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Colon 26 Adenocarcinoma (C26)-Induced Cancer Cachexia Impairs Skeletal Muscle Mitochondrial Function and Content

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Abstract

Introduction.—The present study aimed to determine the impact of colon 26 adenocarcinoma (C26)-induced cancer cachexia on skeletal muscle mitochondrial respiration and content.

Methods.—Twelve male CD2F1 mice were injected with C26-cells (tumor bearing (TB) group), whereas 12 age-matched mice received PBS vehicle injection (non-tumor bearing (N-TB) group). Mitochondrial respiration was studied in saponin-permeabilized *soleus* myofibers.

Results.—TB mice showed lower body weight (~ 20%) as well as lower *soleus*, *gastrocnemius-plantaris* complex and *tibialis anterior* masses versus N-TB mice ($p < 0.05$). *Soleus* maximal state III mitochondrial respiration was 20% lower (10 mM glutamate, 5 mM malate, 5 mM adenosine diphosphate; $p < 0.05$) and acceptor control ratio (state III/state II) was 15% lower in the TB vs. N-TB ($p < 0.05$), with the latter suggesting uncoupling. Lower VDAC protein content suggested reduced mitochondrial content in TB versus N-TB ($p < 0.05$).

Discussion.—Skeletal muscle in C26-induced cancer cachexia exhibits reductions in: maximal mitochondrial respiration capacity, mitochondrial coupling and mitochondrial content.

Keywords

Mitochondrial respiration; Mitochondrial coupling; Mitochondrial homeostasis; Muscle wasting; Mitochondrial content; Oxidative phosphorylation

Introduction

Cancer cachexia is characterized by a loss of skeletal muscle mass that may be accompanied by a loss of fat mass (Fearon et al. 2011; Argiles et al. 2015) and which cannot be fully

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None.

explained by anorexia (Evans et al. 1985; Bosaeus et al. 2001). This condition is frequently characterized by an elevated resting energy expenditure (Bosaeus et al. 2001) which might be suggestive of altered mitochondrial function (see (Argiles et al. 2015; Carson et al. 2016; VanderVeen et al. 2017) for reviews).

Despite the established role of mitochondria in many forms of muscle atrophy (Romanello and Sandri 2010), very few studies have evaluated the extent of mitochondrial impairment in cancer cachexia. Moreover, although studies of mitochondria in cancer cachexia demonstrate lower mitochondrial content (White et al. 2012; White et al. 2011; Brown et al. 2017), reductions in mitochondrial enzymatic activities involved in oxidative phosphorylation (Antunes et al. 2014; Fermoelle et al. 2013), altered mitochondrial morphology (Shum et al. 2012; Fontes-Oliveira et al. 2013), and alterations in mitochondrial dynamics and turnover (White et al. 2011; White et al. 2012; Puppa et al. 2014; Marzetti et al. 2017; Brown et al. 2017; Ballaro et al. 2019), only a few previous studies have assessed mitochondrial function (recently reviewed in (VanderVeen et al. 2017)). One of the major findings from these studies was a reduction in maximal oxygen consumption in permeabilized muscle fibers harvested from cachectic mice which had been injected with breast cancer cells (Gilliam et al. 2016). This is suggestive of a reduced capacity for ATP synthesis, which was recently identified in mice inoculated with Lewis Lung Carcinoma (LLC) cells (Constantinou et al. 2011; Tzika et al. 2013). Furthermore, a recent study in tumor bearing mice found evidence of mitochondrial dysfunction prior to the occurrence of any muscle atrophy, suggesting mitochondrial dysfunction may be a key driver of cancer-induced cachexia (Brown et al. 2017).

Based on this limited data, in the current study we selected to evaluate the impact of colon 26 adenocarcinoma (C26)-induced cancer cachexia on skeletal muscle mitochondrial respiration and content. The C26 model is the most extensively-used and well-characterized cancer cachexia model, and published microarray data using this model demonstrate a down-regulation of mitochondrial transcripts in skeletal muscles compared to controls (Judge et al. 2014).

Material and Methods

Animals

All animal procedures were approved by the University of Florida Animal Care and Use Committee (#201608146).

