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BLUNTED MUSCLE ANGIOGENIC TRAINING-RESPONSE IN COPD PATIENTS VERSUS SEDENTARY CONTROLS

METHODS

Pulmonary function tests

All subjects underwent whole body plethysmography (Transmural Bodybox 2800; Sensomedics, Yorba Linda, CA, USA). The values were compared with normal values [1]. Arterial blood samples were obtained from the radial artery of seated COPD patients while they breathed room air. PaO_2 was measured with a blood gas analyzer (Roche OMNI S, Roche Diagnostics, Mannheim, Germany).

Exercise testing and muscle function assessment

Exercise capacity

The 6-minute walking test (6MWT), which is routinely used by our group [2], was performed in a 30-meter corridor. The distance walked during the test (6MWD) was compared with the reference values [3]. Patients were asked to walk at their own maximal rate. They were asked to cover as much ground as possible in 6 minutes while walking at a steady rate with no running. Arterial oxygen saturation (SpO2) and HR were monitored every minute using a pulse oximeter (Nonin 8500 M; Nonin Medical, Inc., Minneapolis, MN, USA).

Participants performed an incremental cycloergometric test until exhaustion on an electrically-braked cycloergometer (Ergoselect 200P, Ergolyne, Bitz, Germany), following the individualized protocol usually used in our laboratory [4] and according to the

international standards [5]. During the exercise test, heart rate, ECG, blood pressure, and transcutaneous oxygen saturation were monitored. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured and calculated by breath-by-breath analysis (Sensormedics, Vmax 229, Autobox, Yorba Linda, CA, USA). Maximal power output was the maximal workload sustainable, and peak oxygen consumption (VO2SL) was the mean value during the last 20 s of the test. The ventilator threshold was blindly and independently assessed for each subject by two experienced practitioners on the basis of noninvasive methods (ventilator equivalent and V-slope methods), as recommended [5]. The dyspnea threshold was also determined blindly and independently by two experienced practitioners in each patient [6]. After the identification of the descriptor of the dyspnea used by the patient [7], the breathlessness was rated every minute during the test [8], using a visual analogue scale (VAS) [9]. Then, the dyspnea was plotted against the power output. On this slope, regression lines were drawn and the power output of their intercept corresponded to the dyspnea threshold. Last, the heart rate corresponding to this dyspnea threshold was used to target the exercise intensity during the training, in cases where the ventilatory threshold was not determined or not valid [5], as recommended [7].

Muscle function assessment

The maximal voluntary contraction (MVC) and endurance time (T.lim) were assessed with the usual methods of our group [8-10]. Briefly, the MVC was measured at 90° on a bench (Kettler, Germany). Three reproducible measurements (within 10%) of the force of the dominant leg were recorded and the best value was retained as the MVC. The T.lim was then measured on the dominant leg as the time (in s) during which the subjects were able to maintain a contraction at 30% of MVC, and at the rate of 10 movements per minute to exhaustion. A reduction in MVC >10% in 1 min was defined as fatigue and validated the test.

Fat-free mass index and muscle mass were estimated with multi-frequency bio-electrical impedancemetry (BIA) (QuadScan 4000, Bodystat, Isle of Man, UK), using the validated equations [11, 12]

Exercise training

The training protocol followed the recommendations of the ATS/ERS statement on pulmonary rehabilitation [7]. Twenty sessions of endurance exercise (stationary cycling, walking) condensed into 6 weeks were proposed. The exercise training sessions were performed 3 or 4 times per week on a cycloergometer or a treadmill. Outdoor walking sessions were included. The exercise intensity was individualized for each patient and corresponded to the target heart rate at the ventilatory threshold [7, 13] assessed during the exercise test. This intensity was continuously monitored with a cardiofrequency meter. Each session lasted 1h30, with 45 minutes of endurance training (10 min of work at the intensity of the ventilatory threshold followed by 5 min of active recovery, repeated 3 times) completed by 30 minutes of strength-building exercise (8-10 exercises, with sets of 10-15 repetitions). The load for the resistance exercise was initially set at 40% of the isotonic one-repetition maximum (1-RM) of each muscle (deltoid, biceps, triceps and quadriceps), and then progressively increased using a perceived exertion scale (with a target of 5-6 on a 10-point scale [14]). All sessions were supervised by an experienced clinician, and the training intensity was increased during the training protocol. The exercise training sessions were part of a multi-component and comprehensive pulmonary rehabilitation program, including education. Six 1- to 1.5-hour small group education sessions were conducted interactively by an experienced therapist. These sessions were dedicated to the improvement of various health-related behaviors (regarding nutrition, physical activity, cardiovascular risk factors, sleep, etc.).

