Nuclear magnetic resonance in conjunction with functional genomics suggests mitochondrial dysfunction in a murine model of cancer cachexia

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Abstract. Cancer patients commonly suffer from cachexia, a syndrome in which tumors induce metabolic changes in the host that lead to massive loss in skeletal muscle mass. Using a preclinical mouse model of cancer cachexia, we tested the hypothesis that tumor inoculation causes a reduction in ATP synthesis and genome-wide aberrant expression in skeletal muscle. Mice implanted with Lewis lung carcinomas were examined by in vivo \(^{31}\)P nuclear magnetic resonance (NMR). We examined ATP synthesis rate and the expression of genes that play key-regulatory roles in skeletal muscle metabolism. Our in vivo NMR results showed reduced ATP synthesis rate in tumor-bearing (TB) mice relative to control (C) mice, and were cross-validated with whole genome transcriptome data showing atypical expression levels of skeletal muscle regulatory genes such as peroxisomal proliferator activator receptor \(\gamma\) coactivator 1 \(\beta\) (PGC-1\(\beta\)), a major regulator of mitochondrial biogenesis and, mitochondrial uncoupling protein 3 (UCP3). Aberrant pattern of gene expression was also associated with genes involved in inflammation and immune response, protein and lipid catabolism, mitochondrial biogenesis and uncoupling, and inadequate oxidative stress defenses, and these effects led to cachexia. Our findings suggest that reduced ATP synthesis is linked to mitochondrial dysfunction, ultimately leading to skeletal muscle wasting and thus advance our understanding of skeletal muscle dysfunction suffered by cancer patients. This study represents a new line of research that can support the development of novel therapeutics in the molecular medicine of skeletal muscle wasting. Such therapeutics would have wide-spread applications not only for cancer patients, but also for many individuals suffering from other chronic or endstage diseases that exhibit muscle wasting, a condition for which only marginally effective treatments are currently available.

Introduction

Cachexia is a complex metabolic syndrome that can result from adaptation to an underlying illness; it is characterized by loss of muscle mass with or without loss of fat mass (1). Selective targeting of skeletal muscle is a principal feature of cachexia pathophysiology (2), and a major cause of fatigue (3) in patients. Indeed, the condition can rob patients of 30% or more of their body weight (4). As many as half of untreated cancer patients present with cachexia (5,6), which is most commonly associated with cancers of the gastrointestinal tract and lung (6); furthermore, in these cancer types muscle wasting occurs at a faster rate than any other known situation in human subjects (6-8).

Cancer-induced muscle wasting is typically associated with the development of significantly increased resting energy
In muscle cells (21). In addition, it has been shown that kinase (MAPK) pathway, resulting in increased respiration thus eliminating mitochondria function by providing measurements that provide a snapshot of the transcriptome in a specific tissue in response to experimentally defined physiological conditions, provides a systematic approach for studying complex systems, such as cancer-induced muscle wasting, by integrating mitochondrial function data with information about mitochondrial regulation at the cellular and organism levels.

Although mitochondrial dysfunction, has been shown in experimental models of burn injury to lead to skeletal muscle wasting (31-39), it has never been studied in the context of cancer-induced cachexia. The aim of the present study was to study mitochondrial function in cancer cachexia holistically in a suitable preclinical model. To this end, highly cachectic, fast-growing Lewis lung carcinomas, characterized by poorly differentiated cells with a short doubling time (40), were implanted in mice. This model is advantageous owing to the fast rate of muscle wasting produced and lack of anorexia which produce a clear cachetic state characterized by profound muscle wasting (40-44), mimicking closely the pathophysiology of untreated human cancer cachexia. We hypothesize that mitochondrial dysfunction triggered by an altered gene expression program is a major cause of skeletal muscle wasting in cancer. To test this hypothesis, in vivo NMR combined with whole-genome expression analysis was applied to intact Lewis lung carcinoma-inoculated mice and the data were compared to data from control (C) tumor-free mice to assess alterations in the tumor bearing (TB) animals' bioenergetic status and to characterize concomitant gene expression patterns in cancer-induced cachectic versus control skeletal muscle tissue.

