Autophagy in locomotor muscles of patients with chronic obstructive pulmonary disease

Autophagy in Locomotor Muscles of Patients with Chronic Obstructive Pulmonary Disease

Yeting Guo[1,2], Harry R. Gosker[3], Annemie M. W. J. Schols[3], Sophia Kapchinsky[4], Jean Bourbeau[5], Marco Sandri[6], R. Thomas Jagoe[7], Richard Debigaré[8], François Maltais[8], Tanja Taivassalo[4], and Sabah N. A. Hussain[1,2]

[1]Department of Critical Care and [5]Montreal Chest Institute, McGill University Health Centre, Montréal, Québec, Canada; [2]Meakins Christie Laboratories, Department of Medicine, [4]Department of Kinesiology, and [7]Segal Cancer Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University, Montréal, Québec, Canada; [3]Department of Respiratory Medicine, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Center, Maastricht, the Netherlands; [6]Dulbecco Telethon Institute at Venetian Institute of Molecular Medicine, Padova, Italy; and [8]Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval and Hôpital Laval, Laval, Québec, Canada

Rationale: **Locomotor muscle atrophy develops in patients with chronic obstructive pulmonary disease (COPD) partly because of increased protein degradation by the ubiquitin-proteasome system. It is not known if autophagy also contributes to protein degradation.**
Objectives: **To investigate whether autophagy is enhanced in locomotor muscles of stable patients with COPD, to quantify autophagy-related gene expression in these muscles, and to identify mechanisms of autophagy induction.**
Methods: **Muscle biopsies were obtained from two cohorts of control subjects and patients with COPD and the numbers of autophagosomes in the vastus lateralis and tibialis anterior muscles, the levels of LC3B protein lipidation, and the expression of autophagy-related genes were measured in the vastus lateralis muscle. To investigate potential pathways that might induce the activation of autophagy, measures were taken of protein kinase B (AKT), mTORC1, and AMPK pathway activation, transcription factor regulation, proteasome activation, and oxidative stress.**
Measurements and Main Results: **Autophagy is enhanced in the locomotor muscles of patients with COPD as shown by significantly higher numbers of autophagosomes in affected muscles as compared with control subjects. Autophagosome number inversely correlates with FEV_1. In the vastus lateralis, LC3B protein lipidation is increased by COPD and the expression of autophagy-related gene expressions is up-regulated. LC3B lipidation inversely correlates with thigh cross-sectional area, FEV_1, and FEV_1/FVC ratio. Enhanced autophagy is associated with activation of the AMPK pathway and FOXO transcription factors, inhibition of the mTORC1 and AKT pathways, and the development of oxidative stress.**
Conclusions: **Autophagy is significantly enhanced in locomotor muscles of stable patients with COPD. The degree of autophagy correlates with severity of muscle atrophy and lung function impairment.**

Keywords: autophagy; COPD; proteasome; proteolysis; protein kinase B (AKT); atrophy

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Skeletal muscle dysfunction contributes to morbidity and mortality of patients with chronic obstructive pulmonary disease (COPD). Muscle atrophy is a major cause of muscle dysfunction in these patients as a result of enhanced proteolysis. The mechanisms behind enhanced proteolysis remain unclear.

What This Study Adds to the Field

We investigated whether autophagy, a proteolytic pathway, is induced in locomotor muscles of patients with COPD and how it is regulated. We report here that autophagosome formation is significantly elevated in two locomotor muscles of patients with mild-to-severe COPD. We also describe significant increases in the expression of several genes involved in autophagosome formation. Enhanced autophagy is associated with enhanced protein ubiquitination and correlates with the severity of lung disease and the degree of limb muscle wasting. Enhanced autophagy is also associated with activation of the AMPK pathway and FOXO transcription factors, inhibition of the mTORC1 and AKT pathways, and the development of oxidative stress, including increased protein oxidation and up-regulation of antioxidant enzymes. Collectively, these results suggest that enhanced autophagy in locomotor muscles of patients with COPD may play a protective role in the recycling of protein aggregates, lipid globules, and dysfunctional mitochondria.

Locomotor muscle weakness and reduced exercise capacity are associated with increased mortality in chronic obstructive pulmonary disease (COPD) independent of the severity of the airflow obstruction (1–3). Muscle weakness in patients with COPD results from atrophy and intrinsic metabolic abnormalities (2, 4–6). Atrophy has primarily been attributed to increased protein degradation. The ATP-dependent ubiquitin-proteasome (UP) pathway is involved in limb muscle atrophy of patients with COPD, as illustrated by augmented 20S proteasomal activity and significant inductions of three muscle-specific E3 ligases (ATROGIN-1, MURF1, and NEDD4) (7–9).

In addition to the UP, studies have indicated involvement of the autophagy-lysosome pathway in muscle breakdown. Autophagy is significantly induced in skeletal muscles in response to atrophic stimuli, such as denervation, fasting, disuse, oxidative stress, and hypoxia (10–14). Because these stimuli are often present in patients with COPD, it is possible that autophagy is enhanced in their locomotor muscles and that it contributes to the muscle atrophy seen in these patients. However, information regarding the contribution of the autophagy-lysosome system to

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Author Contributions: S.N.A.H. supervised the study. All authors participated in the study design. H.R.G. and A.M.W.J.S. provided muscle samples for cohort 1. S.K., R.D., F.M., J.B., T.T., and R.T.J. were involved in surgical procurement of patient muscle biopsies and in obtaining disease severity and functional characteristics of control subjects and patients with chronic obstructive pulmonary disease in cohort 2. Y.G. performed electron microscopy, real-time polymerase chain reaction, and immunoblotting. S.N.A.H., M.S., and Y.G. analyzed data and prepared figures. S.N.A.H. wrote the manuscript. Y.G., T.T., H.R.G., A.M.W.J.S., R.D., and F.M. edited the manuscript.

