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ORIGINAL ARTICLE

Impact of different methods of induction of cellular hypoxia: focus on protein homeostasis signaling pathways and morphology of C2C12 skeletal muscle cells differentiated into myotubes

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Abstract

Hypoxia, occurring in several pathologies, has deleterious effects on skeletal muscle, in particular on protein homeostasis. Different induction methods of hypoxia are commonly used in cellular models to investigate the alterations of muscular function consecutive to hypoxic stress. However, a consensus is not clearly established concerning hypoxia induction methodology. Our aim was to compare oxygen deprivation with chemically induced hypoxia using cobalt chloride ($CoCl_2$) or desferrioxamine (DFO) on C2C12 myotubes which were either cultured in hypoxia chamber at an oxygen level of 4% or treated with $CoCl_2$ or DFO. For each method of hypoxia induction, we determined their impact on muscle cell morphology and on expression or activation status of key signaling proteins of synthesis and degradation pathways. The expression of HIF-1α increased whatever the method of hypoxia induction. Myotube diameter and protein content decreased exclusively for C2C12 myotubes submitted to physiological hypoxia (4% O_2) or treated with $CoCl_2$. Results were correlated with a hypophosphorylation of key proteins regulated synthesis pathway (Akt, GSK3-β and P70S6K). Similarly, the phosphorylation of FoxO1 decreased and the autophagy-related LC3-II was overexpressed with 4% O_2 and $CoCl_2$ conditions. Our results demonstrated that in vitro oxygen deprivation and the use of mimetic agent such as $CoCl_2$, unlike DFO, induced similar responses on myotube morphology and atrophy/hypertrophy markers. Thus, physiological hypoxia or its artificial induction using $CoCl_2$ can be used to understand finely the molecular changes in skeletal muscle cells and to evaluate new therapeutics for hypoxia-related muscle disorders.

Keywords Hypoxia · Protein homeostasis · Myotube · Atrophy · Cobalt chloride · Desferrioxamine

Background

Hypoxia, a lessening level or tension of O_2, not only is observed in physiological situations, such as people ascending to high altitude, but also occurs in pathological conditions such as myocardial ischemia, chronic obstructive pulmonary disease (COPD) or obstructive sleep apnoea. Hypoxia has deleterious effects on the muscular system, in particular on skeletal muscle mass [20]. This mechanism is particularly highlighted in COPD patients presenting a marked decrease in skeletal muscle mass [29]. This loss of muscle mass results from the disequilibrium between protein synthesis and protein degradation, translating the imbalance of protein homeostasis [12]. Thus, through its strong negative effects on protein homeostasis and on muscle mass, chronic hypoxia worsens the quality of life of COPD patients and is a strong predictor of mortality [25]. This dysregulation of protein homeostasis is also observed in cultured skeletal muscle cells. Indeed, in vitro studies on hypoxic differentiated C2C12 or on muscle cells derived from COPD patients show a reduced diameter and an impaired fusion capacity of myotubes [26, 28].

To date, the precise impact of hypoxic stress in signaling pathways regulating protein homeostasis in skeletal muscle, i.e. the balance between protein synthesis and protein degradation, remains to be clarified. Indeed, the fine understanding of the links between tissue oxygen

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deprivation and protein turnover in skeletal muscle is crucial for the development of future therapeutics aimed to limit or reverse muscle wasting or cachexia consecutive to hypoxic stress. Several studies have examined the effects of hypoxia on the signaling pathways regulating protein synthesis and protein degradation on in vitro models such as human primary myoblasts or animal cell lines (rat L6 or murine C2C12 myotubes) [7, 21, 28]. To create an experimental hypoxic environment, the in vitro experimentation is based on two approaches. The first method, the normobaric physiological hypoxia, consists in reducing the level of oxygen (< 20.9%) of ambient air inside a chamber by adding nitrogen (N_2), leading so to a real oxygen deprivation; this allows simulating high altitude or pathological hypoxia environment. The second approach is based on the use of chemicals; the best-known chemical inducers of hypoxia-like responses are the cobalt chloride ($CoCl_2$) and the desferrioxamine (DFO) [3, 34]. Such as hypoxia, the experimental deprivation in oxygen and the induce-mimetic agents ($CoCl_2$ or DFO applied at normoxic conditions) lead to stabilization of hypoxia-inducible factor 1 (HIF-1α) which blocks its ubiquitination and its proteasomal degradation [4, 11, 18].

