Downregulation of Akt/mammalian target of rapamycin pathway in skeletal muscle is associated with increased REDD1 expression in response to chronic hypoxia

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Favier FB, Costes F, Defour A, Bonnefoy R, Lefai E, Baugé S, Peinnequin A, Benoit H, Freyssenet D. Downregulation 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 298: R1659–R1666, 2010. First published March 17, 2010; doi:10.1152/ajpregu.00550.2009.—Although it is well established that chronic hypoxia leads to an inexorable loss of skeletal muscle mass in healthy subjects, the underlying molecular mechanisms involved in this process are currently unknown. Skeletal muscle atrophy is also an important systemic consequence of chronic obstructive pulmonary disease (COPD), but the role of hypoxemia in this regulation is still debated. Our general aim was to determine the molecular mechanisms involved in the regulation of skeletal muscle mass after exposure to chronic hypoxia and to test the biological relevance of our findings into the clinical context of COPD. Expression of positive and negative regulators of skeletal muscle mass were explored in the soleus muscle of rats exposed to severe hypoxia (6,300 m) for 3 wk and in vastus lateralis muscle of nonhypoxicemic and hypoxicemic COPD patients. In rodentmus, we observed a marked inhibition of the mammalian target of rapamycin (mTOR) pathway together with a strong increase in regulated in development and DNA damage response 1 (REDD1) expression and its association with 14-3-3, a mechanism known to downregulate the Akt/mammalian target of rapamycin pathway. Importantly, REDD1 overexpression in vivo was sufficient to cause skeletal muscle fiber atrophy in normoxia. Finally, the comparative analysis of skeletal muscle in hypoxicemic vs. nonhypoxicemic COPD patients confirms that hypoxia causes an inhibition of the mTOR signaling pathway. We thus identify REDD1 as a negative regulator of skeletal muscle mass during chronic hypoxia. Translation of this fundamental knowledge into the clinical investigation of COPD shows the interest to develop therapeutic strategies aimed at inhibiting REDD1.

—Skeletal muscle atrophy can have a profound effect on overall health and viability. The consequences of a reduction in skeletal muscle mass are multiple and include a decrease in strength and power output, an increased fatigability, and an increase in insulin resistance. Identifying the mechanisms leading to muscle mass loss is therefore a fundamental question to develop therapeutic strategies. The maintenance of skeletal muscle mass results from the balance between protein synthesis and degradation, a disruption in this equilibrium promoting either muscle hypertrophy or muscle atrophy. Protein synthesis and degradation are coordinately regulated by Akt signaling pathways (reviewed in Refs. 14 and 34). When activated by phosphorylation, Akt can phosphorylate and inhibit forkhead transcription factors, thus repressing the expression of atrogenes (35, 39) and the subsequent degradation of targeted proteins by the ubiquitin/proteasome system (UPS). Furthermore, activation of the Akt/mammalian target of rapamycin (mTOR) pathway triggers skeletal muscle hypertrophy (6, 19). The Akt-mediated activation of mTOR is indirect and involves the phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2) by Akt (17). mTOR then stimulates protein translation notably through 4E-BP1 inhibition and ribosomal protein S6 kinase (S6K) activation (16, 21). The Akt/mTOR pathway is subjected to multiple regulatory influences. Particularly, regulated in development and DNA damage response 1 (REDD1) has been recently shown to inhibit mTOR signaling via releasing TSC2 from its inhibitory 14-3-3 partners (9) in response to cellular stress such as DNA damage (13), energy depletion (38), dexamethasone treatment (42), alcohol intoxication (22), or low O2 concentration (7).