A total of 24 eight-week old male CD2F1 mice (Charles River laboratory, Wilmington, United States) housed in a humidity- and temperature-controlled animal facility on a 12:12-h light cycle with water and standard diet provided *ad libitum* were randomly attributed to one of the two groups: tumor- (TB) or non-tumor (N-TB) bearing mice. Mice allocated to the TB group were injected subcutaneously with 5×10^5 C26-cells diluted in 100 μ l of PBS per flank, whereas mice in the N-TB group received an equivalent PBS vehicle injection. Mice were euthanized by a lethal intraperitoneal dose (150 mg/kg) of Euthasol® ~ 26 days (range: 25-27 days) after tumor or vehicle injection. At time of sacrifice, *soleus*, *gastrocnemius-plantaris* complex (GasPL) and *tibialis anterior* (TA) muscles were harvested from both legs.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, United States) except for L(+)-Glutamic acid (Acros Organics, Waltham, United States), MgCl_2 (Honeywell, Morris Plains, United States).

Mitochondrial respiration assay

Mitochondrial respiration was measured in *soleus* muscles (predominantly composed of type I and IIa fibers). Muscle bundles were permeabilized with 0.05 mg/ml of saponin for 30 min as previously described (Gouspillou et al. 2014) and were then placed in the respirometry chamber in buffer B (composed of CaK_2 EGTA (2.77 mM), K_2 EGTA (7.23 mM), MgCl_2 (1.38 mM), K_2HPO_4 (3.0 mM), DTT (0.5 mM), imidazole (20 mM), K-MES (100 mM) and taurine (20 mM), (Gouspillou et al. 2014)) at 37°C. Respiration was recorded with a polar electrode-based high resolution respirometer (Oxygraph-2k; Oroboros, Innsbruck, Austria) upon successive sequential addition of: 10 mM of glutamate (G) and 5 mM of malate (M), 5 mM of adenosine diphosphate (ADP), 20 mM of succinate (S), and 10 μM of cytochrome c. Respiration rates were normalized to muscle bundle wet mass. For each mouse, duplicate measurements were acquired when enough material was collected. Muscle bundles presenting an increase in respiration >10% upon cytochrome c addition were excluded from the analysis (Gilliam et al. 2013). In total, six muscle bundles had to be discarded, including two from a given mouse, resulting in N = 11 instead of 12 for all respiratory parameters in the tumor-bearing group.

Protein analyses

Proteins were extracted from *soleus* and GasPL muscles and electrophoresed through a 4-15% TGX Stain-free gel (Biorad, Hercules, United States). Following protein transfer, the nitrocellulose membranes were blocked in 1% (w/v) non-fat dried milk PBS and subsequently incubated with primary and secondary antibodies at room temperature (antibody characteristics are listed in Table 1). Signals of proteins of interest were visualized using Odyssey Imaging System (LI-COR, Biosciences, Lincoln, USA), quantified with Image Studio Lab software and normalized to total protein content (visualized using the Stain-free technology and quantified with Image Lab software (BioRad, Hercules, United States)).

Statistical analysis

Statistical analyses were performed with SigmaPlot (version 11 for Windows, Systat, Chicago, United States) and the level for statistical significance was set to $p < 0.05$. Unpaired t-tests or Mann-Whitney tests were conducted for all of the comparisons presented, depending on the normality test outcome tested with Shapiro-Wilk. Data are presented as mean $\pm$ SD.

Results

C26 cells inoculation Reduced body weight and skeletal muscle mass

Mice were sacrificed 25 to 27 days post tumor-cell/PBS inoculation, at which time, average tumor weight was 1.7 ± 0.5 g (range: 0.68–2.41 g). At sacrifice, TB mice showed a ~20% lower tumor-free body weight compared to N-TB mice ($p < 0.05$, Fig. 1A). Ulower body weight was accompanied by lower *soleus* (–21%), GasPL (–10%) and TA (–11%) muscle masses ($p < 0.05$, Fig. 1B-D).