**Materials and methods**

**Animals.** C57Bl/6 mice (20-25 g) (Charles River Laboratories, Boston, USA) were used as a representative inbred stock and reliable population for the microarray studies. The animals were maintained at 22±2˚C with a regular light-dark cycle (lights on from 8:00 am to 8:00 pm) and had free access to standard rodent chow and water. The diet consisted of 54% carbohydrate, 17% protein, and 5% fat (the residue was non-digestible material). Food intake was measured daily; food provided daily was pre-weighted. After 24 h, remaining food was weighted and subtracted from the initially provided food. The net value of food consumed every 24 h gave the rate of food intake. Only male mice were used in order to avoid the variability that can result from the female estrous cycle. All animal experiments were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital, Boston.

**Tumor implantation.** Mice were inoculated with tumor according to an established protocol (43) under short-time isoflurane anesthesia (3% in O2) as described previously (43). Animals were randomized into tumor-free control (C) and tumor-bearing (TB). TB-mice received an intramuscular (right hind leg) inoculum of 4x10^5 Lewis lung carcinoma cells obtained from exponential tumors.

**Evaluation of cancer induced cachexia.** Fourteen days after tumor transplantation, the mice were weighed and anesthetized with an intraperitoneal (i.p.) ketamine (87 mg/kg) and xylazine (13 mg/kg) injection. Tumor, tissues of interest and blood were collected. All mice were then administered a lethal dose of pentobarbital (200 mg/kg, i.p.). The cancer-induced cachexia was evaluated by measuring: i) the total body and carcass (muscle + bone + skin) weights, ii) the weight of gastrocneumius, tibialis, soleus and extensor digitorum longus (EDL) muscle weights in the contralateral (left leg) to the tumor bearing leg as described previously (43), and iii) the TNFα, interleukin-6 (IL-6) and interleukin-10 (IL-10) levels in blood using Q-Plex™ Mouse Cytokine/Chemokine kit by Quansys Biosciences laboratory (USA). (www.quansysbio.com/products-services/sample-testing).

**13P NMR spectroscopy**

**Data acquisition.** The theoretical basis of saturation transfer experiments has been described previously by Forsen and...
where \( y \) is the \( z \) magnetization and \( t \) is the inversion time.

31P NMR spectra were analyzed using the Data analysis.

time between 152 and 7,651 ms.

data points) was used to invert \( \text{Pi} \) and \( \text{PCr} \), with an inversion

An adiabatic pulse (400 scans; sweep width, 10 kHz; 4,000

time 2 sec, 400 averages, 4,000 data points). Saturation 90˚ was optimized for detection of phosphorus spectra (repetition

was adjusted using the 1H signal of tissue water. A 90˚ pulse

USA) using a Bruker Advance console. Field homogeneity

was placed into a solenoid coil (four turns; length, 2 cm; diameter, 1 cm) tuned to 31P frequency (162.1 MHz). During

was optimized for detection of phosphorus spectra (repetition time 2 sec, 400 averages, 4,000 data points). Saturation 90˚ selective pulse trains (duration, 36.534 ms; bandwidth, 75 Hz) followed by crushing gradients were used to saturate the \( \gamma \text{ATP} \) peak. The same saturation pulse train was also applied

downfield of the inorganic phosphate (Pi) resonance, symme-

ted onto MOE430A oligonucleotide arrays, which were

Total RNA extraction. The mice were anesthetized by intra-

peritoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg) and the gastrocnemius muscle contralateral (left) to the TB-hind leg was rapidly excised, weighed, and frozen in liquid nitrogen. Left gastrocnemius muscles excised from C animals served as controls specimens. All mice were then administered a lethal dose of pentobarbital (200 mg/kg, i.p.). Frozen biopsies from TB and C mice (n=4) were immersed in 1 ml TRIZol® (Gibco-BRL, Invitrogen, Carlsbad, CA) for RNA extraction. Each muscle specimen was homogenized for 60 sec with a Brinkman Polytron 3000 homogenizer before extraction of total RNA. Chloroform (200 μl) was added to each homogenized muscle specimen and mixed by inverting the tube repeatedly for 15 sec. After centrifugation at 12,000 x g for 15 min, the upper aqueous phase was transferred by pipet to a new tube and precipitated by adding 500 μl of isopropanol.

