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Carbonyl groups: Bridging the gap between sleep disordered breathing and coronary artery disease

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Abstract
Sleep disordered breathing (SDB) is related to coronary artery disease (CAD), but the mechanisms are uncertain. SDB is characterized by periods of intermittent hypoxia and free radical formation. This study tested the hypothesis that carbonylation can be the link between SDB and CAD. It included 14 cases with CAD and 33 controls with <50% coronary narrowing. CAD cases have higher erythrocyte carbonyl levels than controls ($p=0.012$). Positive correlation was observed between apnea-hypopnea index (AHI) and erythrocyte carbonyl concentration ($\rho=0.310$; $p=0.027$). To predict CAD, including as regressors: AHI, erythrocyte carbonyl, gender, age and body mass index, the significant variables in the Poisson multiple regression model were AHI and erythrocytes carbonyl. An increase of 1 pmol/gHb in erythrocyte carbonyl levels increases by 1.8% the risk of CAD and one unit of AHI increases by 3.8% the risk of CAD. The present findings represent the first evidence in humans that SDB may cause CAD through protein carbonylation.

Keywords: *Sleep apnea, coronary artery disease, oxidative stress, carbonyl*

Abbreviations: *AHI, apnea-hypopnea index; BMI, body mass index; CAD, coronary artery disease; CAT, catalase; DNPH, 2,4-dinitrophenylhydrazine; GPx, glutathione peroxidase; HPLC, high-performance liquid chromatography; OSA, obstructive sleep apnea; RBC, red blood cell; SDB, Sleep disordered breathing; SOD, superoxide dismutase.*

Introduction

Studies suggest a link between sleep-disordered breathing (SDB) and coronary artery disease (CAD), but the mechanisms are uncertain [1–5]. SDB included obstructive sleep apnea (OSA) and central sleep apnea. Repeated episodes of hypoxia and reoxygenation experienced by individuals with sleep apnea lead to increased formation of reactive oxygen species [6–8] with consequent oxidative damage of biomolecules [9–12]. Oxidative damage is prevented by enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) or non-enzymatic endogen and dietary antioxidants, such as vitamin C [13]. The imbalance between oxidants and antioxidants triggers oxidative stress and, consequently, protein, nucleic acids and lipid damage. Oxidative damage to proteins, DNA and to lipids can cause alterations in cell membrane, enzymatic systems and cell signalling, processes involved in the pathophysiology of CAD [8,10,14–16].

Carbonylation, both plasmatic and cellular, indicates oxidative damage in proteins [17]. Carbonyl groups (C=O) are formed through the oxidation of the lateral chains of amino acids proline, lysine, arginine and treonine [17] or they can be introduced in proteins through the aldehyde groups reaction produced during lipid peroxidation or through end-products from glucose oxidation reactions [18–20]. In the literature

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there are no studies quantifying carbonyl in haemolysate from patients with CAD or SDB. Increased carbonylation of plasma proteins were observed in patients with CAD [21–23]. In humans only one study was reported showing evidence of augmented carbonylation of intercostal muscle proteins in OSA patients [24].

The purpose of this study is to test the hypothesis that carbonyl formation in erythrocytes and plasma, secondary to increases with the apnea-hypopnea index (AHI), can lead to CAD.

Materials and methods

Patients

A cross-sectional study was conducted between March 2007 and February 2008, screening consecutively patients between 35–65 years of age that were referred by their physicians for diagnostic or therapeutic coronary angiography. The exclusion criteria were: smoking in the previous 6 months; clinical diagnosis, dietary or pharmacological treatment for diabetes mellitus; anginous pain in the previous week; use of anxiolytic medication; treatment for chronic pulmonary disease; use of vitamin supplement; body mass index (BMI) >40 kg/m^2; any physical, psychological or social issue encumbering the attainment of the home polysomnographic test; and previous coronary intervention (myocardial revascularization or angioplasty). A full medical history was taken from all study participants. The project was approved by the institutional ethics committee and all participants signed an informed consent form.