However, the accurate and precise outcome of the effects of oxygen deprivation on the intricate and complex signaling network regulating protein synthesis and protein degradation remains elusive, mainly because of the plethora of hypoxia-inducing protocols. Indeed, several studies have used either process between physiological hypoxia by diminution O_2 level or hypoxia mimetic agents to induce cellular hypoxia. However, despite the fact that these different processes result to a stabilization of HIF-1α, to our knowledge, no study is devoted to the repercussions of each method on activation status of proteins in charge of skeletal muscle homeostasis (considering both protein synthesis and protein degradation) and their impact on the morphology of muscle cell. In other words, it is currently not known if the different methods of hypoxia induction lead to similar impact on skeletal muscle protein homeostasis, so as the alterations of the myotubes morphometry. Thus, the purpose of our study was to determine the precise impact of physiological hypoxia and chemical hypoxia-mimetic agents ($CoCl_2$ and DFO) on the signaling pathways regulating protein synthesis and protein degradation, in order to determine which one is the most reliable mimicker of the in vivo effects of hypoxic stress in skeletal muscle. Thus, on an in vitro model of C2C12 myotubes, we determined the effects of physiological oxygen deprivation, $CoCl_2$, and DFO on protein homeostasis and their consequences on the myotube morphology.

Materials and methods

C2C12 cells culture and induction of hypoxia mediated effects

C2C12 mouse myoblasts (ATCC: American Type Culture Collection, Manassas, VA) were grown in proliferation medium (PM), corresponding to Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum (Gibco) and 1% antibiotics/anti-mycotics (Sigma-Aldrich). During proliferation, the cells were plated at a density of 2×10^5 cells/ml in PM. When C2C12 myoblasts reached 80–90% confluence, they were switched to DMEM containing 2% heat-inactivated horse serum (Gibco) (corresponding to DM, differentiation medium) and 1% antibiotics/anti-mycotics. The shifting time to DM was assigned to day 0 of differentiation. The incubation was performed in humidified atmosphere of 5% CO_2 at 37 °C; media were changed every 48 h, and myotube formation was monitored daily. For the physiological hypoxia experiments, oxygen was replaced by nitrogen (N_2) by the ProOx P110 oxygen controller (BioSpherix, Lacona, NY). After 4 days of differentiation, myotubes were transferred to a chamber maintained at 4% O_2, 5% CO_2 and 91% N_2. Normoxia cells grown at 21% O_2 in the ambient air were directly placed in a 5% CO_2 incubator at 37 °C in a humidified atmosphere (HeraCell, Thermo Fisher Scientific, Loughboroug, UK). To mimic hypoxic conditions, C2C12 myotubes were treated with $CoCl_2$ or DFO (Sigma-Aldrich) from the 4th day of differentiation. Chemical inducers of hypoxia were diluted in DM to reach a final concentration of 200 μM. To assess the potential toxicity of the chemical mimetics of hypoxia ($CoCl_2$ and DFO) in our experimental protocol, we performed a cell viability by MTT assay.

Western blot analysis

C2C12 myotubes were rinsed three times in cold PBS (phosphate buffered saline). They were then scrapped in cold RipA lysis buffer (10 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS) containing anti-proteases (Complete EDTA- free, Roche Diagnostic) and anti-phosphatases (Phos-Stop, Roche Diagnostic). Protein extracts were rapidly sonicated using Ultra-sonic Cell Disruptor and then homogenized with gentle agitation for 1 h at 4 °C. Protein concentration of whole cellular extracts was assayed using Bradford assay (Bio-Rad). Equal amounts of proteins were denatured by boiling at 95 °C for 7 min in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8; 10% glycerol; 2% SDS; 5% ß-mercaptoethanol; 0.02% bromophenol blue).

Proteins were separated on Mini-PROTEAN TGX Stain-Free 10% precast polyacrylamide gels (Bio-Rad); an internal standard was loaded on each gel. Electrophoretic separation

