Muscle weakness precedes atrophy during cancer cachexia and is linked to muscle-specific mitochondrial stress

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Muscle weakness and wasting are defining features of cancer-induced cachexia. Mitochondrial stress occurs before atrophy in certain muscles, but the possibility of heterogeneous responses between muscles and across time remains unclear. Using mice inoculated with Colon-26 (C26) cancer, we demonstrate that specific force production was reduced in quadriceps and diaphragm at 2 weeks in the absence of atrophy. At this time, pyruvate-supported mitochondrial respiration was lower in quadriceps while mitochondrial H$_2$O$_2$ emission was elevated in diaphragm. By 4 weeks, atrophy occurred in both muscles, but specific force production remained reduced in diaphragm. Mitochondrial respiration increased and H$_2$O$_2$ emission was unchanged in both muscles vs control while mitochondrial creatine sensitivity was reduced in quadriceps. These findings indicate muscle weakness precedes atrophy and is linked to heterogeneous mitochondrial alterations that could involve adaptive responses to metabolic stress. Eventual muscle-specific restorations in force and bioenergetics highlight how the effects of cancer on one muscle do not predict the response in another muscle. Exploring heterogeneous responses of muscle to cancer may reveal new mechanisms underlying distinct sensitivities, or resistance, to cancer cachexia.
Muscle weakness precedes atrophy during cancer cachexia and is linked to muscle-specific mitochondrial stress

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Abstract

Muscle weakness and wasting are defining features of cancer-induced cachexia. Mitochondrial stress occurs before atrophy in certain muscles, but the possibility of heterogeneous responses between muscles and across time remains unclear. Using mice inoculated with Colon-26 (C26) cancer, we demonstrate that specific force production was reduced in quadriceps and diaphragm at 2 weeks in the absence of atrophy. At this time, pyruvate-supported mitochondrial respiration was lower in quadriceps while mitochondrial H$_2$O$_2$ emission was elevated in diaphragm. By 4 weeks, atrophy occurred in both muscles, but specific force production increased to control levels in quadriceps such that reductions in absolute force were due entirely to atrophy. Specific force production remained reduced in diaphragm. Mitochondrial respiration increased and H$_2$O$_2$ emission was unchanged in both muscles vs control while mitochondrial creatine sensitivity was reduced in quadriceps. These findings indicate muscle weakness precedes atrophy and is linked to heterogeneous mitochondrial alterations that could involve adaptive responses to metabolic stress. Eventual muscle-specific restorations in specific force and bioenergetics highlight how the effects of cancer on one muscle do not predict the response in another muscle. Exploring heterogeneous responses of muscle to cancer may reveal new mechanisms underlying distinct sensitivities, or resistance, to cancer cachexia.
Introduction

Cancer-induced cachexia is a multifactorial syndrome characterized, in part, by a loss of skeletal muscle mass that cannot be fully reversed by conventional nutritional support (1). This condition leads to progressive reductions in functional independence and quality of life (2). Such declines in muscle mass also reduce tolerance to anticancer therapies and overall survivability (3, 4), and is associated with increased hospitalization time (5). 20-80% of cancer patients are thought to develop cachexia depending on the type and stage of cancer (6). However, the time-dependent relationship between muscle atrophy and weakness remains unclear, as does the degree to which this relationship may vary between muscle types. Exploring the natural divergence of muscle responses to cancer may be an opportunistic approach to identify distinct mechanisms underlying muscle weakness and wasting during cancer cachexia.

Contemporary theories posit that muscle wasting during cachexia is induced by circulating factors generated during cancer which trigger protein degradation and loss of myofibrillar proteins through various mechanisms (4, 7, 8). However, recent literature suggests skeletal muscle mitochondria are also subject to damage during cancer cachexia (9–11) and may be direct contributors to either muscle weakness or atrophy. Current literature suggests oxidative phosphorylation is impaired in the soleus, gastrocnemius and plantaris muscle of tumour-bearing mice, while reactive oxygen species (ROS) - in the form of mitochondrial H$_2$O$_2$ emission (mH$_2$O$_2$) - can be increased or decreased depending on the muscle and duration of cancer (9, 11, 12). This suggests cellular mechanisms contributing to muscle loss during cancer cachexia may be more complicated than previously believed. Moreover, in the Lewis lung carcinoma (LLC) xenograft mouse model, certain indices of skeletal muscle mitochondrial dysfunction preceded the onset of
muscle atrophy, suggesting mitochondria may be a potential therapeutic target (12). This theory was supported by subsequent studies reporting positive effects of the mitochondria-targeting compound SS-31 in preventing certain indices of cachexia in some but not all muscles of the C26 xenograft mouse model (13, 14). However, given the multifactorial contributions to cachexia during cancer, it seems likely the relationship between mitochondria and myopathy may differ between muscle type and throughout cancer progression.

Indeed, skeletal muscle mitochondria are known to be highly adaptable to metabolic stressors and can super-compensate during an energy crisis (15, 16). In this light, the available literature does not provide sufficient information to predict the extent to which cancer will affect individual muscles, particularly in relation to their underlying mitochondrial responses to the systemic stress of this disease. Understanding the time-dependent nature of unique mitochondrial signatures during cancer-induced cachexia might better inform the development of mitochondrial therapies that have so far yielded disparate results across various muscle types in the C26 cancer mouse model (13, 14).

In this study, we compared the time-dependent relationship of muscle dysfunction and mitochondrial bioenergetic responses to cancer between locomotor (quadriceps) and respiratory muscles (diaphragm). In so doing, we employed a careful consideration of mitochondrial substrate titration protocols modeling key parameters governing mitochondrial bioenergetics in vivo. Similar assay design considerations have been essential for identifying precise mitochondrial bioenergetic contributions to cellular function in our previous research (17–20). Using the C26 tumour-bearing mouse model, we reveal muscle weakness precedes atrophy in quadriceps and
diaphragm. Energetic insufficiencies were more pronounced in quadriceps whereas mitochondrial redox stress was more evident in diaphragm, yet both muscles showed a delayed correction, if not super-compensation, as cancer progressed. In quadriceps, increased mitochondrial respiratory control was related to a surprising increase in specific force production at 4 weeks that likely mitigated the magnitude of reduction in absolute force due to atrophy. These findings demonstrate the effects of cancer on one muscle do not necessarily predict the response in another muscle type. Moreover, the heterogeneous muscle-specific and time-dependent mitochondrial relationships to cancer may provide an opportunity for informing a more targeted approach to developing mitochondrial therapies to improve muscle health in this debilitating disorder.
Results

C26 tumour-bearing mice show progressive reductions in body weight, muscle mass, fat mass and grip strength

All results were in male mice. Body weights were reduced 4 weeks after subcutaneous implantations of C26 cells (Figure 1A), while tumour-free body weights progressively decreased beginning at 3 weeks to a net loss of 27% by 4 weeks (Figure 1B, C) at a time of substantial tumour growth (Figure 1D). Tumours grew to ~0.2g at 2 weeks and ~2.2g at 4 weeks (Figure 1E). C26 spleen mass (marker of inflammatory stress) was not different from PBS at 2 weeks but was significantly greater at 4 weeks (Figure 1F). Adipose tissue from the inguinal fat pad was not different at 2 weeks, but significantly lower at 4 weeks (-65%; Figure 1G). A similar pattern was observed with grip strength (Figure 1H). The mass of specific muscles was similar between C26 and PBS at 2 weeks (Figure 1I). At 4 weeks soleus (SOL) mass was similar between C26 and PBS while lower muscle masses were observed in C26 for extensor digitorum longus (EDL; -23%), plantaris (PLA; -20%), tibialis anterior (TA; -26%), gastrocnemius (GA; -21%) and quadriceps (QUAD; -29%) vs PBS (Figure 1J).

Muscle atrophy occurs in both the quadriceps and diaphragm after 4 weeks of tumour bearing.

