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Metabolic capacity of the diaphragm in patients with COPD

J.H. Wijnhoven[a], A.J.M. Janssen[b], T.H. van Kuppevelt[c], R.J.T. Rodenburg[b], P.N.R. Dekhuijzen[a,*]

[a]*Department of Pulmonary Diseases and Institute for Fundamental and Clinical Human Movement Sciences, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands*
[b]*Department of Pediatrics and Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands*
[c]*Department of Biochemistry, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands*

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KEYWORDS
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Summary Chronic obstructive pulmonary disease (COPD) is associated with an increased load on the diaphragm. Chronic loading on skeletal muscles results in metabolic changes and fiber-type shifts. Therefore, we investigated whether the load on the human diaphragm imposed by COPD altered oxidative enzyme activity, glycogenolytic enzyme activity and mitochondrial energy generating capacity and efficiency. Biopsies of the diaphragm from COPD patients and control subjects were obtained and activities of L(+)3-hydroxyacylCoA-dehydrogenase (HADH, marker for β-oxidation capacity) and phosphorylase (marker for glycogenolytic capacity) were measured spectrophotometrically. Mitochondrial energy generating capacity was measured by spectrophotometrical and radiochemical methods. Fiber-type distribution was determined electrophoretically. We found that HADH activity was increased with increasing severity of COPD ($P = 0.05$). No change in glycogenolytic enzyme activity was observed. The activity of the mitochondrial respiratory chain complexes III and IV and oxidation of pyruvate was increased with increasing airflow obstruction. These results suggest that in COPD the diaphragm adapts to a higher workload by increasing the oxidative capacity and mitochondrial function.

Abbreviations: COPD, chronic obstructive pulmonary disease; LDH, lactate dehydrogenase; FEV_1, forced expiratory volume in 1 s; VC, vital capacity; TLC, total lung capacity; FRC, functional residual capacity; HADH, L(+)3-hydroxyacylCoA-dehydrogenase; MHC, myosin heavy chain; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; BMI, body mass index; K_{CO}, diffusion coefficient; FRC, functional residual capacity; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CS, citrate synthase; C-I, complex I; C-III, complex III; C-IV, complex IV; SCC, succinate:cytochrome-*c* oxidoreductase; SDH, succinate dehydrogenase

*Corresponding author. Tel.: +31 24 3614579; fax: +31 24 3610324.
E-mail address: R.Dekhuijzen@long.umcn.nl (P.N.R. Dekhuijzen).

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Introduction

In patients with chronic obstructive pulmonary disease (COPD), adaptations in the diaphragm occur as a result of increased workload. These adaptations include a shift from type II fibers towards type I fibers,[1,2] which is most pronounced in patients with very severe COPD.[1] Levine et al.[3] hypothesized that the diaphragms of severe COPD patients are undergoing endurance-exercise training. However, endurance training programs that elicit these effects, consist normally of training sessions at appropriate intensity which are separated by rest. In the diaphragm, this rest is rather impossible. Therefore, the diaphragm in COPD might come in a state of ''overtraining'', with all the deleterious consequences. In limb muscles, endurance training elicits a hypertrophy of type I fibers,[4] an increase in oxidative capacity,[4–7] and an increase in capillarization.[4] Given the fact that the workload is increased in COPD diaphragm, similar adaptations as elicited by exercise training may occur, for example changes in activity of oxidative enzymes and enzymes involved in anaerobic metabolism. Indeed, Levine et al.[3] found an increase in succinate dehydrogenase (SDH) activity in diaphragm fibers of COPD patients versus control subjects. Sanchez et al.[8] reported a decrease of glycolytic enzyme activity (lactate dehydrogenase (LDH) and hexokinase) in the diaphragm of patients with moderate COPD.

In addition to an upregulation in oxidative enzyme activity, changes in mitochondrial function may occur. Indeed, Ribera et al.[9] showed an increased mitochondrial respiration and efficiency in diaphragm of COPD patients. Besides a change in function, also an increase in mitochondrial density has been reported in COPD diaphragm.[10] However, it is not known which parts of the mitochondrial energy generating system are changed in the diaphragm of COPD patients.

