Nuclear transcription factor κB activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia

Celine M Op den Kamp, Ramon C Langen, Frank J Sneyvangers, Chiel de Theije, Johanna M Schellekens, Fabian Laugs, Anne-Marie C Dingemans, and Annemie M Schols

ABSTRACT

Background: Experimental models of cancer cachexia have indicated that systemic inflammation induces muscle-protein breakdown and wasting via muscular nuclear transcription factor κB (NF-κB) activation. This process may limit the efficacy of nutritional intervention.

Objectives: We assessed muscle NF-κB activity and protein turnover signaling in progressive stages of clinical lung cancer cachexia and assessed whether circulating factors can induce muscular NF-κB activity.

Design: Patients with lung cancer precachexia (n = 10) and cachexia (n = 16) were cross-sectionally compared with 22 healthy control subjects. mRNA transcripts of muscle proteolytic (ubiquitin proteasome system and autophagy lysosomal pathway) and myogenic markers and protein expression of PI3K/Akt, myostatin, and autophagy signaling were measured. A multiplex analysis showed the systemic inflammatory status, whereas plasma exposure to stable NF-κB-luciferase-reporter muscle cells revealed NF-κB inducibility.

Results: Compared with healthy control subjects, cachectic patients had reduced (appendicular) muscle mass (−10%), muscle fiber atrophy (−27%), and decreased quadriceps strength (−31%). Subtle alterations in the muscle morphology were also detectable in precachectic patients, without changes in body composition. Despite increased Akt phosphorylation, downstream phosphosubstrates glycogen synthase kinase 3β, mammalian target of rapamycin, and Forkhead box protein were unaltered. The expression of autophagy effectors B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3 and microtubule-associated proteins 1A/1B light chain 3B gradually increased from precachectic to cachectic patients, without differences in E3 ubiquitin ligases. Systemic and local inflammation was evident in cachexia and intermediate in precachexia, but the plasma of both patients groups caused ex vivo muscle NF-κB activation.

Conclusions: In lung cancer, muscular NF-κB activity is induced by factors contained within the circulation. Autophagy may contribute to increased muscle proteolysis in lung cancer cachexia, whereas the absence of downstream changes in phosphosubstrates despite increased Akt phosphorylation suggests impaired anabolic signaling that may require targeted nutritional intervention.


INTRODUCTION

It has been well established that cachexia is a severely debilitating syndrome that accompanies cancer, and it was recently postulated that cancer cachexia develops in a spectrum that traverses mild to advanced stages (ie, precachexia, cachexia, and refractory cachexia) (1). The syndrome has received growing attention as unmet medical need because it is directly responsible for 20% of cancer-related deaths (2). Patients who suffer from pulmonary malignancies have shown a high prevalence and rapid progression of cachexia, but currently, effective interventions to prevent or reverse cachexia in lung cancer are not available (1, 3).

Studies in the 1990s already showed that it is particularly the loss of skeletal muscle cell mass that is accountable for the poor prognosis and declined performance status in lung cancer cachexia (4–6). Consequently, skeletal muscle wasting can be considered an important indicator of cachexia progression as well as a potent target of nutritional or pharmacologic intervention (1). High-quality protein diets as well as specific amino acids that target the nutrient-sensing signaling pathways have shown promising effects on muscle mass maintenance in chronic lung disease (7). To identify the potential of such anabolic interventions in lung cancer cachexia, which may differ depending on the stage of cachexia, it is crucial to obtain more insight in molecular alterations of skeletal muscle protein turnover in patients who suffer from cancer cachexia.
In muscle-protein metabolism, protein synthesis and degradation are subject to extensive (patho)physiologic regulation, and the balance between synthesis and degradation ultimately determines the net muscle protein turnover (8, 9). Experimental studies have especially postulated a role of ubiquitin 26S-proteasome system (UPS)\textsuperscript{5}\textsuperscript{6}–dependent proteolysis in cancer cachexia and, to a lesser extent, autophagy lysosomal pathway–dependent protein degradation (10–12). Myonuclear turnover may constitute an additional determinant of muscle mass by the accretion (myogenesis) and loss of muscle nuclei (12). Current insights in the mechanisms that dictate muscle-mass plasticity in cancer cachexia have revealed intricate interactions between these processes, but data have been primarily obtained from experimental research (8, 9, 12). A schematic representation of mediators identified in experimental cancer cachexia is shown in Figure 1.

Systemic inflammation is considered an important host-related alteration that induces muscle atrophy in cancer cachexia (13, 14). Systemic inflammation conveys its action via local inflammatory signaling through the nuclear transcription factor \(\text{NF-\kappaB}\) (15–17). However, although increased systemic inflammatory profiles (18, 19) and elevated muscular \(\text{NF-\kappaB}\) signaling have been observed in cancer patients (20), it remains to be addressed whether mediators in the circulation can be causally linked to the activation of muscle \(\text{NF-\kappaB}\) signaling in lung cancer cachexia.