Muscle atrophy is a hallmark of cancer cachexia. In both quadriceps and diaphragm, fibre CSA was similar between C26 and PBS groups for specific MHC isoforms (Figure 2A-D) and when pooling all MHC isoforms (data not shown) at 2 weeks. However, at 4 weeks, quadriceps muscle exhibited lower CSA in pooled fibres (-40%, p<0.05, data not shown) with specific reductions in MHC IIX (-32%) and MHC IIB (-49%) but not the MHC IIA isoform (Figure 2E, F) vs PBS. MHC
I-positive fibres were not detected in the quadriceps (Figure 2B, F). At 4 weeks, diaphragm muscle also showed lower CSA in pooled fibres (-31%, *p*<0.05, data not shown) which mirrored changes in MHC I (-28%), MHC IIA (-21%), MHC IIB (-30%) and MHC IIX (-35%) vs PBS at 4 weeks (Figure 2G, H).

We validated that these reductions in fibre CSA were a result of muscle atrophy by measuring key proteasomal markers of muscle wasting. In the quadriceps, muscle RING-finger protein-1 (MURF1) mRNA content was significantly increased at 4 weeks of tumour bearing compared to PBS controls (Figure 3A). This was also the case for atrogin (Figure 3B), ubiquitin C (UBC; Figure 3C) and growth arrest and DNA damage-inducible 45α (Gadd45a; Figure 3D). The diaphragm demonstrated similar results, such that MURF1, atrogin and Gadd45a were all significantly increased at 4 weeks of tumour-bearing, however this did not occur in UBC (Figure 3E-H).

**Force production is reduced prior to atrophy in quadriceps and diaphragm but returns to normal in quadriceps**

At 2 weeks and 4 weeks, C26 absolute muscle force was lower in quadriceps relative to PBS as a group main effect (Figure 4A). Interestingly, when absolute quadriceps force was normalized to muscle wet weight, specific force production remained decreased at 2 weeks of tumour bearing compared to PBS as a group main effect. However, at 4 weeks of tumour bearing, specific force was higher compared to 2 weeks and not different compared to PBS controls as a group main effect (Figure 4B). In addition, there was an interaction whereby C26 at 2 weeks produced less force at 80Hz, 100Hz and 120Hz compared to both PBS groups and the C26 at 4 weeks (not shown).
We are unable to report absolute force in the diaphragm as dissected muscle strips of diaphragm were used to complete the in-vitro force assay, not the whole diaphragm. At 2 weeks and 4 weeks of tumour-bearing C26 specific muscle force was lower in the diaphragm relative to PBS controls as a group main effect (Figure 4C).

Mitochondrial electron transport chain protein contents are reduced in quadriceps but do not change in diaphragm by 4 weeks of tumour development

At 2 weeks, electron transport chain (ETC) subunit contents in both muscles were unchanged in C26 relative to PBS controls (Figure 5A, B). At 4 weeks, C26 showed lower contents in subunits of complex I (-31%), complex II (-18%), complex IV (-37%), complex V (-11%) and total ETC subunit content (-22%; Figure 5C) relative to PBS that were significant. ETC subunit contents did not change in diaphragm at 4 weeks relative to PBS (Figure 5D).

Mitochondrial respiratory control by ADP is greater in both muscles by 4 weeks of tumour development despite early reductions in the quadriceps

We determined if the central role of ADP in stimulating respiration was impaired in both quadriceps and diaphragm at 2 and 4 weeks after subcutaneous implantations of C26 cancer cells. We stimulated complex I with NADH generated by pyruvate (5mM) and malate (2mM) across a range of ADP concentrations to challenge mitochondria with a spectrum of metabolic demands. The ADP titrations were repeated with (+Creatine) and without (-Creatine) 20mM creatine to model the two main theoretical mechanisms of energy transfer from mitochondria to cytosolic compartments that utilize or bypass mitochondrial creatine kinase (mtCK) respectively (Figure 6).
Briefly, the +Creatine system stimulates mitochondria to export phosphocreatine (PCr) whereas the -Creatine condition drives ATP export.

In both the -Creatine and +Creatine conditions, pyruvate/malate-supported ADP-stimulated respiration normalized per mass of fibre bundles (not corrected for ETC subunit content) was lower 2 weeks after C26 implantations in the quadriceps compared to PBS with a main effect across all ADP concentrations with or without creatine (Figure 7A, B). The general reduction in respiration for C26 normalized per mass of fibre bundle was also seen when data were normalized to ETC subunit content (Figure 7C, D). This suggests respiratory control was reduced within mitochondria due to an inherent property of the ETC not related to ETC abundance. We also evaluated if creatine sensitivity was altered in the C26 tumour-bearing muscle by calculating the +Creatine/-Creatine respiratory ratio. This creatine sensitivity index is a measure of the ability of creatine to stimulate respiration by accelerating matrix ADP/ATP cycling and represents coupling of the creatine kinase system to ATP generation (Figure 6), particularly at sub-maximal ADP concentrations (21, 22) which we have reported previously (17, 18). In quadriceps, creatine sensitivities at 100μM and 500μM ADP were unchanged at 2 weeks in C26 vs PBS at this time point (Figure 7E, F). Collectively, these findings indicate respiration was reduced to similar extents in both -Creatine and +Creatine conditions of energy exchange between mitochondria and cytoplasmic compartments.

These early decrements in quadriceps respiration in C26 at 2 weeks were reversed by 4 weeks. This apparent compensation was seen in both -Creatine and +Creatine conditions. Specifically, respiration was similar to PBS control mice at 4 weeks when normalized per mass of fibre bundle
(Figure 7G, H) and higher than controls when normalized to ETC subunit protein content (Figure 7I, J) despite reductions in ETC content as described above (Figure 5C). These findings suggest mitochondria in quadriceps are highly plastic and can super-compensate by upregulating their responsiveness to ADP to levels exceeding PBS controls. Additionally, at 4 weeks, quadriceps mitochondrial creatine sensitivity was impaired in C26 relative to PBS when considering respiration normalized to ETC subunit content given the ratio did not exceed a value of 1.0 which indicates that creatine could not stimulate respiration above the level elicited by ADP alone (Figure 7L). Thus, while C26 cancer strongly increased ADP-stimulated respiration by 4 weeks (Figure 7I), it compromised the coupling of creatine kinase energy transfer, suggesting that this system did not contribute to restored force at this time point (Figure 4B).

In the diaphragm, respiration was similar between C26 and PBS at 2 weeks (Figure 8A-F) but was greater in C26 compared to PBS at 4 weeks in both the -Creatine and +Creatine conditions (Figure 8G-J). This upregulation by 4 weeks occurred despite no changes in ETC subunit content as noted above (Figure 5D) which suggests mitochondria increase their responsiveness to ADP through mechanisms that may be independent of mitochondrial content. No changes in creatine sensitivity were observed in C26 vs PBS at 4 weeks (Figure 8K, L) suggesting that coupling of creatine kinase to ATP generation was maintained, in contrast to impaired creatine sensitivity seen in the quadriceps as noted above. Lastly, there was a significant interaction whereby respiration was greater in C26 vs PBS at 5000µM and 7000µM ADP when normalized per mass of fibre bundles (Figure 8G, H) and at all ADP concentrations except 25µM and 100µM when normalized to total ETC subunit content (Figure 8I, J).
These alterations were specific to pyruvate/malate-supported ADP-stimulated respiration as there were no changes in respiration in response to glutamate (further NADH-generation) and succinate (FADH2) generation when comparing C26 to PBS at either time point (Figure S1, S2). By 2 weeks of C26, diaphragm showed a decrease in State II respiration (no ADP, supported by pyruvate/malate; Figure S1, S2) which is generally used as a marker of respiration driven by proton leak into the matrix from the inner membrane space through various sites that are not coupled to ATP synthesis (23). However, State II respiration was greater than control by 4 weeks in both muscles suggesting greater uncoupling at occurs as cancer progresses (Figure S2). Lastly, changes in respiration noted above did not result in changes to the phosphorylation of AMPK in C26 relative to PBS at either 2- or 4-week timepoints (Figure S3); albeit increases in AMPK and the p-AMPK/AMPK ratio were trending in the C26 (4wk) group in the quadriceps.