These metabolic enzyme activities and mitochondrial function are in part related to fiber type, since type I fibers contain more oxidative capacity whereas type II fibers contain more glycolytic capacity.[7] Besides, training can also increase oxidative enzyme activities without major shifts in fiber types.[11]

In this study, we tested the following hypotheses: With increasing severity of COPD: (1) the oxidative enzyme activity increases, (2) the glycogenolytic enzyme activity decreases, and (3) the mitochondrial energy generating capacity is enhanced. Furthermore, we determined to what extent the changes in enzyme activities paralleled the shift in fiber type with increasing severity of COPD. To test these hypotheses, we determined oxidative and glycogenolytic enzyme activity, mitochondrial energy generating capacity and fiber-type shifts in the diaphragm of patients with COPD.

Methods

Subjects

Nineteen patients with mild to severe COPD (mean forced expiratory volume in 1 s (FEV_1) = 64 (range 18–114)% predicted; FEV_1/vital capacity (VC) = 50 (range 18–61)%) and seven male non-COPD patients (mean FEV_1 = 102 (range 81–117)% predicted; FEV_1/VC = 77 (range 70–83)%), who were undergoing thoracic surgery for a malign T1 lung tumor participated in this study. General characteristics and pulmonary function data are shown in Table 1. Informed consent for diaphragm biopsies was obtained from each of the subjects, and the study was approved by the ethical committee of our hospital.

Biopsies

For measurements of enzyme activity, biopsies of the anterior costal diaphragm were obtained, directly frozen in liquid nitrogen, and stored at –80 °C until being used. For measurements of mitochondrial function, fresh biopsies were put immediately in ice-cold SETH buffer (0.25 M sucrose, 2 mM EDTA, 10 mM Tris, 5×10^4 U heparin, pH 7.4) and delivered within 1 h in the laboratory.

Table 1 Patient characteristics.

	COPD ($n = 19$)	Non-COPD ($n = 12$)
Age (years)	61 ± 2	60 ± 2
BMI	26 ± 1	25 ± 1
FEV_1 (l)	1.95 ± 0.18**	3.20 ± 0.17
FEV_1 % predicted	64 ± 5**	103 ± 4
FEV_1/VC	50 ± 3**	77 ± 1
TLC % predicted	100 ± 6	100 ± 3
K_{CO}	1.03 ± 0.06*	1.30 ± 0.04
K_{CO} % predicted	68 ± 4*	87 ± 3
FRC % predicted	109 ± 10	86 ± 10

Values are means ± SEM. FEV_1, forced expiratory volume in 1 s; VC, vital capacity; TLC, total lung capacity; K_{CO}, diffusion coefficient; FRC, functional residual capacity. *$P < 0.05$ and **$P < 0.001$ between COPD and non-COPD patients in the same group.

Enzyme activity measurements

Spectrophotometric analyses were performed to determine the activity of L(+)3-hydroxyacylCoA-dehydrogenase (HADH, oxidative enzyme [$n = 15$]) and phosphorylase (glycogenolytic enzyme [$n = 14$]). The procedures to determine biochemical activities of the oxidative and glycogenolytic enzymes were performed as described previously.[12] Briefly, fresh frozen biopsies were thawed in ice-cold SET buffer (0.25 M sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4). In this buffer, muscle homogenates (5% w/v) were prepared using a Potter–Elvehjem glass–teflon homogenizer. Total phosphorylase (*a*+*b*) activity was assayed at 37 °C and expressed as μM NADPH formed · min^{-1} g wet tissue. HADH activity, assessed at 50 μmol acetoacyl-CoA at 37 °C, was expressed in nmol NADH oxidized · min^{-1} g wet tissue. All assays for metabolic enzymes were performed in duplicate.

Mitochondrial energy generating capacity

Equipment and Materials

[1-^{14}C] Pyruvic acid, sodium salt (0.4–1.1 GBq/mmol) was obtained from ICN. Pyruvic acid, sodium salt, L-malic acid, creatine, sodium-*m*-arsenite, atractyloside, potassium salt and p^1,p^5-di(adenosine-5′) pentaphosphate (Ap5A) were obtained from Sigma (St. Louis, MO., USA). Adenosine-5′-diphosphate disodium salt (ADP), hexokinase, glucose-6-phosphate dehydrogenase, L-lactate-dehydrogenase and NADH were obtained from Roche (Ont., Canada). All chemicals were of the highest purity commercially available. Glass incubation vials of 20 ml capacity, with injection caps and rubber septums, hydroxide or hyamine 10-X (hyamine) and Insta Fluor were obtained from Packard Becker BV (Groningen, The Netherlands). All solutions were prepared with ultra-pure water from a Milli-Q_{plus} water purification system.

