Research Paper

Mitochondrial dysfunction and therapeutic approaches in respiratory and limb muscles of cancer cachectic mice

Clara Fermoselle[1,2], Elena García-Arumí[3,4], Ester Puig-Vilanova[1,2], Antoni L. Andreu[3,4], Alejandro J. Urtreger[5], Elisa D. Bal de Kier Joffé[5], Alberto Tejedor[6], Luís Puente-Maestu[7] and Esther Barreiro[1,2]

[1] *Pulmonology Department, Lung Cancer Group, IMIM-Hospital del Mar, Health and Experimental Sciences Department, Universitat Pompeu Fabra, Barcelona Biomedical Research Park (PRBB), Barcelona, Spain*

[2] *Centro de Investigación en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), Bunyola, Majorca, Balearic Islands, Spain*

[3] *Unitat de Patologia Neuromuscular i Mitocondrial, Hospital Universitari Vall d'Hebron Institut de Recerca (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain*

[4] *Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Barcelona, Spain*

[5] *Research Area, Institute of Oncology 'Angel H. Roffo', University of Buenos Aires, Buenos Aires, Argentina*

[6] *Servicio de Nefrología and*

[7] *Servicio de Neumología, Hospital General Gregorio Marañón, Universidad Complutense de Madrid, Madrid, Spain*

New Findings

- **What is the central question of this study?**
  We explored whether experimental cancer-induced cachexia may alter mitochondrial respiratory chain (MRC) complexes and oxygen uptake in respiratory and peripheral muscles, and whether signalling pathways, proteasome and oxidative stress influence that process.
- **What is the main finding and what is its importance?**
  In cancer cachectic mice, MRC complexes and oxygen consumption were decreased in the diaphragm and gastrocnemius. Blockade of nuclear factor-κB and mitogen-activated protein kinase actions partly restored the muscle mass and force and corrected the MRC dysfunction, while concomitantly reducing tumour burden. Antioxidants improved mitochondrial oxygen consumption without eliciting effects on the loss of muscle mass and force or the tumour size, whereas bortezomib reduced tumour burden without influencing muscle mass and strength or MRC function.

Abnormalities in mitochondrial content, morphology and function have been reported in several muscle-wasting conditions. We specifically explored whether experimental cancer-induced cachexia may alter mitochondrial respiratory chain (MRC) complexes and oxygen uptake in respiratory and peripheral muscles, and whether signalling pathways, proteasomes and oxidative stress may influence that process. We evaluated complex I, II and IV enzyme activities (specific activity assays) and MRC oxygen consumption (polarographic measurements) in diaphragm and gastrocnemius of cachectic mice bearing the LP07 lung tumour, with and without treatment with *N*-acetylcysteine, bortezomib and nuclear factor-κB (sulfasalazine) and mitogen-activated protein kinases (MAPK, U0126) inhibitors ($n = 10$ per group for all groups). Whole-body and muscle weights and limb muscle force were also assessed in all rodents at baseline and after 1 month. Compared with control animals, cancer cachectic mice showed a significant reduction in body weight gain, smaller sizes of the diaphragm and gastrocnemius, lower muscle strength, decreased activity of complexes I, II and IV and decreased oxygen consumption in both muscles. Blockade of nuclear factor-κB and MAPK actions restored muscle mass and force and corrected the MRC dysfunction in both muscles, while partly reducing tumour burden.

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Experimental Physiology

Antioxidants improved mitochondrial oxygen uptake without eliciting significant effects on the loss of muscle mass and force or tumour size, whereas the proteasome inhibitor reduced tumour burden without significantly influencing muscle mass and strength or mitochondrial function. In conclusion, nuclear factor-κB and MAPK signalling pathways modulate muscle mass and performance and MRC function of respiratory and limb muscles in this model of experimental cancer cachexia, thus offering targets for therapeutic intervention.

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Corresponding author E. Barreiro: URMAR, IMIM-Hospital del Mar, PRRB, C/ Dr. Aiguader, 88, E-08003 Barcelona, Spain. Email: ebarreiro@imim.es

Introduction

Muscle wasting and cachexia are common systemic manifestations of acute and chronic conditions including sepsis, respiratory disease, cardiovascular disease and cancer, especially those of the lung and gastrointestinal tract. Although the prevalence of cachexia may vary widely, in advanced malignancies it may range from 60 to 80%, thus having a great impact on the patient's quality of life.

Several molecular and cellular mechanisms have been proposed to contribute to the aetiology of cancer-induced cachexia, such as inflammation (Argiles *et al.* 2011), oxidative stress (Barreiro *et al.* 2005*a*, 2009; Marin-Corral *et al.* 2010), insulin resistance and other metabolic disturbances (Evans *et al.* 2008). The mitochondria play a major role in meeting the cellular requirements of energy through oxidative phosphorylation of different substrates. Additionally, mitochondrial content and function are important modulators of physiological muscle mass maintenance and loss in disease. Abnormalities in muscle mitochondrial content, morphology and function have been reported in several muscle-wasting conditions, including limb muscles of chronic obstructive pulmonary disease (COPD) patients (Gosker *et al.* 2007; Puente-Maestu *et al.* 2009*a*,*b*; Naimi *et al.* 2011; Puente-Maestu *et al.* 2012), skeletal muscle in cardiac cachexia (De Sousa *et al.* 2000), the diaphragm in mechanical ventilation (Kavazis *et al.* 2009) and limb muscles in interleukin-6-induced cachexia (White *et al.* 2011, 2012) and cancer-induced cachexia (Constantinou *et al.* 2011; Fontes-Oliveira *et al.* 2012). However, whether the mitochondrial respiratory chain (MRC) may also be specifically affected in skeletal muscles of cancer cachexia models, as shown to occur in other conditions (Puente-Maestu *et al.* 2009*a*, 2012), remains to be fully elucidated. In this regard, despite the relevance of mitochondrial oxidative metabolism as a source of energy production, knowledge about the potential alterations of the muscle MRC in cancer-induced cachexia is still in its infancy.

Although reactive oxygen species (ROS) synthesized within the mitochondria may participate in different adaptive responses, MRC dysfunction also leads to enhanced ROS production in skeletal muscles, which probably contributes to oxidative stress and impaired muscle function in COPD patients (Puente-Maestu *et al.* 2009*a*, 2012), and in the respiratory muscles of rats exposed to mechanical ventilation (Kavazis *et al.* 2009).

Mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) are central regulators of gene expression, redox balance and metabolism. Importantly, these molecules also play a relevant role in oxidative stress-mediated muscle wasting and atrophy in several models of cachexia (Smith & Tisdale, 2003; McClung *et al.* 2010) and in tumour cell growth (Karin, 2006; Galli *et al.* 2008). Moreover, NF-κB was also shown to enhance mitochondrial ROS production, leading to mitochondrial and cardiac dysfunction in diabetic mice (Mariappan *et al.* 2010). The MAPK signalling pathway was also shown to mediate increased oxidative stress markers, such as protein and lipid oxidation, in response to mitochondrial uncoupling in mouse skeletal muscles (Keipert *et al.* 2013).

The ubiquitin–proteasome system plays a major role in conditions characterized by muscle wasting and weakness (Van Surell *et al.* 1992; van Hees *et al.* 2008, 2011; Supinski *et al.* 2009; Fermoselle *et al.* 2011, 2012). Bortezomib, the first proteasome inhibitor used in patients, was shown to restore muscle function and mass in several models (van Hees *et al.* 2008, 2011), while inducing no beneficial effects in sepsis-induced cachexia (Supinski *et al.* 2009). Bortezomib also exerted important antitumour activities in cancer cell lines via oxidative stress-mediated apoptosis (Ling *et al.* 2003). The antioxidant *N*-acetyl cysteine (NAC) was shown to scavenge ROS directly in skeletal muscle fibres by targeting different cellular structures, including mitochondrial proteins (Shindoh *et al.* 1990; Reilly *et al.* 1991; Van Surell *et al.* 1992). Importantly, NAC was also shown to interfere with MAPK activation in response to hypoxia in cardiomyocytes (Van Surell *et al.* 1992; Kulisz *et al.* 2002). Despite this knowledge, it remains to be elucidated whether molecular mechanisms, such as MAPK and NF-κB signalling pathways, oxidative stress and the proteasome system, may modulate the function of the MRC in cancer cachectic muscles. Importantly, investigations conducted so far have focused exclusively on the analysis of limb muscles. Whether alterations in MRC may take place in cachectic respiratory muscles remains unknown.