The objective of the current study was to investigate the expression of signaling molecules involved in protein metabolism during different stages of lung cancer cachexia and assess whether factors within the circulation can induce muscle inflammatory \(\text{NF-\kappaB}\) signaling. These indexes were studied in a cross-sectional study design, in which healthy control subjects and precachetic and cachectic patients with non–small cell lung cancer (NSCLC) were compared.

**SUBJECTS AND METHODS**

**Study population**

Newly diagnosed patients with advanced stage NSCLC admitted to the Department of Respiratory Medicine of the Maastricht University Medical Centre\textsuperscript{7} between July 2007 and July 2010 were eligible for participation in the study. Participants were divided into precachetic and cachetic groups according to the definition in the international cancer cachexia consensus (1). Precachexia is defined as “an early stage in which clinical and metabolic signs such as anorexia and systemic inflammation can precede substantial (ie, >5%) body weight loss” (1). Diagnostic criteria for cancer cachexia are “body weight loss of >5% in the past 6 months or body weight loss of >2% in combination with either BMI (in kg/m\(^2\)) <20 or appendicular skeletal muscle index consistent with sarcopenia” (1). NSCLC was confirmed by pathologic analysis, and tumor stage was determined by using the 6th Tumor-Node-Metastasis International Staging System for Lung Cancer (21, 22).

![Schematic representation of signaling molecules involved in muscle mass turnover in cancer cachexia](https://example.com/schematic.png)

**FIGURE 1.** Schematic representation of signaling molecules involved in muscle mass turnover in cancer cachexia [inhibitory signal (—|); activating signal (\(\rightarrow\))]. The predominant signaling cascade involved in muscle protein synthesis is the PI3K/Akt pathway. The protein kinase Akt is a central mediator that induces stimulatory or inhibitory phosphorylation of downstream mediators such as GSK-3\(\beta\) as well as mTOR. Furthermore, Akt activity blocks catabolic signaling via inhibitory phosphorylation of FOXO 1 and 3a, which are potent inducers of proteolytic cues (ie, UPS and ALP). Experimental research revealed that the UPS and ALP are important proteolytic systems involved in muscle-protein depletion during catabolic conditions. In the UPS, individual proteins are targeted for degradation by the 26S proteasome through covalent binding of a polyubiquitin chain. In experimental cancer cachexia, E3 ubiquitin-ligase enzymes are considered rate limiting in this process of protein targeting, and the activation of \(\text{NF-\kappaB}\) has been linked to the activation of this pathway. In the ALP, autophagosomes fuse with lysosomal structures to degrade cellular components. BNIP3 and LC3B are important markers of autophagosome formation.

Myostatin is a transforming growth factor-\(\beta\) superfamily member and potent negative regulator of muscle mass. Intracellular myostatin signaling in muscle occurs after the binding of myostatin to the ActRIIB receptor and recruitment of ALK. Subsequently the intracellular phosphorylation of Smad 2 and 3 proteins occurs, which, on complex formation, transfer to the nucleus to convey their actions. Impaired myonuclear accretion (myogenesis) might also contribute to muscle atrophy. Experimental research has indicated that the expression of MRFs such as MyoD and myogenin, which are essential in myogenesis, is altered in experimental cancer-induced muscle wasting. ActRIIB activin receptor type II B; ALK, activin receptor like kinase; ALP, autophagy lysosomal pathway; BNIP3, B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3; CRP, C-reactive protein; E3aII, ubiquitin-protein ligase E3aII–FOXO, Forkhead box protein; GSK3\(\beta\), glycogen synthase kinase 3\(\beta\); LC3B, microtubule-associated proteins 1A/1B light chain 3B; MRF, myogenic regulatory factor; mTOR, mammalian target of rapamycin; MuRF1, muscle-specific RING finger 1; Nedd4, neuronal precursor cell expressed developmentally downregulated 4; \(\text{NF-\kappaB}\), nuclear transcription factor \(\kappa\)B; Trim32, tripartite motif-containing protein 32; UPS, ubiquitin 26S-proteasome system; 4EBP1, 4E-binding protein 1.
Smoking is not only an important risk factor for lung cancer but also for chronic obstructive pulmonary disease (COPD), which is another condition associated with cachexia, especially in advanced stages (23, 24). To study a representative sample of lung cancer patients but minimize the interference of advanced comorbidities or drugs that could have potential effects on the studied variables, patients with the following characteristics were excluded: Global Initiative for Chronic Obstructive Lung Disease stage IV COPD, Congestive Heart Failure New York Heart Association stage III–IV, and active infectious disease as well as patients who were taking hormones or continual oral corticosteroids. Additional exclusion criteria were the presence of other malignant disease and the initiation of antitumor therapy.

Healthy control subjects were selected by using advertisements in newspapers. It was confirmed that healthy control subjects had no recent body weight loss or any of the previously mentioned diseases or used any of the earlier described medications.

The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre and conducted according to local ethical guidelines. The study was not registered in an additional public trial registry because inclusion started before the moment that this became an obligation (ie, July 2008). All participants signed informed consent forms. All tests mentioned hereafter were performed on the same day after 8 h fasting.