Muscle biopsy

Muscle biopsies were performed in the vastus lateralis of the quadriceps before the exercise training program and 48 hours after the endurance exercise, with the usual methodology [15]. Specimens were then dissected free of visible connective tissue and fat and the muscle tissue was immediately frozen in isopentane, cooled to freezing point with liquid nitrogen, and stored at -80°C until analysis.

Muscle immunohistochemistry

Muscle fiber type and mean cross sectional area (CSA) were assessed after immunohistochemistry on frozen sections (10 µm thickness) from the muscle biopsies, using antibodies specific to each isotype of MHC. Muscle fiber type and CSA were assessed by immunohistochemistry on frozen sections from the muscle biopsies using a panel of anti-MHCI, anti-MHCIIa and anti-MHCIIx monoclonal antibodies (#A4.951-c, #2F7-c #6H1-c, respectively, University of Iowa, IO, USA) [16, 17]. The fiber number per type (I, I/IIa, IIa, IIa/IIx, IIx) and CSA were identified with a microvision image analysis system (Histolab 6.1.0, Microvision Instruments, Evry, France). A mean of 187±67 fibers/biopsy was analyzed. Capillaries were visualized by immunochemistry with the monoclonal antibody against CD31 (BD Bioscience, #550389, Franklin Lakes, NJ, USA). CSA, capillary density and the capillary-to-fiber ratio were determined after counting capillaries and myofibers on 1 to 2 cryosections from each muscle biopsy and in quintuplet on each of these cryosections. An average of 201±48 fibers and 316±85 capillaries were counted on each counting area.

Western blotting

Protein extraction: Frozen muscle samples were homogenized in a buffer containing 100 mM potassium phosphate buffer with 10 mM EDTA, 0.05% bovine serum albumin (BSA), 0.13mM butylated hydroxytoluene, 0.13 mM desferrioxamine (pH 7.4) and 1% protease/phosphatase inhibitors. The total muscle protein level in each sample was determined with the Bradford technique in triplicate and with BSA as standard (500-006, BioRad, Marnes-la Coquette, France). Denatured samples (30 μg/well) were subjected to SDS-PAGE on 4-15% Criterion precast gels (Biorad, #567-1084, Oakville, ON, Canada) and blotted onto nitrocellulose membranes (Whatman, BA95, Sigma-Aldrich, Oakville, ON, Canada). After blocking with 5% BSA, the blots were probed for VEGF-A (clone VG-1, Millipore #05-1117, Etobicoke, ON, Canada), TSP-1 (clone A6.1, Invitrogen #399300, Burlington, ON, Canada), and β-actin (clone C4, Santa Cruz Biotechnologies sc-47778, CA, USA), and appropriate secondary HRP-conjugated antibodies (anti-mouse, Dako #p0260, Mississauga, ON, Canada; anti-rabbit, Cell Signaling Technology #7074, New England Biolabs, Pickering, ON, Canada). The specificity of the clones against VEGF-A and TSP-1 was tested on our bank of muscle samples from VEGF-A- and TSP-1-deficient mice. Proteins were visualized using an enhanced chemiluminescence procedure (SuperSignal WestPico, ThermoScientific, Mississauga, ON, Canada) and a Kodak Image Station 4000MM Pro. Western blot results were analyzed with Carestream Molecular Imaging Software (version 5).