On this basis, the rationale to conduct the present investigation was to explore specifically whether experimental cancer-induced cachexia may alter MRC complexes and oxygen uptake in both respiratory and peripheral muscles, and to what extent several cellular signalling pathways and oxidative stress may be involved in that process. Accordingly, our main objectives were to explore specifically the function of complexes I, II and IV of the MRC and oxygen consumption in the diaphragm and gastrocnemius muscles of mice bearing the LP07 lung tumour. Furthermore, rodents concomitantly received treatment with selective inhibitors of MAPK, NF-κB and the proteasome, or the antioxidant NAC in order to assess its specific contribution to MRC dysfunction, thus possibly entailing therapeutic value in cachexia. Whole-body and muscle weights and limb muscle force were also assessed in rodents from all groups.

Methods

Ethical approval

All animal experiments were conducted in the animal facilities at Parc de Recerca Biomèdica de Barcelona (PRBB, Spain). This controlled study was designed in accordance with the ethical standards on animal experimentation (EU 609/86 CEE, Real Decreto 1201/05 BOE 252, Spain) at PRBB and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). Ethical approval was obtained by the Animal Research Committee at PRBB and the Catalan Government (Animal welfare department, number of procedure EBP-09-1228AE).

Animal experiments

Tumour. The LP07 cell line is derived from the transplantable P07 lung tumour that appeared spontaneously in the lung of a BALB/c mouse (Diament *et al.* 1998). The LP07 cell line was obtained *in vitro*, after successive passages of a P07 primary culture (Urtreger *et al.* 2001). The LP07 cell line shares identical characteristics regarding lung tumour incidence, tumour histology and cachexia with its parental P07 tumour (Diament *et al.* 1998, 2006; Urtreger *et al.* 2001). It was also consistently demonstrated that 1 month after tumour transplantation, all animals developed lung metastasis, spleen enlargement and cachexia without affecting any other organs (Diament *et al.* 1998, 2006; Urtreger *et al.* 2001).

Experimental groups. Female BALB/c mice, 2 months old (weight ∼20 g), were obtained from Harlan Interfauna Ibérica SL (Barcelona, Spain). In all experimental groups (except for control rodents), LP07 viable cells (4×10^5), resuspended in 0.2 ml minimal essential medium, were subcutaneously inoculated into the left flank of the mice (day 1). All groups ($n = 10$ per group) were studied for a period of 1month. Significant differences in food intake were not observed between cachectic and control mice in an initial pilot study; therefore, no specific pair-fed group was used in the investigation.

Animals were randomly assigned to the following groups: (i) control, inoculation of 0.2 ml minimal essential medium in the left flank; (ii) lung cancer (LC) cachexia group, inoculation of LP07 cells; (iii) LC cachectic mice receiving concomitant treatment with the antioxidant NAC (kindly provided by Dr X. Mateu, Hospital del Mar, Barcelona, Spain), 3 mmol kg^{-1} (24 h)$^{-1}$, administered orally using a 22 gauge, 25 mm needle (gavage, LC cachectic-NAC group); (iv) LC cachectic mice receiving concomitant treatment with the proteasome inhibitor bortezomib (Velcade; Millenium Pharmaceuticals, Cambridge, MA, USA), 0.15 mg kg^{-1}, 0.1 ml every 6 days, I.V. injection into the tail vein (LC cachectic-bortezomib group; Lu *et al.* 2006); (v) LC cachectic mice receiving concomitant treatment with sulfasalazine (Pfizer, Madrid, Spain), 200 mg kg^{-1}, 0.3 ml every 48 h, I.P. (LC cachectic-NF-κB inhibitor group; Olmez *et al.* 2008); and (vi) LC cachectic mice receiving concomitant treatment with the MAPK inhibitor U0126 (a highly selective inhibitor of extracellular signal-regulated kinases (ERK) 1 and 2 proteins; Selleck chemicals, Houston, TX, USA), 30 mg kg^{-1}, 0.1 ml every 48 h, I.P. (LC cachectic-MAPK inhibitor group; Schuh & Pahl, 2009). All pharmacological therapies were administered from day 15 after the inoculation of the LP07 cells until the end of the study period on day 30.

In vivo measurements and sample collection from mice

Body weight and food intake were determined every day during the entire duration of the study. Limb strength was determined on days 0 and 30 using a strength grip meter (Bioseb, Chaville, France) following previously published methodologies (Barreiro *et al.* 2010). In the LC cachexia group of mice, tumour progression was determined using positron emission tomography on days 13 and 20. Mice from all the experimental groups were always killed on day 30 postinoculation of LP07 cells or minimal essential medium (control animals). Before being killed, all mice were anaesthetized with 0.1 ml sodium pentobarbital (60 mg kg^{-1} I.P.). Diaphragm and gastrocnemius muscles and the subcutaneous tumour were harvested from all animals. The weight of both muscles and tumour were determined in each animal using a high-precision scale. For the enzyme activity assays, one half of the diaphragm and the entire gastrocnemius of one of the hindlimbs were immediately frozen and

stored at −80°C for further analyses in all animals. In order to assess oxygen consumption using polarographic studies, the second half of the diaphragm and the entire gastrocnemius of the second hindlimb were immediately placed in cold isolation buffer to perform the *ex vivo* measurements (Puente-Maestu *et al.* 2009*a*, 2012; Naimi *et al.* 2011).

Homogenization procedures

Diaphragm and gastrocnemius muscles were obtained from all mice and snap-frozen to be stored subsequently at −80°C until further use. Muscle homogenization was always performed at 0–5°C. Samples were homogenized using a Potter S Homogenizer (Sartorius Stedim Biotech GmbH, Goettingen, Germany). In general, 50 mg of whole muscle tissue was placed into the homogenizer containing 9 volumes (w/v) mannitol buffer pH 7.2 (225 mM mannitol, 75 mM sucrose, 10 mM Tris–HCl and 0.1 mM EDTA), at 1000 r.p.m., while three strokes up and down were performed. Samples were then transferred into a new tube and centrifuged at 650*g* for 20 min. After centrifugation, the supernatants were collected and placed into new tubes, while the pellets were resuspended with the initial mannitol buffer volume, pH 7.2. The homogenization procedure was repeated again with the resuspended pellets. The supernatants obtained from the second centrifugation were added to the first ones, thus yielding the final sample supernatants. Protein concentrations were measured using the Bradford method (Bradford, 1976).

Mitochondrial citrate synthase (CS) activity

The procedures employed to determine CS activity were previously published (Kavazis *et al.* 2009; Medja *et al.* 2009). Citrate synthase activity was used as a marker of mitochondrial content or density as commonly described (Kavazis *et al.* 2009; Medja *et al.* 2009). Citrate synthase catalyses the acetyl-coenzyme, which is an oxaloacetate reaction resulting in citrate and coenzyme A. The latter compound can be measured using 5,5′-dithiobis-(2-nitrobenzoic acid). The reaction is monitored at 412 nm. The specific activity of CS enzyme was expressed as nanomoles per minute per milligram of protein. Intra- and interassay coefficients of variation were 5.07 and 2.7%, respectively.

Mitochondrial respiratory chain enzyme activities

Mitochondrial complex I activity. All procedures employed in the present investigation have been published previously (Medja *et al.* 2009). Enzymatic determination of NADH ubiquinone oxidoreductase rotenone sensitive is based on the oxidation rate of NADH in the presence of rotenone. It monitors the decrease in absorbance at 340 nm in the presence and absence of rotenone. The rotenone-resistant activity was subtracted from the total activity of NADH ubiquinone oxidoreductase to obtain the activity sensitive to rotenone. The specific activity of complex I was expressed as nanomoles per minute per milligram of protein. Intra- and interassay coefficients of variation were 8.93 and 7.84%, respectively.

Mitochondrial complex II activity. All procedures used for measuring this complex have been published previously (Medja *et al.* 2009). Succinate decylubiquinone reductase activity was measured using 2,6-dichlorophenolindophenol as the electron acceptor. The reaction was monitored at 600 nm. The specific activity of complex II was expressed as nanomoles per minute per milligram of protein. Intra- and interassay coefficients of variation were 7.81 and 5.18%, respectively.