In healthy subjects exposed to high altitude, chronic hypoxia leads to an inexorable loss of skeletal muscle mass, which has been mainly attributed to hypophagia. However, chronic hypoxia also downregulates skeletal muscle mass regardless of the nutritional status (4). Importantly, hypoxia has been shown to trigger hypophosphorylation of mTOR and its downstream effectors 4E-BP1 and S6K in HEK-293 cells (2), indicating that the Akt/mTOR pathway is sensitive to oxygen deprivation. Furthermore, REDD1 is strongly upregulated in response to hypoxia (7, 37, and REDD1 is essential for mTOR signaling inhibition by low O2 concentration in mouse embryonic fibroblasts (7). Altogether, these observations suggest that REDD1 could contribute to skeletal muscle atrophy during prolonged hypoxia via the inhibition of mTOR and the subsequent reduction of protein translation. In addition, inhibition of mTOR by REDD1 may also activate the UPS through the mTOR complex 2-Akt-Foxo pathway (15).

Chronic hypoxia is a common feature of chronic obstructive pulmonary disease (COPD), and loss of skeletal muscle mass has been identified as a major determinant of disability in COPD and an independent predictor of mortality (36). Multiple
factors have been evoked to explain the atrophy of skeletal muscle in COPD patients, including chronic inflammation, oxidative stress, sedentarity, altered hormonal status, and hypoxia (41, 43). Although hypoxemia is generally less severe in COPD patients than in the one observed in subjects exposed to high altitude, the regulation of Akt/mTOR pathway by REDD1 could be one factor contributing to skeletal muscle atrophy in COPD patients.

In the present study, we demonstrated that the Akt/mTOR pathway is downregulated in rat skeletal muscle in response to chronic hypoxia and in skeletal muscle of hypoxicemic COPD patients. Furthermore, while REDD1 has not been shown to be required for muscle atrophy, our data show that REDD1 per se is sufficient to trigger skeletal muscle atrophy in the rodent model. Our study provides molecular insights into the mechanisms involved in the regulation of skeletal muscle mass in response to chronic hypoxia and their potential relevance in COPD.

METHODS

Animals and tissue collection. The protocol was approved by the Comité d’Ethique de la Plate-Forme d’Expérimentation Animale de la Faculté de Médecine (Université Jean Monnet, Saint Etienne). Animals were kept in the Plate-Forme d’Expérimentation Animale (Université Jean Monnet) in accordance with the Helsinki Accords for Human Treatment of animals during experimentation.

Fourteen-week-old male Wistar rats (Charles River Laboratories, L’Arbresles, France; n = 30) were randomly assigned into three groups. Hypoxic animals (H group, n = 10) were housed in a hypobaric chamber to a simulated altitude of 6,300 m (45.2 kPa, 340 mmHg) for 16 days after 5 days of progressive acclimatization. H rats were fed ad libitum. Two other groups of rats (n = 10/group) were maintained in normoxia: control animals (C group) were fed ad libitum, whereas pair-fed animals (PF group) were restricted to have the same food intake as the H group. At the end of the experimental period, soleus (SOL) and extensor digitorum longus (EDL) muscles were removed under general anesthesia (injection of 90 mg/kg ketamine and 10 mg/kg xylazine ip). Animals were then euthanized by pentobarbital sodium overdose.

Histochemical analysis. SOL and EDL muscles were cut transversely at the widest point. Serial transverse sections (12 μm) were cut in a cryostat at −20°C. The tissue slices were then processed by the myosin adenosine triphosphatase method after acid preincubation at pH 4.35 (3). Fiber cross-sectional areas (FCSA) of 3,000; Dako, Carpinteria, CA), or goat anti-rabbit (1:2,000; Dako) antibodies were used for chemiluminescent detection of proteins. Films were scanned and quantified using the NIH Image 1.63 program.