Laboratory measurements

Blood collection. In the morning, 3–6 h after wake up time, 20 mL arterial blood samples were collected from each patient, fasted for at least 8 h, at the site of femoral artery puncture for catheterization. Blood was collected in three vials, containing: coagulation activator, EDTA and citrate. Immediately after the collection, the samples were refrigerated to 0°C, centrifuged for 10 min in a 0°C-cooled centrifuge, aliquoted and stored at -80°C. Hemolysates were prepared by lysing red blood cells (RBC) with ethanol 2% (ratio 1:10) followed by centrifugation to obtain crude extracts. Glucose, high-sensitivity C-reactive protein, total proteins and lactate dehydrogenase were quantified in the routine clinical analysis laboratory and the additional tests at the research laboratory.

Protein carbonyl content. Plasma and haemolysates were used to determine protein carbonyl groups. Carbonyl content was measured using the method described by Levine [25]. Plasma protein (0.025 mL) or RBC haemolysates (0.025 mL) were added with 0.2 mL of 10% trichloroacetic acid. The samples were centrifuged (4300 g, 10 min) and 1 mL of either 2 M chloridric acid or 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl were added to the precipitates and incubated at 37°C for 90 min. After the samples were centrifuged (4300 g, 10 min) the DNPH excess was removed with ethanol-ethyl acetate 1:1. The samples were centrifuged (4300 g, 10 min) and the protein was then dissolved with the addition of 6 M guanidine hydrochloride. The quantification was performed using a spectrophotometer at 370 nm absorbance. The carbonyl content was calculated using a molar absorption coefficient of hydrazone (21.000 M^{-1} cm^{-1}). Plasma carbonyl was normalized by total proteins and erythrocyte carbonyl was normalized by haemoglobin content.

Superoxide dismutase. The SOD activity was measured in erythrocyte haemolysates using RanSOD® (Randox) Kit. The assay principle is based on the reaction of xanthine-xanthine oxidase, to form superoxide radical, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. We measured the inhibition degree of this reaction using a spectrophotometer. The results are given in SOD units/g haemoglobin.

Glutathione peroxidase. To verify the enzymatic kinetic of GPx, one solution was prepared with buffer phosphate 100 mM, pH 7.4, 1 mM EDTA, 0.15 mM NADPH, 1 mM glutathione and 1U glutathione reductase enzyme. The reaction was started with the addition of 10 μL of t-BOOH 70% and the GPx activity was measured by observation of the NADPH decrease with a spectrophotometer at 340 nm [26]. The results are given in units/g haemoglobin.

Catalase. The following was introduced into a quartz cuvette: 2.8 mL phosphate buffer 100 mM, pH 7.4, 100 μL of 300 mM H_2O_2, the reaction was started with the addition of haemolysates. The CAT activity was determined with a spectrophotometer, where we monitored the disappearance of H_2O_2 at 240 nm [27]. The results are given in units/g haemoglobin.

Vitamin C. Vitamin C was measured through high-performance liquid chromatography (HPLC) [28]. Nine hundred microlitres of 0.1 M perchloric acid were added to a 100 μL aliquot portion of human plasma. The samples were centrifuged at 4300 g for 10 min and used for HPLC analysis, LC-18 DB column. The mobile phase was 82.5:17.5 (v/v) 30 mM monobasic potassium phosphate (pH 3.6)-methanol, the flow rate was 1 mL/min and the chromatograms were monitored at 250 nm.

Sleep study

The volunteers underwent portable polysomnography at home using a level III monitor (SomnoCheck, Weinmann, Germany), a procedure, validated by our group, that showed the same variability as two sequential full polysomnographies [29]. Air flow and snoring were measured through a nasal cannula connected to a pressure transducer. In addition, inspiratory effort, pulse oximetry, heart rate and sleep position were measured. The records were made at the patient's home, usually between 11 pm and 7 am. The polysomnography respiratory analyses were made by a board-certified sleep specialist in a different location, blind to the other results.

Apneas were defined as the airflow reduction to 10% or less of the baseline value for 10 s or more; hypopneas as airflow reduction of 50% or more, associated with reduction of oxygen saturation of 3% or more. The AHI was calculated by dividing the total apneas and hypopneas by the hours of recording without artifacts and expressed as apnea/hypopnea episodes per hour.