Pulmonary function
Pulmonary function was tested to assess the extent of airflow obstruction in the current population because the coincident presence of COPD could influence the tests described hereafter. Forced expiratory volume in 1 s and forced vital capacity were assessed by using spirometry.

Body composition
Dual-energy X-ray absorptiometry (DPX-L; Lunar Radiation Corp) was used to measure different body compartments (ie, fat mass, lean mass, and bone mineral content).

Muscle strength
The isometric strength of quadriceps muscle of the contra lateral leg where the muscle biopsy was taken was measured by using a dynamometer (Biodex system version 3.3; Biodex). Subjects were seated upright on the dynamometer, and straps were attached at the level of the thigh and ankle. Isometric muscle-strength testing was performed at an angle of 60° (3 repetitions). Muscle strength was defined as the highest muscular force output (peak torque) in newton meters.

Plasma inflammatory markers
Venous blood sampling was performed by using a blood collection tube containing EDTA (Sherwood Medical). Blood was processed in 2 successive steps. First, the collected samples were centrifuged at 3000 relative centrifugal force for 10 min (4°C). Plasma was collected and subjected to a second centrifugation step (5 min; 3000 relative centrifugal force; 4°C) to remove any remaining cellular constituents. Aliquots of blood samples were stored at −80°C until analyses were performed.

C-reactive protein (CRP) was measured by using a CardioPhase high-sensitivity CRP kit according to the manufacturers’ protocol (Siemens Healthcare Diagnostics). The lower detection limit was 0.2 mg/L. A Human Multiplex Antibody assay was run with the Luminex System (Invitrogen; Life Technologies) to determine plasma TNF-α, soluble TNF (sTNF)–receptor 1 and 2, IL-6, -8, and -10, and interferon-γ concentrations (lower detection limit ranged from 5 to 28 pg/mL). All samples were analyzed in duplicate, and these assays were performed at Invitrogen Luminex Testing Services.

Muscle biopsies
Percutaneous needle biopsies of quadriceps muscle (vastus lateralis muscle) were obtained of all subjects. The technique used for muscle biopsies was described by Bergstrom (25). Muscle specimens were processed for either histochemical (immunohistochemistry) or biochemical [quantitative realtime polymerase chain reaction (Q-PCR) and Western blot analysis] analysis.

Muscle specimens for the histochemical analysis were embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek Europe BV) and subsequently frozen in melting isopentane, which was precooled in liquid nitrogen. Samples were stored at −80°C. Before histochemical analysis, serial cryostat cross-sections (5 μm) were cut on a cryostat (Leica Biosystems) at −20°C and mounted on SuperFrost microscope slides (Menzel-Glaser).

Muscle specimens for biochemical assays were snap frozen in liquid nitrogen and subsequently stored at −80°C. Before analyses, muscle biopsies were crushed with a mortar and pestle in liquid nitrogen.

Cross-sectional area of muscle fibers
Immunohistochemical staining of laminin was used to determine the muscle fiber cross-sectional area. Muscle sections were air dried overnight when the sections were treated with phosphate-buffered saline (PBS) for 30 min and subsequently with 0.5% Triton X-100 (Sigma-Aldrich) solution in PBS for 5 min. Sections were incubated for 45 min with primary antilaminin antibody (L-9393; dilution 1:50;(Sigma). Sections were rinsed once with 0.05% Tween (Sigma-Aldrich) solution in PBS and twice with regular PBS for 5 min. Subsequently, sections were incubated for 45 min with secondary antibody Alexa Fluor 350 (Invitrogen; Life Technologies; A-11069; dilution 1:100). Slides were twice rinsed with 0.05% and regular PBS for 5 min. Images for analysis were obtained by using fluorescent microscopy (objective: ×10). Computer image analysis was performed with Lucia Software (version 4.81; Laboratory Imaging). Per biopsy, an average of 200 fibers were analyzed (≥100). Detached, damaged, or non–cross-sectional fibers were excluded from the analysis.

Messenger RNA abundance
For messenger RNA (mRNA) expression analysis, a ToTALLY RNA Kit (Ambion Ltd) was used according to the manufacturers’ protocol. Muscle specimens (10–30 mg) were homogenized by using a Polytron PT 1600 E (Kinematica AG) sample homogenizer, and total RNA was extracted with the use of the ToTALLY RNA
Kit. Subsequently, an RNase Mini Kit with RNase-free DNase (Qiagen) was used for the elution of contaminated genomic DNA, and RNA concentrations were measured by using spectrophotometry (NanoDrop ND-1000; Isogen Lifescience). A total of 400 ng total RNA was reverse transcribed to complementary DNA (cDNA) with anchored oligo(dT) primers according to the supplier’s protocol (Transcriptor First Strand cDNA Synthesis kit; Roche Diagnostics). Q-PCR primers (Sigma Genosys) were designed for the detection of mRNA transcripts of nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor α (IκBα) ( inhibitory protein of NF-κB); IκBα mRNA expression was considered an indirect measure of NF-κB activity because IκBα constitutes a target gene of NF-κB, which ensures negative feedback regulation to prevent uncontrolled and sustained NF-κB activity (26)).

