

Online Data Supplement

OXIDATIVE STRESS IN THE EXTERNAL INTERCOSTALS OF OBSTRUCTIVE
SLEEP APNEA PATIENTS

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METHODS

Subjects

All individuals were Caucasian. Twelve consecutive male patients [1] with a polysomnographically confirmed diagnosis of OSAS (recently diagnosed and without CPAP therapy) were recruited. All patients were clinic referrals with a clinical history compatible with OSAS. Six male healthy nonsmokers control individuals [1] of similar anthropometric characteristics were also recruited from the general population (relatives and/or friends of the study patients'). All control subjects were recruited on the basis of the absence of a clinical history compatible with OSAS and a negative sleep study (polysomnography). The sample size of both patient and control populations was calculated on the basis of formerly published studies by our group, where similar physiological and biological approaches were used [2-5]. Following criteria established by the United States Adult Treatment Panel III of the National Cholesterol Education Program (ATPIII) [6] the number of individuals with metabolic syndrome did not significantly differ between OSAS patients and controls (42% and 50%, respectively). Both patients and controls were recruited at the Institute of Tuberculosis and Lung Diseases in Warsaw, Poland. Exclusion criteria included previous treatment with CPAP, female sex in order to avoid sex-related effects on muscles [1], COPD, bronchial asthma, cardiovascular disease, chronic metabolic diseases including diabetes, suspected para-neoplastic or myopathic syndromes, and/or treatment with drugs known to alter muscle structure and/or function.

Clinical, Nutritional and Functional Assessment

Clinical evaluation included medical history, a complete physical examination, thorax radiology, and electrocardiogram. Nutritional evaluation included anthropometric and analytical parameters. Forced spirometry (alpha 3 spirometer;

Vitalograph; Maidenhead, UK) was performed and both diffusion capacity and static lung volumes (Masterlab; Jaeger, Würzburg, Germany) were determined in all subjects using standard procedures, and reference values were those of the European Community for Coal and Steel [7]. Arterial blood gases were analyzed using microelectrodes and polarographic techniques (Blood gas analyzer Corning 278, Corning; Medfield, MA, USA). All the study subjects performed these tests. In patients with OSAS, inspiratory muscle strength was assessed through determination of maximal inspiratory pressure at the mouth using a manometer (Vmax Encore, Sensormedics Corp., Yorba Linda, CA) during an occluded maneuver from residual volume. Reference values by Black and Hyatt *et al* [8] were chosen. Specific inspiratory muscle endurance was assessed in both control subjects and patients (only 9 patients gave their consent to perform this test) using a simplified isoflow inspiratory endurance test by means of an inspiratory resistor, with oscilloscope feedback, and electronic pressure monitoring. Patients and controls were fully stimulated by a laboratory technician throughout the entire test. All individuals breathed against a submaximal constant load (equivalent to 70% of their maximal inspiratory pressure, measured at baseline and after the CPAP treatment) until exhaustion. The elapsed time was defined as the inspiratory endurance time (T_{lim}, in minutes). Exercise capacity was evaluated only in patients using the standard 6-minute walking distance test [9] and reference values by Enright *et al* [10] were employed. All patients also performed a handgrip maneuver with the nondominant hand and reference values by Mathiowetz were chosen [11].

Polysomnography

Standard polysomnographic examination was performed in the sleep laboratory according to the standards of the American Sleep Disorders Association [12] using the Somnostar Alpha apparatus (Somnostar Alpha Series, Sensormedics Corp., Yorba

Linda, CA, USA). Electroencephalogram recording was performed by using two electrodes: capital (C4) and occipital (O2). Electrooculogram electrodes were placed lateral to the outer canthus of each eye. Two electromyogram electrodes were placed at submental region. Chest and abdominal wall breathing movements were recorded using inductive plethysmography: Oronasal air flow was monitored by a thermistor. Pulse oxymetry was taken using finger print probe. Heart rate was monitored using three electrocardiogram leads. An obstructive apnea was defined as a complete cessation of airflow for > 10 seconds in the presence of thoracoabdominal breathing movements. Hypopnea was defined as any airflow reduction that lasts > 10 seconds resulting in arousal or oxygen desaturation. A decrease in SaO₂ > 4% was considered to be desaturation. The apnea-hypopnea index was calculated by dividing the total number of both apneas and hypopneas per hour of sleep. The diagnosis of OSAS was established by obtaining an apnea-hypopnea index > 10 per hour of sleep [12]. When the diagnosis of sleep apnea syndrome was clearly established, patients underwent a second polysomnography for manual CPAP titration. The starting pressure was 4 cm H₂O, and the pressure was increased by 1 cm H₂O every 5 minutes until the apneas and snoring disappeared. CPAP therapy was applied to all patients (REMstar, Respironics ®, Murrysville, PA, USA) for at least 6 months. Adherence to CPAP treatment over this period of time was monitored by the built-in time counter in the CPAP device. Good compliance of the treatment was established as a mean usage of CPAP > 4 hours/night. Furthermore, all patients completed the Epworth Sleepiness Scale (ESS) [13] (normal: ESS score < 10, borderline: ESS score 10-12, excessive sleepiness: ESS score > 12) before and after the 6-month treatment period with CPAP. CPAP treatment was administered to all the patients 7-10 days after undergoing the baseline muscle biopsy surgery.

