The Plasminogen System and Transforming Growth Factor-β in Subjects With Obstructive Sleep Apnea Syndrome: Effects of CPAP Treatment

Alessia Steffanina MD, Lidia Proietti MD, Caterina Antonaglia MD, Paolo Palange MD, Elena Angelici MD, and Rita Canipari PhD

BACKGROUND: Obstructive sleep apnea syndrome (OSAS) has emerged as a risk factor for cardiovascular disease. A prothrombotic state may affect coagulation and participate in the atherosclerotic process in subjects with OSAS. These alterations in coagulation seem to involve the plasminogen activation system. We evaluated the imbalances of the plasminogen activation system related to OSAS, and we assessed the effects of CPAP on the plasminogen activation system.

METHODS: Thirty-nine subjects were submitted to a home-based cardiorespiratory sleep study, and 14 healthy subjects (apnea-hypopnea index < 5) were used as controls. Serum levels of urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), and active transforming growth factor-β (TGF-β) were measured. These molecules were reassessed in only 17 of the subjects after 1 month of CPAP.

RESULTS: PAI-1 and tPA were significantly higher in the subjects with OSAS compared with the controls, whereas TGF-β and uPA levels were lower. PAI-1 showed a significant positive correlation with the apnea-hypopnea index, percentage of time spent at O₂ saturation < 90%, and oxygen desaturation index, whereas TGF-β was inversely related to all 3 of these parameters. After the CPAP therapy, PAI-1 significantly decreased, whereas TGF-β showed a significant increase, although the values did not reach those of the controls. uPA and tPA did not show significant differences after the treatment.

CONCLUSIONS: Our results suggest an imbalance of fibrinolysis related to OSAS and an improvement of the prothrombotic state after the CPAP treatment. Key words: sleep apnea; CPAP; PAI-1; TGF-β; uPA; tPA.

Introduction

Obstructive sleep apnea syndrome (OSAS) is the most common form of sleep-disordered breathing and affects thousands of people each year. Its frequency in the general population is approximately 3–7% of middle-age men and 2–5% of middle-age women.1 OSAS is characterized by the repetitive partial or complete closure of the upper airway during sleep that is caused by a coll...
lapse of the pharyngeal airway. It is associated with excessive daytime sleepiness, snoring, recurrent awakenings and gasping episodes. Obstructive sleep apnea may induce severe intermittent hypoxemia and CO2 retention during sleep. Hypoxemia and sleep deprivation appear to be important mechanisms that trigger systemic inflammation. Recurrent hypoxic stress induces the release of vasoactive and trophic substances and increased levels of plasma cytokines, adhesion molecules, and C-reactive protein.\(^2,3\)

Individuals with OSAS have an increased risk for cardiovascular disease and stroke, and OSAS is an independent risk factor for hypertension.\(^4\) Emphasis has been placed on recognizing patients with cardiovascular disease who have coexisting sleep apnea and understanding the mechanisms by which sleep apnea may contribute to the progression of their cardiovascular conditions. Moreover, the prevalence of metabolic syndrome in patients with OSAS reaches 30–77%.\(^5\)

OSAS patients have potential markers of thrombotic risk, such as higher plasma levels of fibrinogen,\(^6,7\) and increased platelet activation, that decrease after CPAP treatment.\(^8\) The hypercoagulability resulting from increased coagulation or impaired fibrinolysis is associated with an increased risk for atherothrombosis and cardiovascular disease in OSAS.\(^9,10\)

In recent years, coagulation in OSAS has received some attention, and different studies have focused on the fibrinolytic activity and the role of the plasminogen system in subjects with sleep apnea.\(^11-15\) There are 2 forms of plasminolytic activators: tissue-type (tPA) and urokinase-type (uPA). Both are serine proteases that convert plasminogen to the active enzyme plasmin. tPA is mostly involved in the dissolution of fibrin in the circulation, whereas uPA is mainly associated with the pericellular proteolysis during tissue remodeling or tumor invasion.\(^16\) tPA is currently used in clinical practice for thrombolysis. tPA and uPA activities are regulated by specific inhibitors. Plasminogen activator inhibitor-1 (PAI-1) is one of the primary regulators of this system, and its principal function in blood is the inhibition of fibrinolysis and the stabilization of a thrombus. PAI-1 is synthesized by adipocytes as well as by several other cell types. It has been clearly demonstrated that plasma PAI-1 levels are increased in obesity and are reduced by weight loss,\(^17,18\) but the exact role of PAI-1 in adipose tissue is still controversial. The first connection between decreased fibrinolytic capacity, increased plasma PAI-1 levels, and cardiovascular disease was reported in young survivors of myocardial infarction.\(^19\)

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is secreted by different cell types as an inactive complex and then is activated by plasmin.\(^20\) TGF-\(\beta\) has been shown to be involved in cardiovascular disease.\(^21\) In fact, it has been observed that subjects with coronary artery disease present with low levels of TGF-\(\beta\) in their serum.\(^22\) Among the 3 different isoforms (TGF-\(\beta\)\(_1\), TGF-\(\beta\)\(_2\), and TGF-\(\beta\)\(_3\)), TGF-\(\beta\)\(_1\) is the most prominent in the cardiovascular system.\(^23\) However, its role in OSAS remains largely unexplored.

