Advanced Glycation Endproducts in Nondiabetic Patients With Obstructive Sleep Apnea

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Subject Objective: The formation and accumulation of advanced glycation endproducts (AGEs) has been implicated in the progression of age-related diseases such as diabetes mellitus and atherosclerosis. We hypothesize that AGE concentrations may be increased in subjects with obstructive sleep apnea (OSA), a condition associated with increased oxidative stress.

Methods: One hundred nineteen nondiabetic patients with OSA and 234 age-matched healthy controls and 134 patients with type 2 diabetes were recruited for participation in the study. Serum AGEs were assayed by competitive enzyme-linked immunosorbent assay using a polyclonal rabbit antisera raised against AGE-RNase.

Results: Serum AGEs were increased in OSA subjects, as compared with controls, but were less increased than the AGEs of patients with type 2 diabetes (control: 3.22 ± 0.54 unit per mL; OSA: 3.68 ± 0.39; diabetes mellitus: 4.11 ± 0.99; analysis of variance p < .01). In the subjects with OSA, serum AGEs correlated with the duration of nocturnal desaturation (r = 0.21, p = .025) and plasma total 8-isoprostane concentration, a biochemical marker of oxidative stress (r = 0.22, p = .015), but not with fasting glucose level. On general linear model univariate analysis, the association between serum AGEs and 8-isoprostane was independent of age, sex, body mass index, smoking status, and glucose.

Conclusion: Serum levels of AGEs were increased in nondiabetic subjects with OSA and were associated with the severity of OSA. Whether increased AGE formation contributes significantly to the high cardiovascular risk associated with OSA remains to be determined.

Keywords: Obstructive sleep apnoea, advanced glycation end products, oxidative stress

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INTRODUCTION

ADVANCED GLYCATATION ENDPRODUCTS (AGES) ARE A COMPLEX AND HETEROGENEOUS GROUP OF COMPOUNDS THAT ARE FORMED WHEN REDUCING SUGARS, such as glucose, react nonenzymatically with the amino groups through the Maillard reaction.1 Glycoxidation is the later stage of the reaction in which the reversible Schiff base proceeds to stable, covalently bonded Amadori rearrangement products, which then undergo further rearrangement reactions to produce AGEs. It is now clear not only that AGEs can form on long-lived, extracellular proteins, but also that they arise on short-lived molecules as well as cytoplasmic proteins and nucleic acids.2 AGEs can cause a number of adverse cellular events, including reduction of enzymatic activity, damage to nucleic acids, cross-linking and impaired degradation of proteins, and induction of cytotoxic pathways.3,4 The accumulation of these AGEs on tissue proteins has been implicated in the aging of proteins and the progression of age-related diseases such as diabetes mellitus, atherosclerosis, chronic renal failure, and Alzheimer disease.5,6,7

The formation of AGEs is enhanced by hyperglycemia. Increased circulating and tissue AGEs have been demonstrated in both animal and human studies of diabetes mellitus.8 Elevated concentrations of serum AGEs have been demonstrated in children and adults with diabetes,7,8 and the increase in serum AGEs concentration is most marked in diabetic subjects with end-stage renal disease.9,10 There also is evidence to suggest that the formation of AGEs is increased by oxidative stress and contributes to the accumulation of tissue AGES in aging.5 Increased production of reactive oxygen species by the mitochondrial electron transport chain leads to intracellular AGEs formation that can be blocked by normalizing mitochondrial superoxide production.11 Since AGEs are formed not only in conditions of hyperglycemia, but also in states of enhanced oxidative stress, we hypothesize that AGE concentration might be increased in subjects with obstructive sleep apnea (OSA). In OSA, repeated episodes of upper-airway obstruction occur during sleep and lead to significant hypoxia. There are cyclic alterations of arterial oxygen saturation, and the hypoxia/reoxygenation causes alteration in oxidative balance through the induction of excess oxygen free radicals.12 An increased production of oxygen-reactive species by granulocytes and monocytes has been demonstrated in subjects with OSA,13,14 and systemic levels of biomarkers of oxidative stress (lipid peroxidation products,15 malondialdehyde,15 F2-isoprostanes16,17 and reactive oxygen metabolites18) are consistently elevated. In this study, we have determined serum AGE levels in a group of nondiabetic subjects with OSA and compared those with levels from a group of healthy controls and a group of patients with type 2 diabetes.