5′-CTACACCCTTGCTTGAGCA-3′ and 3′-CTCTTG-AGCAATGTACAC-5′, myostatin (5′-ACGAAGGTTAGCCACATCGA-3′ and 3′-TGGTGGTTCATTTGAAGCTCCTA-3′AAAGAAGCT-5′), muscle-specific RING finger 1 (MurRF1) (5′-GGAGGTTGGGCCCAT-3′ and 3′-GGATGTCTGACACAGTCT-5′), muscle atrophy F-box (MAFBx/atrogin-1 (atrogin-1) (5′-GGAAAGAACCTCTCTGCAAGGACTCACC-3′ and 3′-CCCTTGCTGAGCAATATACG-5′), tripartite motif-containing protein 32 (5′-ATTGCTGCTTCTATTCCTGCTGTTT-3′ and 3′-CATAATAGTGCTTTGCTGAATTTTGAC-5′), neuronal precursor cell expressed developmentally downregulated 4 (5′-AAAATGCTATCACCTCCCCTGTGT-5′ and 3′-AACCTTCCCAGG-3′).

Plasma transfer experiments

C2C12 murine myoblasts stably transfected with a luciferase reporter construct that contained 3 tandem NF-κB luciferase responsive elements were cultured and differentiated into mature myotubes for 5 d as described before (28). A differentiation medium (Dulbecco’s Modified Eagle’s Medium containing 0.5% fetal bovine serum, 50 U penicillin/mL, and 50 μg streptomycin/mL) was supplemented with plasma (10% final; vol/vol) of individual subjects in the presence of 0.250 mol/L Tris-HCL, 8% SDS, 40% glycerol, 0.4 mol/L dithiothreitol, and 0.02% Bromphenol blue) was added in a 1:4 dilution to the cytoplasmic fraction, and samples were incubated 5 min at 95°C. Equal amounts of protein were loaded per lane of a Wells Criterion XT 4–12% bis-tris gel (Bio-Rad). Two standard samples were included in every blot to correct for blot-to-blot variation. Photoreprobing was performed by using an Electrophoresis Cell System (Bio-Rad). Gels were transferred to nitrocellulose membranes (Whatman; GE Healthcare). Membranes were blocked during 60 min in 2% bovine serum albumin or 5% milk in Tris-buffered saline with 0.05% Tween 20 (Sigma-Aldrich) and exposed to primary antibodies. Primary antibodies of total and phosphorylated Akt-Ser473 (total: 9272; phosphorylated: 9071; Cell Signaling Technology), Forkhead box protein 1 (FOXO 1 (total: 2880; serine 256 phosphorylated: 9461; Cell Signaling Technology)), MyoD (5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), P70S6 kinase (total: 9202; threonine 389 phosphorylated: 2971; Cell Signaling Technology), glycogen synthase kinase 3β (GSK-3β) (total: 27C10; serine 9 phosphorylated: 9336; Cell Signaling Technology), mammalian target of rapamycin (mTOR) (total: 2983; serine 2448 phosphorylated: 2971; Cell Signaling Technology), glycogen synthase kinase 3β (GSK-3β) (total: 27C10; serine 9 phosphorylated: 9336; Cell Signaling Technology), 4E-binding protein 1 (total: 9452 and threonine 37/46 phosphorylated: 9459; Cell Signaling Technology), P70S6 kinase (total: 9202; threonine 389 phosphorylated: 9206; Cell Signaling Technology), Smad 3 (total: 5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), Smad 3 (total: 9523; serine 423/425 phosphorylated: 9520; Cell Signaling Technology), MyoD (5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), MyoD (5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), and LC3B (2775; Cell Signaling Technology) were used. GAPDH (2118; Cell Technology) was used as a loading control. Primary antibodies were incubated overnight at 4°C. Next, membranes were incubated with secondary antibodies (1:5000) of anti-mouse IgG peroxidase (A851P-1000.S1; Bio-Connect) or anti-rabbit IgG peroxidase (A885L-2000.S1; Bio-Connect). Detection was performed by using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s manual. Densitometric quantification was performed with the use of Quantity One software (version 4.6.2; Bio-Rad).