There is a link between PAI-1 and TGF-\(\beta\). In fact, TGF-\(\beta\) induces PAI-1 production, but the increase in PAI-1 may inhibit TGF-\(\beta\) activation by inhibiting plasminogen activators and, in turn, the activation of plasminogen to plasmin.\(^24\)

There is limited information about the effect of CPAP treatment on the plasminogen activation system. Some authors have reported higher levels of PAI-1 in subjects with mild to severe OSAS than in controls. However, contrasting results can be found in the literature concerning the relationship between PAI-1 and the severity of OSAS.\(^11,13,14,25\)

Our study was designed to assess the presence of a hypercoagulability state in OSAS subjects and in control subjects by comparing the levels of uPA, PAI-1, and TGF-\(\beta\). Additionally, we evaluated the effect of 1 month of CPAP treatment on plasminogen system activity.
we performed a full overnight home-based cardiorespiratory sleep study using a portable sleep apnea monitoring device, SOMNOScreen (SOMNOmedics, Randersacker, Germany). The parameters evaluated were the thoracic and abdominal respiratory movements, respiratory air flow by nasal cannula, body position, snoring, and oxygen saturation. Daytime sleepiness was assessed using the Epworth Sleepiness Scale. The cardiorespiratory register was analyzed according to the American Academy of Sleep Medicine 2007 guidelines. In our study, apnea was defined as the cessation of air flow for at least 10 s, and hypopnea was defined as a reduction of at least 30% of the air flow and ≥4% of oxygen desaturation or ≥50% air flow reduction with ≥3% oxygen desaturation for 10 s or more. From each subject, we manually assessed the number of apneas and hypopneas per hour of polygraphic recording (apnea-hypopnea index [AHI]), the percentage of the total polygraphic recording time passed with an oxygen saturation < 90% (time < 90%), and the number of oxygen desaturations of at least 4%/h (oxygen desaturation index [ODI]) as severity parameters. In the treatment arm, the inclusion criteria were an AHI ≥ 5 in symptomatic subjects and the consent to CPAP therapy.

The optimal CPAP level for each subject was determined by a titration with the auto-CPAP device iSleep 20 (MedicAir, Pogliano Milanese, Milan, Italy). The positive airway pressure was automatically adjusted, throughout a 4-night period, to determine the optimal pressure for maintaining upper airway patency.

A fasting venous blood sample was obtained from each subject between 8 and 10 AM after having performed the sleep study. PAI-1, uPA, tPA, and TGF-β were measured.
The levels of the same markers were measured after 1 month of CPAP therapy in the subjects who were diagnosed with OSAS.

**Laboratory Analysis**

To measure uPA, tPA, and PAI-1 antigen levels, venous blood was collected into plastic tubes containing 3.8% sodium citrate (ratio 9:1). The samples were centrifuged at 1,500 g for 15 min at −4 °C, and the supernatant was stored at −80 °C until it was examined. Aliquots of the appropriately diluted samples were assayed by enzyme-linked immunosorbent assay following the instructions of the manufacturer (PAI-1/tPA and uPA IMUBIND ELISA kits, American Diagnostica, Pfungstadt, Germany).

Active TGF-β was assayed by adding 100 μl of serum for 16 h to subconfluent mink lung epithelial cells that were stably transfected with a PAI-1 promoter luciferase construct. At the end of culture, the cells were washed twice with phosphate-buffered saline and lysed in 60 μl of lysis buffer (Promega, Madison, Wisconsin). The cell lysates were assayed for luciferase activity with an assay kit from Promega using a Berthold luminometer. Parallel mink lung epithelial cells were incubated with known increasing concentrations of TGF-β to obtain a standard curve.

**Statistics**

Statistical analyses were performed using analysis of variance followed by the Tukey–Kramer test for comparisons of multiple groups or paired Student t test for the comparison of data derived from the 2 groups. For the assessment of the correlations, Spearman’s rank-order correlation coefficients (r) and their probability (P) levels were calculated. Values of P ≤ .05 were considered statistically significant. All of the analyses were performed using SPSS 18.0 for windows (SPSS, Chicago, Illinois).

