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

Author(s): Yuichi Chihara[1], M.D, PhD; Kazuo Chin[2], M.D, PhD; Kosuke Aritake[3], PhD; Yuka Harada[1], M.D; Yoshiro Toyama[1], M.D; Kimihiko Murase[1], M.D; Chikara Yoshimura[2], M.D, PhD; Takefumi Hitomi[2], M.D, PhD; Toru Oga[2], M.D, PhD; Michiaki Mishima[1], M.D, PhD; Yoshihiro Urade[3],PhD.

Institutions: [1]Department of Respiratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, [2]Departments of Respiratory Care and Sleep Control Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, [3]Departments of Molecular and Behavioral Biology, Osaka Bioscience Institute, Osaka, Japan.

Correspondence to: Kazuo Chin, M.D, PhD, Departments of Respiratory Care and Sleep Control Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-Ku, Kyoto 606-8507, Japan. Telephone: 81-75-751-3852; FAX: 81-75-751-3854; E-mail: chink@kuhp.kyoto-u.ac.jp

Author's contributions

Conception and research design: Y. Chihara, K. Chin.

Data collection: Y. Chihara, Y. Harada, Y. Toyama, K. Murase, C. Yoshimura, T. Hitomi, T. Oga

Data analysis and interpretation, and drafting the article: Y. Chihara, K. Chin, K.Aritake, Y. Urade, M. Mishima.

Critical revision of the article: Y. Chihara, K. Aritake, K. Chin.

Final approval of the article: all the authors.

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

In Japan, patients whose AHI is more than 20 can use CPAP under the health insurance system. In this study, 20 patients were permitted to use the CPAP device (Table 4). Twelve of the 20 patients had severe OSA.

After 2 days of CPAP treatment, morning urinary L-PGDS concentrations were significantly decreased compared with baseline values (Figure 4-A), In contrast, the plasma L-PGDS level, the RH-PAT index (Figure 4-B), and the other biomarkers were not significantly changed (Table 4). In the 12 patients with severe OSA, morning urinary L-PGDS concentrations were significantly decreased compared with baseline values (591.2 at baseline vs. 317.8 ng/mg・Creatinine after 2 days of CPAP, $p = 0.02$). Those decreased L-PGDS concentrations reached the levels present in control subjects ($p = 0.65$).

Circadian variations in urinary L-PGDS concentrations

Circadian variations in urinary L-PGDS concentrations are shown in Figure 5-A. In 25 patients, a multiple analysis of variance with repeated measures revealed a significant time-dependent fluctuation ($p = 0.0002$), with the highest levels at 14:00 and lowest levels at 6:00. Compared to the 6:00 values, urinary L-PGDS concentrations significantly increased at 14:00 ($p < 0.0001$) and 18:00 ($p = 0.02$). Regarding the associations between the AHI and urinary L-PGDS concentrations at each time point, the 6:00 values were only significantly positively correlated with AHI ($r = 0.566$, $p = 0.004$). In the patients with AHI $\geq$ 30, 6:00

values for urinary L-PGDS were significantly increased compared with patients with AHI < 30 (AHI < 30 (n=13) 227 vs. AHI ≥ 30 (n=12) 780.3, p = 0.003) (Figure 5-B).

DISCUSSION

The major findings of our study are that morning urinary L-PGDS concentrations were positively correlated with the severity of OSA as indicated by AHI, 3% ODI, and the arousal index after adjustment for several confounding factors, and that urinary L-PGDS was significantly elevated in patients with severe OSA in comparison with control subjects and those with moderate OSA. Multivariate modeling of L-PGDS determinants revealed that HDL-C and the AHI, 3% ODI, or the arousal index were significantly and independently associated with the morning urinary L-PGDS (Table 3). However, of interest is that urinary L-PGDS levels at 14:00 were the same in non-to-moderate and severe OSA patients. Our results also showed that morning urinary L-PGDS concentrations were decreased to control values after only 2 days of CPAP treatment although the RH-PAT did not change. These results suggest that the urinary L-PGDS concentration might be a moderately useful marker to identify patients with severe OSA.

In the current study, subjects with severe OSA had elevated urinary L-PGDS values and decreased endothelial function determined by the RH-PAT index whereas the relationship between urinary L-PGDS and the RH-PAT index was not significant. Therefore, the

combined measurement of endothelial function and L-PGDS may be a useful tool for finding and managing patients with severe OSA.