Further centrifugation at 12,000 x g for 10 min condensed the RNA pellet, which was then washed with 500 μl of 70% ethanol and centrifuged at 7,500 x g for 5 min prior to air drying. The pellet was resuspended in 100 μl DEPC-H2O. An RNeasy kit (Qiagen, Germantown, MD) was used to purify the RNA according to the manufacturer's protocol. Purified RNA was quantified by UV absorbance at 260 and 280 nm and stored at -70°C for DNA microarray analysis.

Gene array hybridization and analysis. Biotinylated cRNA was generated from 10 μg aliquots of total RNA, and hybridized onto MOE430A oligonucleotide arrays, which were subsequently stained, washed, and scanned. All procedures followed standard Affymetrix protocols (Santa Clara, CA). The hybridized array image data files were converted to cell intensity (CEL) files in Microarray Suite 5.0 (MAS 5.0, Affymetrix). The data were scaled to a target intensity of 500, and Genespring GX (version 7.3) software (Agilent Technologies) was employed for statistical analysis of differential transcript expression using the Welch t-test for multiple testing correction and Benjamini and Hochberg False Discovery Rate (cut-off of 5% false discovery rate and 2-fold change). Probe sets representing the same transcript were ordered on their corresponding unigenes, and the 3’-most probe set was selected

Hoffman (45). Animals were subjected to in vivo 31P NMR spectroscopy 14 days after tumor inoculation. The mice were transiently anesthetized with a mixture of isoflurane (3.0%) and \( \text{O}_2 \) (2.0 l/min) delivered through a nose cone and placed in a customized restraining tube. Each animal's left hind limb was placed into a solenoid coil (four turns; length, 2 cm; diameter, 1 cm) tuned to 31P frequency (162.1 MHz). During the MR imaging, mice were kept anesthetized with a mixture of isoflurane (1.5%) and \( \text{O}_2 \) (0.6 l/min). The rectal body temperature was maintained at 37±1˚C using heated water blankets. All in vivo 31P NMR experiments were performed in a horizontal bore magnet (proton frequency at 400 MHz, 21 cm diameter, Magnex Scientific, Varian, Palo Alto, CA, USA) using a Bruker Advance console. Field homogeneity was adjusted using the 'H signal of tissue water. A 90˚ pulse was optimized for detection of phosphorus spectra (repetition time 2 sec, 400 averages, 4,000 data points). Saturation 90˚ selective pulse trains (duration, 36.534 ms; bandwidth, 75 Hz) followed by crushing gradients were used to saturate the \( \gamma \text{ATP} \) peak. The same saturation pulse train was also applied
downfield of the inorganic phosphate (Pi) resonance, symme-

tically to the \( \gamma \text{ATP} \) resonance. T1 relaxation times of Pi and phosphocreatine (PCr) were measured using an inversion recovery pulse sequence in the presence of \( \gamma \text{ATP} \) saturation. An adiabatic pulse (400 scans; sweep width, 10 kHz; 4,000 data points) was used to invert Pi and PCr, with an inversion time between 152 and 7,651 ms.

Data analysis. 31P NMR spectra were analyzed using the MestRe-C NMR software package (Mestrelab Research. NMR solutions, website: www.mestrec.com). Free induction decays were zero-filled to 8,000 points and apodized with exponential multiplication (30 Hz) before Fourier transformation. The spectra were then manually phased and corrected for baseline broad features. The Levenberg-Marquardt algorithm was used to least-square-fit a model of mixed Gaussian/Lorentzian functions to the data. Similarly, the T1obs relaxation time for Pi and PCr was calculated by fitting the function \( y = A_1[1-A_2e^{-t/T_{1obs}}] \) to the inversion recovery data, where \( y \) is the \( z \) magnetization and \( t \) is the inversion time.

