationships were shown between BMI and IL-8, SFA and IL-8, and SFA and VEGF. PSG measurements of ODI, AHI, and RDI were positively significantly related to serum leptin, IL-6, and TNF-α (|r|=0.34 to 0.63, p<0.05). In contrast, serum IL-8 and VEGF were related to none of the PSG measurements. %TST<90 % was significantly related to serum levels of leptin and IL-6, and the lowest SpO_2 was significantly related only to serum leptin levels.

Next, using the indices significantly related to each serum biomarker, we performed stepwise multiple regression analyses (Table 3). Regarding serum leptin, SFA most significantly explained it [r^2 (coefficient of determination)=0.59] followed by the lowest SpO_2 (r^2=0.10). Serum IL-6 and VEGF were significantly related to VFA (r^2=0.43 and 0.21, respectively), and IL-8 and TNF-α were significantly related to waist circumference (r^2=0.16 and 0.43, respectively).

Relationships of airway inflammation markers with obesity measurements and polysomnographic data and with sputum neutrophil number

Table E2 shows the relationships of sputum biomarker levels with obesity measurements and polysomnographic data. With regard to obesity measurements, significant relationships were only found between SFA and leptin, BMI and IL-6, and VFA and IL-8. Sputum IL-8 and TNF-α were significantly related to all PSG measurements (|r|=0.49 to 0.69, p<0.05), except for nonsignificant relationships between IL-8 and the lowest SpO_2. Sputum IL-6 and VEGF were significantly related to

Table 3 Stepwise multiple regression analyses to predict serum biomarker levels

	Leptin (ng/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF-α (pg/ml)	VEGF (pg/ml)
Obesity measurements					
BMI, kg/m^2	–	–		–	–
Waist circumference, cm	–	–	r^2=0.16 p=0.01	r^2=0.41 p<0.001	–
SFA, cm^2	r^2=0.59 p<0.001	–		–	
VFA, cm^2	–	r^2=0.43 p<0.001	–	–	r^2=0.21 p=0.009
Polysomnographic data					
3 % ODI	–	–		–	
4 % ODI	–	–		–	
AHI, events/h	–	–		–	
RDI, events/h	–	–		–	
SpO_2<90 % time/TST, %	–	–			
Lowest SpO_2, %	r^2=0.10 p=0.02		–		

– data did not show statistical significance, *IL-6* interleukin-6, *IL-8* interleukin-8, *TNF-α* tumor necrosis factor-α, *VEGF* vascular endothelial growth factor, *BMI* body mass index, *SFA* subcutaneous fat area, *VFA* visceral fat area, *ODI* oxygen desaturation index, *AHI* apnea/hypopnea index, *RDI* respiratory disturbance index, *TST* total sleep time

the lowest SpO_2 and RDI, respectively. Sputum leptin was related to none of the PSG measurements.

We also performed stepwise multiple regression analyses with respect to sputum biomarkers (Table 4). Sputum leptin was significantly related to SFA (r^2=0.22). Regarding sputum IL-6, the lowest SpO_2 significantly explained it (r^2= 0.33). Sputum IL-8 and TNF-α were significantly related to %TST<90 % (r^2=0.48 and 0.43, respectively), and sputum VEGF was related to RDI (r^2=0.16).

We then further investigated whether sputum biomarker levels were related to the degree of infiltration of neutrophils. The sputum neutrophil number significantly correlated with sputum levels of IL-6, IL-8, TNF-α, and VEGF (Fig. 1), but not with sputum levels of leptin (r=0.07, p=0.74).

Extravasation in the airways and interrelationships between systemic and airway inflammation markers

The airway vascular permeability index was significantly related to PSG measurements ($|r|$=0.41 to 0.60, p<0.05) (Table 5). Then, to investigate the possible relationships between systemic and airway inflammation, we analyzed the relationships between the same biomarkers in serum and sputum. However, there were no significant relationships with regard to leptin, IL-6, IL-8, TNF-α, and VEGF (Table E3).