METHODS

Patients with OSA were recruited form the Sleep Laboratory at Queen Mary Hospital, and those with a history of diabetes and/or impaired renal function were excluded. An overnight sleep study was performed using a computerized polysomnogram system (Alice 3, Healthdyne, Respirnics, Pittsburgh, PA) as previously described,19 and fasting blood samples were taken the following morning. Measurements of apnea-hypopnea index (AHI, the total...
number of apnea and hypopnea episodes divided by the hours of sleep) and oxygen saturation (SaO₂) during the night were obtained. Hypopnea was defined as an abnormal respiratory event lasting at least 10 seconds with at least a 30% reduction in thoracoabdominal movement or airflow, as compared with baseline, and with at least a 4% oxygen desaturation.²⁰ An AHI ≥ 5 plus characteristic symptoms established the diagnosis of sleep apnea. Patients with type 2 diabetes who were of similar age and with normal renal function were recruited from the diabetes clinics over the same period, and healthy controls were recruited from the community by advertisement. World Health Organization 1999 criteria were used to diagnose diabetes mellitus and impaired fasting glycemia (IFG).²¹ Diabetes mellitus is defined as fasting venous plasma glucose ≥ 7.0 mmol/L, and IFG is defined as fasting venous plasma glucose ≥ 6.1 and < 7.0 mmol/L. Fasting blood samples were taken for the measurement of glucose, serum AGEs, and plasma lipids. All samples were stored at -70°C before analysis. The study was approved by the Ethics Committee of the University of Hong Kong.

Serum AGEs were measured by competitive enzyme-linked immunosorbent assay (ELISA) using a well-characterized polyclonal rabbit antiserum raised by hyperimmunization against AGE-ribonuclease (AGE-RNase), as previously described.⁸ This antiserum recognizes, as a predominant epitope, the AGE crosslink, ALI (arginine-lysine imidazole).²² The 96-well plates were coated with 50 µL per well of AGE-RNase (3.75 µg/mL) in coating buffer (0.1 mol sodium bicarbonate, pH 9.6) overnight at 4°C. After the wells were rinsed 3 times with washing buffer and blocked by SuperBlock™ blocking buffer in phosphate-buffered saline solution for 2 hours at room temperature. After the wells were rinsed 3 times, 50 µL of serum (1:4 dilution) was added, followed by 50 µL of 1:500 diluted anti-AGE antibody in dilution buffer containing 2% goat serum. Plates were incubated at room temperature with gentle agitation for 2 hours. Wells were then rinsed 3 times. Alkaline phosphate-conjugated antirabbit IgG (1:2000) in dilution buffer was added to each well and incubated for 1 hour at 37°C. After washing, color was developed by the addition of 100 µL pNPP substrate (Sigma-Aldrich, St. Louis, MO). Optical density (OD) at 405 nm was determined by an ELISA reader. Results were calculated as 1 - (experimental OD - background OD) / (total OD - background OD). There is no universally established unit of measurement of AGEs. In our assay, 1 unit of AGEs is defined as the amount of AGE present in 1:4 diluted serum that causes 50% competitive inhibition of anti-AGE antibody binding to coated AGE-RNase on the ELISA plate.

Fasting venous plasma glucose was measured by enzyme-coupled spectrophotometric kinetic method using Hexokinase on Hitachi-747 analyzer (Boehringer Mannheim, GmbH, Mannheim, Germany). Plasma total cholesterol and triglyceride levels were determined enzymatically on a Hitachi 912 analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). High-density lipoprotein cholesterol was measured using a homogenous method with polyethylene glycol-modified enzymes and α-cyclodextrin. Low-density lipoprotein cholesterol was calculated by the Friedewald equation. Plasma high-sensitive C-reactive protein was measured by a particle-enhanced immunoturbidimetric assay (Roche Diagnostics). Plasma 8-isoprostanate, a marker of oxidative stress, was measured by a specific enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Since less than half of total plasma 8-isoprostanate was present as the free acid and the remainder was esterified in lipoproteins, total plasma 8-isoprostanate was measured after alkaline hydrolysis prior to enzyme immunoassay.

Results are expressed as mean ± SD or as median and interquartile range if the distribution of the data was found to be skewed. Data that were not normally distributed were logarithmically transformed before analyses were made. Comparisons between the 3 groups were done using analysis of variance and posthoc analysis by Dunnett test. Pearson correlation analyses were performed to determine whether there were associations between 2 variables. Partial correlations analyses were performed to test the relationship between the 2 variables while controlling for the effects of 1 or more additional variables. General linear model univariate analysis was used to assess the relationships between AGEs and various variables simultaneously.

