Leptin is believed to play a significant role in the pathogenesis of obstructive sleep apnea syndrome (OSAS) as well as progression of OSAS-related obesity. It is also known that other factors such as gender and diurnal variations in serum strongly affect the measurement results making repeated blood sampling necessary for leptin precise monitoring. Since renal metabolism and urine secretion are the main elimination mechanism for leptin, in this study we evaluated urine relevance for leptin secretion monitoring. Serum and urine (collected during the day and overnight) sampled from 169 OSAS patients and 41 controls were assayed by immunoenzymatic method specific for human leptin. Only 5 (17%) controls and 10 (5.8%) OSAS patients had undetectable urine leptin. We observed significant relationships between serum and urinary leptin in both day-time (r=0.656, P<0.001) and night-time (r=0.518, P<0.001) samples and between day and night-time urine leptin (r=0.811, P<0.001). Significance values did not alter when urinary leptin levels were expressed as the ratio to urinary creatinine. Gender-related differences in leptin concentrations were present both in serum (P<0.001) and overnight urine (P<0.01) in the OSAS group. However, mean night-time urine leptin was lower in the OSAS patients (P<0.05) and their subgroups stratified according to disease severity (P<0.01), while serum leptin levels were comparable in both groups. We conclude that assaying leptin in urine by immunoenzymatic method is a reliable and useful non-invasive alternative for its serum measurement. However, night-time urine leptin levels better reflect differences in its turnover due to gender and OSAS severity.

**Key words:** leptin, obstructive sleep apnea syndrome, serum, urine
INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) affects approximately 2-4% of middle-aged adults and is characterized by repeated collapse of the pharynx during sleep, causing oxygen desaturation, disorder of sleep, and often excessive daytime sleepiness (1). The syndrome also is associated with increased cardiovascular and cerebrovascular morbidity and mortality rates (2, 3). This association is due to some predisposing features linked to OSA, such as obesity, hypertension, and diabetes mellitus, but is also believed to be related to OSAS itself. OSAS is described as being an additional vascular risk factor (syndrome Z) (4). However, the pathogenesis of OSAS remains largely unknown, despite extensive studies conducted in recent years.

Latest reports suggest that leptin, adipocyte-derived pleiotropic cytokine involved in the control of feeding behavior and regulation of endocrine, reproductive, immune, hematopoietic, and other systems (5, 6, 7), also may play a significant role in the progression of OSA-related obesity and in the development of OSA syndrome itself (8, 9). Several studies reported higher serum leptin levels in OSA patients in comparison to non-apneic controls (10-12). Most importantly, some authors have been able to demonstrate that leptin levels decrease following treatment of OSAS, even without a corresponding change in BMI (10, 11). Sanner et al (10) have shown that independently of BMI variations, a significant relationship exists between leptin serum level and improvement in nocturnal respiration as expressed by the apnea-hypopnea index (AHI).

Several mechanism are thought to be responsible for a different leptin turnover regulation in OSAS, including beta-3 receptor polymorphism and subsequent changes in its affinity to leptin, abnormal sympathetic nervous system activity, disturbed lung and visceral hemodynamics, and interferences in feedback control of leptin secretion (13-15). Till now, however, there are no convincing data proving any of them. Apart from the disturbed leptin regulation due to OSAS, leptin levels are influenced by many other factors, such as gender, body weight, hypertension, and pharmacological treatment (16). It also is known that diurnal and ultradian variations in serum leptin and even the awakening status strongly affect the results of leptin measurements (17, 18). Repeated blood sampling is

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>OSAS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole group</td>
<td>Mild</td>
</tr>
<tr>
<td>AHI</td>
<td>3.9 ±1.9</td>
<td>37.1 ±22.4</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>30/11</td>
<td>140/29</td>
</tr>
<tr>
<td>Age, yr</td>
<td>43.1 ±16.4</td>
<td>53.9 ±10.4</td>
</tr>
<tr>
<td>BMI</td>
<td>31.1 ±4.5</td>
<td>32.9 ±6.7</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P<0.001 vs. control and other groups of OSAS patients.
often necessary for precise monitoring of leptin turnover, which may raise ethical problems for both patients and investigators. Since renal metabolism and urine secretion are the main elimination mechanism for leptin (19, 20), we planned this study with the purpose of evaluating the urine relevance for leptin secretion monitoring.

