ORIGINAL ARTICLE

Study of endothelial dysfunction and vascular inflammation in sleep apnea, obesity and aged humans

Essam El-Shamy a, Samir Eskaros a, Abeer E. Dief a, Seham Z. Nassar a, Anwar Algenady b,* , Nermeen Hossam Eldin c

a Physiology Department, Egypt
b Chest Diseases Department, Egypt
c Clinical and Chemical Pathology Department, Egypt

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KEYWORDS
Obstructive sleep apnea; Nuclear factor κB; Cardiovascular diseases; Endothelial dysfunction; Inflammation

Abstract Background: Obstructive sleep apnea (OSA) has been associated with cardiovascular complications. The overnight repetitive hypoxia represents a form of oxidative stress in the vasculature which may activate the oxidant-sensitive, proinflammatory transcription factor nuclear factor κB (NF-κB), affecting endothelial function and atherosclerosis.

Aim: We investigated whether the endothelial alterations attributed to OSA rather than to other confounding factors. Also, the production of inflammatory cytokine nuclear factor-kappa β (NF-Kβ) was investigated as the molecular mechanism involved in vascular endothelial dysfunction with OSA.

Material and methods: Sixty subjects underwent attended nocturnal polysomnography were grouped by apnea hypopnea index: control (AHI < 5/h) and OSA cases (AHI > 5/h) the cases were further classified according to age and BMI into subgroup IIA: OSA, non-obese, middle age (35–52 y), subgroup IIB: OSA, non-obese, older age group (55–68 y), subgroup IIIA: OSA, obese, middle age group (35–52 y) and subgroup IIIB: OSA, obese, older age group (55–68 y).

Abbreviations: OSA, Obstructive sleep apnea; AHI, Apnea hypopnea index; BMI, body mass index; NF-κB, Nuclear factor κB; sVCAM-1, soluble Vascular cell adhesion molecule; FMD, Flow mediated dilatation
* Corresponding author. Tel.: +20 1223926049.
E-mail address: A.algenady@hotmail.com (A. Algenady).
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A morning venous blood sample was obtained. Neutrophils were isolated, and NF-κB activity was determined. Plasma sVCAM-1 was assayed by enzyme-linked immunosorbent assay and flow-mediated dilation (FMD) was performed.

Results: NF-κB activation and plasma level of sVCAM-1 were significantly increased in OSA patients as compared to the control group and there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases. The degree of NF-κB activation was positively correlated with indices of apnea severity ($r = 0.938; p < 0.001$). FMD was significantly decreased in OSA patients as compared to the control group.

Conclusion: These findings suggested that OSA is an independent risk factor for cardiovascular morbidity also that OSA leads to NF-κB activation, which may constitute an important pathway linking OSA with systemic inflammation and cardiovascular disease.

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inflammatory cytokine nuclear factor-kappa B (NF-κB) as the molecular mechanism involved in vascular endothelial dysfunction with OSA.

Subjects and methods

The present study was carried out on 60 persons referred to sleep laboratory in the chest department in the main University hospital. The inclusion criteria for the studied subjects were; patients with newly diagnosed obstructive sleep apnea (OSA) [defined as an apnea-hypopnea index (AHI) of 5 obstructive events per hour of sleep], no any other clinical diseases as assessed by medical history, physical examination, blood chemistry, and resting ECGs. However, the exclusion criteria were smoking, diabetes mellitus, and subjects taking any medications or herbal supplements. The studied subjects were subdivided according to their age, body mass index and polysomnography data into 3 groups:

Group I: (control group) consisted of 20 nonsmokers healthy volunteers (BMI < 25) (AHI < 5); Group II (OSA, non-obese group) consisted of 20 non-obese OSA patients (BMI < 25) (AHI ≥ 5) and Group III (OSA, obese group) consisted of 20 obese OSA patients (BMI > 25) (AHI ≥ 5). All the 3 groups were subdivided into two subgroups 10 persons each, subgroup A (Middle age group 35–52 y) and subgroup B (old age group 55–68 y).