Mitochondrial complex IV activity. All procedures have been published previously (Medja *et al.* 2009). Complex IV activity was measured using reduced cytochrome *c* as the substrate. Oxidation of cytochrome *c* was monitored at 550 nm. The specific activity of complex IV was expressed as nanomoles per minute per milligram of protein. Intra- and interassay coefficients of variation were 4.13 and 4.03%, respectively.

Mitochondrial oxygen consumption

The methodologies employed have already been published (Puente-Maestu *et al.* 2009*a*, 2012; Naimi *et al.* 2011). The gastrocnemius and diaphragm muscles were obtained from mice and rapidly placed into a cold tube containing isolation buffer (on ice). Muscle samples, approximately 40–80 mg total weight, were placed into a potter glass (Potter S Homogenizer; Sartorius Stedim Biotech GmbH) containing 4 volumes (w/v) of isolation buffer. Muscle specimens were homogenized at 500 r.p.m., applying three strokes with 30 s periods between each stroke in order to avoid sample heating. Homogenates were centrifuged at 1.1*g* for 10 min. Pellets were discarded and supernatants centrifuged at 8.8*g* for another 10 min. Supernatants were discarded and pellets softly resuspended using a finger rod in 1 ml isolation buffer, subsequently to be centrifuged at 8.8*g* for 10 min. Supernatants were again discarded and pellets resuspended, using the finger rod, in 50 μl measurement buffer. The mitochondrial concentration was analysed using Bradford procedures (Bradford, 1976). The whole process of mitochondrial isolation was performed at 4°C. Materials and small equipment were washed with EGTA to inhibit reverse calcium release from uncoupled mitochondria. Furthermore, for the same purpose, the isolation buffer [300 mM mannitol, 1 mM EGTA, 10 mM Tris(hydroxymethyl)aminomethane

hydrochloride-HCl (Trizma-HCl), 1 mM KH_2PO_4, 1.74 mg ml^{-1} phenylmethanesulfonyl fluoride, 0.2% bovine serum albumin and 10 mg l^{-1} amoxicillin, pH 7.4] and a part of the measurement buffer (300 mM mannitol, 10 mM Trizma-HCl and 1 mM KH_2PO_4, pH 7.4) were continuously gassed with nitrogen, which displaces molecular oxygen (O_2) and keeps mitochondria in reducing conditions (avoiding respiration).

Mitochondrial oxygen consumption was measured using a Clark-type electrode, placed in an RC 650 six-cell respirometer attached to a 929 six-channel respirometer system (Strathkelvin Instruments Ltd, Motherwell, UK). The whole system was maintained at 37°C during the entire duration of the protocol. The isolated mitochondria and substrates were introduced into the chambers through a capillary hole in the plunger using a graduated syringe (Hamilton, Bonaduz, Switzerland). In order to quantify complex I (NADH ubiquinone oxidoreductase) oxygen consumption, 0.9 ml measurement buffer containing 5 mM pyruvate, 5 mM malate and the mitochondrial yields were added to the chambers (at this point, the measured rates correspond to State 4 respiration). In order to determine State 3 respiration, 2 mM ADP was added to the chamber. Subsequently, 0.7 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and 7 mM ascorbate were added to the chambers in order to quantify complex IV (cytochrome *c* oxidase) oxygen consumption. The oxygen consumption was expressed as nanomoles of O_2 per minute per microgram of mitochondria. Intra- and interassay coefficients of variation were 8.77 and 8.72%, respectively, for complex I oxygen consumption, and 9.75 and 9.96%, respectively, for complex IV consumption.

In order to verify mitochondrial integrity, NADH was added to the buffer containing the sample, ADP, pyruvate and malate. Oxygen consumption did not increase after the addition of NADH to the working buffer, meaning that mitochondrial integrity was ensured and that MRC was not uncoupled.

Statistical analysis

Results are presented as means (SD). Comparisons of physiological and biological variables among the different study groups were made using one-way ANOVA. For the purpose of the study, two different sets of comparisons were made, as follows: (i) control and LC cachectic mice; and, in contrast, (ii) LC cachectic and LC cachectic-NAC; (iii) LC cachectic and LC cachectic-bortezomib; (iv) LC cachectic and LC cachectic-NF-κB inhibitor; and (v) LC cachectic and LC cachectic-MAPK inhibitor. Dunnett's (two-sided) *post hoc* analysis was used to adjust for multiple comparisons. Statistical significance resulting from these two different sets of comparisons is indicated in the figures and tables. Correlations between physiological and biological variables were explored using Pearson's correlation coefficient. The sample size chosen was based on previous studies, where very similar approaches were employed (Barreiro *et al.* 2005*a*; Marin-Corral *et al.* 2009, 2010; Puente-Maestu *et al.* 2009*a*, 2012; Constantinou *et al.* 2011; Naimi *et al.* 2011; White *et al.* 2011, 2012; Busquets *et al.* 2012; Fermoselle *et al.* 2012; Fontes-Oliveira *et al.* 2012), and on assumptions of 80% power to detect an improvement of >20% in measured outcomes at a level of significance of $P \leq 0.05$.

Results

Physiological characteristics

As shown in Table 1, at the end of the study period, LC cachectic mice exhibited a reduction in body weight that was not observed in control animals. Food intake was similar among the study groups (3 g (24 h)$^{-1}$). Diaphragm and gastrocnemius muscle weights and limb strength were significantly reduced in the LC cachectic mice compared with control rodents, which also showed a significant gain in muscle force (Table 1). The LC cachectic mice treated with the NF-κB and MAPK inhibitors exhibited a significantly smaller reduction in weight gain, a significant improvement in diaphragm and gastrocnemius weights and a significant recovery of muscle strength gain compared with the untreated cachectic animals (Table 1). The proteasome inhibitor or the antioxidant NAC did not induce any significant effects on body or muscle weights or limb muscle strength in the LC cachectic mice (Table 1). Compared with untreated tumour-bearing animals, the weight of the subcutaneous tumour was significantly reduced in the cachectic rodents treated with the inhibitors of the proteasome (36%), NF-κB (29%) and MAPK (50%) pathways, but not NAC (Table 1). Among cachectic rodents, significant correlations were found between body weight gain and limb strength gain ($r = 0.428$, $P = 0.005$), as well as with either diaphragm or gastrocnemius weights ($r = 0.505$, $P = 0.001$, and $r = 0.689$, $P < 0.001$, respectively). In addition, limb strength gain was significantly correlated with gastrocnemius weight ($r = 0.527$, $P < 0.001$) in the same animals.

Citrate synthase enzyme activity

In either diaphragm or gastrocnemius muscles, no significant differences were observed in CS enzyme activity between LC cachectic mice and the control animals (Fig. 1). The CS activity levels were significantly increased in the diaphragms of the LC cachectic mice treated only with NF-κB inhibitor compared with the respiratory muscles of the untreated LC cachectic rodents (Fig. 1). Citrate synthase enzyme activity levels were significantly decreased in the gastrocnemius of LC cachectic mice

Table 1. Physiological characteristics in all groups of animals at the end of the study period

			Lung cancer cachexia			
Characteristic	Control	Lung cancer cachexia	Antioxidant	Proteasome inhibitor	Nuclear factor-κB inhibitor	MAPK inhibitor
Body weight gain (%)	+9.5 (3.9)	−5.74 (8.61)†††	−10.03 (11.9)	−9.72 (9.81)	−0.11 (6.43)**	−0.24 (5.04)**
Diaphragm weight (g)	0.087 (0.01)	0.06 (0.01)†††	0.067 (0.01)	0.07 (0.01)	0.079 (0.01)***	0.074 (0.012)*
Gastrocnemius weight (g)	0.12 (0.008)	0.09 (0.01)†††	0.09 (0.014)	0.085 (0.01)	0.098 (0.01)**	0.098 (0.01)**
Limb strength gain (%)	+10.1 (14.6)	−9.04 (9.3)†††	−6.96 (14.4)	−12.8 (21.4)	+5.12 (13.6)***	+3.47 (12.7)***
Subcutaneous tumour weight (g)	n.a.	1.68 (0.47)†††	1.48 (0.45)	1.08 (0.6)***	1.19 (0.41)***	0.84 (0.57)***

Variables are presented as means (SD). Abbreviations: MAPK, mitogen-activated protein kinase; and n.a., not applicable. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ between any of the treated mouse groups with cachexia and animals with lung cancer cachexia only. $\dagger\dagger\dagger P \leq 0.001$ between lung cancer cachectic and control mice.

treated with NAC, bortezomib or NF-κB inhibitor compared with untreated cachectic animals (Fig. 1).