was done at 200 V for 35 min in migration buffer (25 mM TrisBase; 0.2 M glycine; 1% SDS (p/v)). Stain-free (SF) technology contains a proprietary trihalo compounds which react with proteins, rendering them detectable through UV exposure. SF imaging was performed using ChemiDoc MP Imager and Image Lab 4.0.1 software (Bio-Rad) with a 5-min stain activation time, and total protein patterns were therefore visualized. Proteins were then transferred on 0.2 μm nitrocellulose sheet using the Trans-Blot Turbo Transfer System (Bio-Rad). The quality of transfer was controlled by imaging membranes using the SF technology. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBST 15 mM Tris/HCl, pH 7.6; 140 mM NaCl; 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated at 4 °C overnight or 2 h at room temperature with the following primary antibodies: Akt (#9272, Cell Signaling), p-Akt (Ser473; #9271, Cell Signaling), GSK-3β (#9315, Cell Signaling), p-GSK-3β (Ser9; #9336, Cell Signaling), FoxO1 (#2880, Cell Signaling), p-FoxO1 (Ser256; #9461, Cell Signaling), HIF-1α (#A300-286A-M, Bethyl), MHC (fast; #MY-32, Sigma), Myogenin (#ab80956, Abcam), LC3A/B (#4108, Cell Signaling), P70S6K (#9202, Cell Signaling), p-P70S6K (Thr389; #9205, Cell Signaling) and Ubiquitin (P4D1; #3936, Cell Signaling). After 3 × 10 min washes in TBST, membranes were probed with secondary antibodies (anti-mouse or anti-rabbit IgG-HRP linked; #7076 or #7074, Cell Signaling) in blocking solution for 2 h at room temperature and finally extensively washed with TBST. The dilution of primary and secondary antibodies was optimized for each antibody. Chemiluminescence detection was carried out using ECL Clarity (Bio-Rad), and image capture was done with ChemiDocMP.

All the images were analysed using the Image Lab 4.0.1 software. Normalization of protein signal intensities was carried out following the quantification of respective total protein level on SF images and sample control (normoxia 21% O_2). If membranes needed to be reprobed to use another antibody, membrane stripping was performed using Western reprobe (G-Biosciences).

Morphological analysis

Myotubes were fixed in methanol for 7 min and then stained with May–Grünwald–Giemsa reactive (Sigma-Aldrich); myotubes were visualized using Leica DMLS microscope equipped with a video and Ulead Video Studio software. Images were acquired at × 10 magnification. Fifteen fields were chosen randomly for each experimental condition and all the myotubes (≥ 3 nuclei) from each field were measured. The average diameter per myotube was calculated as the mean of 5 measurements taken along the length of the myotube. Morphological analyses were performed using ImageJ Software. The fusion index was determined by dividing the total number of nuclei in myotubes by the total number of nuclei counted [30].

C2C12 myotube protein content

Total protein content of C2C12 was obtained after cellular extractions steps, scrapped, sonicated and homogenized. Protein concentration in cell lysate was assayed using Bradford assay, and the volume (μl) of each sample was accurately measured three times; the total amount of proteins per sample was thus determined and reported as protein content in milligrams.

Statistical analyses

All treatments were performed at least in biological quadruplicates from 2 or 3 independent cultures. All data are presented as means ± SEM. Data were tested for normality using a Shapiro–Wilk test. The effects of conditions (i.e. normoxia vs physiological hypoxia vs $CoCl_2$ vs DFO) and times (24 h vs 6 h and 72 h vs 48 h) were tested by two-way ANOVAs (conditions and times). If significant main effects and/or interactions were observed with ANOVAs, Duncan's multirange post hoc tests were applied to examine specific pairwise differences. Differences were considered statistically significant when $P < 0.05$. Statistics were calculated using Statistica 8.0 software.

Results

HIF-1α, the marker of intracellular adaptation under hypoxia

To determine the effect of oxygen deprivation and hypoxia-mimetic agents on hypoxia induction in C2C12 myotubes, we quantified the expression of HIF-1α protein, the master regulator of hypoxia-mediated cellular adaptations (Fig. 1a); the representative signals are presented on Fig. 1b. Immunoblotting analysis revealed a significant increase in expression of HIF-1α for muscle cells exposed to an oxygen level of 4% for 24 h compared to normoxia ($P < 0.001$) (Fig. 1a). Both hypoxia-mimetic agents, $CoCl_2$ and DFO at 200 μM, led to a significant increase in expression of HIF-1α as early as 6 h of treatment compared with normoxia, the expression of HIF-1α being maintained after 24 h ($P < 0.001$) (Fig. 1a). It is worth to note that the expression level of HIF-1α was quite similar at 24 h whatever the method of induction of hypoxia.