Homogenization procedure of muscle tissue

Muscle tissue was washed with fresh ice-cold SETH, and fat and connective tissue were disconnected. Tissue was cut in very small pieces of about 0.1 × 0.1 mm with help of a Sorvall TC2 tissue chopper with razor blade. Tissue was homogenized in fresh ice-cold SETH buffer (5–10% w/v) with a Potter–Elvehjem tissue homogenizer according to Fischer et al.[13] Some 100 μl samples of the crude homogenate were frozen immediately in liquid nitrogen and kept at −80 °C for measuring protein content, cytochrome *c* oxidase and citrate synthase (CS). The rest of the crude homogenate was centrifuged for 10 min at 2 °C at 600*g*. The 600*g* supernatant was frozen in 100 μl aliquots in liquid nitrogen and kept at −80 °C for measuring protein content, CS activity and activities of respiratory chain enzymes. CS activity was measured according to Srere[14] with minor modifications. Protein concentration was measured according to Lowry et al.[15] with minor modifications.

Incubations

Incubations were performed in a shaking water bath at 37 °C in 20 ml glass incubation vials closed with injection caps and rubber septums. In the incubations for measuring oxidation rates for ^{14}C-labelled substrates, the glass vial contained a small plastic tube inside a plastic cylinder, the tube containing 0.2 ml hyamine. Incubation volume was 0.5 ml and the incubation time was 20 min. Incubations were carried out in 30 mM potassium phosphate, 75 mM potassium chloride, 8 mM Tris, 1.6 mM EDTA, 5 mM $MgCl_2$, 0.2 mM Ap5A and where indicated 2.0 mM ADP, 20 mM creatine, 1 mM sodium pyruvate, 1 mM malic acid, ±8 kBq [1-^{14}C] sodium pyruvate and 2 mM sodium arsenite. Only incubations in the presence of ADP also contained creatine. In the incubations for measuring ATP production rates, radioactive substrates were omitted. After 20 min the incubations were stopped by adding 0.2 ml 3 M perchloric acid through the rubber septum to the incubation mixture with help of a hypodermic syringe. Incubations for measuring ATP production rates were stopped by opening the vial and adding the same amount of perchloric acid with an Eppendorf pipette. Incubations were allowed to stand for 1 h in ice for fully trapping $^{14}CO_2$ into the hyamine. After 1 h the tubes containing the hyamine were taken out, mixed with 5 ml Insta Fluor and counted in a Wallac 1400 Liquid Scintillation Counter. Incubations for measuring ATP production rates were allowed to stand in ice for 15 min and after that the incubation mixtures were centrifuged for 5 min, 14,000*g*, at 2 °C in an Eppendorf 5402 centrifuge. 0.5 ml of the supernatant were neutralized by adding 0.6 ml ice cold 1 M $KHCO_3$ under thoroughly stirring on a vortex mixer. The mixtures were allowed to stand in ice for 15 min and were frozen at −20 °C.

Incubations for determination of the activity of complex I (C-I) and succinate cytochrome *c* oxidoreductase (SCC) were performed according to Fischer[16,17] with minor modifications. Incubations for determination of complex III (C-III) and complex IV (C-IV) activity were performed according to Bentlage et al.[18] and Cooperstein and Lazarow,[19] respectively.

ATP and creatine phosphate measurement
Neutralized incubation mixtures were thawed and put on ice for 5 min. The mixtures were centrifuged at 2 °C in an Eppendorf 5402 centrifuge. ATP and creatine phosphate were measured spectrophotometrically at 340 nm and 25 °C. A total of 0.4 ml of supernatant were incubated in a semi-micro-acryl cuvette in a total volume of 1.0 ml, containing 0.3 M triethanolamine, 3.3 mM NADP, 1.6 mM ADP, 5.3 mM $MgSO_4$ and 33 mM glucose, pH 7.5.

Absorbance (E_0) was measured and 5 μl of a mixture of hexokinase (6 U) and glucose-6-phosphate dehydrogenase (1.75 U) were added and mixed. After the absorbance had become constant, absorbance was measured (E_1) and 5 μl creatine kinase (1 U) were added and mixed. After absorbance had become constant again, absorbance (E_2) was measured. The increase in absorbance E_1–E_0 was used to calculate ATP content. The increase in absorbance E_2–E_1 was used to calculate creatine phosphate content. An ε_{340} for NADPH of $6.22 \times \text{mmol}^{-1} \times \text{cm}^{-1}$ was used.