Mitochondrial respiratory chain complexes: enzyme activities

Complex I enzyme activity (NADH ubiquinone oxidoreductase activity) was significantly diminished in diaphragm and gastrocnemius of LC cachectic rodents compared with the control animals (Fig. 2*A*). Activity of this enzyme was increased in the diaphragms of the LC cachectic mice treated only with NF-κB inhibitor, while no differences among groups were observed in complex I activity in the cachectic gastrocnemius compared with the untreated cachectic rodents (Fig. 2*A*). The ratio of complex I to CS activities was significantly decreased in both muscles of LC cachectic mice

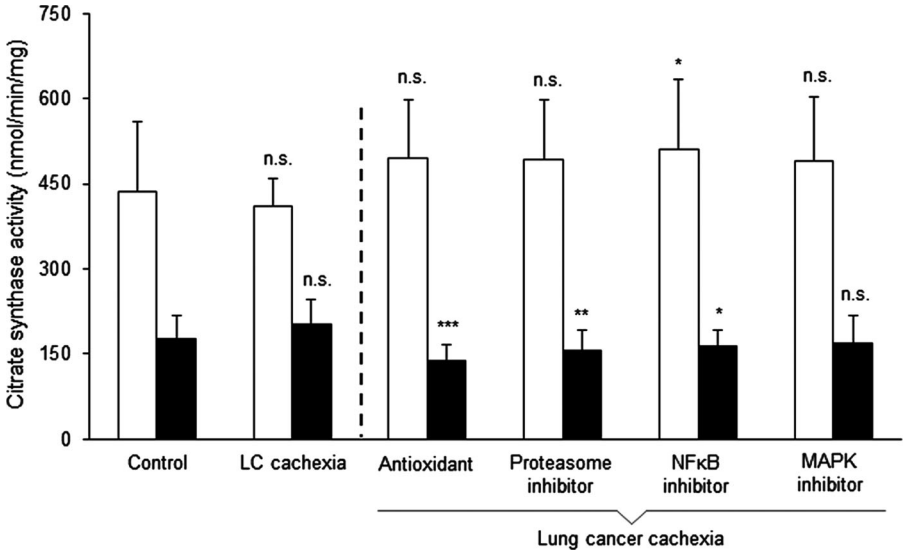


Figure 1. Citrate synthase (CS) activity (expressed as means and SD in nanomoles per minute per milligram of protein) in diaphragm (open bars) and gastrocnemius muscles (filled bars)
Citrate synthase activity levels did not differ significantly between lung cancer (LC) cachectic rodents and the control animals in any of the muscles. A significant increase in diaphragm CS activity was observed among LC cachectic rodents in response to nuclear factor-κB (NF)-κB inhibition, while *N*-acetylcysteine (NAC), bortezomib and sulfasalazine elicited a significant decline in CS activity in the gastrocnemius. Statistical significance is represented as follows: (i) n.s., non-significant differences in CS levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups.

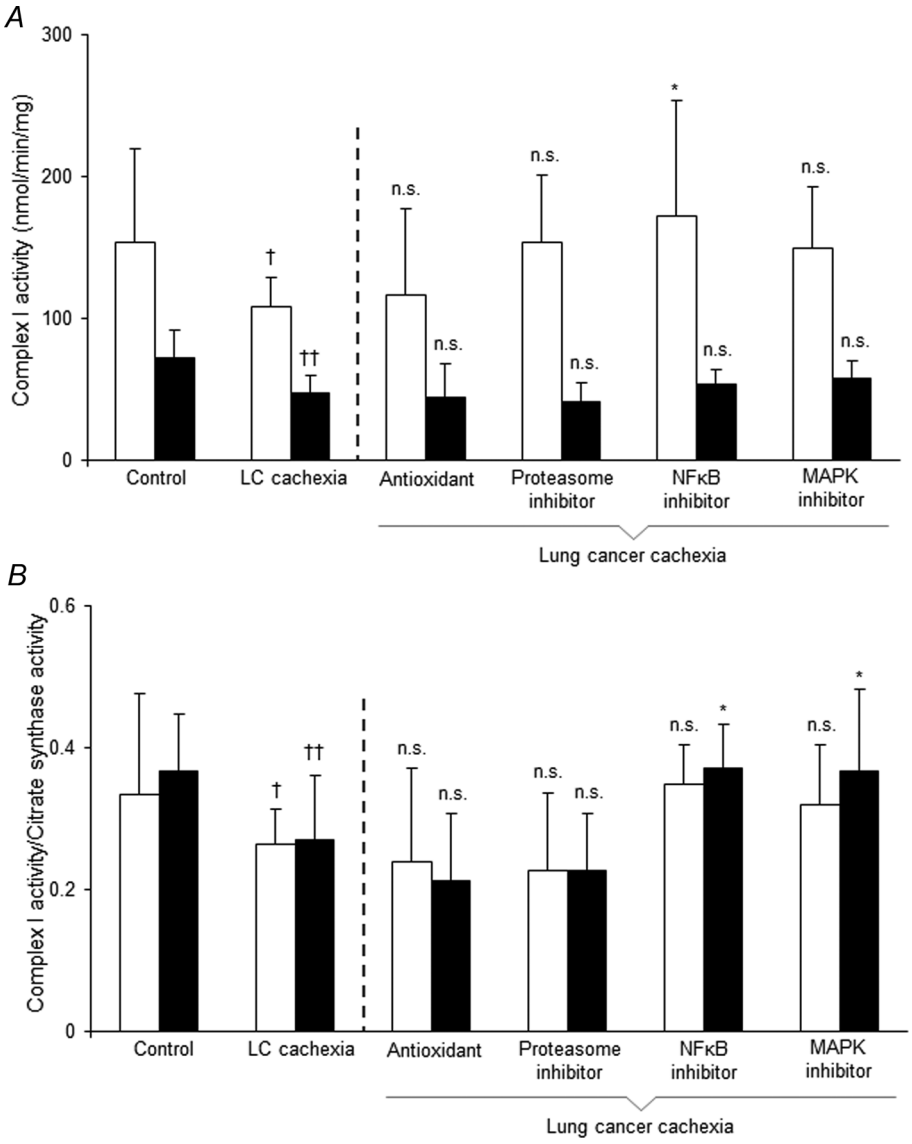


Figure 2. Levels of complex I activities in the study groups in both respiratory and limb muscles
A, mean values and SD of complex I enzyme activity (expressed as nanomoles per minute per milligram of protein) in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex I activity levels were significantly reduced in both diaphragm and gastrocnemius muscles among LC cachectic rodents compared with the control animals. A significant increase in diaphragm complex I activity was observed among LC cachectic rodents only in response to NF-κB inhibition, while no differences were observed in the gastrocnemius among any of the groups. Statistical significance is represented as follows: (i) $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ in complex I activity levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant and $^{*}P < 0.05$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups. *B*, mean values and SD of the ratio of complex I enzyme activity to CS activity in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex I/CS activity levels were significantly reduced in both diaphragm and gastrocnemius muscles among LC cachectic rodents compared with the control animals. A significant increase was observed only in gastrocnemius complex I/CS ratios among LC cachectic rodents in response to NF-κB and mitogen-activated protein kinase (MAPK) inhibitors, while NAC or bortezomib did not induce any significant effect on any of the muscles among the LC cachectic rodents. Statistical significance is represented as follows: (i) $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ in complex I/CS ratio levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant and $^{*}P < 0.05$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups.

compared with control animals (Fig. 2*B*). The ratio of complex I/CS activity was significantly improved only in the gastrocnemius of LC cachectic mice treated with either NF-κB or MAPK inhibitors, but not with the antioxidant or bortezomib, compared with untreated cachectic rodents (Fig. 2*B*). Among cachectic mice treated with sulfasalazine, a significant correlation was detected between limb strength gain and complex I activity in the gastrocnemius ($r = 0.576$, $P = 0.031$). This activity was also significantly correlated with gastrocnemius weight in cachectic rodents treated with MAPK inhibitor ($r = 0.572$, $P = 0.041$).