H2O2 emission is increased in diaphragm early during tumour development and restored to normal by 4 weeks but is unaffected in quadriceps

We stimulated complex I with pyruvate (10mM) and malate (2mM) to generate NADH in the absence of ADP to elicit mH2O2 emission and determined ADP’s ability to attenuate this emission as occurs naturally during oxidative phosphorylation (see schematic representation, Figure 6). At 2 weeks following C26 implantations, quadriceps mH2O2 emission was similar to PBS controls under maximal emission conditions (no suppression by ADP, State II; Figure 9A, C) and during suppression by ADP (Figure 9B, D). By 4 weeks of C26 growth, quadriceps mH2O2 was lower than PBS in both maximal and ADP-suppressive states (Figure 9E, F). However, when mH2O2 was normalized to total ETC subunit content, no differences were observed between C26 and PBS (Figure 9G, H). This finding suggests eventual decreases in quadriceps mH2O2 by 4 weeks were
related to decreased ETC subunit content as shown in Figure 4. Due to limited tissue availability, pyruvate-supported mH$_2$O$_2$ was assessed only in the +Creatine condition.

In contrast to the lower mH$_2$O$_2$ in quadriceps, diaphragm mH$_2$O$_2$ was greater in C26 mice at 2 weeks relative to PBS in the presence of ADP despite no change in maximal mH$_2$O$_2$ (Figure 9I, J). This finding reveals C26 induces early elevations in diaphragm mH$_2$O$_2$ that are likely due to a specific impairment in the ability of ADP to attenuate H$_2$O$_2$ emission. Moreover, when mH$_2$O$_2$ emission was normalized to total ETC subunit content at 2 weeks, maximal mH$_2$O$_2$ emission remained unchanged (Figure 9K), while the higher emissions in the presence of ADP did not reach significance (Figure 9L) but mirrored patterns observed when normalized to wet mass of tissue as noted above. At 4 weeks, there were no differences in diaphragm mH$_2$O$_2$ under maximal or submaximal (presence of ADP) conditions using either normalization approach (Figure 9M-P) suggesting diaphragm mitochondria are plastic and can eventually restore mH$_2$O$_2$ to normal levels.

Succinate-supported mH$_2$O$_2$ emission generally did not change in either muscle in C26 vs PBS at either time point (Figure S4). This finding suggests reverse electron flux to Complex I from Complex II (23) was not altered by C26 cancer, and the responses mentioned above using pyruvate/malate reveal a specific alteration in mH$_2$O$_2$ emission supported by forward electron flux through Complex I.

**Inhibition of mitochondrial ATP synthase directly impairs force production in diaphragm**

These observations suggest mitochondrial stress contributes to early weakness at 2 weeks in both muscles, and that restoration of force in quadriceps by 4 weeks is related to a natural compensatory
increase in mitochondria respiratory control. In order to establish a direct link between mitochondria and force production, we designed an in-vitro experiment whereby we used oligomycin to inhibit ATP synthase in diaphragm strips from of healthy CD2F1 mice. Within each strip, force-frequency relationships were assessed in standard assay media (CTRL) (Figure 10A). Strips were then incubated with 0.2% ethanol (Vehicle) while relaxing in the apparatus for 40 minutes. A 2nd force-frequency assessment was then performed which demonstrated no effect of the vehicle (Figure 10A). We then repeated this experimental design on separate muscle strips but in the presence of 10μM oligomycin during the 40-minute incubation after a control stimulation. There was a main effect whereby oligomycin lowered force production (Figure 10B). Likewise, a main effect of lower force was exhibited within the oligomycin condition when the change in force production relative to control strips was analyzed. (Figure 10C).

Reduced, oxidized and total glutathione are decreased in quadriceps after 4 weeks of tumour-bearing despite an increase in the reduced-oxidized glutathione ratio

Noting the mH₂O₂ patterns were also time- and muscle-dependent, we then determined whether there was a corresponding impact on cellular redox state by assessing the equilibrium of the glutathione redox buffer. At 2 weeks, there were no differences in reduced glutathione (GSH), oxidized glutathione (GSSG), GSH:GSSG ratio and total glutathione in the either muscle (Figure 11A-D, I-L. At 4 weeks, quadriceps demonstrated a lower content of reduced, oxidized and total glutathione but interestingly there was an increase in the GSH:GSSG ratio (Figure 11E-H). No changes in any measure were observed in diaphragm at 4 weeks. (Figure 11M-P).
Certain indices of skeletal muscle mitochondrial stress have been associated with cancer cachexia in various mouse models (11–13, 24, 25), but the time- and muscle-dependent relationship remains unclear. Moreover, the early relationships between force production and atrophy have not been understood. Here, we demonstrate how quadriceps and diaphragm have both common and distinct time-dependent responses to cancer in the C26 colon carcinoma mouse model of cancer cachexia.

First, a reduction in specific muscle force was observed prior to atrophy in both muscles, yet an eventual increase in specific force production to control levels by 4 weeks occurred only in quadriceps. This restoration of specific force indicates an apparent intrinsic compensation given absolute force was lower at this later timepoint due to atrophy. Second, atrophy in most fibre types was preceded by altered mitochondrial bioenergetics but the individual relationship differed between muscles with decreases in respiration occurring in quadriceps in contrast to elevated mH₂O₂ emission in diaphragm. Third, both muscles upregulated mitochondrial respiration supported specifically by pyruvate and malate substrates at 4 weeks which may reflect a hormetic adaptation to maintain energy homeostasis during cachexia. Likewise, the diaphragm restored Complex I-supported mitochondrial H₂O₂ emission to normal lower levels by 4 weeks which demonstrates the transient nature of this potential redox pressure.

Collectively, these findings suggest muscle weakness can occur before atrophy during C26 cancer, and this progression is related to dynamic time-dependent changes in mitochondrial bioenergetics that are unique to each muscle.
Mitochondrial bioenergetic alterations and skeletal muscle force reductions precede skeletal muscle atrophy

Work from Brown et al. suggested mitochondrial degradation and dysfunction precedes muscle atrophy in the LLC xenograft mouse model of cancer cachexia (12). In this study, atrophy markers occurred after earlier indices of mitochondrial degeneration in comparator muscles (flexor digitorum brevis and plantaris) including mitochondrial degradation, respiratory control ratios and H$_2$O$_2$ emission. The findings of the present study support this proposal with a comparison of atrophy, mitochondrial respiration and mH$_2$O$_2$ emission within the same muscle types, namely quadriceps and diaphragm. These findings also extend the proposal by showing muscle-specific mitochondrial alterations occur concurrent to muscle weakness and before atrophy. Specifically, early decreases in respiratory kinetics in quadriceps were not seen in diaphragm suggesting more oxidative muscle might avoid such respiratory decrements. Conversely, early increases in mH$_2$O$_2$ emission seen in diaphragm did not occur in quadriceps. These relationships suggest targeted therapies to counter mitochondrial alterations during cancer cachexia could consider the specific bioenergetic function that is altered at precise timepoints in each muscle type.

This relationship between early mitochondrial stress prior to atrophy in both muscles becomes further complicated when considering force production. Muscle weakness occurred at 2 weeks in both muscles before atrophy which highlights a shared pattern in the progression of muscle dysfunction during cancer. While the purpose of this investigation was not to address other mechanisms regulating force production, reduced fibre sizes cannot be an explanation given atrophy did not occur until after weakness was first observed. In this regard, the reduction in absolute force in quadriceps at 2 weeks was therefore due to the lower specific force. However,
the distinct mitochondrial signatures in both muscles at 2 weeks could guide additional questions. For example, in the quadriceps, the early reductions in specific force were associated with early decreases in mitochondrial respiratory control by ADP. When specific force production was restored to control levels by 4 weeks, respiration actually increased above control levels when normalized to ETC subunit content. This dynamic relationship is intriguing and suggests early quadriceps weakness might be due to impairments in mitochondrial energy provision that is nonetheless plastic and capable of adapting – possibly as a hormetic response to the earlier respiratory deficiency – to correct this weakness through super-compensations in energy supply.