All the participants were subjected to; history taking, clinical examination, routine laboratory investigations, resting ECG, assessment of anthropometric data using fat absorptiometer in the physiology department. Nocturnal polysomnography at sleep lab chest department, Alexandria university hospital were done and flow-mediated dilation of the brachial artery (FMD) was done using high-resolution ultrasonography in the Radiology department [13].

Moreover, fasting blood samples were collected from all participants between 9 and 11 AM within 48 h of polysomnography for estimation of:

NF-κB by ELISA

Neutrophils (95–98% purity) were isolated using Ficoll–Paque centrifugation and were subsequently purified by dextran sedimentation and hypotonic lysis of residual erythrocytes. Purification of cellular nuclear extracts using Cayman’s nuclear extraction kit to isolate nuclear protein. NF-κB was quantified using NF-κB (human P50/P65) combo transcription factor assay kit (Caymann) [14].

Statistics

The values of the measured parameters were expressed as mean ± SD or as median (min–max). The difference between the studied groups was determined using ANOVA test (F-test) and The Kruskal-Wallis test (H-test). Pearson’s correlation coefficient was performed for evaluating the biochemical and radiological variables. P < 0.05 values were considered significant. All statistical analyses were processed using SPSS for windows Version 18.

Results

Clinical characteristics and laboratory values of the patients upon initial admission are summarized in Table 1. All the 3 groups were subdivided into two subgroups, subgroup A (Middle age group 35–52 y) and subgroup B (old age group 55–68 y). It was observed that BMI is significantly increased in group III as compared to the other two groups. There were no significant differences between the 3 groups in the fasting blood sugar and serum cholesterol levels.

Sleep studies data

AHI, ODI, Mini PaO2, and average pulse rate are shown in Table 2. Apnea-hypopnea index (AHI) and oxygen desaturation index (ODI) increased significantly as expected between the control group I with AHI (2.25 ± 1.16) and ODI (1.53 ± 0.32) as compared to OSA patient with AHI (24.07 ± 15.14) and ODI (23.49 ± 22.07).

Mini PaO2 was significantly decreased in OSA patients (76.20 ± 6.76) as compared to the control group I (92.45 ± 1.66) and average pulse rate was significantly increased in OSA patients as compared to the control group.

Endothelial dysfunction and vascular inflammation investigations are shown in Table 3. It is observed that NF-κB activation was significantly increased in OSA patients as compared to the control group. On the other hand there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases.

Serum sVCAM-1 was significantly increased in OSA patients as compared to the control group. On the other hand there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases. However, there was a significant difference in the control group between the middle age as compared to the old age subgroup.

The reactive dilatation of the brachial artery was significantly decreased in OSA patients as compared to the control group. On the other hand there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases. However, there was a significant difference in the control group between the middle age as compared to the old age subgroup.

Correlation study between NF-κB activity, sVCAM-1 and the severity of OSA

Neutrophil NF-κB activity correlates with indices of OSA severity as assessed by AHI. Neutrophil NF-κB activity was positively correlated with AHI (r = 0.938; p < 0.001), ODI (r = 0.927; p < 0.001), and negative with Mini PaO2 (r = −0.888; p < 0.001).

A significant strong positive correlation was found between sVCAM-1 level and AHI (r = 0.914, p < 0.001) and also with the degree of hypoxaemia as assessed by ODI (r = .928, p < 0.001) and strong negatively correlation with Mini PaO2 (r = −.877, p < 0.001) (see Table 4).
The vascular endothelium participates in control of various vascular functions through regulation of vasoactive mediators in response to physical or biochemical stimuli in the body [1]. Endothelial injury, at cellular level or tissue level, is an important initial event in atherogenesis, preceding thickening of intima and formation of atherosclerotic plaques [8]. Endothelial dysfunction was shown to have a predictive value for cardiovascular events in patients with chest pain and/or coronary artery disease [16]. It was shown also to be the early event in accelerated atherosclerosis attributed to OSA [4].