Complex II enzyme activity (succinate ubiquinone oxidoreductase) was decreased in both respiratory and limb muscles of LC cachectic rodents compared with the control animals (Fig. 3*A*). In the diaphragm, complex II activity was greater in LC cachectic rodents treated with NF-κB inhibitor compared with the untreated cachectic mice, but no differences were observed in the limb muscle (Fig. 3*A*). The ratio of complex II to CS was significantly reduced in both limb and respiratory muscles of the cachectic rodents compared with control animals (Fig. 3*B*). The MAPK inhibitor elicited a significant increase in that ratio only in the limb muscle of the cachectic rodents compared with untreated tumour-bearing animals (Fig. 3*B*).

Complex IV activity (cytochrome *c* oxidase) was decreased in the respiratory muscle of LC cachectic rodents compared with control animals (Fig. 4*A*). Interestingly, in the diaphragm the NF-κB and MAPK inhibitors elicited a significant improvement in complex IV activity of LC cachectic mice, but in the limb muscle no differences were observed among groups (Fig. 4*A*). The ratio of complex IV to CS activity was decreased in the diaphragm of cachectic mice compared with the control animals (Fig. 4*B*). Importantly, MAPK inhibitor elicited a significant increase in complex IV/CS in both respiratory and limb muscles of LC cachectic rodents compared with untreated cachectic animals, while the antioxidant NAC and bortezomib also induced an improvement in the limb muscle (Fig. 4*B*).

Mitochondrial respiratory chain oxygen consumption

Oxygen consumption (State 3) was decreased in both diaphragm and gastrocnemius of cachectic rodents compared with the control animals (Fig. 5*A*). Importantly, treatment with NAC or with NF-κB or MAPK inhibitors elicited a significant improvement in oxygen consumption in both respiratory and limb muscles of LC cachectic rodents compared with untreated cachectic animals (Fig. 5*A*). In State 4 respiration, no significant differences were observed between cachectic and control rodents, and the pharmacological agents did not elicit any significant effect on either muscle (Fig. 5*B*). Oxygen consumption by cytochrome *c* oxidase was decreased in both respiratory and limb muscles of LC cachectic mice compared with control animals (Fig. 5*C*). Importantly, in the cachectic rodents the oxygen consumption was increased in both types of muscles in response to concomitant treatment with NAC or MAPK inhibitor compared with untreated animals (Fig. 5*C*). No significant correlations were found between oxygen uptake and physiological variables in any of the study groups.

Discussion

Physiological and molecular results

The study results confirm to a great extent the initial hypothesis; the activities of MRC complexes are depressed in both respiratory and limb muscles in an experimental model of cancer-induced cachexia. In addition, NF-κB and MAPK signalling pathways seem to modulate in part the reduction in the activity of the complex enzymes. The significant correlations observed between complex I activity in the gastrocnemius and either limb strength gain or gastrocnemius weight in cachectic rodents treated with NF-κB and MAPK, respectively, reinforces this conclusion. In the investigation, compared with control animals, at the end of the 1 month study period the LC cachectic mice exhibited a significant reduction in total body weight gain, smaller size of their respiratory and limb muscles and lower limb muscle performance as measured by muscle strength. Interestingly, only treatment with either NF-κB or MAPK inhibitors elicited a significant improvement in body weight gain, muscle mass and performance, while simultaneously decreasing subcutaneous tumour size among the LC cachectic rodents. It should also be mentioned that as the animals were young adults, the reduction in muscle mass and body weight observed in the tumour-bearing rodents could also be the result of a decrease in muscle growth and mitochondrial biogenesis, and not due only to enhanced muscle protein breakdown. Although not specifically quantified on a daily basis, in an initial pilot study no differences in food intake were observed between control and tumour-bearing rodents. Hence, protein catabolism was likely to be a major contributing factor to reduced muscle mass in the model. Future investigations should focus on the assessment of potential differences in protein synthesis and catabolism involving muscle mass maintenance between mature and young adult animals.

Despite the finding that bortezomib induced a significant reduction in subcutaneous tumour size in the cachectic mice, body and muscle weights or force generation did not improve in these animals. These findings suggest that NF-κB and MAPK are predominant pathways in the development of loss of muscle mass and force production, while the role played by the proteasome

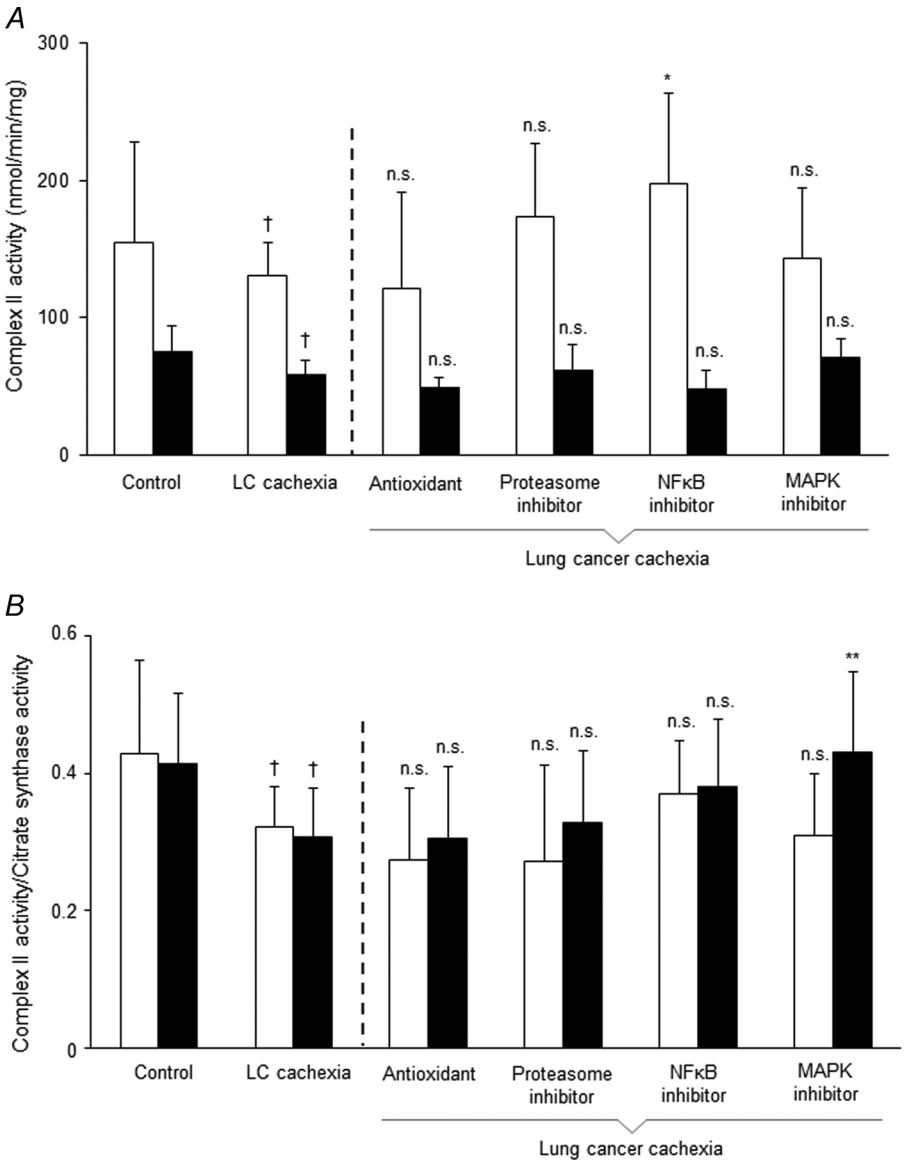


Figure 3. Levels of complex II activities in the study groups in both respiratory and limb muscles
A, mean values and SD of complex II enzyme activity (expressed as nanomoles per minute per milligram of protein) in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex II activity levels were significantly reduced in both diaphragm and gastrocnemius muscles among LC cachectic rodents compared with the control animals. A significant increase in diaphragm complex II activity was observed among LC cachectic rodents in response to NF-κB inhibition, while no differences were observed in the gastrocnemius. Statistical significance is represented as follows: (i) $\dagger P < 0.05$ in complex II activity levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant and $^{*}P < 0.05$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups. *B*, mean values and SD of the ratio of complex II enzyme activity to CS activity in diaphragm (open bars) and gastrocnemius muscles (filled bars). The ratio of complex II/CS was significantly reduced in both diaphragm and gastrocnemius among LC cachectic rodents compared with the control animals. A significant increase in gastrocnemius complex II/CS ratio was observed among LC cachectic rodents in response to MAPK inhibition, in comparison to untreated cachectic mice. Statistical significance is represented as follows: (i) n.s., non-significant and $\dagger P < 0.05$ in complex II/CS ratio levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant and $^{**}P < 0.01$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups.