REDD1 immunoprecipitation. Lysates containing 150 μg of total protein were incubated with pan-14–3–3 mouse monoclonal antibody (cat. no. MS-1504; LabVision, Fremont, CA) overnight at 4°C. Complexes were immunoprecipitated using protein G Sepharose (cat. no. MS-1504; LabVision, Fremont, CA) overnight at 4°C. Gel loading was systematically checked by Coomassie and Ponceau staining (data not shown). With the exception of calpain-2 (1:1,600, cat. no. 39168; Abcam, Cambridge, MA) and -3 (1:100, cat. no. CALP-21A2; Novocastra, Newcastle upon Tyne, UK), glycogen synthase kinase (GSK)3β (1:1,000, cat. no. PAI-26168; Affinity BioReagents, Rockford, IL), REDD1 (1:750, cat. no. 10638–1-AP; Protein Tech Group, Chicago, IL), S6K (1:2,000, cat. nos. A300, 510A; Bethyl Lab, Montgomery, TX), TSC2 (1:200, cat. nos. sc-893; Santa Cruz Biotechnology, Santa Cruz, CA), ubiquitin (1:400, cat. no. PW8810; Biomol International, Plymouth Meeting, PA) and primary antibodies against Akt (cat. no. 9272), Akt T308 (cat. no. 29656), Akt S473 (cat. no. 9271), AMP-activated protein kinase (AMPK) T172 (cat. no. 2531), GSK3β S9 (cat. no. 9336), 4E-BP1 (cat. no. 9452), 4E-BP1 T37/46 (cat. no. 9459), mTOR (cat. no. 2972), mTOR S2448 (cat. no. 2971), S6 ribosomal protein (S6) (cat. no. 2217), S6 S235/236 (cat. no. 2855), S6K (1:2,000, cat. nos. A300, 510A; Bethyl Lab, Montgomery, TX), TSC2 (1:200, cat. nos. sc-893; Santa Cruz Biotechnology, Santa Cruz, CA), ubiquitin (1:400, cat. no. PW8810; Biomol International, Plymouth Meeting, PA) and primary antibodies against Akt (cat. no. 9272), Akt T308 (cat. no. 29656), Akt S473 (cat. no. 9271), AMP-activated protein kinase (AMPK) T172 (cat. no. 2531), GSK3β S9 (cat. no. 9336), 4E-BP1 (cat. no. 9452), 4E-BP1 T37/46 (cat. no. 9459), mTOR (cat. no. 2972), mTOR S2448 (cat. no. 2971), S6 ribosomal protein (S6) (cat. no. 2217), S6 S235/236 (cat. no. 4856), and TSC2 (cat. no. 3611) were all used to detect and quantify the amount of the corresponding protein in the samples. The experiments were repeated at least three times with different animals.

Table 1. Primer sequences used for PCR analysis

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<th>Gene</th>
<th>Primer Sequence</th>
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<td>ACTB</td>
<td>(GenBank_NM_031144)</td>
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<tr>
<td>ARBP</td>
<td>(GenBank_NM_022402)</td>
<td>F: 5′-CCTCAGCACTGGGTTACACTGGAG-3′</td>
<td>R: 5′-TTTCTCGTGGTACATTTGGAGT-3′</td>
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<td>Atrogin-1</td>
<td>(GenBank_NM_133521)</td>
<td>F: 5′-GGAAGTTGAGCGGAGACCTGTTC-3′</td>
<td>R: 5′-GTCCTGAGCACCGGATTTGAC-3′</td>
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<tr>
<td>CypA</td>
<td>(GenBank_NM_017101)</td>
<td>F: 5′-GATAGCTCGGACATGGTGTAG-3′</td>
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<td>HPRT</td>
<td>(GenBank_NM_012583)</td>
<td>F: 5′-GAGCTGCGTTGGAAAGTATGAC-3′</td>
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<td>F: 5′-TTCCCTGGCCGCCGAGCAACAG-3′</td>
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<td>REDD1</td>
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<td>F: 5′-GAGCTGCGTTGGAAAGTATGAC-3′</td>
<td>R: 5′-GTCTTACCTCGGATTTGAC-3′</td>
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ACTB, β-actin; ARBP, acidic ribosomal phosphoprotein P0; CypA, cyclophilin A; HPRT, hypoxanthine guanine phosphoribosyl transferase; MuRF1, muscle-specific RING finger protein 1; REDD1, regulated in development and DNA damage response 1.
previously described (12). The fascia and skin were then closed with sutures. Seven days after gene electrotransfer, SOL muscles were removed under general anesthesia. Serial transverse sections (12-μm) were stained for β-galactosidase activity and counterstained with hematoxylin-eosin-saffron (12). FCSA was measured in transfected blue fibers and nontransfected fibers (100 fibers/condition).