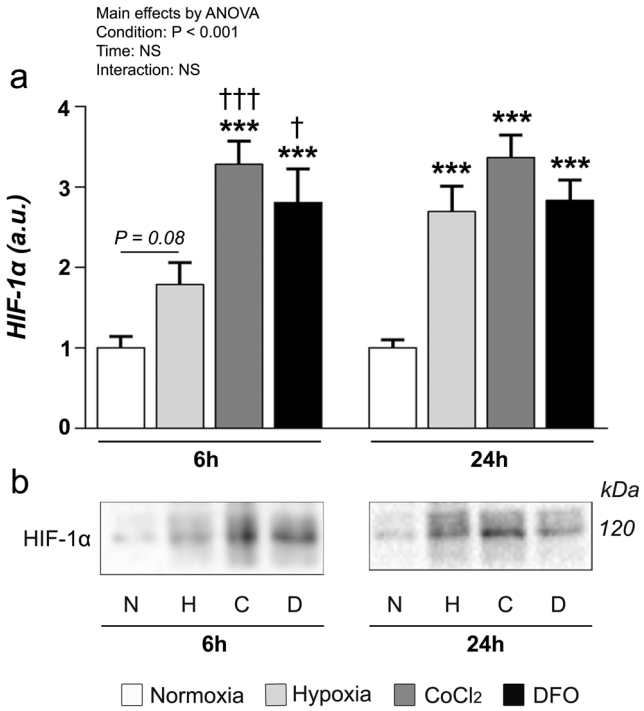

Fig. 1 Effect of hypoxia (4% O_2) and hypoxia-mimetic agents on HIF-1α expression. **a** Immunoblot analysis of HIF-1α expression in C2C12 myotubes exposed to normoxia (21% O_2) (N), normobaric physiological hypoxia (4% O_2) (H) or treated by hypoxia-mimetic agents: $CoCl_2$ (C) or DFO (D) at a concentration of 200 μM for 6 h or 24 h. **b** Representative Western blots to 6 h or 24 h. Protein signal values were normalized to stain-free signal and to normoxia condition (21% O_2). The mean values from the quantification of Western blots are represented in arbitrary units (a.u.). Post-hoc analyses for conditions effect significantly from normoxia (basal) values: ***$P < 0.001$. Significantly different from hypoxia (4% O_2): $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$. Trends are indicated by the specific P value ($n = 8$ per condition)

Hypoxia treatment induced myotube atrophy in muscle cell

Morphological observation of cultured skeletal muscle cells is a suitable indicator of differentiation status and maturation degree of myotubes. The morphological analysis of C2C12 myotubes revealed a significant reduction in myotubes diameter for physiological hypoxia induction (i.e. oxygen deprivation at 4% O_2) (15.61 ± 0.32 μm, $P < 0.001$) and $CoCl_2$ treatment (15.36 ± 0.33 μm, $P < 0.001$) 48 h post-induction, compared to normoxia-control cells (18.06 ± 0.47 μm) (Fig. 2b). After 72 h induction, the reduction of myotubes diameter was exacerbated for hypoxia and $CoCl_2$ treatment compared with 48 h induction (48 h vs 72 h, $P < 0.001$). In contrast, the diameter of DFO-treated myotubes was quite similar to normoxia cells at 48 h and 72 h and significantly greater than in hypoxia condition at 72 h (12.41 ± 0.3 μm and 15.55 ± 0.15 μm, for hypoxia and DFO, respectively, $P < 0.001$) (Fig. 2b). Oxygen deprivation led to a simultaneous decrease number of myotubes and number of nuclei per myotube compared to muscle cells under normoxia (72 h, $P < 0.05$) (Fig. 2c and d). Analysis of all hypoxia induction methods showed a similar myogenic fusion index despite a lower index of fusion of 9% after 48 h ($P = 0.09$) under physiological hypoxia (4% O_2) compared to normoxia (Fig. 2e). The expression of muscle regulatory factors Myogenin was reduced in myotubes incubated at 4% O_2 (0.4 ± 0.05 a.u., at 24 h, $P < 0.001$) and $CoCl_2$ (0.55 ± 0.04 a.u., at 6 h, $P < 0.001$) compared to normoxia myotube control (Fig. 3b). In addition, between 6 and 24 h, physiological hypoxia was the only condition to lead a decrease in Myogenin expression (6 h and 24 h, $P < 0.001$) and MHC II (24 h vs 6 h, $P < 0.05$) (Fig. 3a and b).

We next investigated whether total protein content was altered after the different hypoxia-induced treatments in C2C12 myotubes. Physiological hypoxia (4% O_2) led to a significant decline of total protein content after 24 h (0.4 ± 0.01 mg, $P < 0.01$) and 48 h (0.37 ± 0.01 mg, $P < 0.001$) exposure to reduced level of oxygen, which was exacerbated after 72 h (0.16 ± 0.01 mg, $P < 0.001$) compared to normoxia condition (Fig. 4). Similar to physiological hypoxia, $CoCl_2$ caused a significant decrease of the total amount of proteins (0.4 ± 0.01 and 0.39 ± 0.01 mg/ml for 48 h and 72 h, respectively, $P < 0.001$). It is worth to note that the total protein content was significantly higher for $CoCl_2$-treated cells compared with 4% O_2 exposure cells after 72 h ($P < 0.001$) (Fig. 4). The DFO-treated C2C12 myotubes did not present any change in their total protein content compared to normoxia-control cells, but they had in contrast a significantly higher total protein content compared to hypoxia condition at 24 h, 48 h and 72 h ($P < 0.001$) (Fig. 4).