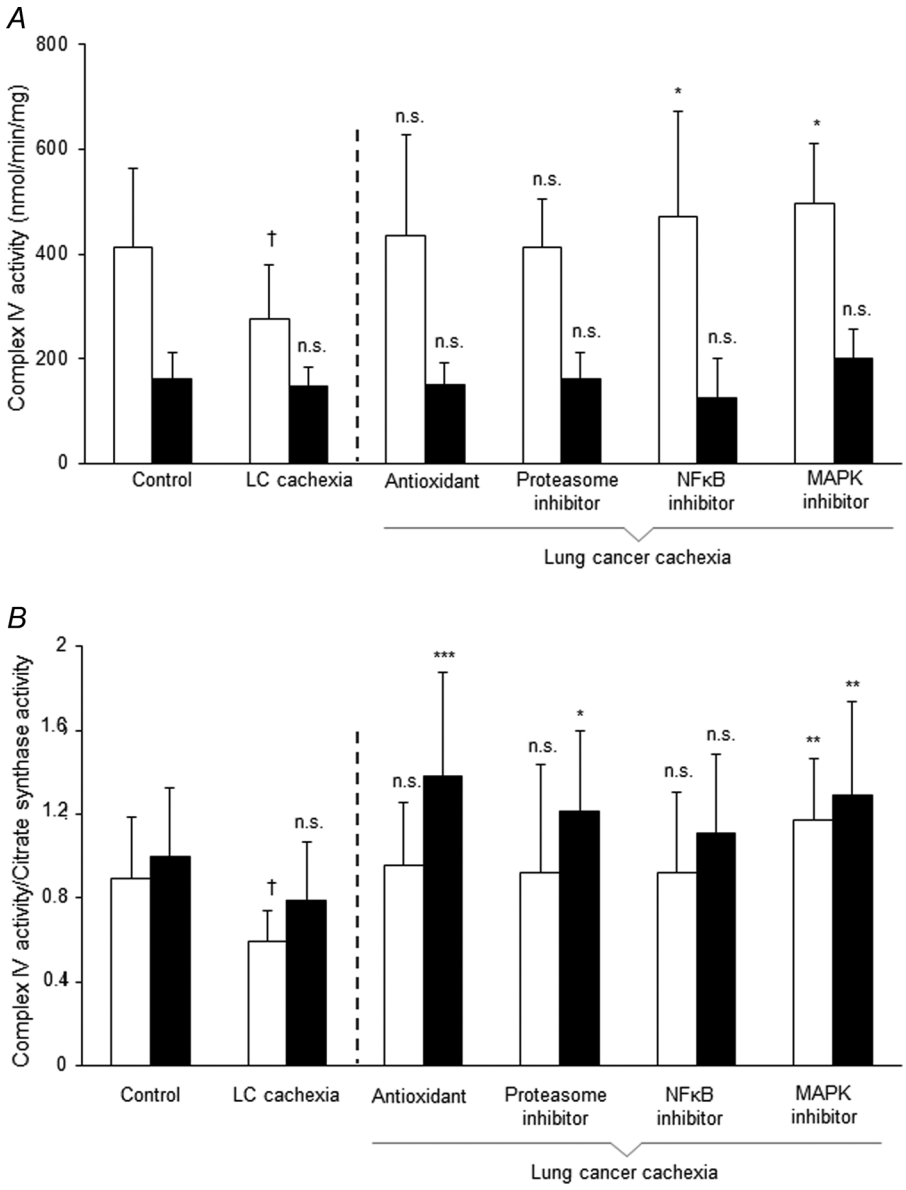
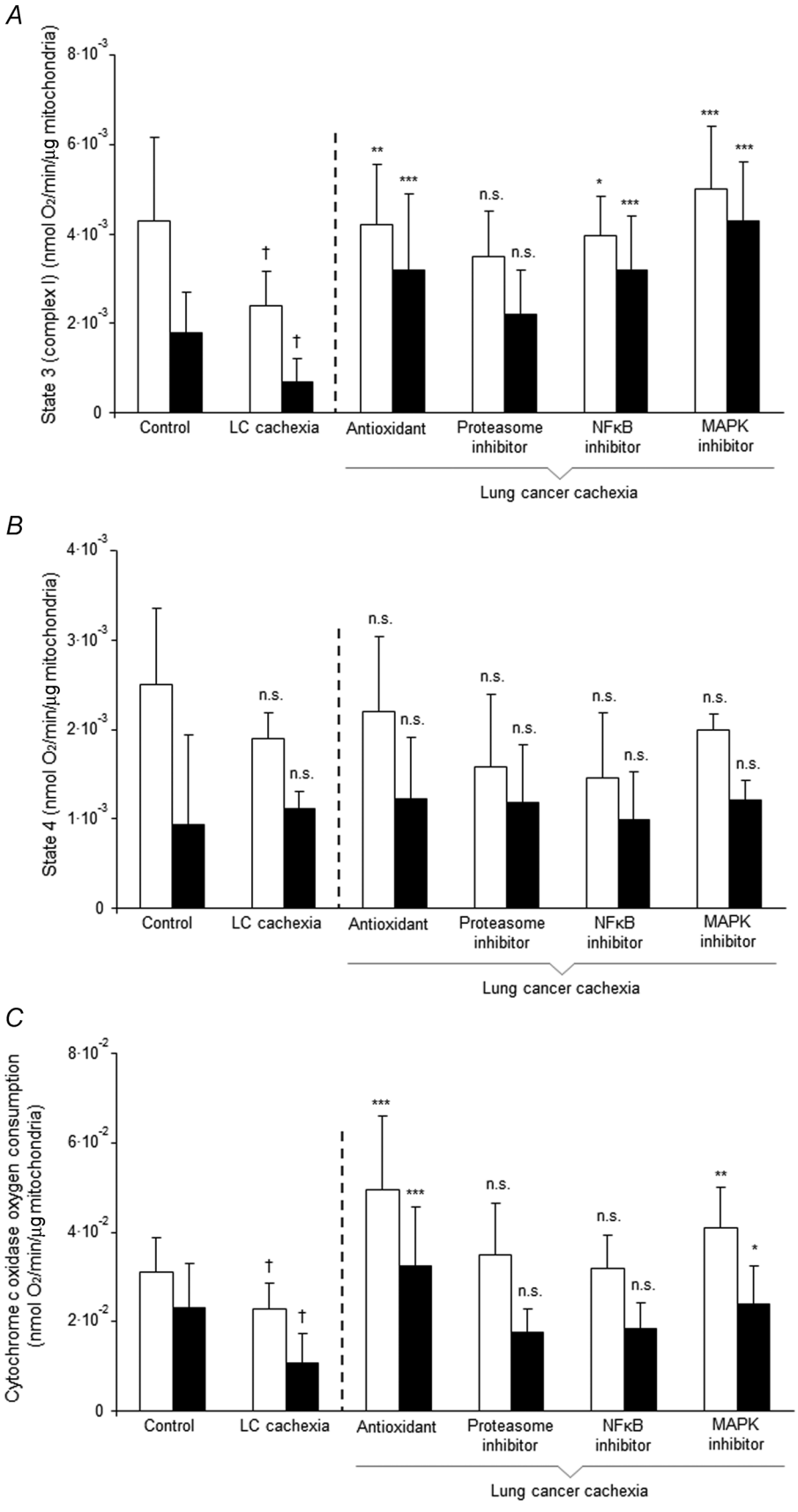