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Correspondence and requests for reprints should be addressed to Sabah N. A. Hussain, M.D., Ph.D., Room L3.05, 687 Pine Avenue West, Montréal, QC, H3A 1A1 Canada. E-mail: sabah.hussain@muhc.mcgill.ca

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TABLE 1. CHARACTERISTICS OF CONTROL SUBJECTS AND PATIENTS WITH COPD WHOSE VASTUS LATERALIS AND TIBIALIS ANTERIOR MUSCLE SAMPLES UNDERWENT ANALYSIS FOR THE PRESENCE OF AUTOPHAGOSOMES USING ELECTRON MICROSCOPY (COHORT 1)

	Vastus Lateralis Biopsy		Tibialis Anterior Biopsy	
	Control Subjects	COPD	Control Subjects	COPD
Subjects, n	4	6	6	5
Male/female, n	3/1	4/2	6/0	5/0
Age, yr	60.5 ± 1.5	60.0 ± 4.8	65.2 ± 2.7	66.5 ± 4.3
Length, m	1.71 ± 0.02	1.69 ± 0.02	1.79 ± 0.02	1.75 ± 0.03
Weight, kg	80.8 ± 4.1	75.8 ± 7.6	84.3 ± 2.0	66.0 ± 4.8*
BMI, $kg \cdot m^{-2}$	27.5 ± 01.8	26.5 ± 2.3	26.3 ± 0.8	21.5 ± 1.5*
FFM, kg	61.0 ± 4.3	53.5 ± 3.4	64.0 ± 1.4	47.4 ± 1.1*
FFMI, $kg \cdot m^{-2}$	20.7 ± 1.0	18.3 ± 1.0	20.0 ± 0.4	15.7 ± 0.4*
FEV_1, % pred	99.5 ± 2.8	53.7 ± 9.3*	111.7 ± 4.3	35.4 ± 5.2*
FVC, % pred	109.3 ± 3.3	88.7 ± 8.7	117.3 ± 0.8	84.6 ± 8.7*
FEV_1/FVC, %	73.3 ± 0.4	47.7 ± 5.6*	73.0 ± 3.6	32.8 ± 3.9*
D_{LCO}, % pred	114.0 ± 5.8	86.1 ± 7.9*	123.4 ± 8.5	65.8 ± 17.7*
Smoking habits, n				
Smoker	0	0	1	1
Nonsmoker	0	0	2	1
Exsmoker	4	6	3	3

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; D_{LCO} = diffusing capacity of the lung for carbon monoxide; FFM = fat-free mass; FFMI = FFM index; % pred = % predicted.

Data are means ± SEM.

* $P < 0.05$, compared with control subjects.

protein degradation and the loss of locomotor muscle mass in patients with COPD is lacking. We sought to test the hypothesis that autophagy is significantly enhanced in locomotor muscles of patients with COPD and to determine whether the activation of autophagy pathways correlates with the severity of muscle atrophy and lung function impairment.

Autophagy is initiated by the ULK1-ATG13-FIP200 protein complex, which is controlled by the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) pathways. In addition, the protein kinase B (AKT) pathway inhibits autophagy through two mechanisms: indirect activation of the mTORC1 complex and direct inactivation of FOXO transcription factors, which are involved in replenishing the short-lived proteins that are required for autophagosome-lysosome fusion (13). We hypothesized that enhanced autophagy in locomotor muscles of patients with COPD is associated with activation of ULK1 protein, that this activation is the result of activation of the AMPK pathway, and, possibly, inhibition of the mTORC1 pathway due to oxidative and metabolic stress (15–18). We also hypothesize that sustained autophagy requires the activation of a FOXO transcriptional program to replenish short-lived proteins that are required for autophagosome formation (13). The second objective of this study, therefore, is to investigate the involvement of these pathways in the regulation of autophagy in locomotor muscles of patients with COPD.

METHODS

Two cohorts of control subjects and patients with COPD were examined.

Cohort 1

Autophagosome formation in the vastus lateralis and tibialis anterior muscles was quantified by transmission electron microscopy using samples obtained from two separate previous studies (Table 1). Vastus lateralis biopsies were obtained using a needle biopsy technique (19) from six patients with COPD with mild-to-severe airflow obstruction and four control subjects, as previously described (20). Tibialis anterior biopsies were obtained using a conchotome (21) from five patients with COPD with mild-to-severe airflow obstruction and six control subjects, as previously described (20). Control subjects were healthy, age-matched volunteers. Specimens were immediately fixed and ultrathin sections were prepared for electron microscopy (*see* the online supplement). Autophagosomes were quantified in 40 separate fields per muscle sample.

Cohort 2

The expression of autophagy-related gene expression was quantified and signaling pathway-associated proteins involved in the regulation of autophagy were identified in the vastus lateralis muscles of 20 patients with severe COPD and 10 control subjects recruited at McGill University and Université Laval hospitals (Table 2). Identical biopsy procedures were followed at each institution. Measurements of pulmonary function, exercise performance, and thigh cross-sectional area (CSA) are described in the online supplement.

RNA extraction. Total RNA was extracted from vastus lateralis muscles using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Oakville, ON, Canada). Expressions of various mRNA transcripts were measured using real-time polymerase chain reaction, as previously described (22). Details are described in the online supplement.

Immunoblotting. Vastus lateralis biopsies were homogenized and loaded onto tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were electrophoretically separated and transferred onto polyvinylidene difluoride membranes, blocked with nonfat dry milk, and incubated overnight with primary antibodies followed by secondary antibody. Proteins were detected using a commercial kit and optical densitometry was performed, as previously described (23). To evaluate protein oxidation, protein carbonyl formation was measured using an OxyBlot kit (Millipore, Billerica, MA), as previously described (23).

Statistical Analysis

Means ± SEM are presented for all data. To account for nonparametric data distribution, all variables between control subjects and patients with COPD were compared using the Mann-Whitney test for unpaired samples. Correlations were tested using Pearson correlation coefficient. *P* values less than 5% were considered significant. Analyses were performed using SigmaStat software (Systat Inc., San Jose, CA).