Physiological hypoxia and $CoCl_2$ showed a decreased protein synthesis and enhanced protein breakdown in skeletal muscle myotubes

The phosphorylation status and/or expression of various markers of the protein synthesis and protein breakdown pathways for each hypoxia induction methods were compared to determine if there was a correlation with the myotubes diameter and the total protein content which were disturbed by both physiological hypoxia and $CoCl_2$. Our results did not show any difference in p-Akt/Akt ratio after 6 h for all hypoxic treatments relative to normoxia (Fig. 5a). In contrast, 24 h after hypoxia induction, physiological hypoxia (4% O_2) and $CoCl_2$ caused a fall in activation level of Akt (0.41 ± 0.05 a.u. and 0.33 ± 0.06 a.u., respectively, $P < 0.01$), whereas muscle cells treated with DFO had a p-Akt/Akt ratio quite similar to normoxia conditions and higher than physiological hypoxia condition ($P < 0.01$) (Fig. 5a). GSK-3β phosphorylation decreases after 6 h (0.83 ± 0.05 a.u., $P < 0.05$) and 24 h (0.72 ± 0.03 a.u., $P < 0.01$) exposure to physiological hypoxia (4% O_2) compared to normoxia (Fig. 5b). C2C12 cells treated over 6 h with $CoCl_2$ observed an increase of p-GSK-3β/GSK-3β ratio compared to normoxia ($P < 0.05$) and hypoxia (4% O_2)