Plasma transfer experiments

For Western blot analysis of signaling proteins, a sample buffer (4X stacking buffer: 0.250 mol/L Tris-HCL, 8% SDS, 40% glycerol, 0.4 mol/L dithiothreitol, and 0.02% Bromphenol blue) was added in a 1:4 dilution to the cytoplasmic fraction, and samples were incubated 5 min at 95°C. Equal amounts of protein were loaded per lane of a Wells Criterion XT 4–12% bis-tris precast gel (Bio-Rad). Two standard samples were included in every blot to correct for blot-to-blot variation. Photoreprobing was performed by using an Electrophoresis Cell System (Bio-Rad). Gels were transferred to nitrocellulose membranes (Whatman; GE Healthcare). Membranes were blocked during 60 min in 2% bovine serum albumin or 5% milk in Tris-buffered saline with 0.05% Tween 20 (Sigma-Aldrich) and exposed to primary antibodies. Primary antibodies of total and phosphorylated Akt-Ser473 (total: 9272; phosphorylated: 9071; Cell Signaling Technology), Forkhead box protein 1 (FOXO 1 (total: 2880; serine 256 phosphorylated: 9461; Cell Signaling Technology)), MyoD (5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), mammalian target of rapamycin (mTOR) (total: 2983; serine 2448 phosphorylated: 2971; Cell Signaling Technology), glycogen synthase kinase 3β (GSK-3β) (total: 27C10; serine 9 phosphorylated: 9336; Cell Signaling Technology), 4E-binding protein 1 (total: 9452 and threonine 37/46 phosphorylated: 9459; Cell Signaling Technology), P70S6 kinase (total: 9202; threonine 389 phosphorylated: 9206; Cell Signaling Technology), Smad 3 (total: 5339; serine 465/467 phosphorylated: 3108; Cell Signaling Technology), Smad 3 (total: 9523; serine 423/425 phosphorylated: 9520; Cell Signaling Technology), and LC3B (2775; Cell Signaling Technology) were used. GAPDH (2118; Cell Technology) was used as a loading control. Primary antibodies were incubated overnight at 4°C. Next, membranes were incubated with secondary antibodies (1:5000) of anti-mouse IgG peroxidase (A851P-1000.S1; Bio-Connect) or anti-rabbit IgG peroxidase (A885L-2000.S1; Bio-Connect). Detection was performed by using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer’s manual. Densitometric quantification was performed with the use of Quantity One software (version 4.6.2; Bio-Rad).

Protein and DNA

For protein analysis, equal amounts of ~50 mg muscle tissue were subsequently dissolved in 400 μL lysis buffer [tris pH 7.4 (50 mmol/L), NaCl (150 mmol/L), glycerol (10%), nonyl phenoxypolyethoxylethanol (0.5%), EDTA (1 mmol/L), Na2VO4 (1 mmol/L), NaF (5 mmol/L), β-glycerophosphate (10 mmol/L), Na-ptyro-P24 (1 mmol/L), dithiothreitol (1 mmol/L), leupeptin (10 μg/mL), aprotenin (1%), and phenylmethylsulfonyl fluoride (1 mmol/L)] and homogenized with Polytron PT homogenizer (Polytron PT 1600 E; Kinematica AG). After 30 min incubation on ice, muscle homogenates were sonicated and centrifuged at 4°C (16000 relative centrifugal force during 30 min). Supernatant fluid (referred to as cytoplasmatic fraction) was separated from the pellet (referred to as the myofibrillar fraction). Protein concentrations of cytoplasmatic and myofibrillar fractions were determined by using a BCA Protein Assay Kit (Pierce, Thermo Fisher), according to the manufacturer’s instructions. A Quant-iT double-stranded DNA assay kit (Promega) was used according to manufacturers’ instructions to measure the DNA content in the myofibrillar fraction.

MUSCLE PROTEIN TURNOVER IN CANCER (PRE)CACHEXIA 741
mL (Leo). Myotubes were incubated for 4 h. This time point was identified in pilot experiments with the pooled plasma of study subjects. Subsequent analyses were performed by using individual samples. For analysis, myotubes were harvested in 500 μL 1× luciferase buffer on ice and stored at −80°C. Luciferase activity was measured according to manufacturer’s protocol (Promega) by using a luminometer (Berthold Technologies). Luciferase activity was corrected for total protein by using the Bradford assay according to the manufacturer’s protocol (Bio-Rad).

Statistics

Data were analyzed with Statistical Package for the Social Sciences software (SPSS version 15 for Windows; SPSS Inc). Except for baseline body weight loss, which represented the weight loss within individual patients in the 6 mo before diagnosis, all data represent comparisons between healthy control subjects and precachectic and cachectic patient groups. When changes are described in percentages (except for baseline weight loss), the change in a specific patient group relative to healthy control subjects is represented. Continuous variables were compared by using 1-factor ANOVA followed by a least-significant difference post hoc analysis. Because post hoc least-significant difference comparisons involved only 3 groups, no additional adjustments for an experiment-wise error rate were performed (29). Pearson’s chi-square test and, in addition, Fisher’s exact test (when expected counts were <5) were used for comparison of categorical variables. Both tests showed the same results for all variables. Correlations were evaluated by using Pearson’s correlation test. Data in tables are represented as means ± SDs. Error bars in figures represent the SEMs. Significance was set at P < 0.05.