RESULTS

The clinical characteristics of the 3 groups of subjects are shown in Table 1. Four out of the 123 subjects with OSA recruited had undiagnosed diabetes and were therefore excluded from the analysis. The 3 groups of subjects were well matched for age, but the diabetic subjects and those with OSA had significantly higher body mass indexes than the controls. The proportion of male subjects was significantly higher in the OSA group. Smok-

Table 1—Clinical Characteristics of 3 Groups of Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n=243)</th>
<th>OSA (n=119)</th>
<th>DM (n=134)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>46.1 ± 9.6</td>
<td>47.8 ± 10.8</td>
<td>46.6 ± 8.4</td>
</tr>
<tr>
<td>Sex, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>52</td>
<td>81</td>
<td>59</td>
</tr>
<tr>
<td>Women</td>
<td>48</td>
<td>19</td>
<td>41</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.4 ± 4.5</td>
<td>28.6 ± 5.3*</td>
<td>27.1 ± 4.4*</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>83.5 ± 12.7</td>
<td>95.6 ± 11.2</td>
<td>89.9 ± 9.4*</td>
</tr>
<tr>
<td>Smoking status, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>85</td>
<td>80</td>
<td>75</td>
</tr>
<tr>
<td>Former</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Current</td>
<td>10</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Serum levels, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FG</td>
<td>5.1 ± 0.4</td>
<td>5.5 ± 0.5*</td>
<td>8.7 ± 2.6*</td>
</tr>
<tr>
<td>TC</td>
<td>5.21 ± 0.92</td>
<td>5.52 ± 0.59</td>
<td>5.47 ± 1.11</td>
</tr>
<tr>
<td>TG</td>
<td>1.2 (0.8-1.7)</td>
<td>1.7 (1.3-2.3)*</td>
<td>1.5 (0.98-2.0)*</td>
</tr>
<tr>
<td>LDL</td>
<td>3.32 ± 0.85</td>
<td>3.32 ± 0.75</td>
<td>3.54 ± 1.01</td>
</tr>
<tr>
<td>HDL</td>
<td>1.26 ± 0.33</td>
<td>1.12 ± 0.25*</td>
<td>1.15 ± 0.39*</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>122 ± 18</td>
<td>130 ± 16*</td>
<td>128 ± 15*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>74 ± 11</td>
<td>76 ± 15</td>
<td>79 ± 9*</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.96</td>
<td>1.49</td>
<td>1.75</td>
</tr>
<tr>
<td>(0.53-1.96)</td>
<td>(0.70-2.63)*</td>
<td>(0.90-3.73)*</td>
<td></td>
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<tr>
<td>AHI, events/h</td>
<td>23.3</td>
<td></td>
<td>(11.3-48.2)</td>
</tr>
<tr>
<td>SaO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration &lt; 90%, min</td>
<td>27.5 (6.5-97.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum, %</td>
<td>71.0 ± 15.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD or median (interquartile range). OSA refers to obstructive sleep apnea; DM, diabetes mellitus; BMI, body mass index; FG, fasting glucose; TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CRP, C-reactive protein; AHI, apnea-hypopnea index; SaO₂, oxygen saturation.

°p < .01 vs controls

²p < .05 vs controls

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The relationship between AGEs and log(8-isoprostane) remained significant \((p = .02)\), and repeating the analyses with the inclusion of fasting glucose in the model did not change our results. Similar results were obtained when the analyses were performed in the whole group of OSA subjects.

**DISCUSSION**

It is well established that AGE levels are elevated in patients with diabetes, and AGEs also are increased in patients with impaired renal function, since the removal of AGEs and their degradation products depends on renal clearance.\(^1\) AGEs have been implicated in the pathogenesis of vascular disease in diabetic patients and those with end-stage renal disease,\(^2,6\) and the accumulation of AGEs has been demonstrated in macrophage-derived foam cells in fatty streaks and atherosclerotic plaques.\(^3\) Recently, it has been recognized that AGEs also may play an important role in the formation and acceleration of atherosclerotic lesions even in normoglycemic nondiabetic patients. In nondiabetic patients with occlusive atherosclerotic disease requiring endarterectomy, AGEs have been detected within atherosclerotic lesions in both extracellular and intracellular locations. Serum levels of AGE-modified apolipoprotein B were increased and correlated with AGE levels in the vessel wall of carotid arteries.\(^24\) Serum AGE levels also were higher in nondiabetic subjects with coronary artery disease than in nondiabetic control subjects, and there was a correlation between serum AGEs and the severity of coronary artery disease.\(^25\) We have shown for the first time that nondiabetic subjects with OSA also have elevated levels of serum AGEs and the levels are intermediate between those of a group of healthy age-matched controls and that of a group of patients with type 2 diabetes.