Figure 4. Levels of complex IV activities in the study groups in both respiratory and limb muscles
A, mean values and SD of complex IV enzyme activity (expressed as nanomoles per minute per milligram of protein) in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex IV activity levels were significantly reduced only in the diaphragm of LC cachectic rodents compared with the control animals. A significant increase in diaphragm complex IV activity was observed among LC cachectic rodents in response to NF-κB and MAPK inhibition, while no differences were observed in the gastrocnemius. Statistical significance is represented as follows: (i) n.s., non-significant and $\dagger P < 0.05$ in complex IV activity levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant and $^{*}P < 0.05$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups. *B*, mean values and SD of complex IV/CS ratio in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex IV/CS ratios were significantly reduced in the diaphragm of LC cachectic rodents compared with the control animals. Among LC cachectic rodents, a significant increase in complex IV/CS ratios was observed in both respiratory and limb muscles in response to MAPK inhibition, while NAC and bortezomib also elicited an increase in complex IV/CS ratios in the limb muscle. Statistical significance is represented as follows: (i) n.s., non-significant and $\dagger P < 0.05$ in complex IV/CS ratio levels in any muscle between LC cachectic and control mice; and (ii) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups.



is questionable in that respect, at least in this specific model of cancer-induced cachexia. Administration of the antioxidant NAC did not induce any beneficial effects on cachexia parameters or subcutaneous tumour size among the tumour-bearing rodents. This also indicates that oxidants are not likely to be involved in muscle mass loss or impaired function in this experimental model of cancer cachexia.

As expected, the activity of the MRC complexes analysed in the present study was in general of greater magnitude in the respiratory than in the limb muscle. Importantly, novel findings in the study were that the diaphragm of cachectic rodents exhibited a significant decrease in the activities of the mitochondrial respiratory chain complexes I, II and IV, as identified by both absolute values and values relative to CS, while in the limb muscle only complex I and II activities were significantly diminished. In addition, inhibition of the NF-κB pathway with sulfasalazine elicited an improvement in the activity (absolute values) of complexes I, II and IV in the diaphragm, while only complex I activity improved in limb muscles of cachectic rodents. Furthermore, selective inhibition of the MAPK pathway induced a significant increase in the activity (relative to CS values) of complexes I, II and IV within the gastrocnemius, whereas in the respiratory muscle only complex IV activity (absolute values) improved in the cachectic mice. Interestingly, NAC and bortezomib also elicited a significant improvement in complex IV activity (relative values) in the peripheral muscle of the cachectic animals.

Oxygen consumption in State 3 respiration and complex IV was significantly decreased in cachectic respiratory and limb muscles, and blockade of the MAPK pathway as well as treatment with the antioxidant NAC elicited a significant improvement in oxygen metabolism in both conditions. Moreover, inhibition of the NF-κB pathway also improved oxygen consumption in State 3 respiration in the diaphragm and gastrocnemius of the cachectic mice.

Mitochondrial respiratory chain enzyme activities in cancer cachectic muscles

In the present investigation, it has been clearly demonstrated, for the first time, that the activities of the MRC complexes I, II and IV are decreased in the diaphragm of mice with cancer-induced cachexia. Besides, similar findings were also observed in the limb muscle of the same animals. This is in line with previous studies in which alterations in mitochondrial function were detected in muscles in different conditions, such as moderate-to-severe COPD (Puente-Maestu *et al.* 2009*a*, 2012; Naimi *et al.* 2011) and mechanical ventilation-induced atrophy of rat diaphragms (Kavazis *et al.* 2009). Furthermore, in limb muscles of interleukin-6-dependent cachectic mice, several aspects of mitochondrial function were also shown to be impaired, such as a reduced content of cytochrome *c* oxidase complex subunit IV and cytochrome *c* protein, a lower number of succinate dehydrogenase-positive fibres,

Figure 5. Levels of oxygen consumption in the study groups in both respiratory and limb muscles
A, mean values and standard deviation of State 3 respiration (expressed as nanomoles of O_2 per minute per microgram) in diaphragm (open bars) and gastrocnemius muscles (filled bars). State 3 levels were significantly reduced in both diaphragm and gastrocnemius muscles among LC cachectic rodents compared with the control animals. A significant increase in State 3 was detected in both respiratory and limb muscles among LC cachectic rodents in response to NAC and either NF-κB or MAPK inhibitors compared with the untreated cachectic rodents. Statistical significance is represented as follows: (i) $\dagger P < 0.05$ in State 3 levels in any muscle between LC cachectic and control mice; and (ii) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups. *B*, mean values and SD of State 4 respiration (expressed as nanomoles of O_2 per minute per microgram) in diaphragm (open bars) and gastrocnemius muscles (filled bars). State 4 levels did not differ significantly among any of the study groups for any of the analysed muscles. Statistical significance is represented as follows: (i) n.s., non-significant in State 4 levels in any muscle between LC cachectic and control mice; and (ii) n.s., non-significant levels in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups. *A*, mean values and SD of complex IV oxygen consumption (expressed as nanomoles of O_2 per minute per microgram) in diaphragm (open bars) and gastrocnemius muscles (filled bars). Complex IV oxygen consumption levels were significantly reduced in both diaphragm and gastrocnemius muscles among LC cachectic rodents compared with the control animals. A significant increase in complex IV oxygen consumption was detected in both respiratory and limb muscles among LC cachectic rodents in response to NAC and MAPK inhibition compared with the untreated cachectic rodents. Statistical significance is represented as follows: (i) $\dagger P < 0.05$ in complex IV oxygen consumption levels in any muscle between LC cachectic and control mice; and (ii) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ levels of complex IV oxygen consumption in muscles between any group of LC cachectic mice treated with each of the inhibitors and the LC cachectic animals without any pharmacological treatment. The dashed line separates both types of comparisons between the groups.

reduced mitochondrial DNA-to-nuclear DNA ratio, and reduced levels of markers of mitochondrial biogenesis and dynamics (fusion and fission; White *et al.* 2011, 2012). Recently, mitochondrial disruption and decreased ATP synthesis rate were also shown in limb muscles of cancer-induced cachectic rats (Fontes-Oliveira *et al.* 2012) and mice (Constantinou *et al.* 2011), respectively.

Other novel findings in the study are the significant improvement observed in the activities of the different MRC complexes analysed, especially in the respiratory muscle, as a result of the blockade of NF-κB and MAPK pathways. These findings suggest that these two signalling pathways may specifically interfere, at different levels, with cellular processes involved in mitochondrial biogenesis and/or protein synthesis, activity, oxidative stress and degradation of the MRC enzymes examined in the investigation. In fact, in the same model, a significant reduction in enhanced protein oxidation levels was attained in both respiratory and limb muscles as a result of the blockade of NF-κB and MAPK pathways and the proteasome system (data not shown). Moreover, sulfasalazine also prevented the increase in total and phosphorylated p65 and p50 levels as well as the transcriptional activity (luciferase assay) observed in both respiratory and limb muscles in the cachectic rodents (data not shown). Total levels of p38 also decreased in response to MAPK inhibitor in both muscles in the cachectic animals (data not shown).

Another aspect that warrants attention is that tumour size was decreased as a result of NF-κB and MAPK pathway inhibition. As also shown to occur in oncological cachectic patients several weeks after cancer surgery (Williams *et al.* 2012), it is likely that the decrease in tumour burden induced by the blockade of NF-κB and MAPK pathways may have contributed to restoration of muscle mass loss and reduction in cachexia in the tumour-bearing mice. Preliminary data from our group (unpublished observations) showed a significant reduction in expression of ki-67, a marker of cell proliferation, in the tumours of animals treated with either NF-κB or MAPK inhibitor (8 and 19%, respectively), while NAC or bortezomib did not induce any significant change in ki-67 expression. On this basis, it is possible to conclude that mechanisms other than retardation of tumour growth (induction of apoptosis, blockade of proteases and angiogenesis, and modulation of redox potential) may also account for the reduction in tumour size detected in the cachectic rodents as a result of treatment with either NF-κB or MAPK pathway inhibitors and bortezomib (Karin, 2006; Chung & Sontheimer, 2009). Overall, it would be plausible to conclude that inhibition of either NF-κB or MAPK pathways seems to exert direct beneficial effects on the loss of muscle mass in the cancer cachectic mice, independently of tumour size and growth. Indeed, the significant reduction in tumour burden observed in response to the proteasome inhibitor, which did not influence muscle mass loss or function, probably because of accumulation of defective proteins in the myofibres or MRC complex activities, further supports such a conclusion.