Muscle Biopsies

Biopsies from the external intercostals (≈ 40 mg) were taken before and after a six-month period of treatment with CPAP. The pre-treatment biopsies were taken either from the nondominant or dominant sides randomly assigned, and the post-treatment biopsies were taken from the contralateral side at identical anatomical region. Biopsies from the external intercostals were taken along the anterior axillary line at the sixth intercostal space following procedures published elsewhere [14-16], and were immediately frozen in liquid nitrogen and stored at -80°C for further analysis or immersed in an alcohol-formol bath for 2h to be thereafter embedded in paraffin. Western-blot analysis required the use of frozen tissues, while paraffin-embedded tissues were used for immunohistochemical analysis, including assessment of the expression of myosin heavy chain isoforms.

Biological Muscle Studies

All the muscle biology analyses were conducted in the same laboratory, at Hospital del Mar-IMIM, in Barcelona in a blinded manner. It should also be mentioned, that both investigators and laboratory technicians in charge of the muscle sample manipulation (molecular biology techniques) were blinded throughout the entire duration of the experimental procedures. Furthermore, the investigators responsible for both patient and control subject recruitment were not involved at any time in the molecular biology experiments conducted on the muscle specimens.

Total Carbonyl groups. Total levels of those highly reactive carbonyl groups in the muscle protein side chains were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of 2,4-dinitrophenylhydrazone (DNP) [17]. The DNP-derivatized proteins were subsequently

separated by electrophoresis and further subjected to immunoblotting with selective antibodies against the DNP moieties.

Aldehyde-protein adducts. The lipid peroxidation product malondialdehyde (MDA) can cause further cellular damage by binding to and modifying proteins, which leads to the formation of aldehyde-protein adducts. The characteristic feature of these adductions is the introduction of carbonyl groups into the modified proteins. MDA reacts with lysine residues to form Schiff base adducts that can also be detected in tissues using a selective antibody [18].

Immunoblotting. The effects of oxidants on muscle proteins and lipids were evaluated according to methodologies published elsewhere [2, 3, 5]. Frozen muscle samples from external intercostals were homogenized in a buffer containing HEPES 50 mM, NaCl 150 mM, NaF 100 mM, Na pyrophosphate 10 mM, EDTA 5 mM, Triton-X 0.5%, leupeptin 2 µg/ml, PMSF 100 µg/ml, aprotinin 2 µg/ml and pepstatin A 10 µg/ml. Samples were then centrifuged at 1,000 g for 30 min. The pellet was discarded and the supernatant was designated as a crude homogenate. Total muscle protein level in each sample was spectrophotometrically determined with the Bradford technique using different runs of triplicates in each case and bovine serum albumin as the standard (Bio-Rad protein reagent, Bio-Rad Inc., Hercules, CA, USA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein from crude muscle homogenates were always loaded (20 µg per sample/lane) onto the gels, as well as identical sample volumes/lanes.

Immunoblotting experiments were specifically designed in such a way that muscle homogenates from control subjects (n=6) and from OSAS patients both before (n=12) and after (n=12) the CPAP treatment were always run together and kept in the

same order. Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with non-fat milk and incubated overnight with selective antibodies. The following antibodies were used to detect the different antigens and phenomena: anti-DNP moiety antibody (Oxyblot kit, Chemicon International Inc., Temecula, CA, USA), anti-MDA antibody (Academy Bio-Medical Company, Inc., Houston, TX, USA), anti-HNE antibody (Alpha Diagnostics International Inc., San Antonio, TX, USA), anti-3-nitrotyrosine antibody (Cayman Chemical Inc., Ann Arbor, MI, USA), anti-Mn SOD antibody (StressGen, Victoria, BC, Canada), and anti-catalase antibody (Calbiochem, San Diego, CA, USA). Tissue homogenates obtained from rat brain mitochondria and rat erythrocytes were used as positive controls of the enzymes Mn-SOD and catalase, respectively. Specific proteins from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, samples from the different groups were always detected in the same film under identical exposure times. Negative control experiments, in which primary antibodies were omitted or samples were not derivatized (carbonyl groups), and membranes were then probed only with secondary antibodies were also conducted. Blots were scanned with an imaging densitometer and optical densities (OD) of specific proteins were quantified with Diversity Database 2.1.1 (BioRad, Philadelphia, PA). Values of total protein carbonylation, total MDA-protein adducts, total HNE-protein adducts, and total protein tyrosine nitration in a given sample were calculated by addition of OD of individual protein bands in each case. Final optical densities obtained in each specific group of subjects corresponded to the mean values of the different samples (lanes) of each of the antigens studied. To validate equal protein loading among various lanes, PVDF membranes were stripped and re-probed with a mouse anti- Glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) HRP conjugated antibody (Abcam plc, Cambridge, England, UK) in all cases. Optical densities in each box and whisker plot were expressed as the ratio of the optical densities of the specific antigen to those of GAPDH.

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