In contrast, the diaphragm weakness seen at 2 weeks might be linked to an early redox pressure given elevated mH₂O₂ was observed. This observation is consistent with prior observations of early and transient increases in H₂O₂ emission in diaphragm in the LLC mouse model of cancer cachexia (24). However, these changes in mH₂O₂ did not alter glutathione in reduced or oxidized forms nor in the static measure of GSH:GSSG equilibrium. While these common measures of glutathione redox buffering are often used to interpret changes in the redox proteome (26), the measures do not necessarily capture the kinetic aspect by which GSH is recycled through glutathione reductases and glutathione peroxidases. As such, a lack of change in these variables does not rule out a signaling role for H₂O₂ in mediating the diaphragm’s adaptive response as one possibility may be that the diaphragm was capable of sustaining greater glutathione recycling rates. These lysate-based measures also do not rule out changes in glutathione-mediated thiol oxidation in specific sub-compartments of the cell including mitochondrial or nuclear fractions (26–28). Likewise, mitochondrial H₂O₂ can also oxidize peroxiredoxins through a thioredoxin-mediated recycling mechanism, the relevance of which to muscle adaptation during diseases is only now
being unravelled in more detail (29). Consideration of such complexities in redox signaling, much of which is H$_2$O$_2$-dependent, demonstrates how no single assay can capture the impact of mH$_2$O$_2$ on redox signaling-mediated adaptive responses yet exemplifies the considerable research required to further explore the impact of the mH$_2$O$_2$ relationships to cancer cachexia reported in this and other studies (12). Lastly, the degree to which mH$_2$O$_2$ influences mitochondrial respiratory control must also be considered more in-depth. For example, the diaphragm did not show reductions in respiration at 2 or 4 weeks yet demonstrated an unexpected increase in respiration at 4 weeks. The explanation for this increase is not apparent but might suggest an earlier energetic deficiency occurred outside of our selected time points. Alternatively, the use of pyruvate as a glucose-derived substrate to stimulate respiration does not rule out a possible change in substrate selection that may be occurring as part of grander adaptive processes. Future studies could compare both pyruvate and fatty acid substrates to determine if such responses are ubiquitous across all substrate oxidation pathways.

The mechanisms by which mitochondria contribute to weakness and atrophy continue to be an area of considerable research and cannot be resolved by a single investigation. However, we attempted to resolve whether mitochondrial stress can acutely suppress force production using an in vitro approach that is removed from potential systemic responses to cancer that might also contribute to weakness. As demonstrated in Figure 10, acute treatment of diaphragm muscle with the ATP synthase inhibitor oligomycin decreased specific force production. Oligomycin inhibits ATP synthesis but theoretically may also increase mitochondrial reactive oxygen species through increased membrane potential-related electron slip (23). As this cannot be verified during contraction in vitro using our system, the findings nonetheless demonstrate that mitochondrial
stress can acutely depress specific force production. This observation matches previous findings of various mitochondrial inhibitors on single fibre contraction performed \textit{in vitro} (30). Likewise, in cultured myotubes, a mitochondrial-targeted antioxidant attenuated cisplatin (chemotherapy) induced atrophy by preventing increases in mitochondrial reactive oxygen species (31). Similarly, a mitochondrial-targeted peptide administered to the same C26 cancer cachexia model used in the present study preserved diaphragm force production and fiber cross sectional area by preventing changes in indices of mitochondrial ATP synthesis and reactive oxygen species (13). By comparing this prior literature to the time-dependent and muscle-specific responses to C26 cancer in the present study, the dynamic relationship between weakness, atrophy and mitochondrial stress-specific responses could now be explored with such mitochondrial enhancing compounds using a design that captures time-dependent responses across muscle types.

\textbf{Perspectives on the potential for mitochondrial hormesis in quadriceps and diaphragm}

The findings of lower respiration and increased mH$_2$O$_2$ emission at 2 weeks is consistent with prior reports at various time points and muscle in the LLC, C26 and peritoneal carcinosis mouse models (11–13, 24, 25). To our knowledge, the eventual increase in pyruvate-supported respiration seen in both quadriceps and diaphragm in the present study is novel, while, the attenuation of mH$_2$O$_2$ emission seen in the diaphragm is consistent with past reports in the plantaris (12) and diaphragm (24) in the LLC mouse model of cachexia. As noted above, mitochondrial respiratory control by ADP increased above control in both muscles despite a stress being observed earlier only in quadriceps. We questioned whether this early reduction in respiration represented an energy crisis triggering compensatory signaling through the energy sensor AMPK – a pathway that triggers compensatory mitochondrial biogenesis or upregulation of substrate-specific oxidation (32). We
did not observe an effect of cancer on AMPK phosphorylation at either time point (Figure S3), although there was a trend in the quadriceps at 4 weeks of tumour bearing whereby AMPK content was increased. Nonetheless, these results do not rule out the potential for AMPK activation at other time points or a more sustained phosphorylation of downstream AMPK- targets. There are also multiple feedback control systems linking metabolic stress to respiratory control that are independent of AMPK which might be considered in future investigations (33).

The design of substrate titration protocols lends insight into the specific mechanisms by which respiration and mH₂O₂ become altered during cancer. For example, as pyruvate/malate were used as the substrates to generate NADH to stimulate complex I-supported respiration, future investigations might consider the potential for cancer to upregulate complex I or pyruvate dehydrogenase activity, albeit maximal activity given saturating pyruvate concentrations were used. Also, the consistent increase in respiration across a wide spectrum of ADP concentrations by 4 weeks in both muscles suggests mitochondrial responsiveness to a wide range of metabolic demands may have been enhanced such that key regulators of matrix ADP/ATP cycling could be considered for future directions (Figure 6). ADP was also more effective at attenuating mH₂O₂ (23) in quadriceps by 4 weeks (Figure 9) which supports this possibility. Collectively, these findings suggest cancer disrupts mitochondrial bioenergetics by specifically desensitizing mitochondria to ADP in both muscles.

Mitochondrial creatine metabolism appeared to be less capable of adapting in quadriceps by 4 weeks (Figure 7L) suggesting mitochondrial creatine kinase-dependent phosphate shuttling is more affected in this muscle than diaphragm which showed no such deficiency. In fact, the
creatine-independent (-Creatine) system showed homogeneous plasticity by upregulating in both
muscles by 4 weeks while the creatine-dependent system upregulated only in the diaphragm. These
findings suggest mitochondrial creatine metabolism may be disrupted in quadriceps muscle during
cancer which may impact energy homeostasis given the importance of this system in certain
muscles (21).

In general, the diaphragm appeared to be superior to quadriceps with respect to maintaining
mitochondrial ETC content markers and respiratory control by ADP at 2 weeks with evidence of
super-compensation in respiratory function at 4 weeks. Furthermore, reductions in ETC protein
contents were observed in quadriceps at 4 weeks after C26 implantation whereas no changes were
observed in diaphragm. This resilience of diaphragm appears to be a unique finding given prior
reports have also shown lower mitochondrial protein markers from various pathways and muscle
types in LLC and APC^{Min/+} mouse models of cancer cachexia (10, 12). While the mechanisms for
this muscle heterogeneity remain unclear, one possibility relates to muscle contractile activity. The
diaphragm constantly contracts in vivo whereas quadriceps is used during locomotion. As
mitochondrial content and substrate oxidation are regulated by contractile activity (33), future
directions might consider whether the diaphragm holds a special mitochondrial ‘resistance’ to
cancer with respect to energy homeostasis which might support the growing notion of chronic
contractile activity in improving muscle health during cancer (34).