Table I. Cancer cachexia effects in C57BL/6 mice.

<table>
<thead>
<tr>
<th>Muscles weights (mg/100 g initial BW)</th>
<th>Control</th>
<th>Tumor-bearing (day 14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>764±16 (n=6)</td>
<td>381±6 (n=8), -50.2%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tibialis</td>
<td>241±8 (n=6)</td>
<td>123±7 (n=8), -49%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Soleus</td>
<td>42±1 (n=6)</td>
<td>37±3 (n=8), -11.9%</td>
<td>NS</td>
</tr>
<tr>
<td>EDL</td>
<td>58±4 (n=6)</td>
<td>39±3 (n=8), -32.8%</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM for the number of animals indicated in parentheses. EDL, extensor digitorum longus; NS, not significant; carcass weight, muscle + bone + skin.

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Final BW (g) 22±1 (n=5) 14±1 (n=8) <0.001

Initial BW (g) 17±0.2 (n=5) 17±0.5 (n=8) NS

% weight change 29% -17% <0.001

Carcass (mg/100 g initial BW) 77±2 (n=5) 56±1 (n=8) <0.001

Muscles weights (mg/100 g initial BW)

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Control Tumor-bearing (day 14) P-value

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Results are expressed as mean ± SEM for the number of animals indicated in parentheses. EDL, extensor digitorum longus; NS, not significant; carcass weight, muscle + bone + skin.

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Table I. Cancer cachexia effects in C57BL/6 mice.
from combined lists of all probe sets. A collection of genes with experimental evidence was compiled using MOE430A chip annotation (Affymetrix, retrieved December, 2009) compiled from GeneSpring GX (version 7.3). Statistically significant sets of functionally related genes were selected using overrepresentation statistics calculated as hypergeometric probabilities using all genes selected in each experiment that had Gene Ontology annotation for biological process (46) using Gene Ontology Analysis (GeneSpring GX, version 7.3). Functions' P-values were estimated using 0.05 as the cut-off point (GeneSpring GX, version 7.3). Functional categories that did not have at least two genes were removed.

Results

Lewis lung carcinoma inoculation decreased body, carcass and skeletal muscle weights. Food intake during the experiment did not differ between TB and C groups. Fourteen days