COPD subjects and Western blot analysis. Nine patients with mild-to-severe COPD were separated according to the absence or presence of resting hypoxemia (PaO₂ < 60 mmHg). They had been free of exacerbation of their disease for at least 2 mo and did not receive oral corticosteroids. Hypoxic patients were treated with long-term oxygen therapy for 15 h/day but breathed ambient air for at least 3 h at the time of the biopsy. Muscle biopsies were taken from the vastus lateralis using Weil-Blakesley forceps.

Written consent in accordance with the policy statement regarding the use of human subjects was obtained from all the subjects. This investigation was approved by the Rhône-Alpes-Loire Regional Consultant Committee on Human Protection from Medical Research (DGS No. 2005/023) in accordance with the Declaration of Helsinki.

Protein extraction from muscle samples was performed as described above. Immunolabeling against Akt, Akt T308, GSK3β, GSK3β S9, S6K, S6K T389 (cat. no. 9205, Cell Signaling Technology) and REDD1 were performed as described above.

Statistical analysis. Data are presented as means ± SE on graphs and means ± SD in tables. Statistical comparisons were performed using two-way or one-way ANOVA. Fisher’s post hoc test or unpaired t-test was then used to determine specific mean difference. All of the statistical analyses were performed using StatView (SAS Institute). The significance level was set at 0.05.

RESULTS

Hypophagia-independent effect of chronic hypoxia on skeletal muscle loss. To test whether hypoxia causes skeletal muscle atrophy regardless of nutritional status, 10 rats were exposed to 6,300 m for 21 days (H group) and compared with

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<tr>
<td>Hematocrit, %</td>
<td>43.1±3.8</td>
<td>67±7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.3±4</td>
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<tr>
<td>Body weight, g</td>
<td>425±13.9</td>
<td>331.4±16.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>353.5±12.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>SOL weight, mg</td>
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<td>134.9±13.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>12.91±2.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.7±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Type I FCSA, μm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4114±668</td>
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<td>3854±281</td>
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<tr>
<td>Type II FCSA, μm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3638±558</td>
<td>2383±728&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3203±704</td>
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SOL, soleus muscle; FCSA, fiber cross-sectional area. Type I fibers represent ~90% of total fibers. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 and <sup>c</sup>P < 0.001: different from C; <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, and <sup>f</sup>P < 0.001: different from PF.

Fig. 1. Ubiquitin/proteasome system is not altered in soleus muscles of hypoxic rats. A: mRNA expression of atrogin-1 and muscle-specific RING finger 1 (MuRF1) ubiquitin ligases. B: representative immunoblots of total ubiquitin-conjugated proteins (result from 2 different animals per group are shown) and Western blot quantification. C: caspase-, trypsin-, and chymotrypsin-like activities of the proteasome 20S. D: cathepsins B+L activities. E: representative immunoblots of calpain-2 and -3 protein content and corresponding quantification. C, control group; H, hypoxic group; PF, pair-fed group (n = 10 per group).
a normoxic control group fed ad libitum (C group) and a normoxic pair-fed group (PF group). Although PaO\textsubscript{2} was not assessed here, previous data show that PaO\textsubscript{2} is \textsim 40 mmHg after 21 days at 5,500 m (31), supporting the existence of marked hypoxemia. Consistently, the adaptation to hypoxic stress was illustrated by the increase in hematocrit in H vs. C and PF animals (Table 2). As expected, body weight of H rats was smaller than their normoxic C and PF counterparts (Table 2). Skeletal muscle mass was also affected by severe hypoxia as muscle weight, total protein content, and FCSA of SOL muscle were lower for the H group compared with C and PF groups (Table 2). The ratio of the difference in muscle mass (C − PF)/(C − H) indicates that \textsim 60% of muscle mass loss in hypoxia was independent of hypoglycemia. It is noteworthy that similar results were obtained in EDL muscle (data not shown). Thus, in agreement with previous studies (4, 33), these data show that hypoxia also exerts a hypoglycemia-independent effect on skeletal muscle mass loss.