RESULTS

Distinct appendicular muscle depletion in cachectic but not precachectic lung cancer patients

Subject characteristics are presented in Table 1. There were no significant differences in sex, age, or tumor stage between study groups. As a result of the smoking history of many lung

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Basic characteristics of the study population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control subjects</td>
</tr>
<tr>
<td></td>
<td>(n = 22)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>M (%)</td>
<td>59</td>
</tr>
<tr>
<td>F (%)</td>
<td>41</td>
</tr>
<tr>
<td>Age (y)</td>
<td>61.4 ± 7.0</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.73 ± 0.10</td>
</tr>
<tr>
<td>Premorbid body (weight 6 mo before diagnosis) (kg)</td>
<td>72.7 ± 11.5</td>
</tr>
<tr>
<td>Body weight at diagnosis (kg)</td>
<td>72.7 ± 11.5</td>
</tr>
<tr>
<td>Body weight loss within patients in 6 mo before diagnosis (kg)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Body weight loss within patients in 6 mo before diagnosis (%)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Disease stage of NSCLC</td>
<td></td>
</tr>
<tr>
<td>IIIB (%)</td>
<td>—</td>
</tr>
<tr>
<td>IV (%)</td>
<td>—</td>
</tr>
<tr>
<td>Histology of NSCLC (%)</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>—</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>—</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>51</td>
</tr>
<tr>
<td>Former</td>
<td>54</td>
</tr>
<tr>
<td>Never</td>
<td>22</td>
</tr>
<tr>
<td>Lung function</td>
<td></td>
</tr>
<tr>
<td>FEV1 (percentage predicted)</td>
<td>115.7 ± 19.3</td>
</tr>
<tr>
<td>FVC (percentage predicted)</td>
<td>125.4 ± 1.1</td>
</tr>
<tr>
<td>Tiffeneau index</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>COPD GOLD stage (on the basis of spirometry in the current study)</td>
<td></td>
</tr>
<tr>
<td>No COPD</td>
<td>73</td>
</tr>
<tr>
<td>I (%)</td>
<td>23</td>
</tr>
<tr>
<td>II (%)</td>
<td>4</td>
</tr>
<tr>
<td>III (%)</td>
<td>0</td>
</tr>
<tr>
<td>IV (%)</td>
<td>0</td>
</tr>
<tr>
<td>No spirometry data (%)</td>
<td>0</td>
</tr>
</tbody>
</table>

1 One-factor ANOVA and LSD post hoc testing: *P < 0.05 (precachexia compared with control subjects), **P < 0.05 (cachexia compared with control subjects), ***P < 0.05 (cachexia compared with precachexia). 2P < 0.05 (Pearson’s chi-square or Fisher’s exact test). COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; LSD, least-significant difference; NSCLC, non–small cell lung cancer tumor-node-metastasis stage

2 Mean ± SD (all such values).
cancer patients, lung function was significantly lower in patients, but no significant correlations were shown between lung function (forced expiratory volume in 1 s) and body weight loss, lean mass index, or other indexes of cachexia in any of the groups (data not shown).

At NSCLC diagnosis, patients with precachexia had a mean within-patient body weight loss of 1.7% in the 6 mo before diagnosis, whereas patients with cachexia showed a mean body weight loss of 12% in the 6 mo before diagnosis \((P < 0.05)\) (Table 1).

Comparisons of healthy control, precachectic, and cachectic groups at the moment of diagnosis revealed that lean mass atrophy was evidently present in cachectic patients compared with in healthy control subjects but not in precachectic patients (Figure 2A), which was in correspondence with the cancer cachexia consensus definition (1). Specifically, the appendicular lean mass index was lower \((-20\%; P < 0.05)\) in cachectic patients compared with in healthy control subjects, whereas trunk lean mass was not significantly different between any of the study groups (Figure 2A). No differences were observed in the fat mass index and bone mineral content (Figure 2A).

**Muscle fiber atrophy and decreased muscle protein content in cachectic and precachectic lung cancer patients**

The muscle fiber mean cross-sectional area \((-27\%)\) and muscle protein concentration per unit DNA \((-30–40\%)\) as well as quadriceps muscle strength \((-31\%)\) were substantially lower in the cachectic group than in healthy control subjects \((P < 0.05)\) (Figure 2B–D). Compared with healthy control subjects, precachectic patients showed a consistent pattern of intermediate values for these indexes of muscle mass without significant changes in muscle strength but a significantly lower muscle fiber cross-sectional area \((-21\%; P < 0.05)\) and muscle protein concentration per unit DNA ratio \((-24\%\) to \(27\%; P < 0.05)\) (Figure 2B, 2D).

**Differential activation of anabolic and catabolic signaling pathways in precachectic and cachectic patients with NSCLC**

To assess the anabolic activity in skeletal muscle, the phosphorylation status of Akt and its downstream phosphosubstrates GSK-3β and FOXO as well as mTOR phosphorylation were assessed. Akt Ser473 phosphorylation to the total protein ratio was significantly increased in cachectic patients compared with in healthy control subjects \((P < 0.05)\), whereas precachectic patients showed intermediate expression (Figure 3A). In contrast, none of the Akt phosphosubstrates (ie, GSK-3β, FOXO, and mTOR) displayed significant alterations in phosphorylation status (Figure 3A).

To assess proteolytic signaling in muscle, the expression of main constituents of the UPS and autophagy lysosomal pathway was determined. No changes in mRNA expression of E3-ubiquitin-
ligases MuRF1, atrogin-1, tripartite motif-containing protein 32 (TRIM32), neuronal precursor cell expressed developmentally downregulated 4 (NEDD-4), or E3a-II were observed between patient groups (Figure 3B). Conversely, significantly higher 

BNIP3 mRNA transcriptional expression and LC3B protein expression was observed in muscle biopsies of patients with cachexia than in healthy control subjects (\(P<0.05\)) (Figure 3, B and C). mRNA transcripts of LC3B were not different between study groups (Figure 3B).