Fiber-type determination

A part of the biopsy was stored in −80 °C to determine myosin heavy chain (MHC) composition. Sections of this sample that were 10-μm-thick were dissolved in sodium dodecyl sulphate (SDS) sample buffer and boiled for 2 min. The MHC composition was determined by SDS polyacrylamide (7%) gel electrophoresis (SDS–PAGE). Electrophoresis was performed at 100 V for 27 h with Tris–glycine electrode buffer at 15 °C (Protean II xi, Bio-Rad, USA). Gels were then silver stained. The gels were scanned with an imaging densitometer and optical densities (OD) of different MHC bands were quantified with GeneTools software (Syngene, UK).

Statistical analysis

Data were analyzed with SPSS for Windows, version 12.0.1 (SPSS, Chicago, IL). A *t* test was used to compare results between groups. To establish the relationship between the metabolic capacity and lung function parameters and between the metabolic capacity, mitochondrial function and MHC composition, Pearson correlation was determined. Significance was set at the 0.05 level.

Results

Patients characteristics

The COPD patients did not differ significantly from the control subjects with respect to age or body mass index (BMI) (Table 1). Clearly, the COPD subjects showed lower values with respect to the FEV_1, FEV_1 percentage of predicted, the ratio of the FEV_1 to forced VC, diffusion coefficient (K_{CO}) and K_{CO} percentage of predicted. The COPD patients in the mitochondrial group had lower values with respect to FEV_1 percentage of predicted and the ratio of FEV_1 to forced VC.

Metabolic changes

The activities of HADH and phosphorylase are presented in Table 2. There was no significant difference in HADH activity between the COPD and non-COPD group ($P = 0.19$). However, there was a significant inverse correlation between HADH activity and FEV_1 % predicted ($r = -0.51$, $P = 0.05$) in the COPD group (Fig. 1A). Between the COPD and non-COPD group there was no significant difference regarding phosphorylase activity ($P = 0.26$). No significant correlation was found between phosphorylase activity and FEV_1 % predicted ($r = 0.54$, $P = 0.13$) in the COPD group (Fig. 1B).

Mitochondrial function

CS activity was not different between the COPD patients and non-COPD patients ($P = 0.47$), nor was there any correlation of CS activity with a pulmonary function parameter. Therefore, it is

Table 2 Enzyme activities and myosin heavy chain distribution.

	COPD ($n = 11$)	Non-COPD ($n = 7$)	*P*-value
Phosphorylase activity (U/g)	0.27 ± 0.04	0.21 ± 0.03	0.26
HADH activity (U/g)	7.24 ± 0.83	4.95 ± 0.87	0.09
% MHC type I	41 ± 2	37 ± 3	0.34
% MHC type IIa	39 ± 2	33 ± 4	0.11
% MHC type IIx	23 ± 2	26 ± 4	0.47

Values are means ± SEM. HADH, L(+)3-hydroxyacylCoA-dehydrogenase; MHC, myosin heavy chain.

permitted that we use CS as reference enzyme for mitochondrial content. There were no significant differences between the COPD group and the non-COPD group regarding any of the mitochondrial parameters (Table 3). However, in the COPD group there were significant correlations between several lung function parameters and mitochondrial parameters, such as oxidation of [1-^{14}C] pyruvate+malate and total lung capacity (TLC) % predicted ($r = 0.92$, $P = 0.01$) (Fig. 2A). In addition, significant correlations were observed between oxidation of [1-^{14}C] pyruvate+malate and VC % pred ($r = 0.806$, $P = 0.04$), functional residual capacity (FRC) % pred ($r = 0.913$, $P = 0.01$) and K_{CO} ($r = -0.836$, $P = 0.04$). Furthermore, significant correlations were found between C-III activity and TLC % predicted ($r = 0.80$, $P = 0.03$) (Fig. 2B) and FRC % predicted ($r = 0.76$, $P = 0.05$). Besides, significant correlations were observed between C-IV activity and K_{CO} % predicted ($r = -0.94$, $P < 0.01$) (Fig. 2C), K_{CO} ($r = -0.847$, $P = 0.01$) and VC % predicted ($r = 0.848$, $P = 0.02$).