Obstructive sleep apnea (OSA) is currently considered to be an inflammatory disorder. Evidence suggests that the chronic intermittent hypoxia and, possibly, sleep loss and fragmentation associated with OSA increase the levels of various markers of inflammation. In particular, increased levels of inflammatory cytokines, adhesion molecules, and activation of circulating neutrophils [8].

The present work was designed to assess the development of endothelial dysfunction and inflammation in patients with OSA compared with age and body mass index (BMI)–matched control subjects.

Regarding the NF-κB activity in circulating neutrophils of OSA patients and the controls the current data demonstrated significant increase NF-κB activity in circulating neutrophils of OSA patients. Moreover, the degree of NF-κB activity is positively correlated with OSA severity. Neutrophil NF-κB activity was positively correlated with AHI, ODI, and mini PaO2. It has been well documented that NF-κB activation is an integral part of the pathophysiology of numerous cardiovascular disorders [11]. our findings suggest that NF-κB could be an important molecule linking the pathophysiological consequences of OSA with cardiovascular disease.

There is ample evidence supporting the essential role of NF-κB activity in the pathogenesis of various cardiovascular diseases. Many genes, whose expression is regulated by NF-κB, have been implicated in atherogenesis and cerebrovascular disease [11,17]. The relevance of NF-κB activation in OSA is demonstrated by the presence of elevated levels of the products of several NF-κB controlled genes in OSA patients. Data from previous studies have shown that OSA patients have increased levels of the adhesion molecules sE-selectin, sVCAM-1, ICAM-1, and L-selectin as well as the cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α, all of which are produced by NF-κB regulated genes [18]. Because these inflammatory proteins have been shown to play a crucial role in the pathogenesis of atherosclerosis [17], these observations collectively suggested that NF-κB may contribute to atherogenesis by stimulating NF-κB, with subsequent activation of cell adhesion and other proinflammatory pathways.

It has been postulated that intermittent hypoxia associated with recurrent obstructive apneas is a crucial pathophysiological feature of OSA which generates increased ROS [19]. These free radicals may then activate oxidant-sensitive transcription factors such as NF-κB. As a result, production of proinflammatory mediators and adhesion molecules is increased, in addition to activation of neutrophils and endothelial cells. Increased adhesion of activated inflammatory cells...
to the endothelium is an important factor contributing to endothelial dysfunction and atherogenesis [8,9].

In Animal studies, Shuo et al. 2011 had studied the systemic production of inflammatory factors and activation of NF-κB in response to different levels of intermittent hypoxia in 160 male Wistar rats, which has a typical breathing pattern of OSA. They have found that the correlation of NF-κB activation under intermittent hypoxia implies an important role of this transcription factor in inflammation-induced cardiovascular damage occurring during OSA [20]. Furthermore, other in vitro studies done by Ryan and colleagues, 2005 reported that, in an in vitro cell culture model, alternating cycles of hypoxia and re-oxygenation resulted in selective and dose-dependent activation of inflammatory NF-κB-dependent pathways [21].

These data go in line with other studies by Aung et al. 2006 who reported that neutrophils demonstrated significantly greater NF-κB activity in the mild to moderate and severe OSA groups than in the control subjects and this increased neutrophil NF-κB activity is reversed with nasal CPAP therapy [23]. Furthermore, the neutrophil is known to play an important role in cardiovascular disease since infiltration of neutrophils into atherosclerotic plaque has been associated with plaque rupture and the occurrence of acute coronary events [24]. However, it is likely that OSA may also activate NF-κB in other cell types such as endothelial cells.

This study also demonstrated that there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases which means that the co-morbid factors are not influencing our findings. This evidence indicates that the observed increase in NF-κB activity is causally related to OSA, rather than to comorbid factors or to differences in BMI between groups. Thus, while obesity has been characterized as a proinflammatory state [25], and we cannot completely rule out a contribution of obesity itself to NF-κB activity, our findings support an independent effect of OSA on NF-κB activation as our study use a lean OSA group to definitely separate effects of obesity from those of OSA.