C26-induced cancer cachexia results in altered mitochondrial respiration

Soleus fiber bundles prepared from TB mice showed a similar state II respiration rate (under saturating concentration of glutamate and malate) but a ~20% lower maximal state III respiration rate under saturating concentration of glutamate, malate and ADP *versus* N-TB mice ($p < 0.05$, Fig. 2A&B). Similar results were obtained when succinate was added to the aforementioned substrates (Fig. 2A&B). When changes between subsequent substrate additions were computed (indicated by (1) and (2) in Fig. 2A), it was found that the increase in respiration rate was smaller in the TB mice ($76 \pm 30\%$) compared to the N-TB mice ($115 \pm 24\%$) when ADP was added following glutamate and malate (Fig. 2C, corresponds to (1) in Fig. 2A). In contrast, similar respiration rate increases were observed after succinate addition ((2) in Fig. 2A) between the two groups ($+22 \pm 11\%$ in TB *versus* $+18 \pm 9\%$ in N-TB mice, $p > 0.05$, Fig. 2C). In addition, a ~15% lower acceptor control ratio (ACR, i.e. an index of mitochondrial oxidative phosphorylation coupling computed as the ratio between respiration rate under saturating concentration of glutamate, malate and ADP and under saturating concentration of glutamate and malate) was observed in TB mice compared to N-TB mice ($p < 0.05$, Fig. 2D).

C26-induced cancer cachexia results in reduced mitochondrial content

All representative subunits of the different mitochondrial complexes showed a reduced content in both the *soleus* and GasPL harvested from TB mice compared to N-TB mice ($p < 0.05$, Fig. 3A). Similarly, VDAC expression was reduced in the TB compared to the N-TB mice ($p < 0.05$, Fig. 3B).

Discussion

The present study aimed to evaluate the extent to which C26-induced cancer cachexia impaired skeletal muscle mitochondrial respiration and content. The results showed that 25 to 27 days post inoculation with C26 tumor cells, TB mice presented a 20% lower tumor-free body weight that was accompanied by reduced *soleus*, GasPL and TA muscle masses. Mitochondrial respiratory function analysis in *soleus* muscle revealed a lower maximal mitochondrial respiration rate, reduced mitochondrial coupling and indices of lower mitochondrial protein abundance.

Evidence suggesting mitochondrial impairments in response to C26 tumors were first inferred from transcriptomics analyses (Judge et al. 2014) and subsequently by proteomics analyses (Barreto et al. 2016) showing down-regulation of mitochondrial transcripts or

proteins, respectively. Interestingly, Tezze et al. (Tezze et al. 2017) recently demonstrated that specific deletion of optic atrophy 1 (known to be downregulated in response to C26 tumors (Barreto et al. 2016)) in skeletal muscle was sufficient to impair muscle force and trigger muscle atrophy. It can therefore be suggested that the tumor-host inflammatory response to C26 inoculation might trigger mitochondrial impairments in skeletal muscles (White et al. 2012) which in turn might lead to reduced muscle force, increased muscle fatigability and wasting (Roberts et al. 2013a; Roberts et al. 2013b) in this model of cancer cachexia. Nevertheless, no studies have examined mitochondrial function and content in this model.

In contrast to the lack of information on C26 cancer cachexia, evidence for the involvement of mitochondrial dysfunction have been reported in other cancer cachexia models. For example, skeletal muscle of mice inoculated with LLC cells (Brown et al. 2017) showed that increased mitochondrial reactive oxygen species production, reduced mitochondrial turnover, reduced mitochondrial content and reduced ACR occurred before reductions of muscle mass. Moreover, two studies conducted in *Apc^{Min/+}* mice (which spontaneously develop colon cancer) also found that mitochondrial content loss preceded muscle atrophy and that the degree of mitochondrial content loss increased with cachexia severity (White et al. 2012; White et al. 2011). Additionally, a reduced maximal mitochondrial respiration rate was observed in a mouse breast cancer cachexia model (Gilliam et al. 2016). The present study, therefore agrees with and extends these previous results to the C26-induced cancer cachexia model, as C26 TB mice showed lower mitochondrial content and maximal mitochondrial respiration. Furthermore, the reduced ACR observed here and in the LLC cancer model previously (Brown et al. 2017) appeared to be due to higher basal respiration per unit of mitochondrial content as suggested by the lack of changes observed in state II respiration per unit of muscle despite lower muscle mitochondrial content in TB mice. This result suggests mitochondrial uncoupling might explain the elevated energy expenditure observed in cachectic cancer patients (Bosaeus et al. 2001).