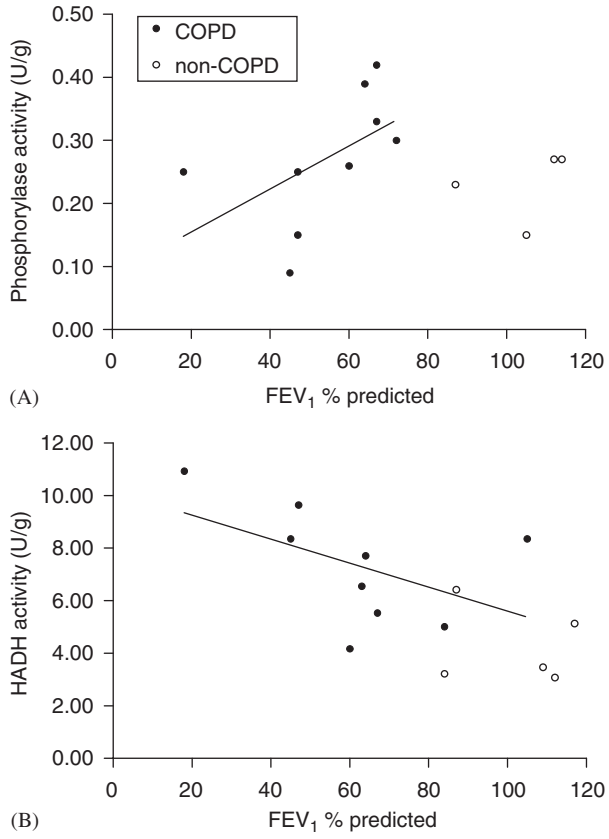

Figure 1 Relationship between FEV_1 and activities of the enzymes phosphorylase (A), and L(+)3-hydroxyacylCoA-dehydrogenase (HADH) (B) within the COPD group. An inverse correlation was found between FEV_1 and HADH ($r = -0.51$, $P = 0.05$). No significant correlation was found between FEV_1 and phosphorylase ($r = 0.54$, $P = 0.13$).

MHC composition

The distribution of the MHC isoforms is presented in Table 2. There was no difference in percentage of the different MHC types between COPD and non-COPD diaphragm ($P = 0.34$). No significant correlation was found between any of the pulmonary function parameters and relative amount of the different MHC isoforms in the COPD group.

Discussion

To our knowledge, this is the first investigation to examine the effects of COPD on bio-energetic enzyme activities combined with mitochondrial capacity and fiber-type changes. The principal findings of this study are that between COPD and non-COPD patients, there were no differences in

Table 3 Mitochondrial parameters.

	COPD ($n = 9$)	Non-COPD ($n = 4$)	P-value
[1-^{14}C] Pyruvate+malate (nmol/h × mU CS^{-1})	2.87±0.35	2.75±0.61	0.86
ATP production (nmol/h × mU CS^{-1})	29.05±4.92	24.28±4.82	0.51
ATP/pyruvate	9.70±0.43	9.00±0.56	0.34
ADP stimulation	4.64±0.24	4.04±0.34	0.17
C-I (mU/U CS)	100±9	81±9	0.18
C-III (mU/U CS)	2749±163	2787±168	0.88
SCC (mU/U CS)	577±54	554±28	0.78
C-IV (mU/U CS)	2162±187	1890±149	0.33
CS (mU/ml)	152±11	136±44	0.47

Values are means±SEM. ADP, adenosine diphosphate; ATP adenosine triphosphate; ATP/pyruvate, ratio between ATP production and [1-^{14}C] Pyruvate+malate; ADP stimulation, ADP stimulation of the pyruvate oxidation; C-I, complex I; C-III, cytochrome b-c_1 complex; SCC, succinate cytochrome *c* oxidoreductase; C-IV, cytochrome *c* oxidase (COX); CS, citrate synthase.