RESULTS

Cohort 1

No significant differences in age, weight, body mass index (BMI), or fat-free mass index were observed between control subjects

TABLE 2. CHARACTERISTICS OF CONTROL SUBJECTS AND PATIENTS WITH COPD WHOSE VASTUS LATERALIS MUSCLE SAMPLES UNDERWENT ANALYSIS FOR INDICES OF AUTOPHAGY INDUCTION AND SIGNALING PATHWAYS INVOLVED IN THE INITIATION OF AUTOPHAGY (COHORT 2)

	Vastus Lateralis Biopsy Control Subjects	Vastus Lateralis Biopsy COPD
Subjects, n	10	20
Male/female, n	7/3	17/3
Age, yr	61.8 ± 2.8	67.6 ± 3.8
Length, m	1.66 ± 0.17	1.69 ± 0.16
Weight, kg	77.0 ± 4.3	71.8 ± 3.6
BMI, $kg \cdot m^{-2}$	27.8 ± 1.5	25.3 ± 1.6
Right thigh CSA, cm^2	102.8 ± 5.8	63.2 ± 4.7*
FEV_1, % pred	123.1 ± 6.1	42.7 ± 2.1*
FVC, % pred	121.4 ± 5.8	86.2 ± 4.1*
FEV_1/FVC, %	75.1 ± 2.0	39.3 ± 2.2*
D_{LCO}, % pred	100.4 ± 6.2	57.2 ± 4.1*
$\dot{V}O_2$max, $ml \cdot min^{-1} \cdot kg^{-1}$	30.47 ± 1.74	13.65 ± 1.29*
Smoking habits		
n	10	20
Smoker	1	10
Nonsmoker	1	0
Exsmoker	8	10

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; CSA = cross-sectional area; D_{LCO} = diffusing capacity of the lung for carbon monoxide; % pred = % predicted.

Data are means ± SEM.

* $P < 0.05$, compared with control subjects.

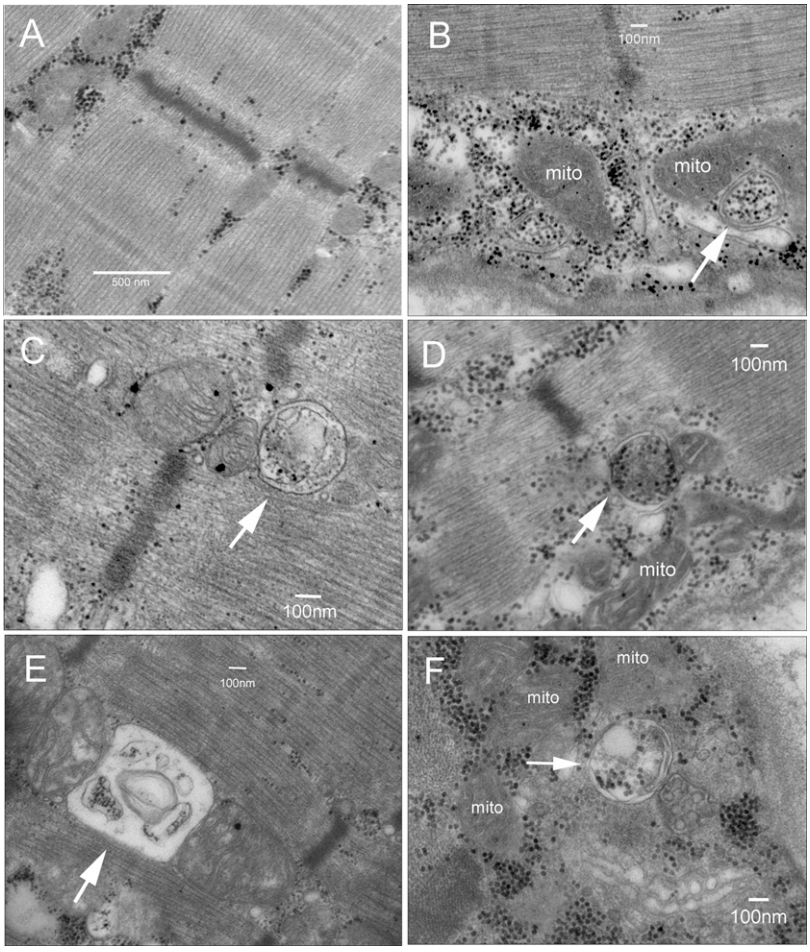

Figure 1. (*A*) Representative electron micrograph of normal mitochondrial and sarcomeric structures in the vastus lateralis of a control subject (cohort 1). (*B*) Representative electron micrograph of an autophagosome (*arrow*) in the tibialis anterior of a control subject. Mito = mitochondria. (*C* and *D*) Representative electron micrograph of an autophagosome (*arrow*) in the vastus lateralis of a patient with chronic obstructive pulmonary disease. (*E* and *F*) Representative electron micrograph of an autophagosome (*arrow*) in the tibialis anterior of a patient with chronic obstructive pulmonary disease.

and patients with COPD from whom vastus lateralis muscle biopsies were taken and analyzed for autophagosome formation (Table 1). However, FEV_1, FVC, FEV_1/FVC ratios, and diffusing lung capacity (D_{LCO}) were significantly lower in patients with COPD as compared with control subjects (Table 1). Weight, BMI, fat-free mass, fat-free mass index, FEV_1, FVC, and D_{LCO} values were significantly lower in patients with COPD as compared with control subjects from whom tibialis