**Chronic hypoxia and the UPS.** The UPS is a main component of the cellular proteolytic machinery, and its activation is associated with muscle mass loss (29). However, the UPS did not seem to be profoundly altered in response to 3 wk of hypoxia. Indeed, mRNA level of MuRF1 and atrogin-1 (Fig. 1A), the amount of ubiquitinylated proteins (Fig. 1B), and the sum of the three activities of the 20S proteasome (Fig. 1C) were not significantly different between H and PF/C groups. It should be noted that the contribution of a transient increase in proteolysis earlier during hypoxia exposure has not been investigated in this study. Similarly, activity of cathepsin B + L (Fig. 1D) and expression of calpain-2 and -3 (Fig. 1E) were not altered by oxygen reduction, suggesting that lysosomal and Ca\textsuperscript{2+}-dependent systems were not stimulated by hypoxia. Also noteworthy is that the C and PF groups did not differ for any of these parameters.

**Chronic hypoxia downregulates the Akt/mTOR pathway.** Bodine et al. (6) characterized the Akt/mTOR pathway as a key regulator of skeletal muscle mass and observed that both phosphorylated and total protein contents are altered during skeletal muscle atrophy. Hypoxia-induced atrophy of SOL muscle was associated with significant decreases in the protein content of Akt (23%), mTOR (50%), S6K (25%), and S6 (33%) compared with the PF group (Fig. 2, A and B). Moreover, hypoxia strongly reduced the phosphorylation of Akt on T308 (36%) and S473 (46%) (Fig. 2, C and D). Consistently, the phosphorylation of GSK3\textbeta\ S9 and TSC2 T1462 by Akt was also significantly reduced by \textsim 30%. mTOR phosphorylation on S2448 and S6 phosphorylation on S235/236 were also significantly reduced. (It should be noted that we did not succeed in detecting S6K phosphorylation on T389 in rodent muscles). However, 4E-BP1 phosphorylation on T37/46 was unaffected. Phosphorylated AMPK can directly activate TSC2 (18) and thus indirectly inhibit mTOR (8). Nevertheless, and in agreement with previous studies (2, 7), AMPK did not seem to participate to mTOR signaling inhibition during hypoxia as phosphorylation of AMPK\textalpha on T172 was reduced by \textsim 50% (P < 0.001) in H group (Fig. 3). Overall, these data showed that severe hypoxia caused a marked inhibition of the Akt/mTOR pathway.