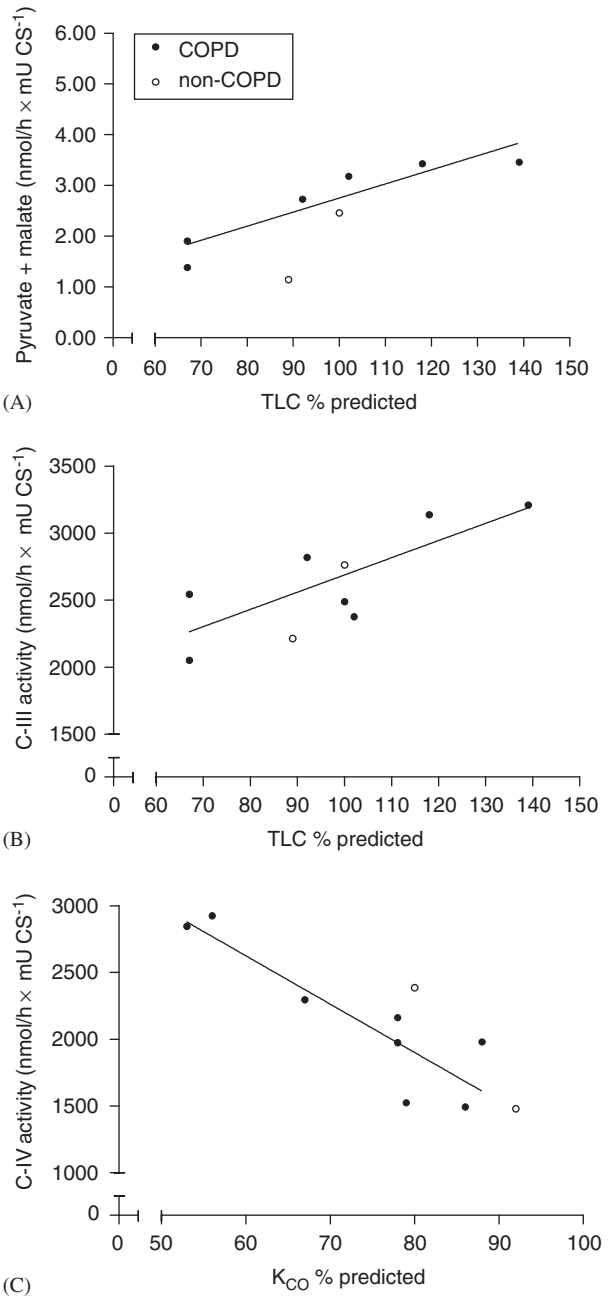

Figure 2 Relationship between several pulmonary function parameters and mitochondrial parameters. A significant relationship was found between TLC % predicted and pyruvate+malate+ADP ($r = 0.92$, $P = 0.01$) (A), TLC % predicted and C_{III} ($r = 0.80$, $P = 0.03$) (B) and between K_{CO} % predicted and C_{IV} ($r = -0.94$, $P < 0.01$) (C).

HADH activity, phosphorylase activity, MHC composition and mitochondrial energy generating capacity, but with increasing airflow obstruction (1) the activity of HADH was increased, (2) the activity of phosphorylase was not changed and (3) the activities of C-III and C-IV of the mitochondrial respiratory chain were increased, as well as the oxidation of pyruvate in the presence of malate. The distribution of MHC isoforms was not different, but tended to shift towards the oxidative isoforms in COPD diaphragm.

Several metabolic changes can occur in the diaphragm of COPD patients. First, due to overloading, a compensatory mitochondrial energy generating mechanism in energy production from the mitochondrial respiratory chain can be induced. Ribera et al.[9] demonstrated that mitochondrial oxidative capacity in the diaphragm of COPD patients is increased. They found an increase in the maximal respiration of mitochondria and an improvement in the coupling of oxidation to phosphorylation in the diaphragm of COPD patients. The latter finding suggests an increased efficiency of mitochondrial ATP production.[9] In the present study we did not find such an increased efficiency, as reflected by the ATP/pyruvate ratio and ADP stimulation ratio. This discrepancy in the findings could be due to the fact that the patients in the study of Ribera et al.[9] had more severe airflow obstruction, namely FEV_1 64% of predicted in our study versus FEV_1 33% of predicted in the study of Ribera et al.[9]

In contrast to the unchanged mitochondrial efficiency, we did find an increase in C-III and C-IV activity and an increase in [1-^{14}C] pyruvate+malate oxidation rate with increasing severity of airflow obstruction, suggesting an adaptive response to increased loading. Sauleda et al.[20] found an increase of C-IV activity in the quadriceps femoris of COPD patients, which was inversely related to the arterial PO_2 value. We postulate that the increase in C-IV activity (and perhaps also other parts of the mitochondrial respiratory chain) might be related to chronic hypoxemia, which could affect the diaphragm. The increase in [1-^{14}C] pyruvate+malate oxidation suggests an increase in the function of the whole mitochondrial energy generating capacity in diaphragm of COPD patients. The finding that there was no difference in mitochondrial function in direct comparison between COPD and non-COPD diaphragm could be due to the low number of patients.