Table 2 sleep studies data AHI, ODI, Mini PaO2, and average pulse rate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control Mean ± SD</th>
<th>Cases Mean ± SD</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHI (events/h)</td>
<td>2.25 ± 1.16</td>
<td>24.07± 15.14</td>
<td>9.06</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>ODI (events/h)</td>
<td>1.53 ± 0.32</td>
<td>23.49± 22.07</td>
<td>6.29</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Mini PaO2 (mmHg)</td>
<td>92.45 ± 1.66</td>
<td>76.20± 6.76</td>
<td>14.35</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Average pulse (beats/min)</td>
<td>66.90 ± 3.67</td>
<td>84.13± 10.39</td>
<td>9.38</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

Table 3 The NF-κB activity, serum s-VCAM-1 and FMD in the studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB (ng/ml)</td>
<td>5.50± 3.0–8.0</td>
<td>21.00± 15.0–65.0</td>
<td>19.50± 15.0–58.0</td>
</tr>
<tr>
<td>sVCAM (ng/ml)</td>
<td>538.00± 470–708</td>
<td>1617.0± 1224–2304</td>
<td>1375.0± 1260–2574</td>
</tr>
<tr>
<td>FMD%</td>
<td>9.10± 8.10–9.60</td>
<td>4.30± 3.10–5.60</td>
<td>4.20± 2.90–4.60</td>
</tr>
</tbody>
</table>

Values are median (min–max). The groups with the same small letter have no significant difference.

Table 4 Correlation between NF-κB activity, sVCAM-1 and severity of OSA.

<table>
<thead>
<tr>
<th>AHI</th>
<th>ODI</th>
<th>Mini PaO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>r(p-value)</td>
<td>.938**</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>R(p-value)</td>
<td>.955**</td>
</tr>
</tbody>
</table>

* p < 0.05 vs control group I.
To confirm the biologic relevance of the observed change in NF-κB, we measured serum levels sVCAM-1, an important NF-κB controlled gene product, in OSA patients and in control subjects. There was a significant increase in the biochemical marker (sVCAM-1) in OSA patients as compared to age matched control groups.

Furthermore our findings have shown that the concentration of circulating adhesion molecules correlated with the severity of sleep apnea. The current study also showed a significant relation between cellular adhesion molecules and the severity of hypoxemia as indicated by the ODI and mini PaO₂ suggesting that the risk of cardiovascular events is perhaps related to the intermittent hypoxia observed during sleep and the level of hypoxaemia. It is plausible that intermittent hypoxia promotes oxygen radical formation [26] that leads to activation of transcriptional factors that upregulate the genetic expression of adhesion molecules [27].

Although the pathologic role of these cellular adhesion molecules in OSA is uncertain, studies have shown elevated circulating levels of adhesion molecules in subjects with OSA compared to those without sleep-disordered breathing [18]. Various circulating markers of endothelial dysfunction, including nitric oxide, soluble cell adhesion molecules, fibrinogen, and plasminogen activator inhibitor, have been reported to be altered in OSA [19,28]. This coincides with recent studies of Bernabe Jurado-Gomez et al, 2012 who found that patients with OSA and greater intermittent hypoxia had worse endothelial function, and higher levels of ICAM-1 and P-selectin [29]. Hypoxia has been implicated in the induction of interleukin-1 and cellular adhesion molecules gene expression [30].

The present data confirm previous results that OSA syndrome is associated with a rise in circulating levels of adhesion molecules. Aung et al, 2006 have found that the circulating levels of VCAM-1 are significantly increased in patients with OSA compared with those of a matched control group. Ohga and coworkers, 1999 reported increased levels of ICAM-1, VCAM-1, and L-selectin levels in seven patients with OSA compared to a control group of “normal subjects”. The authors suggested that the repetitive hypoxic stress during sleep might induce activation and provoke sustained release of these inflammatory mediators. Since cellular adhesion molecules mediate cellular interactions and transmigration of leukocytes across the vascular endothelial wall [31]. Our findings suggest that OSA could contribute potentially to the inflammatory process implicated in atherogenesis.