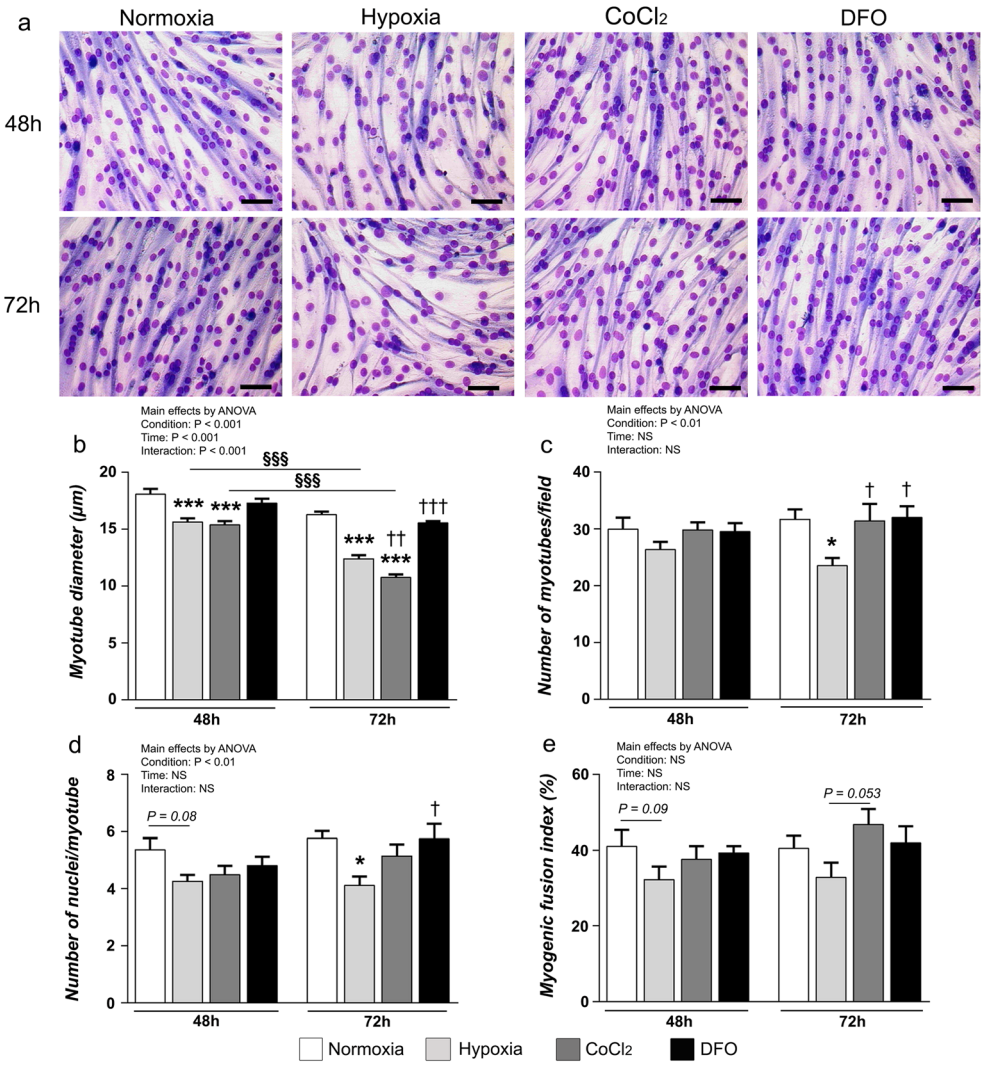

Fig. 2 Atrophy C2C12 cells submitted to oxygen deprivation or after chemically induction of hypoxia. **a** Representative images of myotubes from C2C12 myotubes under normoxia (21% O_2), normobaric physiological hypoxia (4% O_2) or treated by hypoxia-mimetic agents: $CoCl_2$ or DFO at a concentration of 200 μM for 48 h or 72 h. Bright field images were taken at the same magnification (20×). Scale bar = 100 μm. **b** Measurement of the diameter of cultured myotubes, expressed in micrometers. **c** Analysis of the number of myotubes per field. **d** Analysis the number of nuclei per myotubes. **e** Analysis of the myogenic fusion index of cultured myotubes, expressed in percentage. At least, 300 myotubes per condition were analysed. Post-hoc analyses for conditions effect significantly from normoxia (basal) values: $^{*}P < 0.05$, $^{***}P < 0.001$. Significantly different from hypoxia (4% O_2): $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. Post-hoc analyses for times effect significantly different from 72 h vs 48 h values: $^{§§§}P < 0.001$. Trends are indicated by the specific P value ($n = 6$ per condition)

($P < 0.001$), while we quantified a decrease after 24 h of exposure (0.79 ± 0.12 a.u., $P < 0.05$). GSK-3β activation level under DFO was quite similar to normoxia values and was superior to physiological hypoxia (24 h, $P < 0.01$) (Fig. 5b). The increase of protein synthesis requires the activation by phosphorylation of mTOR targets so as P70S6K [35]. The protein activation of P70S6K decreased exclusively for physiological hypoxia (0.86 ± 0.02 a.u., $P < 0.05$) and $CoCl_2$ (0.73 ± 0.03 a.u., $P < 0.001$) conditions compared to normoxia values. In addition, after treatment with DFO, p-P70S6K/P70S6K ratio was significantly higher than the physiological hypoxia condition (6 h and 24 h, $P < 0.05$) (Fig. 5c).

Hypoxia-induced protein degradation in C2C12 myotubes by activation of autophagy and proteasome systems

FoxO1 phosphorylation decreased in response to long-term physiological hypoxia exposition (4% O_2) (24 h, $P < 0.01$) (Fig. 6a). $CoCl_2$ induced a decreased activation level of FoxO1 once 6 h of treatment ($P < 0.05$), which extended after 24 h treatment ($P < 0.01$). In contrast, DFO did not induce any change of FoxO1phosphorylation compared to untreated cells (21% O_2), and it has higher values than physiological hypoxia condition (24 h, $P < 0.01$) (Fig. 6a). The activation of autophagy was analysed using the expression of two markers involved in the biogenesis of autophagosomes: the LC3-I and its lipidated form, LC3-II. Quantitation by Western blotting showed that physiological hypoxia (4% O_2) initiated an increase expression of LC3-II compared with normoxia such as the two methods of chemical induction of hypoxia (6 h and 24 h, $P < 0.001$) (Fig. 6b). As we observed for oxygen deprivation (4% O_2), we quantified an increase of LC3-II/LC3-I ratio after 24 h exposure to $CoCl_2$ compared with normoxia ($P < 0.01$) (Fig. 6b). The ubiquitination of skeletal muscle proteins did not vary after 6 h of physiological hypoxia (4% O_2) or hypoxia-mimetic agent exposure (Fig. 6c). In contrast, ubiquitination of proteins significantly increased following