A second metabolic alteration which can occur in the diaphragm of COPD patients, is an increase in oxidative enzyme activity. Indeed, we found in this study an increase in HADH activity with increasing severity of COPD. Levine et al.[3,21] reported an increase in activity of the mitochondrial enzyme SDH in patients with COPD. Tikunov et al.[22] found increased activities of HADH and CS in patients with chronic heart failure, a condition which elicits changes in the diaphragm which are comparable with the changes found in COPD.

A third alteration which can occur is a decrease in glycogenolytic enzyme activity, due to a shift

towards less anaerobic and more oxidative fibers occurring in COPD.[1] However, in this study we did not find a change in glycogenolytic enzyme activity in COPD. After exercise training, Noble and Ianuzzo[7] also found no significant changes in phosphorylase activity in skeletal muscle of healthy rats. With the exception of hexokinase, which behaves similarly to the majority of mitochondrial enzymes, most glycolytic enzymes do not change markedly in response to endurance exercise in animals. In slow-twitch muscle, endurance training may under certain conditions result in a slightly increased glycolytic potential.[23] Endurance training does not alter diaphragmatic glycolytic capacity.[24,25] Baldwin et al.[23] and Gollnick et al.[26] found that endurance training appears to result in rather minor changes in glycolytic enzyme activity in skeletal muscle. These observations are in accordance with the results found in the present study. However, Sanchez et al.[8] reported a decrease of glycolytic enzyme activity (LDH and hexokinase) in the diaphragm of moderate COPD patients. The latter study did not show an increase in oxidative enzyme activity in the diaphragm of COPD patients, possibly because the load on the diaphragm in their patients was different from ours. Besides, the low number of patients might have contributed to the fact that we found no significant difference in glygenocolytic activity.

We found no relationship between COPD and MHC type I content in the diaphragm. This is in contrast to the findings by Levine et al.[1] and Mercadier et al.[2] who found an increase in percentage type I MHC in COPD patients compared to controls. In our study the COPD group showed a tendency to an increase in percentage types I and IIa MHC and a decrease in % type IIx MHC compared to the non-COPD group. Possible explanations for this discrepancy could be less severe airflow obstruction in our patients, namely FEV_1 64% of predicted in our study versus FEV_1 33% of predicted in the study of Levine et al.[1]

In conclusion, this study shows that with increasing severity of COPD (1) the activity of oxidative enzymes is increased, (2) the glycogenolytic activity is not changed and (3) the mitochondrial capacity is increased. The distribution of MHC isoforms tended to shift towards the oxidative isoforms in COPD diaphragm. These alterations may help the diaphragm dealing with a higher workload associated with increased airflow limitation.