Relationships between airway inflammation and proximal airway resistance

To assess the impact of airway inflammation on proximal airway resistance in OSA, we investigated the relationships of proximal airway resistance with sputum biomarker levels (Table 6). R20 determined by IOS had significant positive correlations with sputum levels of IL-6, IL-8, and TNF-α ($|r|$=0.43 to 0.62, p<0.05), but not with sputum leptin and VEGF. Even after adjustment for BMI, which was significantly associated with R20 (r=0.52, p<0.001), the correlation of R20 with sputum IL-8 and TNF-α remained statistically significant (β coefficient=0.47, p=0.003 and β coefficient=0.46, p=0.004, respectively).

Discussion

This study focused on the relationships between systemic inflammation, airway inflammation, and OSA. We found that (1) regarding systemic inflammation, multiple regression analyses indicated that all measured serum markers were significantly related to obesity measurements, and serum leptin was also significantly related to OSA severity (lowest SpO_2); (2) regarding airway inflammation, multiple regression analyses showed that sputum IL-6, IL-8, TNF-α, and VEGF were significantly related to OSA severity, whereas sputum leptin was related to an obesity measurement (SFA); (3) sputum IL-6, IL-8, TNF-α, and VEGF were significantly related to sputum neutrophil number; (4) the airway vascular permeability index was significantly related to OSA severity but there were no significant direct interrelationships between the same biomarkers in serum and induced sputum; and (5) sputum IL-8 and TNF-α were significantly related to proximal airway resistance independently of BMI.

We simultaneously investigated multiple biomarkers both in serum and induced sputum of patients with OSA.

Table 4 Stepwise multiple regression analyses to predict sputum biomarker levels

	Leptin (ng/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF-α (pg/ml)	VEGF (pg/ml)
Obesity measurements					
BMI, kg/m^2		–			
Waist circumference, cm					
SFA, cm^2	r^2=0.22 p=0.03				
VFA, cm^2			–		
Polysomnographic data					
3 % ODI			–	–	
4 % ODI			–	–	
AHI, events/h			–	–	
RDI, events/h			–	–	r^2=0.16 p=0.04
SpO_2<90 % time/TST, %			r^2=0.48 p<0.001	r^2=0.43 p=0.001	
Lowest SpO_2, %		r^2=0.37 p<0.001		–	

– data did not show statistical significance, *IL-6* interleukin-6, *IL-8* interleukin-8, *TNF-α* tumor necrosis factor-α, *VEGF* vascular endothelial growth factor, *BMI* body mass index, *SFA* subcutaneous fat area, *VFA* visceral fat area, *ODI* oxygen desaturation index, *AHI* apnea/hypopnea index, *RDI* respiratory disturbance index, *TST* total sleep time