On the other hand, other studies have found no significant change. As Klein et al, 1995 who studied the in vitro influences of hypoxia on endothelial cell proliferation and expression of cell adhesion molecules. They found that the presentation of ICAM-1, VCAM-1, and E-Selectin was not affected by hypoxia or even reduced and they are not appear to be the major aspect in hypoxic injury [32].

The reactive dilatation of the brachial artery was assessed as a physical marker of endothelial dysfunction. FMD was significantly decreased in OSA patients as compared to the control group. On the other hand there was no significant difference between the obese and non-obese cases also no significant difference between the middle and old age cases. This indicates impaired reactive hyperaemia in OSA cases which indicates endothelial dysfunction.

The mechanisms of vascular response to vessel occlusion followed by reactive hyperemia are clearly complex, involving myogenic, neurogenic, and vasculogenic components, mediated by a variety of metabolic alterations and vasoactive factors. The exact mechanisms apparently differ with vascular beds and evoking stimuli [33]. Flow-mediated dilatation refers to nitric oxide-mediated vasodilatation resulting from shear-mediated activation of endothelial nitric oxide synthase in response to an acute increase in blood flow [13]. The test has been shown to be accurate and reproducible [34].

Previous studies on vascular endothelial function in OSA have shown conflicting data. These studies have used different methods for evaluation of vascular endothelial function. For instance, Blunted vasodilatation in response to infusion of acetycholine, a vasodilator that stimulates endothelial release of nitric oxide, was demonstrated in forearm resistance vessels using occlusion plethysmography [35], but not confirmed in another study [36]. Impaired relaxation response to bradykinin in the forearm venous vasculature has been reported, suggesting endothelial dysfunction in the venous vasculature [37].

Using ultrasound Doppler method similar to ours, Mathias Grebe et al, 2006 had measured flow-mediated dilatation (FMD) of the brachial artery by ultrasound in 10 otherwise healthy, untreated patients with OSA and 10 age-and sex-matched control subjects without sleep-disordered breathing before and after intravenous injection of the antioxidant vitamin C. They found that the reduced endothelial-dependent vasodilation in untreated patients with OSA acutely improves by the free radical scavenger vitamin C. These results are in favor of oxidative stress being responsible for the endothelial dysfunction in OSA [38].

In contrast to our findings, another study found no significant difference in conduit–vessel dilatation between sleep apnea and control subjects. In that study, subjects underwent conduit–vessel studies at least 1 hour after resistance–vessel studies, which involved intra-arterial infusion of acetycholine, nitroprusside and verapamil, and residual effect of drugs on the vasculature, which affected their response to reactive hyperemia, cannot be completely excluded [39].

To exclude the effect of obesity, we compare the FMD in lean and obese OSA patients, and there was no significant difference between both groups. In another study, OSA and subjects without OSA were matched for obesity, and the body mass index was not a significant independent determinant of FMD on multiple regression analysis. The change in FMD with treatment of OSA without any concomitant change in body weight and the use of a control group who did not receive nCPAP treatment provided further evidence that the improvement in endothelial function in this study sample, most of whom were obese, was attributed to the use of nCPAP, whereas a placebo effect could not be definitively excluded. OSA, therefore, may be associated with functional impairment (“a premature aging effect”) on the endothelium and on arterial stiffness [40].

Conclusions

The findings of endothelial dysfunction in otherwise healthy subjects with OSA lend strong circumstantial evidence to an independent contribution of OSA to atherosclerosis. The present data also provide evidence for activation of the proinflammatory transcription factor NF-κB in OSA. This finding lends further support to an emerging hypothesis which postulates
that OSA contributes to cardiovascular disease by increasing systemic inflammation.

References