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Table II. Results of \textit{in vivo} 31P-NMR saturation transfer experiments performed on the hindlimb skeletal muscle of mice.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>Tumor-bearing (n=6)</th>
<th>Δ(%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP synthesis flux (reaction Pi→γATP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔM/M₀</td>
<td>0.48±0.036</td>
<td>0.30±0.051</td>
<td>-37.2</td>
<td>0.011</td>
</tr>
<tr>
<td>T₁obs (s)</td>
<td>1.59±0.14</td>
<td>1.50±0.14</td>
<td>-5.6</td>
<td>NS</td>
</tr>
<tr>
<td>κᵣ (s⁻¹)</td>
<td>0.30±0.026</td>
<td>0.20±0.038</td>
<td>-33.2</td>
<td>0.038</td>
</tr>
<tr>
<td>ATP (mmol/g)</td>
<td>1.19±0.14</td>
<td>0.87±0.19</td>
<td>-26.9</td>
<td>0.054</td>
</tr>
<tr>
<td>Pᵢ (mmol/g)</td>
<td>0.28±0.062</td>
<td>0.22±0.079</td>
<td>-20.7</td>
<td>NS</td>
</tr>
<tr>
<td>ATP synthesis rate (mmol/g/s)</td>
<td>0.08±0.013</td>
<td>0.04±0.013</td>
<td>-47.1</td>
<td>0.029</td>
</tr>
<tr>
<td>ATP synthesis flux (reaction PCr→ATP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔM/M₀</td>
<td>0.24±0.02</td>
<td>0.26±0.02</td>
<td>+8.3</td>
<td>(NS)</td>
</tr>
<tr>
<td>T₁obs (s)</td>
<td>1.59±0.20</td>
<td>1.50±0.27</td>
<td>-5.7</td>
<td>(NS)</td>
</tr>
<tr>
<td>κᵣ (s⁻¹)</td>
<td>0.15±0.02</td>
<td>0.17±0.03</td>
<td>+13.3</td>
<td>(NS)</td>
</tr>
<tr>
<td>ATP (μmol/g)</td>
<td>1.19±0.28</td>
<td>0.87±0.19</td>
<td>-26.9</td>
<td>(NS)</td>
</tr>
<tr>
<td>PCr (μmol/g)</td>
<td>4.13±0.99</td>
<td>2.65±0.59</td>
<td>-35.8</td>
<td>0.015</td>
</tr>
<tr>
<td>ATP synthesis rate (μmol/g/s)</td>
<td>0.62±0.17</td>
<td>0.45±0.13</td>
<td>-27.4</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Values are means ± SEM; ΔM/M₀ is the fractional change in Pᵢ or PCr magnetization as a result of saturation transfer; T₁obs is the observed spin lattice relaxation time of Pᵢ or PCr during γATP saturation in seconds; κᵣ is the rate constant for the reactions Pᵢ→ATP and PCr→ATP, calculated as (1/T₁obs) x (ΔM/M₀). ATP synthesis is calculated as [Pᵢ] x κᵣ. A bioluminescence assay kit was used to assess ATP concentration. Δ (%), percent change. NS, not significant. Unpaired Student's t-test was used for the comparisons.
after tumor implantation mean total body weight (BW) of TB mice had decreased by 17% relative to pre-inoculation (Table I). BW loss was associated with a 27.3% decrease in carcass weight (muscle + bone + skin) (Table I). Muscle mass was decreased 14 days after tumor implantation in all tissues studied, with the most substantial decreases being observed in the gastrocnemius (50.2% decrease), tibialis (49.0% decrease), and EDL (32.8% decrease) muscles (Table I).

Lewis lung carcinoma inoculation increased blood levels of cytokines. TB mice had elevated levels of the pro-cachectic cytokines TNFα (77.2% greater than C; P=0.02) and IL-6 (8,120.1% greater than C; P=0.01) 14 days after tumor implantation. Moreover, IL-10, which was undetectable in C mice, was also present at high levels (26.12±4.06 pg/ml) in TB mice.

Cancer cachexia reduced ATP synthesis rate. 31P NMR spectra acquired from TB and C mice, before and after saturation of the γATP resonance, with the mean results and their percent change (Δ) presented in Table II. The unidirectional synthesis rate of the P_i → γATP reaction in TB mice was 47% lower than that observed in C mice (P=0.029). This synthesis rate involves measurements from NMR and from a biochemical assay (ATP concentration measurement), and both were significantly decreased in the cancer mice. The NMR-measured fractional change ΔM/M_0 was decreased by 37% in TB mice relative to the C group (percent change in ΔM/M_0, Table I). ATP concentration (14 days post-inoculation) was lower in TB than in C mice by approximately 27% (Table II), a difference that approached significance (P=0.054) in the unidirectional (one-tailed) t-test. ATP synthesis rate was significantly reduced in TB mice (47% lower than in C) in the unidirectional (one-tailed) t-test (P=0.029). The fractional change, ΔM/M_0, and the observed spin lattice relaxation time, T_1obs, were used to calculate the κ_f rate constant using the equation (1/T_1obs) x (ΔM/M_0). The ATP synthesis flux was then obtained as the product of κ_f and P_i concentration. Accordingly, the unidirectional synthesis rate of the PCr → γ-ATP reaction was also 27% lower in TB mice compared to C, a difference that was significant according to a unidirectional (one-tailed) t-test (P=0.036).
Cancer-induced cachexia affected several cellular functions in skeletal muscle. Analysis of transcriptome studies identified 611 genes as differentially expressed in skeletal gastrocnemius muscle in TB versus C mice (P<0.05). Comparison of these 611 differentially expressed genes to annotations in the Gene Ontology Consortium enabled functionally related sets of genes to be identified and subsequent analysis of these data indicated that several cellular functions were altered by cancer-induced cachexia (Fig. 2). The four most prominently affected functions were related to inflammation and immune response, and these effects predominantly involved significant increases in transcription. The four next most prominently affected functional categories involved genes whose products mediate protein metabolism, especially protein degradation. Specifically, as shown in Fig. 2, transcription of molecules involved in protein catabolism, protein depolymerization, and proteasome-related was altered, mostly in the direction of upregulation. The ubiquitin-proteasome pathway in particular was implicated as a major process of protein degradation in cancer-induced cachexia. Transcription of protein maturation related genes was universally down-regulated in TB mice.