**REDD1 associates with 14-3-3 during chronic hypoxia and causes muscle fiber atrophy in vivo.** REDD1 has been shown to be a negative regulator of mTOR signaling in mouse embryonic fibroblasts during hypoxia (7). Here, hypoxic animals exhibited a 370% (H vs. C, P < 0.001) and 210% (H vs. PF, P < 0.001) increase in REDD1 protein level (Fig. 4, A and B). Importantly, no variation was observed at the mRNA level (Fig. 4C), suggesting a posttranscriptional regulation of REDD1 expression by hypoxia. Recently, REDD1 was shown to bind to 14-3-3 family proteins in vitro, thus leading to the dissociation of the TSC2/14-3-3 complex and ultimately to the inhibition of mTOR signaling (9). We therefore investigated the biological relevance of such a mechanism in hypoxia-induced muscle mass loss. Immunoprecipitation of 14-3-3 revealed an increase in...
REDD1/14-3-3 association in H vs. C (P < 0.05) and PF (P = 0.06) muscles (Fig. 4, A and B), suggesting that TSC2 dissociation from 14-3-3 may contribute to inhibit mTOR signaling during chronic hypoxia. These data suggest that REDD1 could negatively regulate skeletal muscle mass. Thus, we determined whether ectopic expression of REDD1 was sufficient to cause skeletal muscle fiber atrophy in normoxia. After ensuring that our expression vector was sufficient to induce a large increase in REDD1 protein level in HEK-293 cells (Fig. 5A), SOL muscles were electroporated with the REDD1 expression vector. Histo-morphometric analysis evidenced that REDD1 overexpression caused a 10% reduction in muscle FCSA compared with fibers electroporated with the empty vector (P < 0.05; Fig. 5, B and C). Therefore, REDD1 overexpression is sufficient to cause muscle fiber atrophy in normoxia.

Akt/mTOR signaling and REDD1/14-3-3 association in hypoxemic COPD patients. The biological relevance of these findings was explored in a human model of chronic hypoxemia. COPD patients were divided into two groups, depending on the presence or the absence of resting hypoxemia. With the exception of the arterial O2 pressure, nonhypoxemic and hypoxemic patients did not differ for any anthropometric and ventilatory parameters (Table 3). While total protein content of Akt, GSK3β, and S6 remained unchanged (data not shown), hypoxemia significantly reduced the phosphorylation of Akt T308, GSK3β S9, and S6K T389 (Fig. 6, A and B). In agreement with these observations, 14-3-3/REDD1 association was nonsignificantly increased (38%) in hypoxemic vs. nonhypoxemic patients (Fig. 6, A and C). These results suggest

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**Fig. 3.** AMPKα phosphorylation is reduced during chronic hypoxia. Representative immunoblots and corresponding quantification of AMPKα T172 in the soleus muscle of C, H, and PF rats. **P < 0.01, different from C group; ‡‡P < 0.01, different from PF group (n = 10 per group).

**Fig. 4.** Chronic hypoxia increases regulated in development and DNA damage response 1 (REDD1) protein expression and promotes its binding to 14-3-3 in soleus muscle. A: representative immunoblots of REDD1 protein level in lysate. Representative immunoblots of REDD1 and 14-3-3 protein level after 14-3-3 immunoprecipitation (IP 14-3-3). Note that 14-3-3 expression remained unchanged. B: quantification of REDD1 protein content and REDD1/14-3-3 association in the soleus muscle of C, H, and PF groups (columns identified in C). C: REDD1 mRNA expression. *P < 0.05 and ***P < 0.001, different from C group; ‡‡‡P < 0.001, different from PF (n = 6 per group).

**Fig. 5.** REDD1 gene electrotransfer induces muscle fiber atrophy in normoxia. A: immunoblots of REDD1 protein expression in HEK-293 cells nontransfected (control) or transfected with 1 μg of an empty vector or a REDD1 expression vector. Cells were harvested 48 h after transfection. B: mean fiber cross-sectional area (FCSA) of β-galactosidase positive fibers normalized to negative fibers in muscles electroporated with empty vector and REDD1 expression vector. Absolute values are 3,780 ± 114 and 3,417 ± 116 μm² in fibers electroporated with empty and REDD1 expression vectors, respectively. Note that β-galactosidase expression per se has no effect on muscle fibers size. C: representative images of hematoxylin-eosin-saffron-stained muscles electroporated with empty vector (blue) or REDD1 expression vector (blue) (scale bar = 50 μm). *P < 0.05, different from fibers electroporated with empty vector.
that REDD1 may contribute to skeletal muscle atrophy in hypoxemic COPD patients.