MATERIAL AND METHODS

Subjects characteristics

Subjects were recruited from those who had been referred to our hospital for suspected OSAS, snoring or excessive daytime sleepiness. There were no shift workers in the population. A total of 169 patients with polysomnographically verified OSAS, AHI ≥ 10 per hour of sleep, were selected for the study (Table 1). There were no significant differences between the groups studied except for the mean AHI and BMI values in severe OSA patients. These patients considerably more obese than the controls and other OSA patients with milder symptoms (P<0.001). The control group consisted of 29 subjects with AHI<10. Informed consent was obtained from all subjects participating in the study.

Polysomnography

All subjects underwent an overnight sleep study using a computerized polysomnography system (Somnostar Alpha, SensorMedics Yorba Linda, CA, USA) as described before (21). The variables recorded included electroencephalography (C3/A2, O1/A1), bilateral electrooculography (ROC/A1, LOC/A2), submental electromyography, oronasal airflows measured by thermistors, snoring detected by microphone, electrocardiography, thoracic and abdominal movements registered by inductive plethysmography, and pulse oximetry taken from a finger probe. Apnea was defined as an airflow cessation lasting >10 s. Hypopnea was considered when there was a >50% reduction of airflow and breathing movements that lasted for >10 s. The mean number of apneas and hypopneas per hour of sleep was calculated as the AHI.

Serum and urine samples

All subjects had fasting blood samples taken between 7:30 and 8:30 a.m. Blood was collected on heparin and centrifuged immediately at 2000 g for 20 min. Serum was than aliquoted and stored at -80°C for further processing.

Urine was collected from 8:00 a.m. till 8:00 p.m. (daytime urine) and from 8:00 p.m. till 8:00 a.m. (nighttime urine) into preservative-free containers. Afterward, a number of 5 ml random urine samples was centrifuged at 2000 g for 5 min and stored at -20°C for further measurements.

Neutralization of human leptin with anti-human leptin antibodies

Absorption of sera samples was performed using monoclonal mouse anti-human leptin antibodies and mouse IgG1 as a control (R&D Systems, Minneapolis, MN, USA). Neutralizing concentration of anti-leptin antibody was chosen according to neutralization curve provided by the manufacturer. The reagents, resuspended in PBS (500 µg/ml), were mixed with respective serum samples to obtain the final concentration of 20 µg/ml and incubated for 60 min in 5% CO₂ atmosphere at 37°C. Samples were stored at -20°C for further measurements.
Leptin and creatinine measurements

Serum and urine leptin was measured in duplicate using commercially available quantitative sandwich enzyme immunoassay specific for human leptin (R&D Systems, Minneapolis, MN). Tests were performed according to the manufacturer’s protocol except that urine samples were not diluted prior to measurements. Optical density was measured at 450 nm using an Elx800 spectrophotometric reader (Biotek Instruments, USA). The sensitivity of the assay was 7.8 pg/ml and the intraassay and interassay coefficients of variations were 3.0-3.3 and 3.5-5.4, respectively. The standards ranged from 15.6 pg/ml to 1000 pg/ml.

Serum and urine creatinine was measured using the standard Jaffe technique on a Hitachi 747 analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

All analyses were performed using a commercially available SPSS package. Serum and urine leptin concentrations and total overnight and daytime urine leptin levels were not normally distributed. Therefore, comparisons and correlations were based on the log-transformed data. Results are expressed as means ±SD. An unpaired t-test and one-way analysis of variance (ANOVA) were used to determine differences between groups. Pearson correlation coefficients were used to examine relationships between variables. A P value <0.05 was used to indicate statistical significance.