Mitochondrial respiration

Dissection and permeabilisation of fibre bundles with saponin were performed as described previously [18, 19] and then they were stored on ice in respiratory solution until determination of mitochondrial respiration activity. Mitochondrial respiratory function in permeabilised myofibres was determined as previously described [19, 20], in the presence of different substrates: the pyruvate, the succinate and the palmitoyl-carnitine. The rates of oxygen

consumption were expressed as micromoles of O2 per minute per gram of dry weight (μmolO2/min/g) and normalized by the proportion of the type I fibers for differences in mitochondrial content between individuals [21].

Statistical analysis

Our main outcome measure was the quadriceps capillary-to-fiber ratio (C/F). The sample size for the COPD patient group was determined with a power of 95% and a two-sided 0.05 significance level, on the assumption that the exercise training induced an increase of the individual C/F of the type I fibers from 1.41 to 1.70 (+21%) and a standard deviation of 0.33 in non-cachectic COPD patients [22]. Assuming an upper or at least similar improvement in the SHS, and a 20% drop-out, the critical sample size was 24 per group.

Data are presented as mean ± SD. Distribution normality was tested by Kolmogorov-Smirnov test. Pearson coefficients describe the correlations. We used a multiple linear regression model. Comparisons of the training effect between groups were performed by mixed linear regression model [23]. Each patient/subject was evaluated two times (repeated measures). Thus, measured variables were adjusted using a linear mixed-effects model for repeated measures, allowing to take into account repeated measures as random variables. Measure time and patient group are used as fixed effect in the model. Then, the group effect can be tested by the mixed-linear model. A p-value of <0.05 was considered statistically significant. Data were analyzed using the R.2.13.0 software.

RESULTS

Baseline characteristics of the COPD patients and SHS

The COPD patients had significantly impaired exercise capacity ($p<0.001$) and muscle endurance ($p<0.05$).

The fiber CSA tended to be not significantly greater in COPD patients compared with SHS ($p=0.14$). Yet we observed a gender effect for fiber CSA ($p<0.001$) in both COPD patients and SHS (group effect: $p= 0.96$). The fiber CSA area and the type I fiber proportion were not correlated in either COPD patients ($p=0.57$) or SHS ($p=0.29$). The capillary-to-fiber ratio was significantly reduced in COPD patients compared with SHS, but the difference in capillary density did not reach significance ($p=0.057$, Table 2).

Training-induced responses of the muscle mitochondrial respiration

In COPD patient (n=10), we observed no increase of the oxygen consumption of the fibers in the presence of pyruvate and after normalization by the type I fiber proportion [in micromoles of O2 per minute per gram of dry weight (μmolO2/min/g): 11.52±5.77 to 6.59±2.71, $p=0.13$. We even observed decreased values of the maximal oxygen consumption with other substrates (15.72±9.16 to 9.07±7.73; $p<0.05$ for the succinate, and 7.51±4.21 to 4.03±2.65; $p<0.01$ for the palmitoyl-carnitine, respectively)

DISCUSSION

Effect of the training stimulus

Our program of exercise training at moderate intensity increased the exercise capacity in both COPD patients and SHS, and the improvement was significant in both groups. Because the endurance time on cycloergometer increased greatly in SHS (+146%; $p<0.001$), their modest increase in 6MWD (5.3±6.1%) might be explained by a ceiling effect. The improved 6MWD of the COPD patients (37±35m) was consistent with a previously published meta-analysis (95% confidence interval: 26-72 m [24]) and was even greater than that observed in a recent study (25±52m [25]). The changes were clinically significant in 8 out of 23 COPD patients

(35%) using the classical 54-m threshold [26] and in even more patients (16/23) if we use a more recently published threshold (26 m [27]).

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FIGURE LEGENDS

Figure E1. Representative transverse cryosections of the *vastus lateralis* stained with type I, type IIa and type IIx myosin heavy chain isotype antibodies at a magnification of * 10, in A) a COPD patient before training and B) an SHS before training. Note the reduction in the proportion of type I MHC fibers in the COPD patient.