**No marked alterations in regulators of myogenesis**

mRNA transcript expression of the negative muscle mass regulator myostatin as well as protein phosphorylation of its downstream signaling constituents (ie, Smad 2 and 3) was not altered in lung cancer patients compared with in healthy control subjects (see Supplemental data Figure 1, A and B, under “Supplemental data” in the online issue). With respect to myogenic signaling, MyoD and myogenin transcripts were unaltered in lung cancer patients compared with in control subjects, whereas myogenin mRNA transcripts differed between precachectic and cachetic patients (\(P<0.05\)) (see Supplemental data Figure 1C under “Supplemental data” in the online issue).

**Factors in plasma of both precachectic and cachetic patients induce inflammatory signaling in skeletal muscle**

To investigate the systemic and local inflammatory status in the cachexia spectrum, inflammatory markers in plasma and muscle were determined. Significantly higher concentrations of various systemic inflammatory mediators such as CRP, IL-6 and -8, and sTNF receptor 1 were observed in plasma of cachetic patients (\(P<0.05\)) (Figure 4A). The plasma concentrations of TNF-\(\alpha\) were not significantly different between precachectic and cachetic patients (Figure 4A). In precachectic patients, similar trends toward increased systemic and local inflammatory signaling were observed, albeit to a lesser extent, an only reached significance for sTNF receptor 1 plasma concentrations (\(P<0.05\)) (Figure 4, A and B).
To explore the notion that inflammatory mediators present in the circulation can induce local inflammatory signaling, ex vivo plasma transfer experiments were conducted on cultured NF-κB–luciferase reporter muscle cells. These experiments showed increased NF-κB activation when cultured muscle cells were exposed to plasma of both precachectic and cachectic patients (P, 0.05) (Figure 4C).

DISCUSSION

This study was conducted to identify whether mechanisms in the control of cachexia in experimental models are evident in patients with progressive stages of lung cancer cachexia. To the best of our knowledge, this was the first study comprehensively assessing regulation of muscle protein metabolism and transition of inflammatory signaling in precachectic and cachectic patients stratified according to the recent international cancer cachexia consensus (1). Insight in the molecular mechanisms responsible for alterations of nutritional status in cancer cachexia is essential for the design and timing of tailored (nutritional) intervention strategies that alleviate the negative consequences of this destructive syndrome.

As shown by the anthropometric data, cachectic patients predominantly exhibited lean mass depletion, especially in the appendicular body compartment. Because the appendicular lean body compartment primarily consists of muscle mass (30, 31), this result indicated a specific loss of skeletal muscle. The wasting of muscle mass despite a similar fat mass index is indicative of an active catabolic state (1). As a result of appendicular muscle mass depletion, quadriceps muscle strength was significantly declined in cachectic patients (Figure 2D). In line with and to a similar extent as the impaired muscle strength, muscle morphologic analyses revealed a substantial lower muscle fiber cross-sectional area and muscle protein per unit DNA ratio in the muscle of cachectic patients (Figure 2, B and C). Subtle alterations in muscle morphology were already detectable in the precachectic group, which were not identified by the body composition and muscle-function analysis (Figure 2, B and C). Future research is indicated to study if other noninvasive imaging techniques (ie, MRI and computed tomography) may be more sensitive to show these subtle but clinically relevant changes in muscle morphology.

With respect to signaling pathways of muscle protein turnover, the PI3K/Akt pathway is considered essential for muscle protein synthesis and, more importantly, sensitive to nutritional
modulation (8, 9). The current data showed that Akt phosphorylation gradually increased in precachexia and was significantly elevated in cachexia (Figure 3A), which might have implied that there was increased anabolic activity as a compensatory response to muscle mass loss. However, Akt activation was not accompanied by phosphorylation of its downstream substrates (GSK-3β and FOXO), and correspondingly, no increases in mTOR phosphorylation were observed (Figure 3A) (8, 9). This discrepancy between Akt activation and downstream signals suggested impaired Akt activity, which might have implied a resistance to anabolic stimuli at the level of Akt. This resistance can have important consequences on muscle mass regulation because downstream molecules such as mTOR and FOXO are important regulators of muscle protein turnover (8, 9). Because nutritional interventions with branched chain amino acids have been shown to stimulate the anabolic activity by directly affecting these mediators downstream of Akt, these findings implied a potential for nutritional interventions to circumvent putative anabolic resistance at the level of Akt in cancer cachexia (32). To our knowledge, only one other report comprehensively studied PI3K/Akt signaling in cancer cachexia and showed a general decrease of the PI3K/Akt signaling cascade in patients with pancreatic cancer (33). Previous studies in lung cancer patients only assessed isolated mediators (GSK-3β) of the PI3K/Akt pathway or myofibrillar protein fractional synthetic rate, which both were unaltered (34, 35).