**Results**

The serum PAI-1 levels were significantly higher in the subjects compared with the control group (Fig. 1A). The values ranged between 37 and 110 ng/mL in the healthy subjects and between 75 and 156 ng/mL in the OSAS subjects. When the PAI-1 values were plotted in relation to the severity of OSAS (Fig. 2), they demonstrated a positive correlation with the AHI (r = 0.46, P = .01), time <90% (r = 0.42, P = .02), and ODI (r = 0.43, P = .01).

On the contrary, the TGF-β levels were statistically lower in the OSAS subjects than in the controls (Fig. 1B). The values ranged between 0.3 and 3.6 ng/mL in the healthy subjects and between 0.3 and 2.3 ng/mL in the subjects with OSAS. Moreover, TGF-β had a negative correlation with the same parameters examined with PAI-1, the AHI (r = 0.23, P = .05), time <90% (r = 0.39, P = .03), and ODI (r = 0.30, P = .04) (Fig. 3).

The uPA values were significantly lower (Fig. 4A), whereas the tPA values were significantly higher (Fig. 4B), in the subjects with OSAS compared with the controls (P = .05 and P = .04, respectively). Neither uPA nor...
tPA showed a correlation with OSAS severity. However, there was a positive correlation between the tPA and PAI-1 values (Fig. 5) ($r = 0.62, P = .01$).

Only 17 of 39 subjects underwent CPAP therapy. However, as shown in Tables 1 and 2, all of the characteristics of these subjects were not statistically different from those of the whole cohort of subjects. One month of CPAP treatment was effective and led to a significant decrease in the AHI ($2.8 \pm 2.4$ events/h, $P < .001$), time < 90% ($1.5 \pm 0.8$, $P < .001$), and ODI ($3.6 \pm 0.6$, $P < .001$); these values were not significantly different from the healthy controls ($P = .63$ and $P = .15$ versus control, for AHI and time < 90%, respectively). Although the ODI values were significantly different from the pretreatment values, they did not reach the values of the healthy subjects ($P = .02$). The subjects’ weight/BMI did not change during the treatment period. After CPAP therapy, we observed a significant decrease in the PAI-1 levels ($-21\%$, $P = .01$) and an increase in active TGF-β ($+49\%$, $P = .04$) (Fig. 6, A and B). The uPA and tPA levels moderately changed after the treatment ($7\%$ and $7\%$, respectively) but did not reach statistical significance (Fig. 6, C and D). Among the different molecules, only PAI-1 levels were not significantly different from the levels of the control subjects after CPAP.
Patients with OSAS have an increased cardiovascular risk. This may be due to several pathophysiological mechanisms, one of which is a hypercoagulable state. PAI-1 has a well-known antifibrinolytic role. High levels of this molecule have been associated with cardiovascular disease,\textsuperscript{27} and it could be responsible for the increased cardiovascular risk in OSAS.

Several studies have reported higher PAI-1 levels in subjects with OSAS compared with healthy subjects.\textsuperscript{11-15}
However, in the literature, contrasting results can be found on the relationship between levels of PAI-1 and AHI, the index of the severity of OSAS. von Känel et al\textsuperscript{13} showed a positive correlation, whereas Zamarro\textsuperscript{a}n et al\textsuperscript{25} found an inverse correlation.

In our subjects with OSAS, we observed higher PAI-1 levels than in the control subjects. Moreover, we have shown a significant positive correlation between PAI-1 and the severity of OSAS, as measured by AHI, time <90\%, and ODI.

In the subjects with OSAS, endothelial cells are exposed to intermittent hypoxia/reoxygenation, which leads to the production of many vasoactive substances that intervene in the fibrinolysis and coagulation processes in response to different hypoxic stimuli. It has been demonstrated that the molecular response to intermittent hypoxia/reoxygenation results in the activation of nuclear factor kappa B with the consequent production of inflammatory mediators, such as tumor necrosis factor alpha.\textsuperscript{2} Therefore, the PAI-1 increase could be due to the activation of inflammatory pathways. In fact, PAI-1 is induced by hypoxia via hypoxia-inducible factor-1 or -2 in sustained hypoxia as well as via the activation of nuclear factor kappa B or TGF-\beta during intermittent hypoxia.\textsuperscript{2}

The total proteolytic activity is controlled by modulating the levels of protease inhibitors and/or the local concentrations of plasminogen activators. Therefore, we also investigated the presence of the 2 plasminogen activators tPA and uPA. In our subjects with OSAS, the tPA antigen was significantly higher than that observed in the control subjects, whereas the uPA levels were slightly lower.