In conclusion, this investigation reports muscle weakness precedes atrophy in quadriceps and
diaphragm in the C26 colon carcinoma mouse model of cancer cachexia. This progression was
associated with heterogenous muscle-specific and time-dependent mitochondrial responses in both
muscles. Specifically, an early energetic stress (reduced respiratory control by ADP) was more apparent in quadriceps in contrast to a mitochondrial redox pressure in diaphragm. These early mitochondrial stressors were seemingly corrected as cancer progressed despite the development of atrophy in both muscles and a unique increase in specific force production in quadriceps. This apparent intrinsic compensation in quadriceps may have partially mitigated the contribution of atrophy to the reduced absolute force seen at 4 weeks. Moreover, C26 cancer caused a unique impairment in the coupling of the mitochondrial creatine kinase system to ATP generation in quadriceps whereas this system was not affected in diaphragm. This dynamic plasticity across time demonstrates how the effects of cancer on one muscle may not predict the response in another muscle type. The findings also highlight how understanding heterogeneity may identify mechanisms that determine whether a given muscle might be sensitive, or resistant, to cancer cachexia.
Methods

Animal Care

48 eight-week-old male CD2F1 mice were purchased from Envigo (Indianapolis, USA). Upon arrival, mice were housed and given a minimum of 72 h to acclimatize before cancer implantations. All mice were provided access to standard chow and water ad libitum. While food intake was not measured, reductions in food intake reported in this model of cachexia did not contribute to reduced muscle weights, fibre cross sectional area or muscle force given pair-fed mice retained normal muscle parameters compared to C26 mice (35, 36). Mice were monitored daily for general well-being, tumour ulcerations and tumour size. If mice demonstrated signs of extreme distress, mice would be sacrificed as soon as possible, however, this was never required.

C26 Cell Culture and Tumour Implantation

C26 cancer cells (Purchased from NCI – Frederick, MD USA) were plated at passage 2-3 in T-75 flasks in DMEM supplemented with 10% foetal bovine serum plus 1% penicillin and streptomycin. Once confluent, cells were trypsinized, counted and diluted in PBS. C26 cells (5 x 10^5) suspended in 100 μL sterile PBS were implanted subcutaneously to both flanks of mice at 8 weeks of age (11). For control, mice received identical subcutaneous injections of 100 μL sterile PBS and aged for 2 weeks (PBS (2wk); n= 8) and 4 weeks (PBS (4wk); n= 16). Tumours developed for 14-17 days (C26 (2wk); n= 8) and 26-29 days (C26 (4wk); n= 16). Tumours were measured daily, recording the length and width of tumours with digital calipers using the following formula to obtain tumour volume (volume of a sphere): \(\frac{4}{3}\pi\frac{(length/2)^2\cdot(width/2)}{2}\) in accordance with York University Animal Care Committee guidelines. The same investigator was responsible for measuring tumour sizes throughout the length of the study as preliminary work demonstrated that
tumour size measurements can vary between individuals (data not shown; CV – 7.2% between 3 individuals, CV – 1.3 within designated individual).

Surgery Procedure

Quadriiceps, soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitorum longus and spleen were quickly collected under isoflurane anesthesia prior to euthanasia. Tissues were weighed and snap-frozen in liquid nitrogen and stored at -80°C. Quadriiceps and diaphragm muscles were placed in BIOPS containing (in mM) 50 MES Hydrate, 7.23 K2EGTA, 2.77 CaK2EGTA, 20 imidazole, 0.5 dithiothreitol, 20 taurine, 5.77 ATP, 15 PCr, and 6.56 MgCl2·6 H2O (pH 7.1) to be prepared for mitochondrial bioenergetic assays. Quadriiceps from one leg and diaphragm strips were harvested for mitochondrial bioenergetic assays while the quadriiceps from the contracted leg and a separate diaphragm strip were used for force measurements. The diaphragm strip used for force measurements was cut within 30 seconds of the entire diaphragm being placed in BIOPS prior to transferring the strip to Ringer’s solution as noted below.

In Situ Quadriiceps Force and In Vitro Diaphragm Force

In situ force production for quadriiceps muscle was partially adapted from previous literature (37). Mice were anesthetized with isoflurane and shaved of all hair on their hindlimb. An incision was made above the patella to expose the femoral tendon which was then tightly secured with suture. Once the knot was in place, the tendon was carefully severed, and the suture was attached to an Aurora Scientific 305C muscle lever arm with a hook (Aurora, Ontario, Canada). The knee was secured with a vertical knee clamp immobilizing the knee joint with a 27G needle. Contraction of the quadriiceps was controlled through percutaneous stimulation of the femoral nerve anterior to
the hip joint. Optimal resting length (L₀) was determined using single twitches (pulse width = 0.2ms) at varying muscle lengths. Once L₀ was established, force as a function of stimulation frequency was measured during 8 isometric contractions at varying stimulation frequencies (1, 20, 40, 60, 80, 100, 120, 140 Hz). The quadriceps muscle was then weighed and used for normalization of force production (‘specific force’).

In vitro force production for diaphragm muscle was partially adapted from previous literature (38). Briefly, the diaphragm strip used for force production was placed in a petri dish of ~25°C Ringer’s solution containing (in mM): 121 NaCl, 5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaHPO₄, 24 NaHCO₃, 5.5 glucose and 0.1 EDTA; pH 7.3 oxygenated with 95% O₂ and 5% CO₂. Diaphragm strips were cut from the central region of the lateral costal hemidiaphragm. Silk suture was tied to the central tendon as well the ribs, and the preparation was transferred to an oxygenated bath filled with Ringer solution, maintained at 25°C. The suture secured to the central tendon was then attached to a lever arm while the suture loop secured to the ribs was attached to a force transducer. The diaphragm strip was situated between flanking platinum electrodes driven by a biphasic stimulator (Model 305C; Aurora Scientific, Inc., Aurora, ON, Canada). Optimal L₀ was determined using twitches (pulse width = 0.2ms) at varying muscle lengths. Once L₀ was established, force as a function of stimulation frequency was measured during 10 isometric contractions at varying stimulation frequencies (1, 10, 20, 40, 60, 80, 100, 120, 140, 200 Hz). Force production was normalized (‘specific force’) to the calculated cross-sectional area (CSA) of the muscle strip (m/l*d) where m is the muscle mass, l is the length, and d is mammalian skeletal muscle density (1.06mg.mm⁻³). Absolute force for whole muscle was reported for quadriceps but not diaphragm given the latter was assessed with muscle strips.
Mitochondrial Bioenergetic Assessments

Preparation of Permeabilized Muscle Fibres. The assessment of mitochondrial bioenergetics was performed as described previously in our publications (17, 19, 32). Briefly, the quadriceps and diaphragm from the mouse was removed and placed in BIOPS. Muscle was trimmed of connective tissue and fat and divided into small muscle bundles (~1.2 – 3.7 mg wet weight for quadricep and 0.6 – 2.1 mg for diaphragm). Each bundle was gently separated along the longitudinal axis to form bundles that were treated with 40 µg/mL saponin in BIOPS on a rotor for 30 min at 4°C. Following permeabilization, the permeabilized muscle fibre bundles (PmFB) for respiration assessments were blotted and weighed in ~ 1.5mL of tared pre-chilled BIOPS (muscle relaxing media) to ensure PmFB remained relaxed and hydrated rather than exposed to open air. Wet weights were used given small pieces of muscle can detach during respirometry assessments, albeit greatly reduced by blebbistatin (described below). Mean ± SEM wet weights (mg) were 2.4 ± 0.07 for quadriceps and 1.3 ± 0.04 for diaphragm. The remaining PmFB for mH2O2 were not weighed at this step as this data was normalized to fully recovered dry weights taken after the experiments. All PmFB were then washed in MiRO5 on a rotator for 15 minutes at 4°C to remove the cytoplasm. MiRO5 contained (in mM) 0.5 EGTA, 10 KH2PO4, 3 MgCl2•6H2O, 60 K-lactobionate, 20 Hepes, 20 Taurine, 110 sucrose, and 1 mg/ml fatty acid free BSA (pH 7.1).