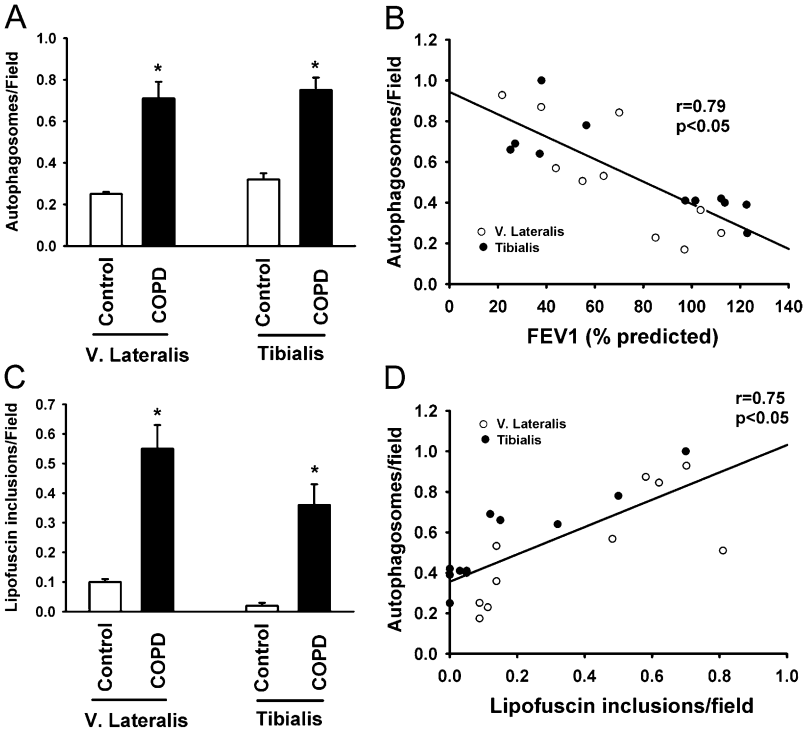

Figure 2. (*A*) Autophagosome formation in the vastus lateralis and tibialis anterior of control subjects and patients with chronic obstructive pulmonary disease (COPD) (cohort 1). Values (means ± SEM) are expressed as number of autophagosomes per field. $*P < 0.05$, compared with control subjects. (*B*) Correlation between FEV_1 (% predicted) and autophagosome number per field in the vastus lateralis and tibialis anterior of control subjects and patients with COPD (cohort 1). (*C*) Lipofuscin inclusion formation in the vastus lateralis and tibialis anterior of control subjects and patients with COPD (cohort 1). Values (means ± SEM) are expressed as number of autophagosomes per field. (*D*) Correlation between lipofuscin inclusion number per field and autophagosome number per field in vastus lateralis and tibialis anterior muscles of control subjects and patients with COPD (cohort 1).

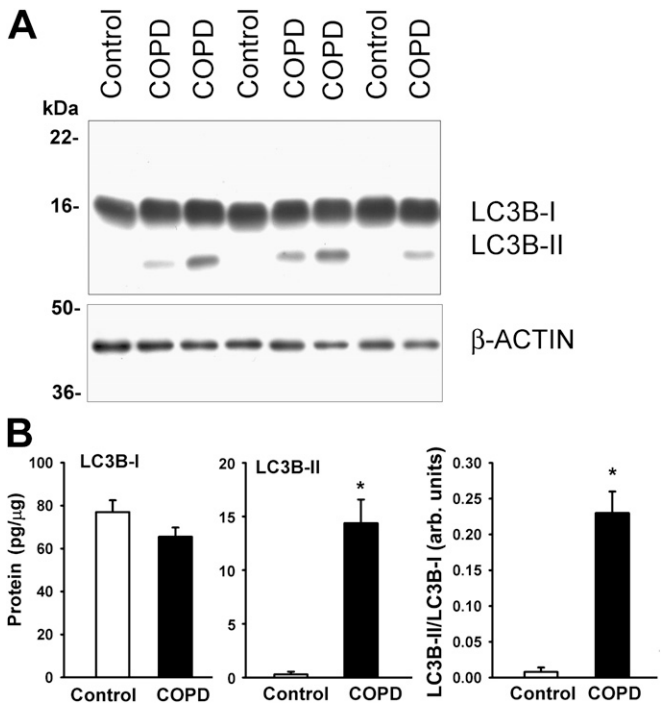

Figure 3. (*A*) Representative immunoblots of LC3B and β-ACTIN proteins in the vastus lateralis of control subjects and patients with chronic obstructive pulmonary disease (COPD) (cohort 2). LC3B-I refers to cytosolic form of LC3B protein, LC3B-II refers to lipidated form of LC3B protein. LC3B-II is incorporated into autophagosome membranes. (*B*) Optical densities of LC3B-I and LC3B-II detected in the vastus lateralis of control subjects and patients with COPD (cohort 2). Values (means ± SEM) are expressed as picogram of LC3B per microgram of total muscle protein. LC3B-II/LC3B-I ratios are also shown. $*P < 0.05$, compared with control subjects.

anterior muscle biopsies were taken and analyzed for autophagosome formation (Table 1).

Autophagosome formation. In control subjects, few autophagosomes were visualized and those were primarily in the subsarcolemmal region (Figure 1B). Significantly higher numbers of autophagosomes (*white arrows*) containing diverse cargo were seen in the subsarcolemmal and intermyofibrillar regions of the vastus lateralis (Figures 1C and 1D) and tibialis anterior (Figures 1E and 1F) of patients with COPD (Figure 2A) (*see* online supplement). Within control subjects and patients with COPD, no significant differences in autophagosome number were detected between the vastus lateralis and tibialis anterior (Figure 2A). The number of autophagosomes significantly and inversely correlated with FEV_1 (% predicted) (Figure 2B) and FEV_1/FVC ratios (*see* online supplement). Lipofuscin inclusions (markers of oxidative cellular damage) were also detected (*see* online supplement), with the vastus lateralis and tibialis anterior muscles of patients with COPD showing significantly higher numbers of inclusions as compared with control subjects (Figure 2C). A significant linear correlation was observed between number of lipofuscin inclusions and number of autophagosomes (Figure 2D). A significant increase in the number of lipid globules was seen in the tibialis anterior of patients with COPD, but not in the vastus lateralis, as compared with control subjects (*see* online supplement).

Cohort 2

No significant differences in age, weight, or BMI were observed between control subjects and patients with COPD from whom vastus lateralis biopsies were taken and analyzed for presence of autophagy (Table 2). However, thigh CSA, FEV_1, FVC, FEV_1/FVC ratios, $D_{L_{CO}}$, and $\dot{V}_{O_2}$max, were significantly lower in patients with COPD as compared with control subjects (Table 2).

Lipidation of LC3B protein. LC3B-II (lipidated LC3B) and LC3B-II/LC3B-I ratio was significantly increased in the muscles of patients with COPD as compared with control subjects (Figures 3A and 3B), indicating enhanced mobilization of LC3B protein to autophagosome membranes. LC3B-II level and LC3B-II/LCB3-I ratio significantly and negatively correlated with thigh CSA and FEV_1/FVC (Figure 4). LC3B-II level and LC3B-II/LC3B-I ratio also significantly and negatively correlated with FEV_1 (% predicted) albeit with lower correlation coefficients than those associated with thigh CSA and FEV_1/FVC (*see* online supplement). In addition, LC3B-II protein levels positively correlated with Global Initiative for Chronic Obstructive Lung Disease stage of COPD (*see* online supplement). No correlations were observed among LC3B-II level, LC3B-II/LC3B-I ratio, and BMI.