It has been shown in epidemiological studies that despite being cardioprotective, high plasma tPA concentrations positively predict future coronary events. The increase of tPA in our study might be a physiological response to endothelial damage. In fact, it has been demonstrated that hypoxia can induce the release of tPA from injured endothelial cells as well as from other cells, such as cerebral cortical neurons. This mechanism suggests that the elevation of tPA antigen is a result rather than a cause of atherosclerotic coronary disease. However, the increase of a factor able to activate the fibrinolytic system, such as tPA, being associated with conditions with increased cardiovascular risk, such as OSAS, may appear paradoxical.\textsuperscript{28} We can hypothesize that higher levels of tPA may represent an attempt to compensate for the hypercoagulable state in subjects with OSAS. However, the concomitant elevation of PAI-1 and the interaction between plasminogen activators and PAI-1 leads to the formation of an enzyme-inhibitor complex, which inhibits plasminogen activation activity.\textsuperscript{11} Many authors suggest a protective role of TGF-\beta on the cardiovascular system because low levels of this molecule are related to coronary artery disease with a poor prognosis.\textsuperscript{22,29,30}

TGF-\beta has been shown to induce PAI-1 synthesis in several cell systems. Therefore, an increase of PAI-1 should be correlated with a concomitant increase in TGF-\beta. Instead, we found lower levels of active TGF-\beta and higher levels of PAI-1 in the subjects with OSAS. This apparent discrepancy can be explained by the fact that we measured active TGF-\beta. Although an increase in active TGF-\beta triggers PAI-1 synthesis, the increase in PAI-1 and the consequent inhibition of plasminogen activation activity lead to a decrease in plasmin formation and, as a consequence, an impaired TGF-\beta activation.\textsuperscript{24,31} In fact, it has been shown that TGF-\beta is secreted in a latent form and can be activated by the uPA/plasmin system.\textsuperscript{20} In our subjects, TGF-\beta levels were inversely correlated with AHI, time <90\%, and ODI, supporting the hypothesis that the decrease in TGF-\beta activity could be involved in the increased cardiovascular risk related to OSAS.

CPAP is the first-line treatment for OSAS. Evidence in the literature shows that CPAP therapy reduces cardiovascular events,\textsuperscript{32} most likely through a variety of mechanisms. For example, it may reduce inflammatory cytokine levels, platelet activity, sympathetic nervous system hyperactivity, and blood pressure.\textsuperscript{33,34} In agreement with previous studies,\textsuperscript{15,35} after CPAP treatment, we observed a significant decrease of PAI-1 levels that was associated with a significant increase in active TGF-\beta levels and a moderate, but not significant, increase in uPA. We are aware of the possible limitations of our results due to the relatively small number of subjects and that the control subjects had lower BMI values compared with those of the subjects with OSAS. However, the fact that after 1 month of CPAP therapy, we observed a decrease in the levels of PAI-1 but not relevant changes in BMI suggests that the decrease of PAI-1 should not be related to BMI.

Another confounding variable in our study is the comorbidity of OSAS with metabolic syndrome, a condition often associated with OSAS.\textsuperscript{5} In subjects with metabolic syndrome, an increase in plasma PAI-1 can be observed, and weight loss is associated with a decrease of PAI-1. However, in our subjects, all of the parameters and molecules examined were not significantly different between the 2 groups, with or without metabolic syndrome (data not shown), probably due to the low number of subjects with OSAS and metabolic syndrome (13 of 39). Therefore, these results need to be evaluated in a larger number of subjects.

The significant decrease of PAI-1 and the increase of TGF-\beta that we observed after the CPAP treatment suggest a decrease in the thrombotic state after treatment, which indicates that CPAP treatment has beneficial effects on cardiovascular disease in subjects with OSAS. However, because in our subjects with OSAS, the TGF-\beta values did not reach the values observed in the control group and the...
PAI-1 values did, we can speculate that longer CPAP treatment may be needed.

Conclusions

Our study, although performed on a limited number of subjects, highlights the importance of focusing on these molecules, especially the important link between PAI-1 and TGF-β, to better clarify the pathophysiological mechanisms underlying the hypercoagulability state in patients with OSAS.

ACKNOWLEDGMENTS

Mink lung epithelial cells were kindly provided by Dr DB Rifkin (New York University School of Medicine).

REFERENCES

31. Sato Y, Tsuboi R, Lyons R, Moses H, Rifkin DB. Characterization of the activation of latent TGF-β by co-cultures of endothelial cells