**DISCUSSION**

In this study, we show that severe hypoxia causes hypophagia-independent muscle mass loss and downregulates the Akt/mTOR pathway, together with an increase in REDD1/14-3-3 association. A similar inhibition of Akt/mTOR pathway was observed in skeletal muscle of hypoxemic COPD patients. Finally, we also demonstrated that REDD1 overexpression was sufficient to induce skeletal muscle fiber atrophy in normoxia. The present findings are compatible with a mechanism according to which REDD1 reduces muscle mass in hypoxia by releasing the inhibitory action of 14-3-3 on TSC2, thus promoting mTOR inhibition.

Three weeks of severe hypoxia led to a reduction in skeletal muscle mass. However, it is questionable whether the reported decrease in skeletal muscle mass is due to atrophy per se, or inhibition of postnatal muscle growth. In other words, did the animals lose muscle mass or did they simply not gain muscle mass at the same rate as they would under normoxic conditions? Analysis of muscle growth curve according to muscle weight (See supplemental Fig. 1 available online at the *American Journal of Physiology–Regulatory, Integrative and Comparative Physiology* website) indicates that muscle growth was not completely achieved at the beginning of the experiment (animal weight was 350 g), suggesting that inhibition of muscle growth and atrophy per se are contributing mechanisms to hypoxia-induced muscle mass loss.

The lower muscle mass was associated with a sharp decrease in Akt/mTOR signaling, while the UPS was not altered after 3 wk of severe hypoxia. However, we cannot rule out the contribution of a transient increase in proteolysis earlier during hypoxia exposure. Indeed, some transitory adaptations may be back to baseline values after 3 wk. This could be particularly relevant for the ubiquitin proteasome system, as a transitory activation of this proteolytic pathway has been frequently reported at the onset of skeletal muscle atrophy (5, 23). Our observations are consistent with previous in vitro studies showing that acute hypoxia inhibits Akt/mTOR pathway in HEK-293 cells (2, 25). Moreover, we recorded a diminution in Akt, mTOR, S6K, and S6 protein expression, corroborating the decrease in mTOR expression reported in subjects exposed to 4,559 m for 7–9 days (40). Altogether, these data show that hypoxia triggers molecular events that result in the inhibition of Akt/mTOR signaling, while the UPS was not altered after 3 wk of severe hypoxia. Indeed, some transitory adaptations may be back to baseline values after 3 wk. This could be particularly relevant for the ubiquitin proteasome system, as a transitory activation of this proteolytic pathway has been frequently reported at the onset of skeletal muscle atrophy (5, 23).