The transcriptome analysis revealed cancer-induced alterations in cellular lipid metabolism and lipid catabolism in TB mice relative to C mice (Fig. 2), indicating that cancer-induced cachexia also begets abnormal lipid metabolism. Among the down-regulated genes (data not shown) was stearoyl-Coenzyme A desaturase 1, which encodes for the first (step-limited) enzyme in unsaturated fatty acid biosynthesis. Phospholipase A2 and phospholipase C, which have roles in several lipid metabolic and signaling pathways, were also differentially affected. Genes related to skeletal muscle antioxidative capacity were affected by cancer cachexia as well (last two functional categories listed in Fig. 2). While increased transcription of genes related to oxidative stress response was evident, superoxide metabolism genes were down-regulated.

Differential expression of key mitochondrial genes in cancer cachexia. Fig. 3 shows the effect of cancer-induced cachexia on the expression levels of selective genes related to mitochondrial function. Specifically, uncoupling protein 3 (UCP3), and forkhead box O 3a (FoxO3a) expression levels were significantly upregulated (P=0.03 and P=0.04 respectively). As well as pyruvate dehydrogenase kinase 4 (PDK4), an inhibitor of pyruvate dehydrogenase complex (P=0.01). Meanwhile, peroxisome proliferator activated receptor-γ coactivator-1β (PGC-1β) was significantly down-regulated (P=0.04).

It is noteworthy that the present experiments revealed also altered expression of genes known to be involved in the IGF-1/AKT pathway which regulates: a) signaling pathways responsible for FoxOxs post-translational activation; and b) protein synthesis and degradation (Fig. 3). More specifically, our data suggest that cachexic animals had decreased AKT activation since insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), tumor necrosis factor α; NFκB, nuclear factor-κB.

Figure 4. Putative PGC-1-mediated mechanism of cancer-induced skeletal muscle wasting. In this model, significant down-regulation of PGC-1β expression causes dysregulation of mitochondria, resulting in reduced ATP synthesis and increased ROS production, most likely due to PGC-1-dependent upregulation of UCPs. Meanwhile, reduced PGC-1β gene expression levels result in a failure to down-regulate expression of FoxOxs, which normally suppresses atrogen expression and proteolysis. Insulin-stimulated IGF-1/AKT pathway signaling is also significantly attenuated. Thus, AKT does not phosphorylate FoxOxs and, consequently, favors further upregulation of atrogens, thus leading to skeletal muscle wasting. Moreover, reduced IGF-1/AKT signaling further facilitates protein translation inhibition due to mTOR inactivation which also results in cachexia. Meanwhile, PGC-1β may affect IGF-1 and IRS-1 expression whereby producing insulin resistance in skeletal muscle, IRS-1, insulin receptor substrate 1; IGF-1, insulin-like growth factor 1; PGC-1s, peroxisome proliferator-activated receptor-γ coactivators-1; UCPs, uncoupling proteins; FoxOxs, forkhead box O proteins; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; NFκB, nuclear factor-κB.
negligible influence of the coupled glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions for ATP production (28,51). Because these enzymes occur at near equilibrium, unidirectional production of ATP can be high. Furthermore, since cancer cachexia upregulates expression of PDK4 (in mouse Fig. 3; in rat, see (23), a well established inhibitor of the pyruvate dehydrogenase complex which is involved in controlling the use of glucose-linked substrates as sources of oxidative energy via glycolysis (52), we can assume that the contribution of glycolytic reactions to unidirectional ATP synthesis flux is negligible.