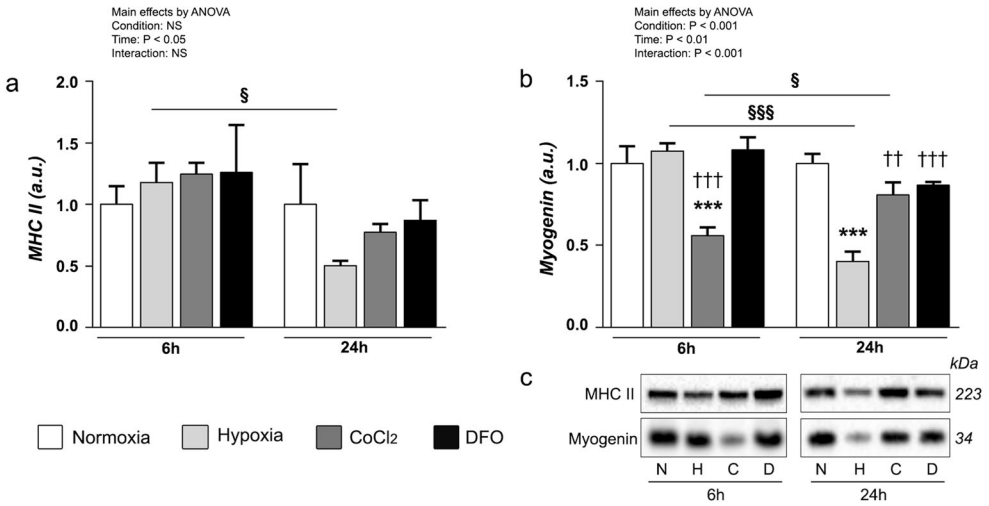

Fig. 3 Hypoxia induction represses skeletal muscle progenitor differentiation in C2C12 myotubes. Expression level of myosin heavy chain type II (MHC II) and muscle regulatory factors Myogenin were determined by Western blot in C2C12 myotubes under normoxia (21% O2) (N), hypoxia (4% O2) (H) or treated by hypoxia-mimetic agents: $CoCl_2$ (C) or DFO (D) at a concentration of 200 μM for 6 h or 24 h. **a** Quantification of MHC II protein. **b** Quantification of Myogenin protein. **c** Representative signals of proteins of interest after 6 or 24 h after induction of hypoxia. The mean values from the quantification of Western blots are represented in arbitrary units (a.u.). Post-hoc analyses for conditions effect significantly from normoxia (basal) values: $^{***}P < 0.001$. Significantly different from hypoxia (4% O2): $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. Post-hoc analyses for times effect significantly different from 24 h vs 6 h values: $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$ ($n = 4$ per condition)

24 h of $CoCl_2$ treatment (2.15 ± 0.31 a.u.) compared with normoxia-control cells and physiological hypoxia cells (4% O_2) ($P < 0.001$). At the same time, there was no change in the level of protein ubiquitination after DFO treatment compared to control cell (Fig. 6c).

Discussion

In vitro muscle models under hypoxia are essential in order to well establish the intracellular mechanisms induced by hypoxia.

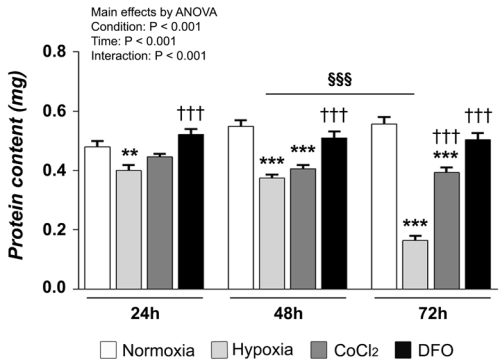

Fig. 4 Lowering of protein content of C2C12 myotubes subjected to hypoxia induction. Total protein content of C2C12 myotubes exposed to normoxia (21% O_2) (N), normobaric hypoxia (4% O_2) (H) or treated by hypoxia-mimetic agents: $CoCl_2$ (C) or DFO (D) at a concentration of 200 μM for 24 h, 48 h or 72 h. Post-hoc analyses for conditions effect significantly from normoxia (basal) values: $^{**}P < 0.01$, $^{***}P < 0.001$. Significantly different from hypoxia (4% O_2): $^{\dagger\dagger\dagger}P < 0.001$. Post-hoc analyses for times effect significantly different from 72 h vs 48 h values: $^{\S\S\S}P < 0.001$ ($n = 12$ per condition)