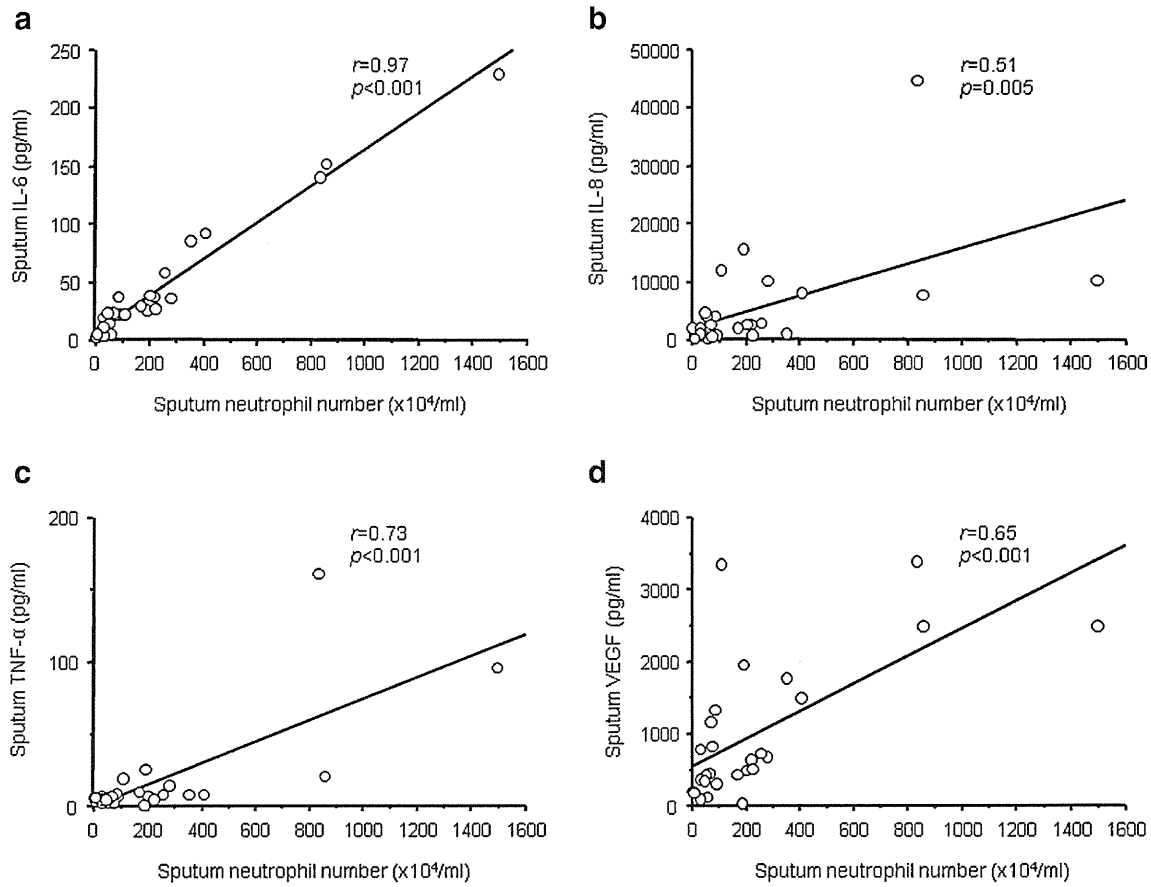

Fig. 1 Scatter diagrams showing the correlation of sputum neutrophil number with sputum levels of IL-6 (**a**), IL-8 (**b**), TNF-α (**c**), and VEGF (**d**). The *r* value indicates a correlation coefficient. *Lines* indicate regression lines

Comparative analyses of relationships of systemic and airway inflammation markers with obesity and OSA severity suggest that inflammatory processes in these two compartments are not similarly regulated in OSA. Systemic inflammation markers were predominantly related to obesity, especially fat accumulation around the abdomen, rather than the severity of OSA. Many previous reports pointed out increased levels of circulating inflammatory markers in OSA [5–14], whereas recent studies suggest that obesity is also an important determinant of systemic inflammation [15, 16]. Because obesity is associated with OSA, the relative contributions of obesity and of OSA itself to systemic inflammatory responses are difficult to clearly distinguish. However, obesity and OSA, both of which are associated

Table 5 Relationships of airway vascular permeability index with polysomnographic data

	Permeability index[a]	
	r value	*p* value
3 % ODI	0.41	0.03
4 % ODI	0.42	0.03
AHI, events/h	0.44	0.02
RDI, events/h	0.44	0.02
SpO_2<90 % time/TST, %	0.41	0.03
Lowest SpO_2, %	−0.60	<0.001

[a] Permeability index indicates albumin concentration in induced sputum/serum

ODI oxygen desaturation index, *AHI* apnea/hypopnea index, *RDI* respiratory disturbance index, *TST* total sleep time

Table 6 Relationships of proximal airway resistance with sputum biomarker levels

	Unadjusted		Adjusted[a]	
	β coefficient	*p* value	β coefficient	*p* value
Leptin, ng/ml	0.18	0.40		
IL-6, pg/ml	0.43	0.02	0.23	0.18
IL-8, pg/ml	0.62	<0.001	0.47	0.003
TNF-α, pg/ml	0.60	<0.001	0.46	0.004
VEGF, pg/ml	0.34	0.07		

IL-6 interleukin-6, *IL-8* interleukin-8, *TNF-α* tumor necrosis factor-α, *VEGF* vascular endothelial growth factor

[a] Adjusted for body mass index

with future cardiovascular risks, may mutually enhance a systemic inflammatory response, although their contributions may differ depending on the type of cytokine [40]. On the other hand, airway inflammation markers were predominantly related to the severity of OSA. While recent evidence has suggested that obesity can provoke or worsen airway inflammation [41], less is known regarding the possible role of comorbid OSA. The results of the present study suggest that airway inflammation in OSA is likely to be affected by OSA itself, unlike systemic inflammation.