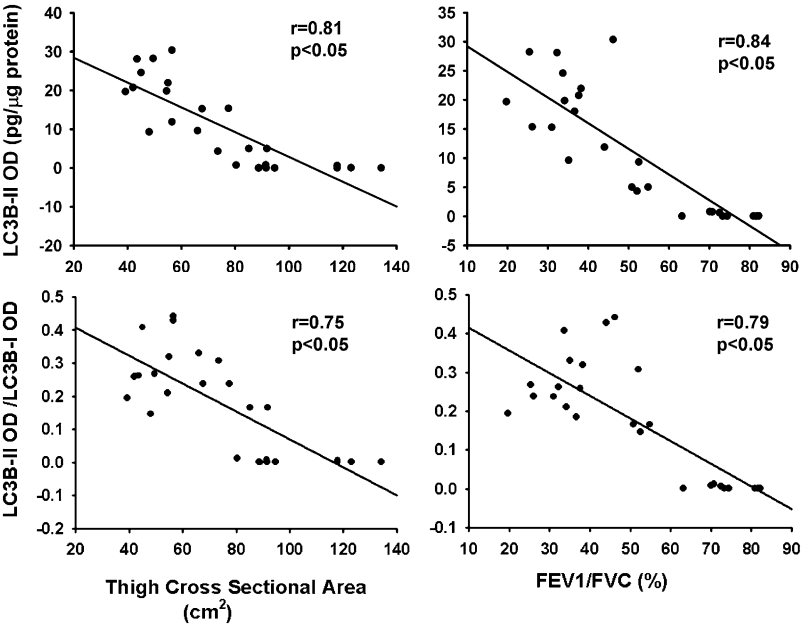

Figure 4. Correlations between thigh cross-sectional area, FEV_1/FVC ratio, and indices of autophagosome formation (LC3B-II intensity and LC3B-II/LC3B-I ratio) in the vastus lateralis of control subjects and patients with chronic obstructive pulmonary disease (cohort 2).

Expression of autophagy-related genes. mRNA and protein levels of several autophagy-related genes involved in autophagosome formation (BECN1, PI3KCIII, UVRAG, AMBRA1, GABARAPL1, ATG7), selective targeting of mitochondria by autophagosomes (SQSTM1, BNIP3, PARKIN), and degradation of autophagosome cargo (CTSL1) significantly increased in the muscles of patients with COPD as compared with control subjects (Figure 5).

Regulation of autophagy initiation. AKT phosphorylation on Ser^{473}, but not total AKT protein, was significantly decreased in the muscles of patients with COPD as compared with control subjects (Figure 6A). Phospho-AKT/AKT ratios in the muscles of patients with COPD did not differ from those of control subjects (Figure 6B). To confirm these results, phosphorylation of proline-rich AKT substrate 40 (PRAS40) was measured. PRAS40 is a downstream effector of AKT that interacts with Raptor in the mTORC1 complex to inhibit its activation (24). Phosphorylation of PRAS40 on Thr^{246} by AKT counteracts PRAS40 inhibition of mTORC1 (25). Phospho-PRAS40 (Thr^{246})/PRAS40 ratios in the muscles of patients with COPD did not differ from those of control subjects (*see* online supplement). To evaluate mTORC1 activity, phosphorylation of ribosomal protein S6 on $Ser^{235/236}$ was measured. mTORC1 activates the kinase P70S6K1, which in turn phosphorylates the ribosomal protein S6 (26). S6 phosphorylation, but not total S6 protein, significantly decreased in the muscles of patients with COPD as compared with control subjects (Figures 6C and 6D). Phospho-AMPKα and total AMPKα significantly increased in the muscles of patients with COPD as compared with control subjects (Figure 6). ULK1 phosphorylation, but not total ULK1, significantly decreased in the muscles of patients with COPD as compared with control subjects (Figure 7).

Regulation of FOXO transcription factors. FOXO1 mRNA and protein levels significantly increased in the muscles of patients with COPD as compared with control subjects (*see* online supplement). Phosphorylation of FOXO1 on Ser^{256} significantly increased in patients with COPD (*see* online supplement). Phospho-FOXO1/total FOXO1 ratios did not differ from those of control subjects (*see* online supplement). FOXO3 mRNA and protein levels and FOXO3 phosphorylation on Ser^{253} did not differ from those of control subjects (*see* online supplement).

Proteasome activation. The intensity of ubiquitin-protein conjugates significantly increased in the muscles of patients with COPD as compared with control subjects (*see* online supplement). Three muscle-specific E3 ligases were measured. MURF1 was the most abundant and NEDD4 the least abundant (*see* online supplement). mRNA levels of ATROGIN-1 and MURF1 did not differ between patients with COPD and control subjects. NEDD4 significantly increased (*see* online supplement).

Oxidative stress. The development of oxidative stress was indirectly assessed by measuring protein carbonyl formation and expressions of three important antioxidant enzymes (SOD1, SOD2, and CATALASE). Total protein carbonyl formation and mRNA levels of SOD1 and SOD2, but not CATALASE, significantly increased in the muscles of patients with COPD as compared with control subjects (*see* online supplement).

DISCUSSION

The most important findings of this study are (*1*) the number of autophagosomes is significantly greater in the vastus lateralis and tibialis anterior muscles of patients with COPD than in control subjects; (*2*) lipidation of LC3B protein and the expression of several autophagy-related genes are significantly increased in the vastus lateralis of patients with COPD; (*3*) both the number of autophagosomes and degree of LC3B lipidation correlate with degree of lower limb atrophy and severity of lung function impairment in patients with COPD; and (*4*) ULK1 phosphorylation in the vastus lateralis of patients with COPD is significantly decreased and this is associated with up-regulation of the AMPK pathway and inhibition of the mTORC1 pathway.