In addition to hyperglycemia, it has been suggested that oxidative stress also can increase the formation of AGEs.\(^5,11\) In patients with OSA, serum concentration of AGE was associated with the severity of OSA and with 8-isoprostane, a biochemical marker of oxidative stress. The correlation coefficients were significant but weak, suggesting that the magnitude of the effect was small. The association between AGEs and 8-isoprostane was independent of age, smoking status, and fasting glucose, suggesting that oxidative stress may be a determinant of serum AGEs in OSA. Hence, increased oxidative stress in OSA may lead to an increase in the formation of AGEs even in the absence of hyperglycemia. However, we can only demonstrate associations and not causal relationships because our study is limited by its cross-sectional nature. We cannot exclude the possibility that increased AGE levels in OSA subjects may contribute to oxidative stress because the binding of AGEs to cell surface receptors like the receptor for AGE (RAGE) can, in turn, stimulate cells to produce reactive oxygen species.\(^26\)

Our data are hypothesis generating rather than being conclusive and prospective studies to determine whether lowering oxidative stress in OSA patients with continuous positive airway pressure therapy will reduce concentrations of AGEs are warranted. Another potential limitation of our study is that we have only measured fasting glucose in our subjects, and measuring HbA1c would be a better marker of overall glucose levels. We have not performed sleep studies in the controls and diabetic patients, and there may not be a significant relationship between the duration of nocturnal desaturation and serum AGEs, we proceeded to further investigate whether serum levels of AGEs were related to oxidative stress in subjects with OSA. Plasma total 8-isoprostane concentration was measured as a marker of oxidative stress. Plasma total 8-isoprostane level was 124.0 pg/mL (95.5-256.1) (median and interquartile range) in the OSA subjects. Plasma log(8-isoprostane) correlated with serum AGEs \((r = .22, p = .015)\) in the whole group of OSA subjects and remained significant after excluding those with IFG \((r = .24, p = .01)\). The association between AGEs and log(duration of SaO₂ < 90%) was no longer significant after adjusting for log(8-isoprostane). To determine whether the association between AGEs and log(8-isoprostane) was independent of age, sex, body mass index, waist circumference, and smoking status, general linear model univariate analysis was performed after excluding subjects with IFG. The relationship between AGEs and log(8-isoprostane) remained significant \((p = .02)\), and repeating the analyses with the inclusion of fasting glucose in the model did not change our results. Similar results were obtained when the analyses were performed in the whole group of OSA subjects.

**Table 2**—Serum levels of AGEs in 3 Groups of Subjects

<table>
<thead>
<tr>
<th>Control</th>
<th>Obstructive Sleep Apnea</th>
<th>Diabetes Mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, no.</td>
<td>243</td>
<td>119</td>
</tr>
<tr>
<td>AGEs (advanced glycation endproducts)</td>
<td>3.22 ± 0.54</td>
<td>3.68 ± 0.39*</td>
</tr>
<tr>
<td>unit/mL</td>
<td>4.11 ± 0.99*</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**—Serum levels of AGEs in 3 Groups of Subjects

AGEs (advanced glycation endproducts) are presented as mean ± SD. IFG refers to impaired fasting glycemia. *p < .01 vs controls.
have been subjects with undiagnosed OSA in these groups. This would not affect our findings because inclusion of subjects with undiagnosed OSA in the control and diabetic groups would only reduce the power to detect a difference between groups.

Does the small increase in serum AGEs in OSA that we have identified reflect a clinically significant tissue burden of AGEs? Serum measurement of AGEs may not necessarily reflect cumulative tissue levels of AGEs, although there are data suggesting that serum levels correlate with AGE levels in the vessel wall. Measurement of tissue AGEs, for example, skin-associated AGEs, may be helpful in future studies. Experimental studies have shown that AGEs play an important role in the pathogenesis of atherosclerosis. AGEs cause endothelial dysfunction by increasing vascular permeability, stimulate cell adhesion molecule expression, and reduce nitric oxide dependent vasodilation. AGEs also cause increases in arterial stiffness and formation of atherosclerotic lesions. Breaking preformed cross-links in AGEs has been shown to reverse the diabetes-related increase in large artery stiffness. Prevention of interaction of AGEs with their cellular receptors suppresses the development of atherosclerotic plaques in apolipoprotein E-deficient diabetic mice independent of glycemic or lipid effects. Since OSA is known to be associated with an increased rate of cardiovascular morbidity, whether increased AGE formation contributes significantly to the pathogenesis of cardiovascular disease in OSA remains to be determined.

In conclusion, serum levels of AGEs were increased in nondiabetic subjects with OSA, albeit to a lesser degree when compared to those seen in age-matched type 2 diabetic patients, and serum AGEs levels were associated with the severity of OSA.

ACKNOWLEDGEMENT

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