Mitochondrial Respiration. High-resolution O2 consumption measurements were conducted in 2 mL of respiration medium (MiRO5) using the Oroboros Oxygraph-2k (Oroboros Instruments, Corp., Innsbruck, Austria) with stirring at 750 rpm at 37°C. MiRO5 contained 20 mM Cr to saturate mitochondrial creatine kinase (mtCK) and promote phosphate shuttling through mtCK or was kept void of Cr to prevent the activation of mtCK (40) as described in Figure 5. For ADP-
stimulated respiratory kinetics, our previously published procedures to stimulate complexes I and II-supported respiration were employed (17–19). 5 mM pyruvate and 2 mM malate were added as complex I-specific substrates (via generation of NADH to saturate electron entry into complex I) followed by a titration of sub-maximal ADP (25, 100 and 500 μM) and maximal ADP (up to 5000 μM in the presence of Cr or 30000 μM in the absence of Cr). 25 μM and 100 μM are close to low and high points of previous estimates of free ADP concentrations in human skeletal muscle in resting and high intensity exercise states and therefore allow the determination of mitochondrial responsiveness to a physiological spectrum of low to high energy demands (41–45). Saturating [ADP] were different depending on the muscle and presence or absence of creatine in the experimental media. Mitochondrial respiration was normalized to mass of fibre bundles as well as total content of ETC subunits detected with the antibody cocktail listed below. This normalization was performed on a separate piece of the same muscle from the same mouse to evaluate whether changes in respiration per mass were due to alterations in mitochondrial content or intrinsic mitochondrial respiratory responses. While normalizing bioenergetic fluxes to ETC content markers within the same bundle would theoretically reduce variability, the occasional separation of 1-2 fibres in chambers after an experiment may cause under-estimations of total bundle ETC marker contents.

Kmapp for creatine to ADP was not established as we have observed that many permeabilized fibres from past studies do not fit Michaelis-Menten kinetics with these assay conditions (low to modest R²). Creatine accelerates matrix ADP/ATP cycling at submaximal [ADP] and lowers the Kmapp for ADP in some muscles (21, 33). Therefore, in order to evaluate mitochondrial creatine sensitivity, 100 and 500 μM ADP were used to calculate a creatine sensitivity index. Following
the ADP titration, cytochrome c was added to test for mitochondrial membrane integrity. Finally, succinate (20 mM) was then added to saturate electron entry into Complex II.

All experiments were conducted in the presence of 5 μM blebbistatin (BLEB) in the assay media to prevent spontaneous contraction of PmFB, which has been shown to occur in response to ADP at 37°C that alters respiration rates (40, 46). Polarographic oxygen measurements were acquired in 2 second intervals with the rate of respiration derived from 40 data points and expressed as pmol/s/mg wet weight. PmFB were weighed in ~1.5 mL of tared BIOPS to relax muscle as noted above.

Mitochondrial H$_2$O$_2$ Emission (mH$_2$O$_2$). mH$_2$O$_2$ was determined spectrofluorometrically (QuantaMaster 40, HORIBA Scientific, Edison, NJ, USA) in a quartz cuvette with continuous stirring at 37°C, in 1 mL of Buffer Z supplemented with 10 μM Amplex Ultra Red, 0.5 U/ml horseradish peroxidase, 1mM EGTA, 40 U/ml Cu/Zn-SOD1, 5 μM BLEB and 20mM Cr. Buffer Z contained (in mM) 105 K-MES, 30 KCl, 10 KH$_2$PO$_4$, 5 MgCl$_2$ • 6H$_2$O, 1 EGTA, and 5mg/mL BSA (pH 7.4). State II mH$_2$O$_2$ (maximal emission in the absence of ADP) was induced using the Complex I-supporting substrates (NADH) pyruvate (10mM) and malate (2mM) to assess maximal (State II, no ADP) mH$_2$O$_2$ as described previously (18). Following the induction of State II mH$_2$O$_2$, a titration of ADP was employed to progressively attenuate mH$_2$O$_2$ as occurs when membrane potential declines during oxidative phosphorylation (Figure 5). After the experiments, the fibres were rinsed in double deionized H$_2$O, lyophilized in a freeze-dryer (Labconco, Kansas City, MO, USA) for > 4h and weighed on a microbalance (Sartorius Cubis Microbalance, Gottingen
Germany). The rate of mH$_2$O$_2$ emission was calculated from the slope (F/min) using a standard curve established with the same reaction conditions and normalized to fibre bundle dry weight.

**Western Blotting**

A frozen piece of quadriceps and diaphragm from each animal was homogenized in a plastic microcentrifuge tube with a tapered Teflon pestle in ice-cold buffer containing (mm) 20 Tris/HCl, 150 NaCl, 1 EDTA, 1 EGTA, 2.5 Na$_4$O$_7$P$_2$, 1 Na$_3$VO$_4$, 1% Triton X-100 and PhosSTOP inhibitor tablet (Millipore Sigma, Burlington, MA, USA) (pH 7.0) as published previously (47). Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Life Technologies, Carlsbad, CA, USA). 15-30 μg of denatured and reduced protein was subjected to 10-12% gradient SDS-PAGE followed by transfer to low-fluorescence polyvinylidene difluoride membrane. Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Lincoln NE, USA) and immunoblotted overnight (4°C) with antibodies specific to each protein. A commercially available monoclonal antibody was used to detect electron transport chain proteins (rodent OXPHOS Cocktail, ab110413; Abcam, Cambridge, UK, 1:250 dilution), including V-ATP5A (55kDa), III-UQCRC2 (48kDa), IV-MTCO1 (40kDa), II-SDHB (30 kDa), and I-NDUFB8 (20 kDa).

Commercially available polyclonal antibodies were used to detect AMP-activated protein kinaseα (AMPKα) (rabbit, CST, 2532; 62kDa; 1:1000) and Phospho-AMPKα Thr172 (P-AMPK) (rabbit CST, 2535, 62kDa; 1:500) as used previously (47).

After overnight incubation in primary antibodies, membranes were washed 3x5 minutes in TBST and incubated for 1 hour at room temperature with the corresponding infrared fluorescent secondary antibody (LI-COR IRDye 680nm or 800nm) at a dilution previously optimized (1:20
Immunoreactive proteins were detected by infrared imaging (LI-COR CLx; LI-COR) and quantified by densitometry using ImageJ. All images were normalized to Amido Black total protein stain (A8181, Sigma) using the entire lane corresponding to each sample.

**Immunofluorescence Analysis**

Quadriceps (cut longitudinally to include half the vastus intermedius & full vastus lateralis) and diaphragm muscle samples embedded in O.C.T medium (Fisher Scientific) were cut into 10-μm-thick sections with a cryostat (HM525 NX, Thermo Fisher Scientific, Mississauga, ON, Canada) maintained at -20°C. Muscle fibre type was determined as previously described (48), with minor modifications. All primary antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa), and secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). Briefly, slides were blocked with 5% goat serum (Sigma Aldrich) in PBS for 1 hour at room temperature. Next, slides were incubated with primary antibodies against myosin heavy chain (MHC) I (BA-F8; 1:25), MHC IIA (SC-71; 1:1000) and MHC IIB (BF-F3; 1:50) for 2 hours at room temperature. Afterwards, slides were washed 3x in PBS for 5 minutes and then incubated with secondary antibodies (MHCI; Alexa Fluor 350 IgG2b; 1:1000), (MHCIIa; Alexa Fluor 488 IgG1; 1:1000), (MHC Iib; Alexa Fluor 568 IgM; 1:1000) for 1 hour at room temperature. Slides were then washed 3x in PBS for 5 minutes and mounted with ProLong antifade reagent (Life Technologies, Burlington, ON, Canada). Images were acquired the next day using EVOS FL Auto 2 Imaging System (Invitrogen, Thermo Fisher Scientific, Mississauga, ON, Canada). Individual images were taken across the entire cross section and then assembled into a composite image. 20-30 muscle fibres per fibre type were selected randomly throughout the cross section and traced.
with ImageJ software to assess CSA after calibrations with a corresponding scale bar. Muscle fibres that appeared black were recorded as MHC IIX (48).