RESULTS

Serum leptin concentration

Fasting serum leptin concentrations are presented in Fig. 1. There were no statistical differences between the serum leptin levels in controls and OSAS patients regardless of the disease severity. However, sex-related differences were observed. Serum leptin was significantly higher in females in both control and entire patient groups (P<0.001) and in the mild and moderate OSA patients (P<0.001). A significant positive correlation between the serum leptin and BMI was also observed (controls r=0.44, P<0.001; OSA patients r=0.42, P<0.001).

Urine leptin concentration

Leptin concentrations in controls and OSAS patients stratified according to the disease severity during daytime and nighttime urine are presented in Fig. 2. Five samples (17%) from controls and 10 samples (5.8 %) from the OSA patients had undetectable urine leptin levels. The mean overnight leptin concentration in the urine samples collected from controls was considerably higher than that in daytime samples (1.52 ±0.40 pg/ml vs. 0.97 ±0.33 pg/ml, respectively; P<0.01), while in the OSAS group no significant difference between the daytime and nighttime mean urine leptin levels was observed (Fig. 2). Also, the mean daytime urine concentration did not differ between both groups (0.97 ±0.33 pg/ml in controls vs. 1.14 ±0.44 pg/ml in OSA patients), whereas in the overnight urine from the whole of OSA
Fig. 1. Mean log serum leptin concentrations in controls and OSAS patients. Gender-related differences were observed in most groups with the leptin serum levels higher in females, while stratification according to disease severity revealed no differences. Values are means ± SD, ***P<0.001.

Fig. 2. Mean daytime and nighttime log urine leptin concentrations in controls and OSAS patients. The daytime and nighttime leptin concentrations differed significantly from each other in controls but not in OSAS subjects. The nighttime leptin concentration was lower in the whole OSA group and in the mild and moderate OSAS patients. There were no inter-group differences in the daytime urine leptin levels. Values are means ±SD, *P<0.05, **P<0.01.
subjects and in mild and moderate disease subgroups leptin was considerably lower compared with controls (control 1.52 ±0.40 vs. total OSA 1.28 ±0.55, P<0.05; mild OSA 1.27 ±0.57, P<0.01; moderate OSA 1.21 ±0.59, P<0.01).

Concerning gender-related serum leptin differences observed in controls, the mean urine leptin value was higher in women in both daytime and nighttime urine (1.36 ±0.62 vs. 1.10 ±0.32, P<0.05; 1.62 ±0.53 vs. 1.23 ±0.48, P<0.001, respectively). In contrast, in the OSAS subgroups the gender-related differences were observed only in the overnight samples from mild and moderate patients (1.70 ±0.54 vs. 1.18 ±0.39, P<0.001; 1.75 ±0.36 vs. 1.11 ±0.32, P<0.05, respectively).

There was a significant relationship between the serum and urinary leptin levels in both examined groups. A positive correlation was found between the daytime serum vs. urine leptin (r=0.66, P<0.001); nighttime serum vs. urine leptin (r=0.52, P<0.001), and between daytime and nighttime urine leptin concentrations (r=0.81, P<0.001) (Fig. 3). The significance of these correlations remained unaltered when the urinary leptin levels were expressed as the ratio to urinary creatinine. The respective data are the following: serum daytime leptin vs. urine leptin/creatine - r=0.62, P<0.001; serum nighttime leptin vs. urine leptin/creatine - r=0.56, P<0.001; daytime urine leptin/creatine vs. nighttime urine leptin/creatine - r=0.76, P<0.001. There were no differences between the control and OSAS groups in serum creatinine levels and creatinine clearance ratio (data not shown). Interestingly, a rather weak correlation between the urine leptin concentration and BMI was observed in both control (daytime urine leptin r=0.33, nighttime leptin urine r=0.36) and OSAS groups (day leptin urine r=0.22, night leptin urine r=0.20).