In agreement with our NMR findings, our genomic experiments revealed reduced expression of PGC-1α, a mitochondrial biogenesis transcription factor. Reduced expression of PGC-1α has also been observed in murine burn-induced skeletal muscle wasting models (31,37), while increased PGC-1α protein levels have been previously reported in a rat cancer cachexia model (23), strongly supporting the view that PGC-1α plays a key role in cancer-induced muscle wasting. Specifically, it has been suggested that PGC-1α protects skeletal muscle from atrophy (53) while PGC-1β expression has been associated with an increase in ATP-consuming reactions (24). Moreover, reduced PGC-1 expression levels have also been correlated to profoundly reduced mitochondrial content and activity (22); these effects may be due to the action of UCPs (34) given that down-regulation of PGC-1s is accompanied by increased UCP expression in murine models of both cancer- (12,14,23) and burn-related cachexia (31,54). Increased levels of UCPs dissipate the proton gradient and lower the mitochondrial membrane potential, a process known to increase energy expenditure by dissipating energy as heat (7). The presently observed upregulation of UCP3 in cachexic animals corroborates previous findings (12,14,16).

Indeed, this increase in UCP3 gene expression suggests that there was mitochondrial uncoupling in TB mice since increased levels of UCPs lower the mitochondrial membrane potential (55) and thus greatly reduce the amount of ROS in mitochondria (56,57), a major site of ROS production in the cell (58,59), especially in conditions characterized by high TNFα levels (60), such as cancer. To this end, it has been proposed that upregulation of UCPs in cancer may be directly related to the antioxidative capacity of skeletal muscle, thus involving UCPs in a counter-regulatory mechanism to lower ROS production; this process is thought to involve the muscle anabolic cytokine IL-15 (16). UCP expression has been reported to be upregulated by proinflammatory cytokines (TNFα and IL-6) via p38MAPK-dependent post-translational activation of PGC-1s (21). Our data agree with this notion, as we observed increased levels of the proinflammatory cytokines TNFα and IL-6 in TB mice relative to C mice (by 77.2% and 8,120.1%, respectively).

A principal finding of the present study was that down-regulation of PGC-1β in TB mice was accompanied by a down-regulation of SOD2 as well as an upregulation of GPX. Interestingly, it has previously been shown that PGC-1s increase gene expression of SOD2, GPX and enzymes responsible for glutathione biosynthesis (24), and both SOD2 and GPX have been implicated in cancer-induced oxidative stress response (61-63). Our results indicate that muscle defense against oxidative stress in cancer is mediated largely by...
in Fig. 4. Specifically, we propose that cancer induction of regulator of cancer-induced bioenergetic dysfunction and facilitate protein translation inhibition due to mTOR inactivation in our experimental cancer cachexia model should further the IGF-1/AKT pathway, attenuation of AKT activity as observed (75). Given that mTOR activation is regulated mainly by the eIF4E, leading to an increase in cap-dependent translation (73) via binding to eukaryotic initiation factor 4E (eIF4E). Hypophosphorylated EIF4Ebp1 binds to eIF4E and prevents recruitment of the 40S ribosomal subunit (74). Specifically, hypophosphorylated EIF4Ebp1 binds to EIF4E and prevents recruitment of the 40S ribosomal subunit to mRNA, thus inhibiting cap-dependent translation. EIF4Ebp1; hyperphosphorylated EIF4Ebp1 is released from the nucleus, where it is activated and promotes transcription of genes related to protein degradation (53,66,68,69), thereby leading to cachexia. Genes related to the structure of the proteolytic system (proteasome activity in Fig. 2) were also upregulated in TB mice, further implicating activation of the ubiquitin-proteasome pathway as a major generator of protein degradation in cancer-induced cachexia, as previously reported in cancer patients exhibiting weight loss (70).