Prostaglandin systems, including PGD_2, are very important in health maintenance and disease prevention, but they are either chemically or metabolically unstable. Therefore, substitutions such as L-PGDS are used as parameters for identifying the functions and effects of prostaglandin systems. Previous studies demonstrated that L-PGDS levels in serum, cerebrospinal fluid, or urine were elevated in patients with cardiovascular, neurological, and renal diseases [21, 23, 29, 30]. The elevation in L-PGDS concentration occurs at an early stage of CVD, and the concentrations of serum or urinary L-PGDS were shown to increase when diseases such as coronary heart disease or type 2 DM worsen [21-23], although the activity of L-PGDS is not well known. OSA induces multi-organ damages such as hypersomnolence and CVD. Therefore, it is supposed that L-PGDS could have significant associations with the pathophysiology and severity of OSA. In addition, a urinary biomarker is attractive because urine is easily obtained and the results of urine testing are easy to follow. Although our present data might not be definitive because of the small sample size, the information gained from this study appears to be of interest and the topic warrants further study.

It was said that serum L-PGDS levels increased with age and were higher in men than in women [19] while urinary L-PGDS was also shown to be higher in males than in females and

was weakly correlated with age in female subjects [22]. In this study, control subjects were slightly younger but not with significance, and those in the moderate and severe OSA groups were almost the same age (Table 1). In addition, morning urinary L-PGDS values were still correlated with the severity of OSA after adjustment for BMI, age, and gender. Therefore, BMI, gender, and age could not be considered to have significant effects on our results. Although L-PGDS concentrations were shown to be increased in essential hypertension [21], morning urinary L-PGDS values were still correlated with the severity of OSA after adjustment for blood pressure. The BMI in individuals with severe OSA was slightly but not significantly higher than in the other groups. It was reported that the BMI [21] was not associated with elevations in blood or urinary L-PGDS. In addition to the fact that morning urinary L-PGDS levels were significantly higher after adjustment for BMI, morning urinary L-PGDS concentrations were also significantly decreased after 2 days of CPAP, which is a specific treatment for OSA, although other factors remained the same. Furthermore, from the viewpoint of circadian measurements, morning urine L-PGDS levels were higher in patients with severe OSA than in the other subjects while values at 14:00 were the same. These findings indicate that L-PGDS is related to OSA independently, especially to severe OSA.

Several causes for elevated L-PGDS concentrations in OSA have been considered, especially in severe OSA. Firstly, intermittent hypoxia induces COX 2 expression and activity, which in turn accelerate arachnoid acid metabolism to PGH_2 and PGE_2 [31, 32]. Han et al.

noted that the expression levels of L-PGDS mRNA and protein in the heart of C57BL/6 mice were significantly increased after 14 days of hypoxia (10% O_2) compared with mice kept under normoxia [33]. OSA is characterized by repeated episodes of oxygen desaturation; however, in OSA there is also sustained hypoxemia, which is expressed by $SpO_2 < 90\%$, %TST. Thus, both sustained and intermittent hypoxemia could possibly increase urinary L-PGDS. Secondly, hypoxia causes pulmonary vasoconstriction and increases pulmonary artery pressure [34]. It has been reported that fluid shear stress increases the expression of L-PGDS in vascular endothelial cells [35, 36] and that urinary PGD_2 metabolites were increased in primary pulmonary hypertension [37]. Semenza reported that intermittent hypoxemia induced systemic hypertension and that sustained hypoxemia induced pulmonary hypertension [38]. Both systemic and pulmonary hypertension induced by OSA might increase L-PGDS values [21, 37].

It is said that sleep fragmentation and arousals caused by sleep apnoea induce an elevation of sympathetic activation [39]. If the severity of OSA might be associated with urinary or plasma L-PGDS concentrations, we thought that it would be important to determine whether there is a significant relationship between sympathetic activation and urinary or plasma L-PGDS. Therefore, we wanted to measure urinary catecholamine concentrations as indicators of sympathetic activity. However, it was difficult to obtain urine in which L-PGDS and catecholamine levels could be measured at the same time. Therefore, even though we