We found no direct interrelationships between systemic and airway inflammation markers in OSA. However, the airway vascular permeability index was significantly related to OSA severity. We recently reported that KL-6, which is predominantly produced in the lung, was elevated in the serum of patients with OSA and that its concentration was related to disease severity [28]. These findings indicate the presence of increased vascular permeability in the airways and protein movement from the airway to the circulation. Conversely, leptin, which is synthesized and secreted mainly by adipose tissue and has been known to be elevated in the serum of OSA patients [8–10], was detected in sputum at about one fourth of the serum level. This suggests that leptin in sputum might in part be derived from the systemic circulation, although leptin was recently reported to be expressed in bronchial epithelial cells, type II pneumocytes, or alveolar macrophages [42]. Thus, there may be some interactions between local and systemic compartments, but the degree of leakage might be subtle compared to the differently regulated inflammatory response in each compartment. In addition, simple correlations of biomarker levels between the two compartments might not be enough to confirm or refute this concept [43], and further studies are needed.

Notably, sputum levels of IL-6, IL-8, TNF-α, and VEGF were significantly related to sputum neutrophil number, indicating that they might play important roles in neutrophil recruitment or increasing airway inflammation. Although airway inflammation in OSA often has been neglected, recent studies indicate its importance in association with respiratory symptoms [19], airway wall thickness, and hyperreactivity [21] or with comorbidities such as asthma [22] and COPD [23]. A further finding in the present study is the significant correlation between airway inflammation and proximal airway resistance. Increased proximal airway resistance, an important clinical characteristic of OSA [20], is considered to be mainly due to the mechanical or functional effects of obesity. However, our current results are the first to show that the magnitude of airway inflammation was associated with airway resistance independently of obesity. We thus demonstrated not only the presence but also the pathophysiological relevance of airway inflammation in OSA.

The present study has some limitations. Firstly, the sample size was small. This is because we made an effort to exclude smokers or subjects with comorbidities that can affect systemic inflammation. Hence, there remains a need for further studies with larger samples to elucidate the relative contributions of obesity and OSA to the inflammatory process in both compartments. Secondly, we could obtain adequate sputum samples from only 73.7 % of subjects, although the clinical characteristics and polysomnographic data did not differ according to the presence of sputum samples. This is partly because the majority of our study subjects were never smokers [32]. The rate of successful sputum induction was comparable with a previous report from our institute in patients with asthma [32].

In conclusion, systemic and airway inflammation in OSA might be differently regulated by OSA itself and comorbid obesity, depending on the type of cytokine. Although we did not find apparent interrelationships between systemic and local compartments, further studies are needed to clarify this concept. Further knowledge of inflammatory processes in both compartments would provide a better understanding of the respiratory as well as cardiovascular consequences of OSA.

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Title: A urine biomarker for severe OSA patients: Lipocaline-type prostaglandin D synthase

Running Head: The relation between prostanoids and OSA

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Abstract

Lipocalin-type prostaglandin D synthase (L-PGDS), which is responsible for the biosynthesis of prostaglandin D_2, has been reported to have a close connection with cardiovascular disease and sleep regulation. This study aimed to test the hypothesis that the L-PGDS level is a useful marker to identify patients with obstructive sleep apnoea (OSA).

Sixty-four subjects were enrolled in this prospective study. Urinary concentrations of L-PGDS were measured in the morning. Measurements were made every 4 hours in 25 of the 64 patients. Endothelial function was assessed by the reactive hyperemia peripheral arterial tone index.

Circadian variations in L-PGDS concentrations had a significant time-dependent fluctuation (p=0.0002). L-PGDS was higher in the subjects with severe OSA (median, n=23, 784.7 ng/mg・Creatinine) than in control subjects (n=16, 262.1, p=0.004) and in those with moderate OSA (n=25, 371.7, p=0.0008). After 2 days of CPAP treatment, L-PGDS concentrations in severe OSA (n=12) decreased significantly (p=0.02) to levels present in control subjects whereas endothelial function did not change significantly. Morning urinary L-PGDS concentrations had significant correlations with the apnoea-hypopnoea index (R^2=13.9%) and serum high-density lipoprotein cholesterol (R^2= 6.2%) but not with sleepiness.

Urinary L-PGDS might be a moderately useful marker to identify patients with severe

OSA.

Keywords: cardiovascular disease, circadian rhythm, hypoxia, sleep.