Our data including electron microscopy analyses, LC3B protein immunoblotting, and measurements of autophagy-related

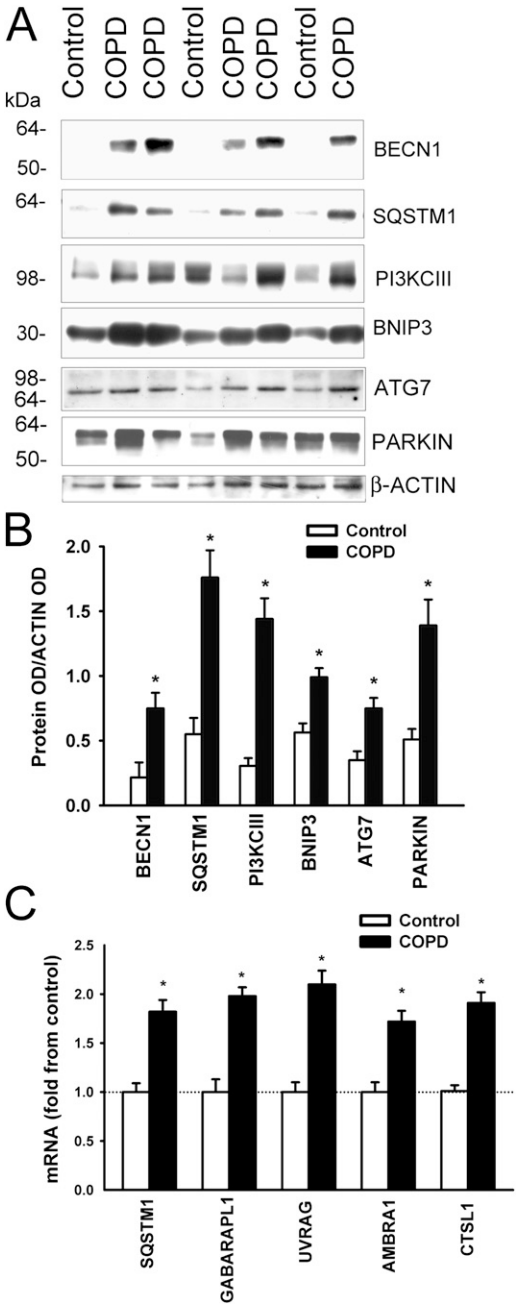

Figure 5. (*A*) Representative immunoblots of BECN1, SQSTM1, PI3KCIII, BNIP3, ATG7, PARKIN, and β-ACTIN proteins in the vastus lateralis of control subjects and patients with chronic obstructive pulmonary disease (COPD) (cohort 2). (*B*) Protein expressions of BECN1, SQSTM1, PI3KCIII, BNIP3, ATG7, and PARKIN in the vastus lateralis of control subjects and patients with COPD. Values (means ± SEM) are expressed as ratio of specific protein optical density to β-ACTIN optical density. $*P < 0.05$, compared with control subjects. (*C*) mRNA expressions of SQSTM1, GABARAPL1, UVRAG, AMBRA1, and CTSL1 in the vastus lateralis of control subjects and patients with COPD. Values (means ± SEM) are expressed as fold change relative to control group. $*P < 0.05$, compared with control subjects.

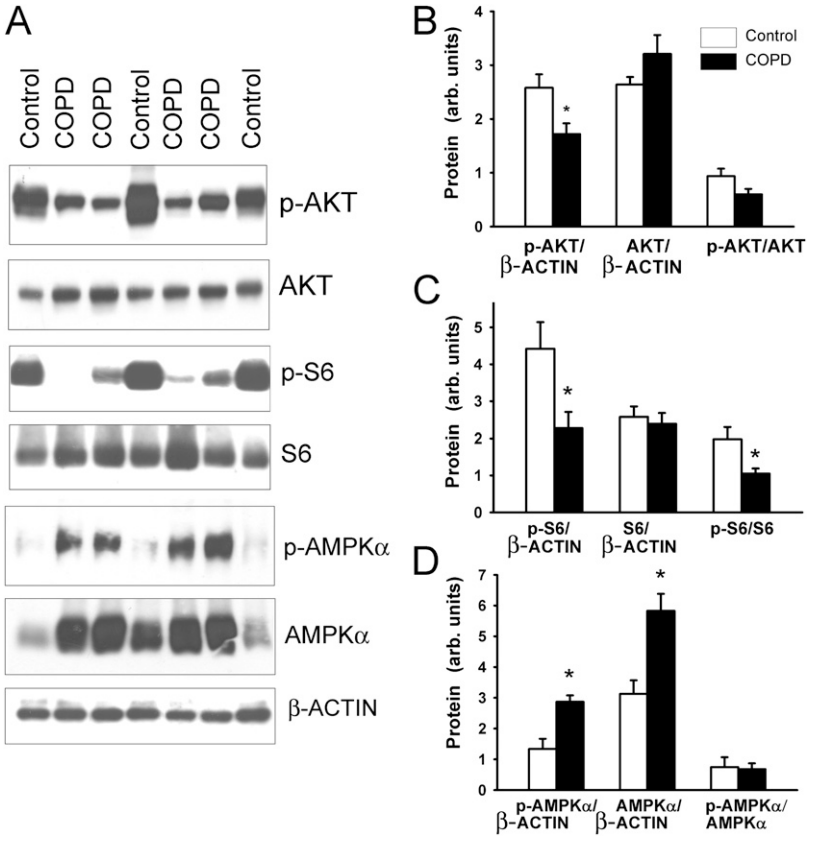

Figure 6. (*A*) Representative immunoblots of phosphorylated and total AKT, ribosomal protein S6, AMPKα, and β-ACTIN in vastus lateralis muscles of control subjects and patients with chronic obstructive pulmonary disease (COPD). (*B–D*) Protein expressions of phosphorylated and total AKT, S6, and AMPKα (normalized to β-ACTIN) in vastus lateralis muscles of control subjects and patients with COPD. Ratios of phosphorylated to total protein for AKT, S6, and AMPKα also shown. Values are means ± SEM. $*P < 0.05$, compared with control subjects.

gene expression suggest that autophagy is significantly enhanced in locomotor muscles of COPD. However, we acknowledge that the limited sample size of the study population warrants further investigation using larger populations of patients with COPD with varying degrees of disease severity.

In their study comparing the vastus lateralis muscles of nine patients with COPD and nine control subjects, Plant and coworkers (7) observed no significant differences in the expression of the autophagy genes BECN1 and LC3B. The discrepancy between their study and ours may simply be an effect of our larger experimental population and our use of multiple techniques to detect activation of autophagy pathways.

Our observational study can only suggest potential mechanisms that lead to the activation of autophagy in the skeletal muscles of patients with COPD: (*1*) alterations in the balance between the AMPK and mTORC1 pathways; (*2*) inhibition of the AKT pathway; and (*3*) enhanced production of reactive oxygen species (ROS).