**Real-time RT-PCR analyses of mRNA**

Measurement of mRNA was completed as previously described (49). Briefly, frozen tissues were homogenized with TriZol agent (Life Technologies, Cat# 15596026) and RNA isolated with a commercial kit. RNA concentrations were quantified with a Biotek Take3 micro-volume microplate with a BioTek PowerWave XS microplate reader (BioTek Instruments Inc., Winooski, VT). RNA was only used if the sample was of sufficient quality (260/280 ratio >2). Isolated RNA was then reverse transcribed into cDNA using commercial reagents (Superscript Vilo, Cat#11755500; Life Technologies). cDNA was then serially diluted to 1:100 and used for quantitative real time PCR analysis. cDNA was combined with appropriate probes, master mix and amplified in a reaction of 40 cycles of denaturation, annealing, and extension at 95, 60, and 72°C. Data were quantified with the ΔΔCt method. Data were normalized to control groups. 18s was used as an internal housekeeping gene (clone #Mm03928990_g1) and did not differ between groups. Probes of interest included: Atrogin/FBXO32 (Clone #Mm00499523_m1), Murf1/Trim63 (Clone #Mm01185221_m1), Gadd45a (Clone # Mm00432802_m1), and Ubc (Clone #Mm02525934_g1).

**Glutathione Analysis**

Glutathione was measured as previously published (18,20). Briefly, reduced (GSH) and oxidized (GSSG) glutathione were measured using the Shimadzu Nexera X2 UHPLC system (Mandel Scientific Company Inc. Guelph, Canada). Quadriceps and diaphragm were homogenized in
50mM Tris buffer with 10mM boric acid, 2mM L-serine, 20 µM acivicin, and 5 mM N-ethylamide, and acidified using TCA (for GSH) and PCA (for GSSG). Samples were separated with a Zorbax C18 column (Agilent Technologies, Mississauga, ON, Canada). GSH was assessed by UV-HPLC (265 nm wavelength) monitoring of NEM-GSH using a 0.25% glacial acetic acid mobile phase with 6% acetonitrile at 1.05ml/min flow rate detected at 265nm. GSSG was assessed by fluorescent-HPLC (excited at 350nm and detected at 420nm emission) using HPLC/UHPLC fluorescence detector (Mandel scientific). GSSG samples were diluted in 0.5M NaOH and run using 25mM Na₂HPO₄ in HPLC-grade water with 15% methanol mobile phase at a 0.5mL/min flow rate, by tracking O-pthalymide (OPA) tagged GSH.

Statistics

Results are expressed as mean ± SD. The level of significance was established at $P < 0.05$ for all statistics. The D’Agostino – Pearson omnibus normality test was first performed to determine whether data resembled a Gaussian distribution, and all data was subject to the ROUT test to identify and exclude outliers. Western blot results for proteins in the electron transport chain subunit complexes I, IV and V in quadriceps failed normality as did proteins in complexes I, II, IV and V for diaphragm. In addition, quadriceps and diaphragm delta glutamate respiration failed normality and were analyzed using a non-parametric Mann-Whitney t-test. Quadriceps and diaphragm mRNA content markers at 4 weeks all failed normality (MURF1, atrogin, UBC, Gadd45a) and were assessed by a non-parametric Mann-Whitney t-test. All other data passed normality. A two-tailed unpaired t-test was used to compare C26 to PBS within each time point with respect to muscle mass, fibre cross-sectional area, remaining western blots, qPCR, and glutathione measures. A two-way ANOVA with factors of timepoint (2 vs 4 week) and treatment
(C26 vs PBS) was used for mitochondrial respiration, mH2O2 and all force-frequency experiments followed by Benjamini, Krieger and Yekutieli’s post-hoc analysis. (50) when a significant interaction was observed. All statistical analyses were performed with GraphPad Prism Software 8.4.2 (La Jolla, CA, USA).

**Study Approval**

All experiments and procedures were approved by the Animal Care Committee at York University (AUP Approval Number 2019-10) in accordance with the Canadian Council on Animal Care.