In conclusion, our results are consistent with the view that defects in mitochondrial respiration represent a common mechanism between various cancer types that is linked to muscle atrophy. However, future studies are warranted to better understand the role of mitochondrial alterations in muscle atrophy seen with cancer cachexia, the mechanisms responsible for cancer-induced mitochondrial dysfunction, and development of future therapeutic treatments.

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Abbreviations

ACR	acceptor control ratio
ADP	adenosine diphosphate

BSA	bovine serum albumin
C26	colon 26 adenocarcinoma
DTT	dithiotreitol
EGTA	ethylene glycol- <i>bis</i> -(2-aminoethylether)-N, N, N', N'-tetraacetic acid
G	glutamate
GasPL	<i>gastrocnemii-plantaris</i> complex
K-MES	2-(<i>N</i> -morpholino) ethanesulfonic acid potassium salt
LLC	Lewis lung carcinoma
M	malate
N-TB	non-tumor bearing
PBS	phosphate buffer saline
TB	tumor bearing
TBS-T	Tris-buffered saline containing 0.1% of Tween

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Figure 1. C26-tumor cell inoculation induces (A) body, (B) soleus, (C) gastrocnemius-plantaris (GasPL) and (D) tibialis anterior (TA) weight losses.

N-TB = non-tumor bearing mice, TB = tumor bearing mice. Data are mean $\pm$ SD. The effects of C26-tumor cell inoculation was evaluated by performing unpaired t-tests. N = 12 per group except for N-TB GasPL where N = 11 and TB and N-TB TA where N = 8.

Figure 2. C26-induced cancer cachexia is associated with (A&B) similar basal (in presence of 10 mM of glutamate (G), 5 mM of malate (M)) but reduced maximal respiration rates measured (in presence of 10 mM of G, 5 mM of M, 5 mM of adenosine diphosphate (ADP) as well as in addition of 20 mM of succinate (S)), (C) smaller state 2 to state 3 respiration increases and (D) lower acceptor control ratio (ACR) in tumor-bearing mice (TB, filled bars) compared to nontumor bearing mice (N-TB, clear bars).

+ GM $\rightarrow$ ADP = relative respiration change induced by ADP addition in presence of GM (indicated on the representative trace in panel A by '(1)') and + GM + ADP $\rightarrow$ + S = relative respiration change induced by S addition in presence of GM + ADP (indicated on the representative trace in panel A by '(2)'). Data are mean $\pm$ SD. The effects of C26-tumor cell inoculation were evaluated by performing unpaired t-tests. N = 12 for N-TB and N = 11 for TB.

Figure 3. C26-cancer cachexia alters the content of (A) several mitochondrial complex representative subunits and of (B) the voltage-dependent anion channel (VDAC). N-TB = non-tumor bearing mice (clear bars), TB = tumor bearing mice (filled bars), CI = representative subunit of mitochondrial complex I, CII = representative subunit of mitochondrial complex II, CIII = representative subunit of mitochondrial complex III, CIV = representative subunit of mitochondrial complex IV and CV = representative subunit of mitochondrial complex V. Data are mean $\pm$ SD. The effects of C26-tumor cell inoculation was evaluated by performing unpaired t-tests. For GasPL, N = 12 per group except for TB CV where N = 11. For *soleus* N = 8 per group.

Table 1.

List of the antibodies used for Western blots.

	Company	Reference #	Dilution
<i>Primary antibodies</i>			
VDAC (rabbit)	Abcam	ab154856	1:10'000
Total OXPHOS Cocktail (mouse)	Abcam	ab110413	1:250
<i>Secondary antibodies</i>			
Goat anti-mouse IgG	LICOR	926-68070	1:20'000
Goat anti-rabbit IgG	LICOR	926-32211	1:20'000