Autophagy initiation is regulated by ULK1 kinase, mTORC1, and AMPK activity (27–29). We found that the AMPK pathway is significantly up-regulated in the vastus lateralis muscles of patients with COPD, that the mTORC1 pathway is significantly inhibited, and that these changes are associated with reductions in ULK1 phosphorylation (Figures 6 and 7). This suggests an imbalance between the AMPK and mTORC1 pathways, favoring the former, which may contribute to autophagy induction. AKT inhibits autophagy through activation of mTORC1 (30), inactivation of FOXO (13), and phosphorylation of BECN1 (Figure 8) (31). To evaluate the relationships between autophagy, AKT phosphorylation, and muscle atrophy, we divided patients from cohort 2 into two groups based on thigh CSA (32). Patients with greater muscle atrophy had significantly lower phospho-AKT/total AKT ratios and significantly greater levels of autophagy (*see* online supplement). Based on these observations, we speculate that decreased AKT phosphorylation may also contribute to autophagy induction in locomotor muscles of patients with COPD. Autophagy is also activated by oxidative stress under various conditions (14, 33–36).

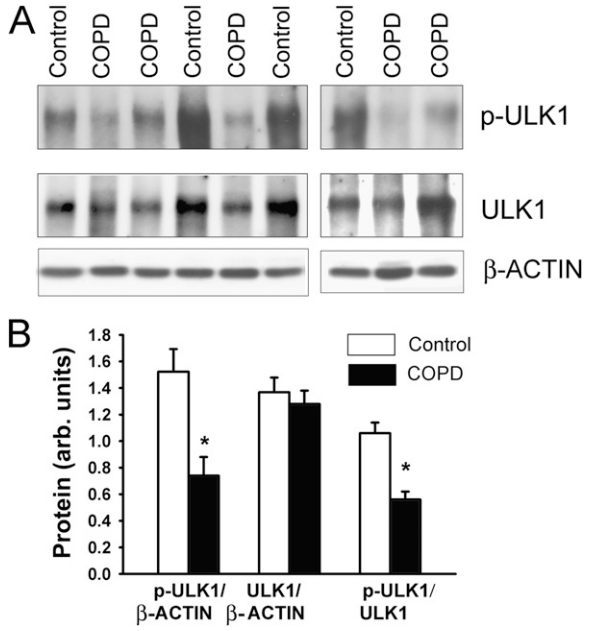

Figure 7. (*A*) Representative immunoblots of phosphorylated and total ULK1 and β-ACTIN in the vastus lateralis of control subjects and patients with chronic obstructive pulmonary disease (COPD). (*B*) Protein expressions of phosphorylated and total ULK1 (normalized to β-ACTIN) in the vastus lateralis of control subjects and patients with COPD. Ratios of phosphorylated to total ULK1 also shown. Values are means ± SEM. $*P < 0.05$, compared with control subjects.

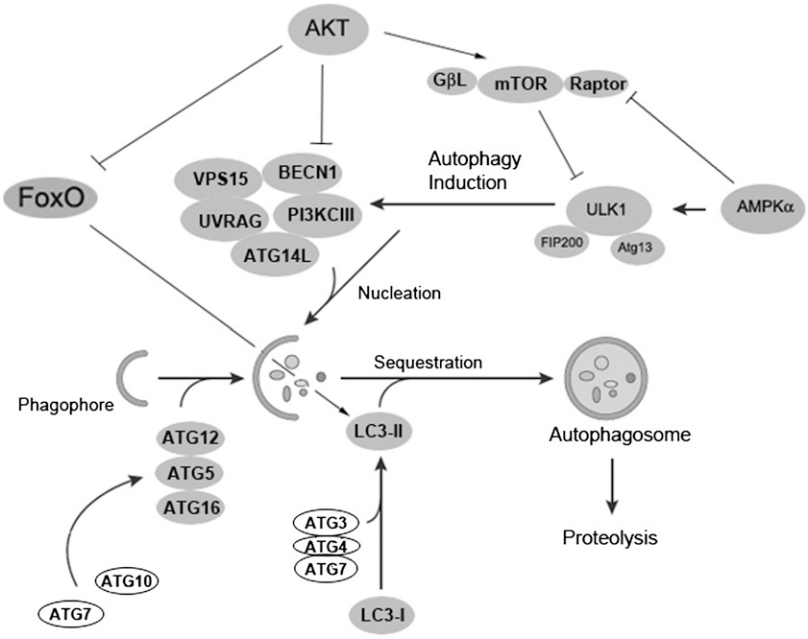

Figure 8. Schematic depiction of various steps involved in autophagosome formation and the signaling pathways that regulate autophagy in skeletal muscles.

ROS influence autophagy through activation of AMPK, inhibition of mTORC1, recruitment of BNIP3 to the mitochondria (37), and activation of FOXO (12). We found that ROS production is enhanced in the vastus lateralis muscles of patients with COPD and suggest that this oxidative stress may also contribute to autophagy induction.

We should emphasize that that our findings regarding the induction of autophagy in locomotor muscles of patients with COPD are descriptive and observational and do not directly delineate the functional contributions of autophagy to muscle dysfunction in these patients. Currently, this question is difficult to answer because there is a dearth of safe and selective autophagy inhibitors that can be administered in humans without affecting other critical processes. We speculate that enhanced autophagy is a protective mechanism designed to preserve muscle function by removing protein aggregates, lipid globules, and dysfunctional mitochondria. Such a protective role has recently been identified in a murine model of ATG7 deficiency where muscle force generation is impaired, leading to the development of muscle atrophy (38). It can also be speculated that autophagy and UP protein degradation are somehow coupled: as myofilament proteins degrade, ratios of cellular organelles to myofilament proteins need to be readjusted, a process for which autophagy is thought to be an important mechanism. Enhanced autophagy was indeed associated with increased UP activity in patients with COPD in the current study. Moreover, the observed increased levels of SQSTM1, BNIP3, and PARKIN are indicative of enhanced mitochondrial clearance through autophagy.