Akt functions as a nodal point between anabolic and proteolytic pathways, and its control of protein degradation relies on the regulation of members of the FOXO transcription factors family. Different FOXO subtypes can activate proteolytic pathways when not phosphorylated by Akt (9, 12) by inducing an increased expression of regulatory constituents of the UPS and autophagy lysosomal pathway (ALP) proteolytic systems (36, 37). However, the current study showed no differences in mRNA expression of a comprehensive set of UPS E3 ligases in any of the cachexia stages (Figure 3B). This seems discrepant because E3 ligases are involved and even considered rate limiting for UPS-mediated proteolysis in experimental cancer cachexia, especially MuRF1 and atrogin-1 (38, 39). It is probable that the rapid development and disproportionate high tumor-to-total mass ratio resulting in more acute and more unrestrained host responses contributed to the significant UPS activation in experimental models (40). As concerns clinical cachexia, a general problem of studies that investigated UPS activity was that they were conducted in small and heterogeneous populations (41–43). With respect to E3 ligases, in line with the current study, expression is almost consistently unaffected in clinical cancer cachexia (35, 44–48). Therefore, we conclude that, when lung cancer-related muscle atrophy involves increased proteolysis, it is unlikely to rely on sustained elevations in mRNA transcripts of E3 ubiquitin ligases.

In contrast to E3 ubiquitin ligases, the expression of ALP-associated effectors was increased in cachectic patients (ie mRNA transcripts encoding BNIP3 and protein expression of LC3B were upregulated in cachectic patients) (Figure 3, B and C). The activation of ALP may imply that, in contrast to highly regulated degradation of individual proteins in UPS-mediated proteolysis, cancer cachexia is characterized by bulk degradation of cytoplasmic components via ALP-dependent proteolysis. The observation that expression of LC3B mRNA transcripts were not increased (Figure 3B) may be explained because its activation is not necessarily accompanied by increased mRNA expression but is rather regulated on the protein level (49). Autophagic flux measurement would be required to ultimately determine ALP activity because the increased expression of BNIP3 and LC3B markers might also reflect accumulation as a result of an obstruction of the ALP at some point, which would not result in increased autophagic activity (49). However, because the patient population exhibited distinct muscle atrophy that was not accompanied by increased UPS activity, it is plausible that the current findings reflect a role for the ALP in cancer-induced muscle catabolism.

An evaluation of important myogenic regulators revealed no marked alterations at the level of myogenesis. Our data suggested a limited role for myostatin in the catabolic process in lung cancer because neither the expressions of myostatin mRNA transcripts nor Smad 2 and 3 protein signaling were altered in different stages of cachexia (see Supplemental data Figure 1, A and B, under “Supplemental data” in the online issue). This result was consistent with findings in non–weight-losing lung cancer patients (34) and cachectic colorectal cancer patients (35). Contrarily, increased myostatin expression was shown in the skeletal muscle of patients with gastrointestinal cancer, which may have indicated tumor-specific effects (34).

No consistent alterations in myogenic regulatory factor expression in the muscle of lung cancer patients were observed compared with in control subjects (see Supplemental data Figure 1C under “Supplemental data” in the online issue) despite affected MyoD and myogenin expression in experimental models (50, 51) and decreased muscular MyoD expression in a set of colorectal patients (52).

Because systemic factors can induce local muscular inflammatory signaling and could be a suitable target for intervention strategies, another aim of this study was to verify whether systemic to local transition of inflammatory signaling also occurs in clinical cachexia. In analogy with many studies, the current data showed a prominent systemic inflammatory response in cachectic patients with lung cancer (18, 20, 53), whereas intermediate increases were apparent in precachectic patients (Figure 4A). In addition, our findings extended these observations because the target gene expression reflective of muscle NF-κB activation was elevated (Figure 4B). This local inflammatory response and subsequent proteolytic signaling appear to be derived from the circulation because the current study showed, for the first time to our knowledge, that factors contained within the plasma of patients with lung cancer can induce local muscular inflammation (Figure 4C).

In conclusion, this study reveals that muscle atrophy in cachectic patients with lung cancer is accompanied by increased systemic and local muscle inflammation, whereas precachectic patients show intermediate expression. Moreover, factors contained within the circulation of both precachectic and cachectic patients with lung cancer can induce inflammatory signaling in skeletal muscle. As concerns muscle protein turnover, increased ALP signaling and Akt phosphorylation without alterations in downstream Akt phosphosubstrates are observed in cachectic patients. This finding implies impaired anabolic signaling that could, in combination with increased proteolytic activity, contribute to the net loss of muscle protein in cancer cachexia and provides further support for a more-targeted nutritional modulation beyond merely macronutrient supplementation.
We thank all study subjects and their families for participation in the study and K Roushop for help with the analysis and interpretation of LC3B protein expression.

The authors’ responsibilities were as follows—RCL, A-MCD, and AMS: designed the research; CMOdK, FJS, JMS, and FL: conducted research and analyzed data; CMOdK, RCL, A-MCD, and AMS: wrote the manuscript and had primary responsibility for the final content of the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

REFERENCES