The hypothesis that IGF-1/AKT attenuation contributes to cancer cachexia is supported by the presently observed elevated levels of the EIF4Ebp1 in TB mice, as EIF4Ebp1 levels have been found to correlate with cancer development and cachexia (71,72) due to inhibition of protein synthesis (73) via binding to eukaryotic initiation factor 4E (eIF4E) (74). Specifically, hypophosphorylated EIF4Ebp1 binds to eIF4E and prevents recruitment of the 40S ribosomal subunit to mRNA, thus inhibiting cap-dependent translation. Binding of EIF4Ebp1 to eIF4E is regulated by mTOR-mediated phosphorylation (73). Upon its activation, mTOR phosphorylates EIF4Ebp1; hyperphosphorylated EIF4Ebp1 is released from eIF4E, leading to an increase in cap-dependent translation (75). Given that mTOR activation is regulated mainly by the IGF-1/AKT pathway, attenuation of AKT activity as observed in our experimental cancer cachexia model should further facilitate protein translation inhibition due to mTOR inactivation and the consequent hypophosphorylation of EIF4Ebp1.

In light of the above-mentioned lines of evidence and previous reports, we propose here that PGC-1 acts as a key regulator of cancer-induced bioenergetic dysfunction and muscle wasting in experimental cancer cachexia, as illustrated in Fig. 4. Specifically, we propose that cancer induction of cachexia involves: i) decreased expression of PGC-18, which normally inhibits FoxOs; and ii) attenuated AKT activity, which both enable activation of FoxO3a and upregulate the expression of atrogenes, which then facilitate muscle degradation. In addition, attenuation of AKT can inhibit protein synthesis via the IGF-1/PI3K pathway, as previously observed in cancer cachexia (67) was confirmed by our data showing decreased AKT activation as evidenced by down-regulated IGF-1, IRS-1 and IGFBP5. Consequently, decreased AKT activation could permit dephosphorylation of FoxOs and thus translocation of FoxOs to the nucleus, where they are activated and promote transcription of genes related to protein degradation (53,66,68,69), namely atrogin-1 and ubiquitin-conjugating enzyme E3, thus leading to cachexia. Genes related to the structure of the proteolytic system (proteasome activity in Fig. 2) were also upregulated in TB mice, further implicating activation of the ubiquitin-proteasome pathway as a major generator of protein degradation in cancer-induced cachexia, as previously reported in cancer patients exhibiting weight loss (70).

In conclusion, because hallmarks of skeletal muscle wasting can be detected by in vivo NMR, our findings are clinically relevant in as much as they can be used as molecular medicine biomarkers that can be translated to humans. Moreover, if PGC-1s mediate key regulatory functions in cancer-induced cachexia as suggested by the present findings, they could serve as funnels where several oncogenic/cachectic signals (cytokines, ROS, IGF-1) converge to drive the downstream physiological response (mitochondrial dysfunction, antioxidative and immune-inflammatory response, protein synthesis and degradation) of skeletal muscle to malignancy. Because PGC-18 is a highly inducible factor in most tissues that responds to common calcium and cAMP signaling pathways, it is conceivable that drugs could be developed with the ability to induce PGC-18 and thereby directly alleviate mitochondrial dysfunction. Such a novel therapeutic approach could revolutionize cancer cachexia treatment.

Acknowledgements

This study was supported in part by a National Institutes of Health (NIH) Center Grant (P50GM021700) to Ronald G. Tompkins (A. Aria Tzika, Director of the NMR core), and Shriner’s Hospital for Children research grants (no. 8893) to A. Aria Tzika, and (no. 8892) to Laurence G. Rahme. Cibely C. Fontes de Oliveira was supported by a Programme Alban scholarship (E05D059293BR). We also thank Ann Power Smith Ph.D. of Write Science Right for editorial assistance.

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