In summary, existing research has tended to focus on the contribution of the UP pathway to muscle breakdown in COPD. In our study, the autophagy-lysosome pathway is clearly enhanced in locomotor muscles of patients with COPD and is associated with muscle atrophy. Understanding how the various proteolytic pathways are intertwined in the process of atrophy may increase our chances of developing improved interventions to prevent or reverse muscle wasting in COPD.

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References

1. Gosselink R, Troosters T, Decramer M. Peripheral muscle weakness contributes to exercise limitation in COPD. *Am J Respir Crit Care Med* 1996;153:976–980.
2. Gosker HR, van Mameren H, van Dijk PJ, Engelen MP, van der Vusse GJ, Wouters EF, Schols AM. Skeletal muscle fibre-type shifting and metabolic profile in patients with chronic obstructive pulmonary disease. *Eur Respir J* 2002;19:617–625.
3. Allaire J, Maltais F, Doyon JF, Noel M, LeBlanc P, Carrier G, Simard C, Jobin J. Peripheral muscle endurance and the oxidative profile of the quadriceps in patients with COPD. *Thorax* 2004;59:673–678.
4. Mador MJ, Deniz O, Aggarwal A, Kufel TJ. Quadriceps fatigability after single muscle exercise in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;168:102–108.
5. Saey D, Debigare R, LeBlanc P, Mador MJ, Cote CH, Jobin J, Maltais F. Contractile leg fatigue after cycle exercise: a factor limiting exercise in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;168:425–430.
6. Gosker HR, Kubat B, Schaart G, van der Vusse GJ, Wouters EF, Schols AM. Myopathological features in skeletal muscle of patients with chronic obstructive pulmonary disease. *Eur Respir J* 2003;22:280–285.
7. Plant PJ, Brooks D, Faughnan M, Bayley T, Bain J, Singer L, Correa J, Pearce D, Binnie M, Batt J. Cellular markers of muscle atrophy in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2010;42:461–471.
8. Doucet M, Russell AP, Leger B, Debigare R, Joanisse DR, Caron MA, LeBlanc P, Maltais F. Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;176:261–269.
9. Debigare R, Maltais F, Cote CH, Michaud A, Caron MA, Mofarrahi M, LeBlanc P, Hussain SN. Profiling of mRNA expression in quadriceps of patients with COPD and muscle wasting. *COPD* 2008;5:75–84.
10. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol* 2009;29:2570–2581.
11. Sandri M. Autophagy in skeletal muscle. *FEBS Lett* 2010;584:1411–1416.
12. Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab* 2007;6:472–483.
13. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del PP, Burden SJ, Di LR, Sandri C, Zhao J, *et al*. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* 2007;6:458–471.
14. Dobrowolny G, Aucello M, Rizzuto E, Beccafico S, Mammucari C, Boncompagni S, Belia S, Wannenes F, Nicoletti C, Del PZ, *et al*.

Skeletal muscle is a primary target of SOD1G93A-mediated toxicity. *Cell Metab* 2008;8:425–436.
15. Barreiro E, Rabinovich R, Marin-Corral J, Barbera JA, Gea J, Roca J. Chronic endurance exercise induces quadriceps nitrosative stress in severe COPD patients. *Thorax* 2009;64:13–19.
16. Steiner MC, Evans R, Deacon SJ, Singh SJ, Patel P, Fox J, Greenhaff PL, Morgan MD. Adenine nucleotide loss in the skeletal muscles during exercise in chronic obstructive pulmonary disease. *Thorax* 2005;60:932–936.
17. Favier FB, Costes F, Defour A, Bonnefoy R, Lefai E, Bauge S, Peinnequin A, Benoit H, Freyssenet DG. Down-regulation of Akt/mammalian target of rapamycin pathway in skeletal muscle is associated with increased REDD1 expression in response to chronic hypoxia. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R1659–R1666.
18. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, *et al*. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 2001;3:1014–1019.
19. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 1975;35:609–616.
20. Gosker HR, Hesselink MK, Duimel H, Ward KA, Schols AM. Reduced mitochondrial density in the vastus lateralis muscle of patients with COPD. *Eur Respir J* 2007;30:73–79.
21. Dietrichson P, Coakley J, Smith PE, Griffiths RD, Helliwell TR, Edwards RH. Conchotome and needle percutaneous biopsy of skeletal muscle. *J Neurol Neurosurg Psychiatry* 1987;50:1461–1467.
22. Mofarrahi M, Brandes RP, Gorlach A, Hanze J, Terada LS, Quinn MT, Mayaki D, Petrof B, Hutchinson DS. Regulation of proliferation of skeletal muscle percursor cells by NADPH oxidase. *Antioxid Redox Signal* 2008;10:559–574.
23. Hussain SN, Mofarrahi M, Sigala I, Kim HC, Vassilakopoulos T, Maltais F, Bellenis I, Chaturvedi R, Gottfried SB, Metrakos P, *et al*. Mechanical ventilation-induced diaphragm disuse in humans triggers autophagy. *Am J Respir Crit Care Med* 2010;182:1377–1386.
24. Vander HE, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* 2007;9:316–323.
25. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* 2007;25:903–915.
26. Dufner A Thomas G. Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res* 1999;253:100–109.
27. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 2011;13:132–141.
28. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, *et al*. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* 2011;331:456–461.
29. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH. ULK-Atg13–FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* 2009;20:1992–2003.
30. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell* 2002;10:151–162.
31. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J, Levine B. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science* 2012;338:956–959.
32. Marquis K, Debigare R, Lacasse Y, LeBlanc P, Jobin J, Carrier G, Maltais F. Midthigh muscle cross-sectional area is a better predictor of mortality than body mass index in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;166:809–813.
33. Djavaheri-Mergny M, Amelotti M, Mathieu J, Besancon F, Bauvy C, Souquere S, Pierron G, Codogno P. NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy. *J Biol Chem* 2006;281:30373–30382.
34. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. *Cell Death Differ* 2009;16:1040–1052.
35. Kirkland RA, Saavedra GM, Franklin JL. Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome c redistribution. *J Neurosci* 2007;27:11315–11326.
36. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, Semenza GL. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 2008;283:10892–10903.
37. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci* 2011;36:30–38.
38. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, Metzger D, Reggiani C, Schiaffino S, Sandri M. Autophagy is required to maintain muscle mass. *Cell Metab* 2009;10:507–515.