INTRODUCTION

Obstructive sleep apnoea (OSA) is one of the most important medical conditions identified in the last 50 years and is a major cause of morbidity and mortality throughout the world [1].

Most patients with OSA that should be treated are undiagnosed. One reason is that the diagnostic methods for OSA, such as polysomnography (PSG), are difficult to perform. Therefore, a reasonable biomarker for OSA would be extremely helpful, especially in identifying patients who have OSA with a degree of severity that would put them at risk for cardiovascular disease (CVD).

Prostaglandin D_2 (PGD_2) is formed by the action of PGD synthases on the cyclooxygenase (COX) product PGH_2. PGD_2 is widely distributed in rat and human brain [2]. In peripheral tissues, PGD_2 executes a wide range of functions, including vasodilatation, inhibition of platelet aggregation, glycogenolysis, vasoconstriction, allergic reaction mediation, and intraocular pressure reduction [3-8]. In the brain, PGD_2 has been shown to contribute to sleep induction, modulation of body temperature, olfactory function, hormone release, nociception, and neuromodulation [9-12]. Thus, since PGD_2 has significant effects on platelet aggregation, vasodilation and vasoconstriction, it has been supposed that PGD_2 is relevant to the occurrence of CVD. Although the prostanoids, including PGD_2, are released from the cells immediately after synthesis, it is believed that prostanoids work only locally, near their site of production because they are either chemically or metabolically unstable [13].

One of the enzymes characterized as a PGD synthase, which catalyzes the isomerization of PGH_2 to PGD_2, the lipocalin-type prostaglandin D synthase (L-PGDS) [14] is responsible for the biosynthesis of PGD_2 in the brain and heart (cardiovascular system). L-PGDS is a unique protein with enzyme activity and ligand-binding properties. L-PGDS binds various lipophilic compounds, such as retinoids, bilirubin, biliverdin, gangliosides, and amyloid-β peptides, with high affinity, acting as an extracellular transporter of these compounds and serving as an endogenous amyloid-β chaperone to prevent amyloid deposition in vivo [15]. The half-life of L-PGDS in canines was reported to be 0.77 hour [16].

L-PGDS was confirmed to be secreted into blood and urine [14], and we have established a system to measure its urine, serum, or plasma levels by an enzyme-linked immunosorbent assay (ELISA) system [14, 17-19]. L-PGDS is a very stable enzyme and is highly resistant against heat treatment [14] and protease digestion [20], whereas PGD_2 is an unstable substance as mentioned above [13]. L-PGDS is localized where PGD_2 would work, that is, in the central nervous system, male genital organs, and the human heart. In the human heart, L-PGDS is localized in myocardial and atrial endocardial cells, smooth muscle cells in the arteriosclerotic intima, and in the atherosclerotic plaque of severely stenotic coronary arteries. In addition, the chemical properties of L-PGDS are similar to those of serum albumin; however, its molecular weight is much smaller than that of serum albumin (26000 vs. 66000 Da). Thus, L-PGDS more easily passes through glomerular capillary walls of the kidney than

serum albumin. Indeed, it has been reported that urinary L-PGDS excretion increased in the microalbuminuric stage in patients with type 2 diabetes mellitus (DM) and in hypertensive patients who were apparently free from overt proteinuria [21, 22]. Although the enzymatic activity of L-PGDS cannot be determined, the amount of serum or urinary L-PGDS increased when conditions such as coronary heart disease, hypertension, or type 2 DM worsened [21-23].

Since OSA induces multi-organ damage and diseases such as hypertension, DM, renal insufficiency, coronary disease, and cerebral-cardiovascular diseases, the degree of severity of OSA in individuals with OSA would have significant associations with morbidity and mortality from these conditions [24]. In addition, serum L-PGDS levels were slightly elevated in individuals with OSA with excessive daytime sleepiness [25]. Thus, we hypothesized that L-PGDS could be a biomarker for OSA because of its close relationships with sleep and CVD. In consideration of this hypothesis, we tested whether plasma or urine L-PGDS would be a powerful biomarker for OSA.

METHODS

Study subjects

Sixty-four clinically stable adults (age >20 years old) with suspected OSA were consecutively enrolled in the present prospective study **(Clinical Trial**

Registration—URL:http://www.clinicaltrials.gov. Unique identifier: NCT01096433).