The TSC1/2 complex is an important crossroad of mTOR signaling that integrates multiple regulatory influences (14). Akt prevents TSC1/2 dimerization by phosphorylating TSC2 on S939 and T1462 (17, 28). In the present study, TSC2 phosphorylation at T1462 was reduced in hypoxia, indicating a decrease in Akt activity. Consistently, Akt phosphorylation at T308 was reduced by hypoxia, suggesting a decrease in Akt activation by the phosphoinositide-dependent protein kinase-1. Full activation of Akt also requires to the phosphorylation of S473 by the mTOR complex 2 (15). The decrease in Akt phosphorylation at S473 thus indicates that mTOR complex 2 activity is also affected by hypoxia (see below). This reinforces the hypothesis that Foxo-induced proteolysis may have been stimulated during hypoxic exposure. Akt-mediated inhibition of TSC2/1 complex involves the sequestration of TSC2 by 14-3-3 (26) when TSC2 is phosphorylated by Akt on S939 and T1462 (9). Moreover, Deyoung et al. (9) showed in HEK-293T cells that REDD1 binds to 14-3-3, leading to the release of TSC2 (9). A similar mechanism involving REDD2 has recently been demonstrated (30), but REDD2 is not sensitive to hypoxia (32, 37). Here, REDD1 expression was dramatically increased, further supporting that REDD1 is highly responsive to hypoxia (20, 24, 37). In...
addition, chronic hypoxia induced a strong increase in REDD1/14-3-3 association. Together with the decrease in TSC2 phosphorylation on T1462, this mechanism likely contributes to release TSC2 from 14-3-3 in muscles from hypoxic rats. In turn, this regulation probably results in a greater TSC1/TSC2 association, therefore contributing to the strong reduction in the phosphorylation of mTOR on S2448 and S6 on S235/236 in H group. Regarding mTOR phosphorylation, one should note that mTOR activity is also regulated by protein interactions (15). Therefore, and although mTOR phosphorylation at Ser2448 was statistically decreased, supporting a response of this site-specific phosphorylation of Ser2448 to hypoxia, we cannot also exclude the possibility that mTOR could be also specifically regulated by protein interaction in response to chronic hypoxia. It is noteworthy that it has been shown that REDD1 overexpression caused Akt inhibition by decreasing T308 and S473 phosphorylation in neurons (27). Therefore, the decrease in Akt phosphorylation in SOL muscles from hypoxic animals may also result from an inhibition of mTOR complex 2 by REDD1. Altogether, these data identify REDD1 as a critical regulator of the Akt/mTOR pathway during hypoxia in vivo and point to the potential role of REDD1 as an atrophic factor. To test this hypothesis, we determined the effect of REDD1 overexpression in normoxia on skeletal muscle fiber area. This experiment demonstrates that ectopic REDD1 expression decreases skeletal muscle fiber area. Additional information regarding the role of REDD1 would be provided by the use of dominant-negative models aimed at determining whether REDD1 is required for muscle atrophy.

Loss of skeletal muscle mass has been identified as a major determinant of disability in COPD patients (36). Many causes have been proposed to explain muscle atrophy in COPD patients (41, 43), but the knowledge of the biochemical pathways involved is limited. One possibility would be a reduction in Akt/mTOR pathway activation. However, Doucet et al. (10) showed that nonhypoxic COPD patients (P\text{AO}2 = 76 mmHg) with low muscle mass exhibited an increase in Akt/mTOR activation compared with COPD patients with preserved muscle mass. In the present study, we hypothesized that the severity of hypoxemia could be a factor controlling the down-regulation of Akt/mTOR signaling pathway. We observed that skeletal muscle of hypoxic COPD patients (P\text{AO}2 = 54 mmHg) displayed a significant reduction in Akt/mTOR signaling compared with nonhypoxic COPD patients (P\text{AO}2 = 73 mmHg). In agreement with our observations in rodents, REDD1 protein was detectable in human skeletal muscle and its binding to 14-3-3 was nonsignificantly increased in skeletal muscles of hypoxic COPD patients (38%). One should note that this observation was obtained with a limited number of subjects (n = 4/group). Additional subjects must be included to definitively conclude on the regulation of REDD1 by 14-3-3 in skeletal muscle of COPD patients.

Perspectives and Significance

This work represents a significant advance in the understanding of the mechanisms leading to skeletal muscle atrophy in response to chronic hypoxia. The present data reveal the existence of a mechanism by which hypoxia-induced expression of REDD1 actively contributes to the inhibition of mTOR pathway in rat skeletal muscle. Our preliminary data also suggests that this mechanism could be relevant in skeletal muscle of hypoxic COPD patients. Importantly, REDD1 expression is also stimulated by glucocorticoid administration (42) and nutritional deprivation (38), two common features of COPD. Defining strategies aimed at disrupting REDD1/14-3-3 association may thus represent a promising way to counteract skeletal muscle loss in COPD patients. Future research should be aimed at delineating the potential mechanism(s) responsible for the increase in REDD1 expression, as well as examine the importance of REDD1 in mediating skeletal muscle adaptations in response to other forms of stress.

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DISCLOSURES

No conflicts of interest, financial, or otherwise, are declared by the author(s).

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