Table 2. Human leptin immunoassay specificity in serum and urine samples.

<table>
<thead>
<tr>
<th>Serum samples (ng/ml)</th>
<th>Urine samples (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>71.41</td>
<td>45.39</td>
</tr>
<tr>
<td>62.76</td>
<td>164.21</td>
</tr>
<tr>
<td>38.7</td>
<td>44.81</td>
</tr>
<tr>
<td>34.66</td>
<td>121.64</td>
</tr>
<tr>
<td>59.43</td>
<td>44.17</td>
</tr>
</tbody>
</table>

Serum and urine samples were preincubated with monoclonal anti-human mouse leptin antibodies or mouse IgG₁ as a control and then were assayed. The inhibition ratio was estimated as follows: $[1-\left(\text{leptin concentration following preincubation/baseline leptin concentration}\right)] \times 100\%$. Bottom line shows means ±SD of the inhibition ratio.
**Leptin neutralization assay**

To confirm the specificity of leptin measurements, random serum and urine samples from 5 patients were incubated with anti-human leptin monoclonal antibodies and then assayed in duplicate using human leptin immunoassay. In the samples incubated with monoclonal antibodies, a significant interference was observed which resulted in a considerable reduction of leptin levels measured in these serum and urine samples compared with those in the baseline and control preincubated with IgG conditions (*Table 2*).

*Fig. 3.* Significant correlations were observed between: A - log leptin concentrations measured in serum and daytime urine; B - log leptin concentrations measured in serum and nighttime urine; C) log leptin concentrations measured in daytime and nighttime urine.
DISCUSSION

In recent years leptin has become an increasingly studied protein due to its role in obesity, cardiovascular and cerebrovascular diseases, and possible participation in the pathogenesis of OSAS and a number of other disorders (22). However, the mechanisms of leptin action and its metabolism in humans are still not well understood.

It is well established that leptin is produced mostly by the adipocytes, more actively in the subcutaneous than in the omental adipose tissue (22). Leptin exerts its biological effect via several forms of specific receptors (Ob-R) expressed in most peripheral organs and tissues, such as brain, lungs, skeletal muscles, and the kidney (22). The main physiological pool of leptin is in the peripheral blood, where it circulates in two forms: free and bound to carrier proteins (23). Accordingly, leptin clearance from the organism is described by the two-pool model. The free form is rapidly removed from serum with half-life of 3-4 min, while the bound form (both serum and tissue-protein bound) is retained for a much longer period (half-life - 71 min) (24). It has been calculated that ~80% of total body leptin clearance can be attributed to the kidney (19, 25). Importantly, leptin extraction from blood to urine is relatively constant, not appreciably affected by its concentration, at least up to 30 ng/ml, high renal blood flow, and even a moderate decline in renal function (26). Consequently, in patients with end-stage renal failure of various etiologies, serum leptin is within normal range or slightly increased (27, 28). Also, no correlation has been found between serum leptin and creatinine levels or creatinine clearance (26). Since molecular weight of circulating leptin is approximately 16 kDa, which allows its crossing through the glomerular membrane, it is believed that leptin is eliminated by glomerular filtration alone (29). However, Zeng et al (19) showed that \(^{125}\text{I}-\text{leptin}\) fails to appear in urine immediately after bolus injection (delay in radioactivity appearance in urine is 56.4 ±10.8 min), which shows that leptin clearance is more complicated and involves also tubular reabsorption and metabolism by renal tissue. This dual elimination model is highly consistent with the above mentioned leptin pharmacokinetic characteristics. Most probably, glomerular filtration is the main elimination mechanism of the pool of free serum leptin, hence explaining its short half-life time and justifying the presence of leptin in the urine that we demonstrated and quantified in the present study.