Coronary angiography study

All patients were assessed by quantitative angiography, using the same equipment and projection, with the table and image intensifier kept at constant height. Image quantification was carried out in all cases by the same investigator, who was blinded to carbonyl levels. A magnification of seven inches was used for all images. Significant CAD was defined as ≥50% luminal narrowing of at least one coronary segment. Controls were patients with no lesion or with lesions < 50% luminal narrowing.

Statistical analysis

Categorical variables are presented as absolute values and analysed by chi-square test. Variables with normal distribution are presented as mean ± SD, means were compared by Student's *t*-test. Variables with no normal distribution are presented as median (minimum–maximum), median were compared by Mann-Whitney test. Spearman coefficient was employed to test correlation between variables with no normal distribution. Regression model, mode Poisson was employed to predict CAD. The following regressors were tested in the model: AHI, erythrocytes carbonyl, age, gender and BMI. A p-value ≤ 0.05 was considered statistically significant.

Results

Individuals attending the cardiac catheterization laboratory due to suspected coronary artery disease were consecutively screened; 47 were included in the study. Fourteen patients (group 1) had significant obstructive CAD (≥ 50% luminal narrowing of at least one major coronary or segment) and 33 patients served as controls (without lesion or lesion less than < 50% luminal narrowing; group 2). Table I shows the clinical and anthropometric characteristics of the individuals of both groups. The AHI was significantly higher in group 1 than that of individuals of group 2 ($p < 0.004$).

The number of patients taking medication in groups 1 and 2 differ in statin use (respectively, $n=8$ and $n=9$; $p=0.041$). Other treatments did not differ statistically and include, respectively: anti-hypertensives ($n=10$ and $n=22$), acetylsalicylic acid ($n=8$ and $n=11$) and anti-depressants ($n=2$ and $n=5$). The biochemical data from groups 1 and 2 are presented in Table II. Plasma carbonyl levels, SOD, CAT and GPx activities did not differ in the two groups (Table II). Lower levels of erythrocyte carbonyl were observed in group 2 than in group 1 (Table II). In the same form the levels of high-density lipoprotein are major in patients from group 2 (Table II) and correlate inversely with the AHI ($\rho=-0.377$; $p=0.009$). AHI does not correlate with enzymatic antioxidants activity, SOD, CAT and GPx.

A positive correlation was observed between AHI and erythrocyte carbonyl ($\rho=0.310$; $p=0.027$) and AHI and age ($\rho=0.419$; $p=0.003$; Figure 1). In the Poisson multiple regression model, to predict CAD including as regressors AHI, erythrocytes carbonyl, gender, age and BMI, these variable entered in the model were AHI and erythrocytes carbonyl. The model explain that 1 pmol/gHb of carbonyl increases

Table I. Clinical and anthropometric characteristics of included patients.

Variables	Group 1 CAD ($n=14$)	Group 2 Controls ($n=33$)	p
Male gender (n)	9 (64)	15 (46)	ns
Age (years)	54 ± 6	52 ± 7	ns
Body mass index (kg/m^2)	28 ± 4	28 ± 4	ns
AHI (events/h)	23 (7–56)	10 (1–48)	0.004
Lowest O_2 saturation (%)	85 ± 4	86 ± 3	ns
Medication use (n)	14 (100)	26 (78)	ns
Hypertension (n)	11 (78)	25 (75)	ns
Other diseases (n)	2 (14)	9 (27)	ns
Past smoking (n)	8 (64)	20 (62)	ns

Categorical data are presented as n (%), variables compared by χ^2 test. Variables with normal distribution are presented as mean ± SD, means were compared by Student's *t*-test. Variables with non-normal distribution are presented as median (minimum–maximum), medians were compared by Mann Whitney test. ns: $p>0.05$. Body mass index: weight divided by the square of height (Kg/m^2); other diseases: hypothyroidism, depression, multiple sclerosis, aortic aneurism, heart valve disease, stroke; AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep).

Table II. Biochemical data of patients from groups 1 and 2.