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References

1. Levine S, Kaiser L, Leferovich J, Tikunov B. Cellular adaptations in the diaphragm in chronic obstructive pulmonary disease. *N Engl J Med* 1997;**337**:1799–806.
2. Mercadier JJ, Schwartz K, Schiaffino S, Wisnewsky C, Ausoni S, Heimburger M, et al. Myosin heavy chain gene expression changes in the diaphragm of patients with chronic lung hyperinflation. *Am J Physiol Lung Cell Mol Physiol* 1998; **274**(4):L527–34.
3. Levine S, Gregory C, Nguyen T, Shrager J, Kaiser L, Rubinstein N, et al. Bioenergetic adaptation of individual human diaphragmatic myofibers to severe COPD. *J Appl Physiol* 2002;**92**(3):1205–13.
4. Proctor DN, Sinning WE, Walro JM, Sieck GC, Lemon PW. Oxidative capacity of human muscle fiber types: effects of age and training status. *J Appl Physiol* 1995;**78**(6): 2033–8.
5. Puente-Maestu L, Tena T, Trascasa C, Perez-Parra J, Godoy R, Garcia MJ, et al. Training improves muscle oxidative capacity and oxygenation recovery kinetics in patients with chronic obstructive pulmonary disease. *Eur J Appl Physiol* 2003;**88**(6):580–7.
6. Green HJ, Jones S, Ball-Burnett M, Farrance B, Ranney D. Adaptations in muscle metabolism to prolonged voluntary exercise and training. *J Appl Physiol* 1995;**78**(1):138–45.
7. Noble EG, Ianuzzo CD. Influence of training on skeletal muscle enzymatic adaptations in normal and diabetic rats. *Am J Physiol* 1985;**249**(4 Part 1):E360–5.
8. Sanchez J, Bastien C, Medrano G, Riquet M, Derenne JP. Metabolic enzymatic activities in the diaphragm of normal men and patients with moderate chronic obstructive pulmonary disease. *Bull Eur Physiopathol Respir* 1984;**20**(6): 535–40.
9. Ribera F, N'Guessan B, Zoll J, Fortin D, Serrurier B, Mettauer B, et al. Mitochondrial electron transport chain function is enhanced in inspiratory muscles of COPD patients. *Am J Respir Crit Care Med* 2002.
10. Orozco-Levi M, Gea J, Lloreta JL, Felez M, Minguella J, Serrano S, et al. Subcellular adaptation of the human diaphragm in chronic obstructive pulmonary disease. *Eur Respir J* 1999;**13**(2):371–8.
11. Gollnick PD, Armstrong RB, Saltin B, Saubert CW, Sembrowich WL, Shepherd RE. Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J Appl Physiol* 1973;**34**(1):107–11.
12. van Balkom RH, van der Heijden HF, van Moerkerk HT, Veerkamp JH, Fransen JA, Ginsel LA, et al. Effects of different treatment regimens of methylprednisolone on rat diaphragm contractility, immunohistochemistry and biochemistry. *Eur Respir J* 1996;**9**(6):1217–23.
13. Fischer JC, Ruitenbeek W, Stadhouders AM, Trijbels JM, Sengers RC, Janssen AJ, et al. Investigation of mitochondrial metabolism in small human skeletal muscle biopsy specimens. Improvement of preparation procedure. *Clin Chim Acta* 1985;**145**(1):89–99.
14. Srere PA. Citrate synthase, EC 4.1.3.7, citrate oxaloacetate-lyase (CoA-acetylating). Lowenstein JM, editor. *Methods in*

Enzymology, vol. XIII. London: Academic Press; 1969. p. 3–11.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**(1):265–75.
16. Fischer JC, Ruitenbeek W, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, et al. Estimation of NADH oxidation in human skeletal muscle mitochondria. *Clin Chim Acta* 1986;**155**(3):263–73.
17. Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders AM, et al. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. *Clin Chim Acta* 1985;**153**(1):23–36.
18. Bentlage HA, Wendel U, Schagger H, ter Laak HJ, Janssen AJ, Trijbels JM. Lethal infantile mitochondrial disease with isolated complex I deficiency in fibroblasts but with combined complex I and IV deficiencies in muscle. *Neurology* 1996;**47**(1):243–8.
19. Cooperstein SJ, Lazarow A. A microspectrophotometric method for the determination of cytochrome oxidase. *J Biol Chem* 1951;**189**(2):665–70.
20. Sauleda J, Garcia-Palmer F, Wiesner RJ, Tarraga S, Harting I, Tomas P, et al. Cytochrome oxidase activity and mitochondrial gene expression in skeletal muscle of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;**157**(5 Part 1):1413–7.
21. Levine S, Nguyen T, Shrager J, Kaiser L, Camasamudram V, Rubinstein N. Diaphragm adaptations elicited by severe chronic obstructive pulmonary disease: lessons for sports science. *Exerc Sport Sci Rev* 2001;**29**(2):71–5.
22. Tikunov B, Levine S, Mancini D. Chronic congestive heart failure elicits adaptations of endurance exercise in diaphragmatic muscle. *Circulation* 1997;**95**(4):910–6.
23. Baldwin KM, Winder WW, Terjung RL, Holloszy JO. Glycolytic enzymes in different types of skeletal muscle: adaptation to exercise. *Am J Physiol* 1973;**225**(4):962–6.
24. Moore RL, Gollnick PD. Response of ventilatory muscles of the rat to endurance training. *Pflugers Arch* 1982;**392**(3): 268–71.
25. Powers SK, Criswell D, Lieu FK, Dodd S, Silverman H. Diaphragmatic fiber type specific adaptation to endurance exercise. *Respir Physiol* 1992;**89**(2):195–207.
26. Gollnick PD, Armstrong RB, Saubert CW, Piehl K, Saltin B. Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *J Appl Physiol* 1972;**33**(3): 312–9.