In the past, there were only few publications regarding urine leptin measurements (19, 20, 26). Those studies either failed to detect leptin in the urine or reported few positive results. However, radioimmunoassay tests used in those studies were characterized by sensitivity of ~500 pg/ml, while the ELISA method we applied in this study allowed the detection of leptin concentration down to 7.8 pg/ml. Still, Dimitriou et al (30), who also applied enzyme immunoassay (range of 20-160 pg/ml), detected leptin only in just 2 of 181 urine samples. That poor outcome might be due to lower sensitivity, but in our opinion it is rather the effect
of excessive dilution of urine samples before the measurement. On the other hand, the sensitivity of the immunoassay applied in this study seems adequate and satisfactory, although we found that 17% of the urine samples from controls and 5.8% from OSAS patients did not contain detectable leptin levels. This is consistent with results published by Zaman et al (31) who measured urine leptin in children and adults by immunoassay and reported similar problems in samples of 27% children and 18% of adults. It seems that assaying urine samples concentrated prior to leptin measurement might be worth considering in the future.

Our study showed without doubt that it is possible to effectively measure urinary leptin using a quantitative sandwich enzyme immunoassay specific for the human leptin. The only modification we introduced into the assay protocol was the exclusion of the urine dilution step prior to measurements (instead of 1:100 according to the manufacturer’s protocol). The parallelism and recovery experiments confirmed the assay’s sensitivity and resistance to nonspecific interferences. To validate specificity of the immunoassay for detecting urine leptin, we performed additional neutralization experiments. We demonstrated that following preincubation of samples with specific mouse anti-human leptin antibodies the levels of leptin detected by the immunoassay in the urine and serum decreased considerably (in serum by 64 ±5% vs. 18 ±10% IgG1 control; in urine by 83 ±4% vs. 17 ±11% IgG1 control). Therefore, we confirmed that protein detected in the urine was indeed leptin.

A highly significant relationship observed between the serum leptin concentration and urinary leptin excretion during both daytime and nighttime further proved the applicability of urine measurements for the monitoring of leptin secretion. Similarly, a strong association between the daytime and nighttime leptin urine levels shows that the urine leptin levels measured by the immunoassay were not accidental. Most importantly, the correlations mentioned above remained significant even when the urinary leptin levels were expressed as a ratio to urinary creatinine, a marker of glomerular filtration, proving that the major source of leptin we were able to measure was the peripheral blood.

Interestingly, gender-related differences detected in sera of the subjects examined were observed only in the urine samples collected overnight. That diversity in urine leptin excretion was seen also in patients stratified according to AHI, except for the most severe OSAS. It is likely that the diversity in urine leptin excretion was a result of OSAS-related disturbances in leptin turnover and was not related to any glomerular filtration disturbances, since urine creatinine levels and creatinine clearance were similar in all analyzed groups. Similarly, comparable concentrations of leptin in daytime and nighttime urine detected in OSAS patient were distinctly different from those in the control group in which overnight samples contained significantly more leptin. Moreover, OSAS patients had considerably lower leptin levels in the overnight urine than healthy subjects. The same was true for the mild and moderate OSAS subgroups. Again, in severe OSAS patients the overnight urine concentrations were higher and comparable.
with those in controls, showing once more that the OSAS severity affects leptin metabolism and excretion with urine.

In conclusion, our study clearly demonstrated the practical potential and technical simplicity of the leptin measurement in urine samples. We showed that leptin in the urine was closely related to its serum concentration. Moreover, we were able to demonstrate a significant difference in the leptin overnight urine level in the OSAS subgroups stratified according to the gender and AHI values. We conclude that assaying leptin in the urine by an immunoenzymatic method is possible, easy, and reliable and might be a useful noninvasive alternative for its serum measurements.

REFERENCES


Author’s address: Joanna Chorostowska-Wynimko, Laboratory of Molecular Diagnostics, National Institute of Tuberculosis and Lung Diseases, 26 Plocka St, 01-138 Warsaw, Poland; phone +48 22 4312386, fax +48 22 4312358, e-mail: j.chorostowska@igichp.edu.pl