	Group 1, CAD ($n=14$)	Group 2, Controls ($n=33$)	p
Risk factors			
Glucose (mg/dL)	104±8	106±13	ns
Low-density lipoprotein (mg/dL)	94±40	110±35	ns
High-density lipoprotein (mg/dL)	38±11	50±12	0.003
Triglycerides (mg/dL)	152 (60–295)	93 (20–640)	ns
High-sensitivity C-Reactive protein (mg/L)	2.3 (0.4–18.4)	1.9 (0.2–21.5)	ns
Correction factors			
Total protein (g/dL)	6.9±0.6	6.8±1	ns
Haemoglobin (g)	13±1.2	13±1.2	ns
Lactate dehydrogenase (mmol/L)	1.03±0.4	1.15±0.4	ns
Oxidative damage markers			
Erythrocyte Carbonyl (pmol/gHb)	110±32	85±29	0.012
Plasmatic carbonyl (nmol/g total proteins)	0.012±0.004	0.012±0.003	ns
Antioxidants			
SOD (U/gHb)	1 893±242	1 692±424	ns
CAT (U/gHb)	20 706±4866	20 144±5676	ns
GPx (U/gHb)	200±26	203±22	ns
Vitamin C (μM)	88 (5–178)	149 (14–527)	0.029

Variables with normal distribution are presented as mean±SD, means were compared by Student's *t*-test. Variables with no normal distribution are presented as median (minimum–maximum), median were compared by Mann Whitney test. ns: $p>0.05$. SOD: superoxide dismutase; CAT: catalase; GPx : glutathione peroxidase.

by 1.8% the risk to development of CAD and one unit of AHI/h increases by 3.8% the risk to develop CAD (Table III). We did not observe any parametric or non-parametric correlation between plasma carbonyl and CAD or AHI. Plasma carbonyl showed

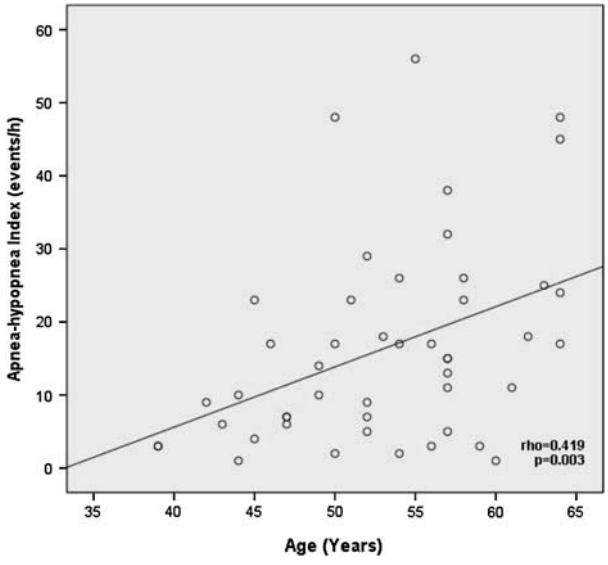

Figure 1. Correlation between apnea-hypopnea index/events hour of sleep and age/years.

Table III. Results from Poisson stepwise regression model to predict coronary artery disease.

Dependent variable Regressor	Obstructive coronary artery disease	
	RR (CI)	p
AHI	1.038 (1.003–1.075)	0.034
Erythrocyte Carbonyl	1.018 (1.005–1.032)	0.005
Gender (male 1)	1.570 (0.552–4.466)	ns
Age	0.964 (0.898–1.036)	ns
Body mass index	0.981 (0.831–1.157)	ns

ns: $p > 0.05$. Body mass index (weight divided by the squares height; Kg/m^2). AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep).

a negative correlation with vitamin C ($\rho=-0.436$; $p=0.003$) and vitamin C level is lower in group 1 than in group 2 ($p=0.029$; Table II).

Discussion

Growing evidence suggests that oxidative stress, especially oxidative damage, such as lipid peroxidation, carbohydrate glycation and protein oxidation are linked to pathogenesis of CAD [11,23,30,31]. Our study is the first to show that the carbonylation of erythrocytic proteins associated to AHI is a predictor of CAD, controlling for known confounders such as age, gender and obesity as well as excluding smoking and diabetes from the roll of causes of oxidative stress.