N-Acetylcysteine may act as a direct antioxidant in skeletal muscle fibres by protecting specific cellular sites, such as the sarcoplasmic reticulum, sarcolemma and/or mitochondrial proteins, from oxidative damage in several models (Shindoh *et al.* 1990; Reilly *et al.* 1991; Van Surell *et al.* 1992; Khawli & Reid, 1994; Reid *et al.* 1994; Barreiro *et al.* 2005*b*). In limb muscles of the cachectic animals, NAC elicited a significant increase in complex IV activity as quantified by the ratio to CS, but not as absolute values. Total mitochondrial content (CS activity) and ROS production in the muscle specimens could play a more relevant role in complex IV activity than in the other MRC complexes. However, these findings warrant further attention in future investigations.

Importantly, CS enzyme activity did not differ in either respiratory or limb muscles between LC cachectic rodents and control animals. The activity of CS enzyme was used as a marker of mitochondrial content in the muscle preparations (Kavazis *et al.* 2009; Medja *et al.* 2009). Hence, mitochondrial content seems to be preserved equally in both diaphragms and gastrocnemius muscles of the cachectic and control animals. These interesting findings suggest that the decline in MRC complex activities is likely to be due to alterations occurring in the enzyme active sites rather than to a decrease in mitochondrial content within the cancer cachectic muscles. An additional relevant finding in the study was that there was a decline in CS activity observed in the limb muscle of the cachectic mice treated with NAC, bortezomib or sulfasalazine, while an increase in CS activity was observed only with the last inhibitor in the diaphragm. Differences in the activity of each type of muscle could account for this differential pattern of CS activity in response to the inhibitors administered to the cachectic animals. Potential depression of mitochondrial biogenesis in the limb muscles elicited by the different treatments could also partly explain those findings.

Mitochondrial respiratory chain oxygen consumption

State 3 respiration was significantly decreased in the respiratory and limb muscles of the LC cachectic rodents compared with control mice. This finding has relevant implications, because it may partly account for the metabolic derangements and energy inefficiency characteristic of cachectic muscles (Evans *et al.* 2008). The results of the present study are also consistent with previous investigations, in which very similar findings were reported in muscles of COPD patients (Puente-Maestu *et al.* 2009*a*, 2012; Naimi *et al.* 2011), in

diaphragms of mechanically ventilated rats (Kavazis *et al.* 2009) and in muscles of senescent mice (Figueiredo *et al.* 2009). Importantly, for each specific muscle the basal mitochondrial respiration (State 4) was similar among all the study groups, irrespective of the disease status or treatment with the different pharmacological agents. Also, State 4 respiration levels were in general of greater magnitude in the respiratory than in the limb muscles for each of the study groups. These are relevant findings, indicating that mitochondrial integrity and stability were achieved equally in both types of muscles of all animal groups. This is a relevant methodological feature that ensures the quality and reliability of the results encountered in the investigation.

Oxygen consumption by MRC complex IV (cytochrome *c* oxidase) was also diminished in both types of muscles of LC cachectic rodents compared with control animals. These findings are in line with the above-mentioned studies (Figueiredo *et al.* 2009; Puente-Maestu *et al.* 2009*a*, 2012; Kavazis *et al.* 2009; Naimi *et al.* 2011), emphasizing the consistency of the study results. Importantly, the antioxidant NAC and the MAPK inhibitor elicited a significant increase in oxygen consumption by complex IV and State 3 respiration (also sulfasalazine) in both diaphragms and gastrocnemius muslces of the tumour-bearing animals compared with the untreated cachectic rodents. These are novel findings, suggesting that the different pathways and cellular processes blocked by the different inhibitors are somehow involved in MRC dysfunction in cancer cachexia.

It has been well established that the antioxidant NAC and other antioxidants influence muscle fibre function by scavenging ROS, thereby exerting beneficial effects in several models, during repetitive isometric contractions in both experimental animal (Shindoh *et al.* 1990; Khawli & Reid, 1994) and human studies (Reid *et al.* 1994), in limb muscles of patients with severe COPD (Koechlin *et al.* 2004), in endotoxaemic rats (Van Surell *et al.* 1992) and in the diaphragmatic dysfunction of streptozotocin-induced diabetic rats (Hida *et al.* 1996). In view of these published findings, it would be possible to conclude that ROS directly synthesized within the MRC, as shown to be the case in other conditions (Figueiredo *et al.* 2009; Kavazis *et al.* 2009; Puente-Maestu *et al.* 2009*a*, 2012; Naimi *et al.* 2011), could exert direct deleterious effects on MRC function in muscles of cancer cachectic rodents. Indeed, the rise in oxygen consumption detected among the cachectic muscles in response to concomitant treatment with the antioxidant NAC further supports this conclusion. Nevertheless, it remains to be understood why whole body or muscle weight and muscle force did not improve in response to treatment with NAC in the cachectic rodents, despite the significant increase observed in mitochondrial oxygen consumption. In this regard, knowledge about the potential links between oxidative stress and muscle protein degradation and maintenance, especially of structural proteins, is still in its infancy, and controversial results have been reported so far (MacMillan-Crow *et al.* 1998; Souza *et al.* 2000; Gomes-Marcondes & Tisdale, 2002; Marin-Corral *et al.* 2009, 2010; Fermoselle *et al.* 2012). While NAC could exert direct positive effects on mitochondrial oxygen consumption, via ROS scavenging, oxidants synthesized by other cellular sites could perhaps not be scavenged sufficiently by NAC or not affect the turnover of structural proteins in the cachectic muscles. A relevant finding was that the antioxidant NAC improved oxygen consumption equally in both respiratory and limb muscles. Future investigations will shed light on the specific mechanisms by which attenuation of ROS may improve mitochondrial function, while exerting no effects on the loss of muscle mass or force generation.

Study limitations

A first limitation in the investigation has to do with the process of mitochondrial isolation using *in vivo* preparations. Procedures previously employed in other studies were followed meticulously in order to attain a reasonable yield of intact mitochondria. A second limitation may be related to the fact that the specific implications of the different cellular pathways on muscle wasting mechanisms have not been assessed in the present study. Ongoing investigations in the group aim at specifically exploring those questions (unpublished observations). A third limitation refers to the fact that young rodents were used for the purpose of the investigation. Thus, it is likely that reduced muscle growth and anabolism may also have played a role, regardless of enhanced protein degradation, in the loss of muscle mass observed in the cachectic rodents. Indeed, this was a major reason to define the decrease in muscle mass and body weights detected in the tumour-bearing mice as 'reduced body weight gain' and not muscle wasting. Although we understand that conducting experiments on young animals entails these limitations, the use of rodents of older ages brings about many other disadvantages that could have interfered with the study results, as mitochondrial dysfunction participates in the aetiology of sarcopenia in ageing (Kujoth *et al.* 2005; Figueiredo *et al.* 2009). Finally, most of the investigations in the field have been conducted in laboratory animals of similar ages (Barreiro *et al.* 2005*a*; Diament *et al.* 2006; Marin-Corral *et al.* 2010; Romanello *et al.* 2010; Constantinou *et al.* 2011; Fermoselle *et al.* 2011; Busquets *et al.* 2012; Fontes-Oliveira *et al.* 2012).

Conclusions

Novel findings in this investigation are that in cancer cachectic mice the diaphragm and gastrocnemius muscles

exhibited a significant depression of the MRC complexes and oxygen consumption. Blockade of NF-κB and MAPK actions partly restored the loss of muscle mass and force as well as the MRC dysfunction, while concomitantly reducing, at least in part, the tumour burden. Antioxidants improved mitochondrial oxygen consumption without eliciting significant effects on the loss of muscle mass and force or the size of the tumour, whereas the proteasome inhibitor reduced tumour burden without significantly influencing muscle mass and strength or mitochondrial function. In conclusion, NF-κB and MAPK signalling pathways modulate muscle mass and performance and MRC function of respiratory and limb muscles in this model of experimental cancer cachexia, thus offering targets for therapeutic intervention.

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Additional information

Competing interests

None declared.

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