Major exclusion criteria were history of CVD, DM under treatment with hypoglycemic agents or insulin, use of glucocorticoid or non-steroidal anti-inflammatory drugs, and being a current smoker. This study was approved by the Ethics Committee of Kyoto University. All patients gave written informed consent to participate. The other exclusion criteria in detail are shown in the supplemental file.

Study design

At baseline, the subject's medical history was recorded and a physical examination was performed. In the medical history, hypertension was defined as a systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or use of an antihypertensive medication. Dyslipidemia was defined as serum low-density lipoprotein cholesterol (LDL-C) ≥ 140 mg/dl or high-density lipoprotein cholesterol (HDL-C) < 40 mg/dl or triglycerides ≥ 150 mg/dl [26] or use of an antilipidemic medication.

All patients underwent attended diagnostic overnight PSG. Before the patient slept, urine samples were collected at 22:00. In addition, the first urine voided in the morning following the overnight PSG was collected at 6:00. Peripheral venous blood samples were collected from 6:00 to 7:00 following a 12-hour overnight fast and PSG. Morning endothelial dysfunction measured by reactive hyperemia peripheral arterial tone (RH-PAT) [27] was

measured. After the overnight PSG, 5 blood pressure measurements, each 1 min apart, were taken in the morning after the patient had rested for at least 5 min in the supine position. The average of the latter two readings was calculated.

After 2 days of continuous positive airway pressure (CPAP) treatment, urine and blood samples were collected at the same time as during and after PSG. In all of the patients, adequate CPAP pressure was determined to have overcome obstruction and all flow limitations by a full night's titration.

During the PSG day, we investigated the circadian variations in urinary L-PGDS concentrations in 25 patients who were randomly selected from all 64 subjects at 4-hour intervals except for midnight (14:00, 18:00, 22:00, 6:00, and 10:00). Urinary sampling was not done at midnight in order not to disturb physiologic sleep.

PSG

PSG was done according to recommendations in American Academy of Sleep Medicine's manual. (See the supplemental files). Apnoea-hypopnoea index (AHI) values were expressed as the number of episodes of apnoea and hypopnoea per hour over the total sleep time. OSA severity was defined by the AHI as follows: control (AHI < 15), moderate OSA ($15 \leq$ AHI $<$ 30), and severe OSA (AHI ≥ 30).

Measurements of plasma and urinary L-PGDS concentrations

In the present study, plasma samples were centrifuged immediately at 3000 rpm at 4℃ for 15 min and urine samples were pooled (not centrifuged) in the present study as described previously [15, 17-19]. The separated samples were stored at -80 ℃ until assay. Concentrations of urinary or plasma L-PGDS were measured by an ELISA using 2 monoclonal antibodies, Mab-7F5 and Mab-1B7, as described previously [15, 17-19] (See the supplemental files).

With this ELISA system, it has been demonstrated that intra- and interassay coefficients of variation in urine samples ranged from 3.2% to 5.8% and from 7.6% to 8.3%, respectively. The intra- and interassay coefficients of variation in serum samples were 3.6% and 5.8%, respectively. The ELISA showed no significant interference by a variety of urinary constituents [18]. In addition, it was shown that serum and plasma L-PGDS values in individual subjects were almost the same [15]. All the samples were measured in duplicate and the results were averaged.

Other parameters

Venous blood samples were taken in the fasting state in the morning after one-night PSG and examined for markers of glucose and lipid metabolism and C-reactive protein. As it was difficult to measure urinary catecholamine and L-PGDS levels at the same time, we measured

plasma catecholamine levels.

Measurements of the RH-PAT

The RH-PAT is a newly established method to measure endothelial function [27]. Endothelial dysfunction as measured by RH-PAT has been reported in patients with OSA [28]. Morning endothelial function assessed by a finger plethysmographic device (Itamar Medical Ltd., Caesarea, Israel) that allows the isolated detection of pulsatile arterial volume changes [27] was measured after an overnight PSG and after 2 days of CPAP.