This finding emphasizes the role of hypoxia-reoxygenation episodes seen in SDB [32,33] and formation of erythrocytes protein damage as a cause of CAD. Vitamin C inversely correlating with plasma carbonyl indicates that this circulating antioxidant is a possible protective factor, explaining the absence of differences of plasma proteins damage in the two groups. Despite a narrow range of age (12–14 years old) correlation was observed between AHI and age. It is possible that the strong exclusion criteria used in this study prevented the masking of the effect of age on SDB.

Hypoxia but not sleep deprivation [34] leads to ROS increase [6], which, in excess or unbalanced with respect to antioxidants, promotes oxidative damage to biomolecules [13,35]. This suggests that it is the number of hypoxia-reoxygenation episodes that determines the amount of damage and not the intensity of hypoxia. Several studies have implicated the AHI in oxidative damage [1,9,24].

In the literature there are no studies that quantify carbonyl in haemolysate from patients with CAD or SDB. Our data agrees with Barreiro et al. [24], the only study in patients with OSA, correlating carbonylation findings of further carbonylation in intercostal muscle cells of 12 male individuals with severe OSA compared to six controls without OSA. In relation, CAD only had been observed to increase in carbonyl groups formation in the plasma of patients with this disease [21,23].

AHI and CAD did not correlate with the intra-erythrocyte antioxidant enzymes we measured: SOD, CAT and GPx. The literature on this topic is controversial [7,9,15,36,37]. The antioxidant enzymes act promptly on oxygen free radicals, preventing their linkage to structural biomolecules and the consequent oxidative damage [13]. In our study, arterial blood was collected during cardiac catheterization, up to 3 h after the exposure to hypoxia episodes during sleep. Probably, at the time we collected blood, the antioxidant enzymes were no longer reflecting AHI severity. However, independent of the time, the antioxidant enzymes were insufficient to inhibit the oxidative damage, reflecting in protein damage. Mildly oxidized proteins are readily degraded ($t_{½}$ human serum albumin (HAS) = 273 ± 6.25/min; $t_{½}$ carbonylated HAS = 150 ± 5.09/min), whereas severe oxidation stabilizes proteins due to aggregation, cross-linking and/or decreased solubility, thus increasing their half-lives [38,39]. Thus, the fact that they still contain carbonylated proteins up to 3 h after the exposure to hypoxia episodes suggests that the protein oxidative damage is severe.

We observed a negative correlation between plasma carbonyl and vitamin C, but not between plasma carbonyl and AHI. Vitamin C is a powerful water-soluble antioxidant, with plasmatic activity [40] that reacts with reactive oxygen species, forming the ascorbyl radical, a less reactive compound [41]. Ascorbate can also contribute as an antioxidant, regenerating glutathione, β-carotene and alpha-tocopherol, inhibiting the lipid peroxidation progress [13]. The main mechanisms through which ascorbate prevents protein oxidation are the reduction of free radicals and the inhibition of oxidative damage propagation. Plasma carbonyl and AHI might not have been correlated due to the vitamin C action, protecting plasma proteins from carbonylation.

Previous investigations, one by our group, showed increased circulating carbonyl in individuals with sickle-cell anaemia [42] and high erythrocyte fragility [43]. Erythrocyte fragility could have increased plasma carbonyl in the present study, due to outflow of oxidative damage products to plasma. Ozturk et al. [36], in a study enrolling six patients with OSA and 10 controls, did not discern differences in erythrocyte fragility between the two groups. In our study, erythrocyte fragility was not measured, but the integrity of the erythrocyte membrane in individuals with sleep apnea is vouched for by normal values of lactate dehydrogenase in both groups.

The evidence here obtained supports our initial hypothesis that repeated episodes of hypoxia encountered in sleep apnea increase the oxidative damage to erythrocyte proteins, suggesting, therefore, involvement of sleep apnea in CAD pathogenesis through oxidative stress mechanisms. Vitamin C may assist in preventing plasmatic protein damage in patients with sleep apnea.

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