**Author Contributions**

L.J.D., C.A.B, M.R.C., N.P.G. and C.G.R.P. contributed to the rationale and study design. L.J.D., C.A.B., S.G. and C.G.R.P. conducted all experiments and/or analyzed all data. C.G.R.P. and L.J.D. wrote the manuscript. All authors contributed to the interpretation of the data and manuscript preparation. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure 1 The effects of C26 colon cancer cells implantation on body size, tumour size, muscle mass and force. Analysis of CD2F1 mice with subcutaneous C26 implantations or with PBS were performed. Body weights (A, n=8-16) and tumour-free body weights (B, n=8-16) were analyzed every week (2 wk mice were measured at a 14-17 day window and 4 wk mice were measured on a 26-29 day window). Percent change in tumour free body weights were analyzed from day 0 to end point (C, n=8-16). In vivo tumour volume measurements were made using calipers (D, n=16). Tumour mass (E, n=7-16) and spleen mass (F, n=8-16) measurements were also completed. Subcutaneous fat from the inguinal fat depot was weighed (G, n=10). Grip strength was assessed in all groups (H, n=10). Evaluation of hindlimb muscle wet weights were made in the 2-week cohort (I, n=8) and 4-week cohort (J, n=16). Results represent mean ± SD; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). One-way ANOVA was used to determine the difference between PBS(4wk) vs. C26(4wk) tumour growth. # P<0.05 PBS(2wk) vs C26(2wk); * P<0.05 PBS(4wk) vs C26(4wk); **** P<0.0001 PBS(4wk) vs C26(4wk).
**Figure 2** Evaluation of quadriceps and diaphragm fibre-type atrophy in skeletal muscle from C26 tumour-bearing mice. Analysis of fibre histology on MHC isoforms of PBS and C26 mice was performed. Cross-sectional area of MHC stains was evaluated in the quadriceps (A, n=8; B, representative image, magnification x 20) and diaphragm at 2 weeks of tumour bearing (C, n=6; D, representative image, magnification x 20). The same was completed for the quadriceps (E, n=9; F, representative image, magnification x 20) and diaphragm at 4 weeks of tumour bearing (G, n=9; H, representative image, magnification x 20). Results represent mean ± SD; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). * P<0.05 PBS (4wk) vs C26(4wk).
Figure 3 The effects of C26 colon cancer on the activation of atrophy markers in quadriceps and diaphragm muscle. mRNA content of atrophy markers muscle RING-finger protein-1 (MURF1) (A, n=10), Atrogin (B, n=10), Ubiquitin C (UBC) (C, n=10) and growth arrest and DNA damage-inducible 45α (Gdd45a) (D, n=10) were measured using qt-PCR in the quadriceps of all groups. This was also completed in the diaphragm (E-H; n=10). Results represent mean ± SD; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). # P<0.05 PBS(2wk) vs C26(2wk); * P<0.05 PBS(4wk) vs C26(4wk); *** P<0.001 PBS(4wk) vs C26(4wk); **** P<0.0001 PBS(4wk) vs C26(4wk).
Figure 4 The effects of C26 colon cancer on quadriceps and diaphragm force production. In situ quadriceps force production was assessed using the force frequency relationship (A absolute force, B normalized force to total quadriceps weight; n=6-14) and in vitro diaphragm force production was also measured using the force frequency relationship (C normalized force; absolute force is not included as the method was performed on muscle strips; n=6-12). Results represent mean ± SD; A two-way ANOVA was used to determine the difference between PBS(2wk) vs. C26(2wk) vs. PBS (4wk) vs. C26(4wk). # P<0.05 PBS(2wk) vs C26(2wk); * P<0.05 PBS(4wk) vs C26(4wk); $ P<0.05 C26 (2wk) vs C26 (4wk).
Figure 5 Muscle-specific changes in markers of electron transport chain complexes in C26 tumour-bearing skeletal muscle. Protein content of electron transport chain subunits were quantified in the quadriceps (A, n=8) and diaphragm at 2 weeks (B, n=8) and 4 weeks (C, D n=12). E, representative image for quadriceps and F, representative image for diaphragm. Results represent mean ± SD; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). * P<0.05 PBS (4wk) vs C26 (4wk).
Figure 6 Schematic representation of energy homeostasis in low metabolic (left) vs. high metabolic (right) demand states. When ADP is low, less ATP is produced. A concomitant accumulation of [H+] in the inner membrane space (IMS) increases membrane potential (∆Ψ), attenuates H+ pumping, induces premature electron slip and generates superoxide (O•−) which is dismutated to H2O2 by manganese superoxide dismutase (MnSOD; top left). Only Complex I-derived superoxide is displayed. When ADP is high, more ATP is produced as [H+] diffuse from the IMS to the mitochondrial matrix through ATP synthase. The decrease in ∆Ψ lowers premature electron slip, generating less O•− and H2O2 (top right). ADP generated by ATPases throughout the cell enter the matrix through the voltage dependent anion channel (VDAC) on the outer mitochondrial membrane (OMM) and the adenine nucleotide translocase (ANT) on the inner mitochondrial membrane (IMM; bottom left). Creatine accelerates matrix ADP/ATP cycling and ATP synthesis by reducing the diffusion distance of the slower diffusing ADP and ATP while shuttling phosphate to the cytoplasm through rapidly diffusing phosphocreatine which is used by cytosolic creatine kinase (cCK) to recycle local ATP to support the activity of various ATPases. Rapidly diffusing creatine returns to the IMS to be re-phosphorylated by mitochondrial creatine kinase (mtCK). Non-ATPase sites of ATP hydrolysis are not displayed but also contribute to net metabolic demand (kinases, and other ATP-dependent processes). The net effect of metabolic demand (global ATP hydrolysis) on matrix ADP/ATP cycling is displayed under the context of creatine independent (−Creatine) and creatine dependent (+Creatine) conditions. Figure adapted from Aliev et al., 2011, Güzün et al., 2012, Wallimann et al., 2011 and Nicholls 2013(23, 43–45). Created with BioRender.com
Figure 7 Complex I-supported mitochondrial respiration in quadriceps muscle of C26 tumour-bearing mice. ADP-stimulated (State III) respiration, supported by complex I supported (NADH) substrates pyruvate (5mM) and malate (2mM), was assessed in the absence (-Creatine) and presence (+Creatine) of 20mM creatine at a range of [ADP] until maximal respiration was achieved to model a spectrum of metabolic demands. Respiration was assessed in the quadriceps normalized to bundle size at 2 weeks (A, B) and normalized to ETC subunit content (C, D) to permit comparisons of intrinsic mitochondrial respiratory responses in each group. Creatine sensitivity was assessed by calculating the +creatine/-creatine ratio (E, F) given creatine normally increases ADP-stimulated respiration. The same measurements were completed at 4 weeks (G-L). Results represent mean ± SD; n=8-16; A two-way ANOVA was used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). # P<0.05, PBS(2wk) vs C26(2wk); * P<0.05, PBS(4wk) vs C26(4wk).
Figure 8 Complex I-supported mitochondrial respiration in diaphragm muscle of C26 tumour-bearing mice. ADP-stimulated (State III) respiration, supported by complex I supported (NADH) substrates pyruvate (5mM) and malate (2mM), was assessed in the absence (-Creatine) and presence (+Creatine) of 20mM creatine at a range of [ADP] until maximal respiration was achieved to model a spectrum of metabolic demands. Respiration was assessed in the diaphragm normalized to bundle size at 2 weeks (A, B) and normalized to ETC subunit content (C, D) to permit comparisons of intrinsic mitochondrial respiratory responses in each group. Creatine sensitivity was assessed by calculating the +creatine/-creatine ratio (E, F) given creatine normally increases ADP-stimulated respiration. The same measurements were completed at 4 weeks (G-L). Results represent mean ± SD; n=8-16; A two-way ANOVA was used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). * P<0.05, PBS(4wk) vs C26(4wk)
Figure 9 Complex I stimulated mH₂O₂ emission in quadriceps and diaphragm muscle of C26 tumour bearing mice. At 2 and 4 weeks, quadriceps mH₂O₂ emission supported by pyruvate (10mM) and malate (2mM) (NADH) was assessed under maximal State II (no ADP) conditions in the presence of 20mM creatine (A, E) and under a range of [ADP] to model metabolic demand (B, F). These measures were also normalized to total ETC subunit content (C, D, G, H) to permit comparisons of intrinsic mitochondrial respiratory responses in each group. These measures were repeated in the diaphragm (I-P). Results represent mean ± SD; n=8-16; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk) for state II H₂O₂ emission. A two-way ANOVA was used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). # P<0.05, PBS(2wk) vs C26(2wk); * P<0.05, PBS(4wk) vs C26(4wk).
Inhibition of mitochondrial oxidative phosphorylation acutely lowers *in vitro* diaphragm force production. Diaphragm strips underwent an *in vitro* force-frequency protocol (CTRL) and were then incubated with 0.2% ethanol (Vehicle) while relaxing for 40 minutes followed by a 2nd assessment (A, \( n = 4 \)). A similar approach was used with 10\( \mu \)M oligomycin in separate strips. (B, \( n = 6 \)). The relative change in force production in both vehicle and oligomycin conditions compared to their respective CTRL was also analyzed (C, \( n = 4-6 \)). Results represent mean ± SD; A two-way ANOVA was used to determine the difference between CTRL vs. Vehicle, CTRL vs. Drug and Vehicle vs. Drug. \( n = 4-6 \); \( \delta P < 0.05 \), CTRL vs Drug; \( \Phi P < 0.05 \), Vehicle vs Drug.
Figure 11 Glutathione redox buffering in quadriceps and diaphragm muscle of C26 tumour-bearing mice. Reduced glutathione was measured in the quadriceps of the 2 and 4 week cohort (A, E) along with oxidized glutathione (B, F). The ratio of reduced to oxidized glutathione was also analyzed (C, G) along with the total glutathione (D, H). This was repeated in the diaphragm (I-P).

Results represent mean ± SD; n=8-10; Two tailed T-tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). * P<0.05, PBS(4wk) vs C26(4wk); ** P<0.01 PBS(4wk) vs C26(4wk); *** P<0.001 PBS(4wk) vs C26(4wk).
Figure 12 Summary of the time-dependent and muscle-specific adaptations to C26 xenografts in CD2F1 mice. At 2 weeks, early impairments in force generating capacity are associated with reductions in mitochondrial pyruvate/malate-supported ADP-stimulated respiration in quadriceps and elevated mH\textsubscript{2}O\textsubscript{2} emission in diaphragm. These distinct mitochondrial responses precede atrophy in both muscles by 4 weeks. At this time, quadriceps and diaphragm responses to C26 become heterogeneous. The restoration of force generating capacity in quadriceps in spite of atrophy is not observed in the diaphragm even though both muscles demonstrate apparent compensatory increases in mitochondrial ADP-stimulated respiration. The mitochondrial responses to cancer are more diverse in quadriceps than diaphragm, with increases in respiration by 4 weeks occurring as a potential compensation for reductions in mitochondrial electron transport chain markers. Mitochondrial creatine metabolism is impaired in quadriceps by 4 weeks. ↓ represents decrease; ↑ represents increase; ↔ represents no change. Created with Biorender.com