Statistical Analysis

Data were analyzed using JMP 9.0 (SAS Institute, Inc. Cary, NC, USA). Continuous variables were expressed as mean ± standard error (SE) or median values and ranges because the sample size of each group was small. The associations between patients' characteristics, PSG data, biomarkers (blood and urine), and OSA severity were assessed by the Kruskall-Wallis test. When a significant difference was observed, we used the Bonferroni corrected t test to identify where differences were significant. We evaluated the sensitivity and specificity of the cut off value of L-PGDS for predicting severe OSA with the use of receiver operating characteristic (ROC) curve analysis, estimating the area under the curve (AUC). Relationship between L-PGDS concentrations (urine, plasma), RH-PAT index, and

other parameters were analyzed by Pearson's correlation coefficient test. Multiple regression analysis was performed to adjust for confounders such as age, gender, BMI, and morning systolic and diastolic blood pressure. Next, multiple regression analyses, with a p value < 0.10 required for entry into the models, were performed to identify those variables that could best predict morning urinary L-PGDS.

To investigate changes in L-PGDS concentrations, the RH-PAT index, and other parameters before and after 2 days of CPAP, comparisons of data between those two time points were tested by a paired *t* test. Multiple analysis of variance with repeated measures was performed to analyze urinary L-PGDS concentrations across the 24-hour period. In all analyses, p value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of study subjects, L-PGDS concentration, and RH-PAT index according to OSA severity

Patient characteristics, PSG data, and laboratory data are shown in Table 1. There were significant differences among the groups in morning urinary L-PGDS concentrations (p = 0.0009) but not in night urinary L-PGDS concentrations (p = 0.19) and plasma L-PGDS levels (p = 0.09) (Table 1 and Figure 1-A). After adjustment for age and body mass index (BMI), subjects with severe OSA had significantly higher morning urinary L-PGDS values

than control subjects ($p = 0.007$) and subjects with moderate OSA ($p = 0.002$). There were significant differences among the groups in the RH-PAT index (Figure 1-B).

The cut-off value for predicting severe OSA with minimal false negative and false positive errors was 621.8 ng/mg · Creatinine (sensitivity 65.2%, specificity, 85.4%). This cut-off value had moderate accuracy for predicting severe OSA (area under curve 0.78) (Figure 2).

Relation between urinary L-PGDS concentrations, RH-PAT index, and clinical indices

Morning urinary L-PGDS concentrations were positively correlated with several parameters, including the AHI (Table 2 and Figure 3). Morning urinary L-PGDS concentrations were positively correlated with AHI after adjustment for age, gender, BMI, and morning systolic and diastolic blood pressure (Figure 3). There was a strongly positive correlation between morning and night urinary L-PGDS concentrations ($p < 0.0001$). The Epworth Sleepiness Scale did not correlate significantly with morning urinary L-PGDS (Table 2).

After adjustment for age, gender, BMI, and morning systolic and diastolic blood pressure, morning urinary L-PGDS concentrations were still positively correlated with the AHI (β coefficient = 0.373, $p = 0.006$), 3% oxygen desaturation index (ODI) ($\beta = 0.322$, $p = 0.02$), arousal index ($\beta = 0.370$, $p = 0.007$), and plasma noradrenaline ($\beta = 0.258$, $p = 0.04$). The RH-PAT index was negatively correlated with the AHI ($\beta = -0.305$, $p = 0.04$) and arousal

index (β = -0.359, p = 0.01) after adjustment.

Relation between plasma L-PGDS concentrations and clinical indices

Plasma L-PDS levels were positively correlated with several parameters (Table 2). Plasma L-PGDS also tended to have a positive correlation with morning urinary L-PGDS (p = 0.07). After adjustment for age, gender, BMI, and morning systolic and diastolic blood pressure, plasma L-PGDS levels were only positively correlated with serum creatinine levels (β = 0.361, p = 0.03).

Clinical determinants of morning urinary L-PGDS concentrations

Table 3 shows results of multiple regression analyses to identify those variables (morning systolic and diastolic blood pressure, arousal index, AHI, 3% ODI, serum HDL-C, plasma adrenalin, plasma noradrenalin, and plasma L-PGDS) that could predict morning urinary L-PGDS concentrations. Then, among the variables that had very strong co-linearity (r > 0.70) with each other, such as the arousal index, AHI, and 3% ODI, one was selected.

In these three models, morning urinary L-PGDS had a significant and independent correlation with the AHI or the 3% ODI, or the arousal index and serum HDL-C (Table 3).

Effects of CPAP treatment on urinary L-PGDS concentrations