A better comprehension of alterations in muscular function is essential to develop new therapeutics. Indeed, impaired muscle function related to respiratory diseases (e.g. COPD) seems to be a more important mortality predictor factor than respiratory disorders themselves [25]. Different strategies exist to induce hypoxia in in vitro muscle models. This study searches to determine the similarities and differences between induction of physiological hypoxia and the use of hypoxia mimetic agents such as cobalt chloride or desferrioxamine on muscular morphology and intracellular mechanisms responsible of atrophy/hypertrophy pathways. Our results demonstrated that physiological hypoxia (4% O_2) and $CoCl_2$ led to similar responses of muscle atrophy, loss of protein content, decreased myogenic markers of differentiation (Myogenin), reduction of phosphorylation level of protein synthesis (Akt/GSK-3β/P70S6K) and increased expression of autophagy marker (LC3). Although DFO induced the same stabilization of HIF-1α as physiological hypoxia, this hypoxia mimetic agent did not concord with the morphological and molecular responses found in physiological hypoxia and $CoCl_2$.

In humans, O_2 tension in skeletal muscle is approximately 30–40 mmHg in normoxia, i.e. 5% O_2 [31]. Thus, O_2 concentration lowered to 4% as in our in vitro study would correspond to a comparable physiological hypoxia condition found in vivo [9]. Although physiological hypoxia (decrease oxygen level of the ambient air) remains the reference model to reproduce most closely the in vivo phenomena of hypoxia [2], pharmacological treatment such as $CoCl_2$ and DFO (defined as hypoxia-mimetic agents) is commonly used to mimic hypoxia [3, 15, 16, 34]. Indeed, by replacing the iron ion in

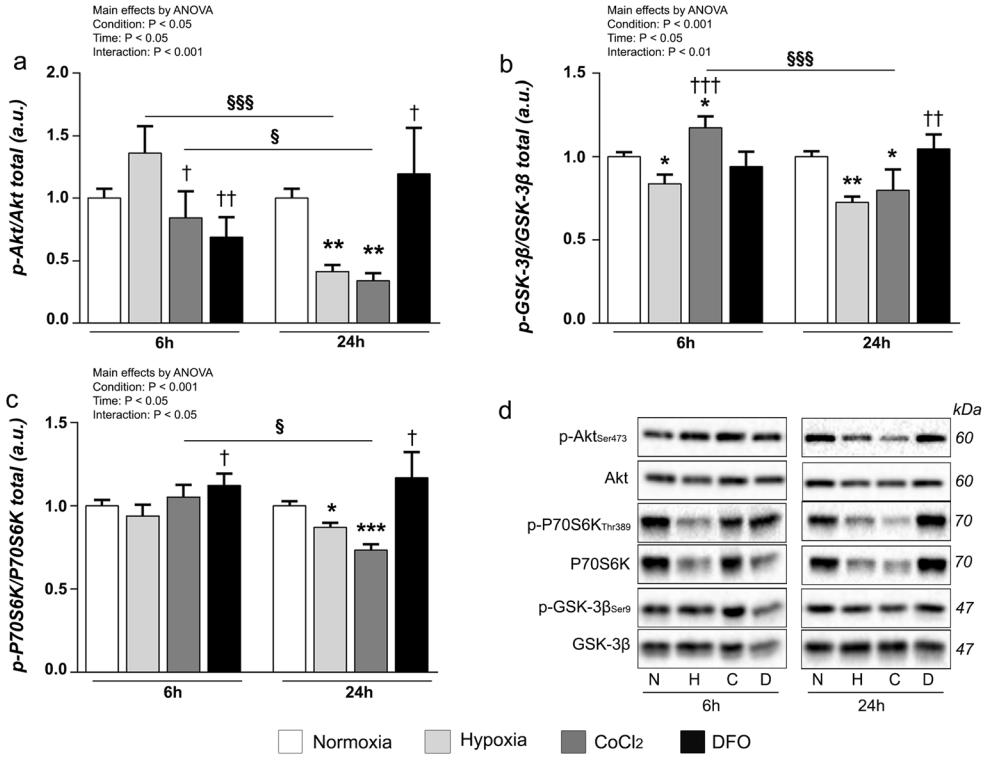

Fig. 5 Effect of different methods of hypoxia induction on protein synthesis through downregulation Akt signaling activity. Activation level of protein synthesis pathway determined by Western blot in C2C12 myotubes under normoxia (21% O_2) (N), hypoxia (4% O_2) (H) or treated by hypoxia-mimetic agents: $CoCl_2$ (C) or DFO (D) at a concentration of 200 μM for 6 h or 24 h. **a** Quantification of the ratio of phosphorylated Akt (Ser473) over total Akt after 6 or 24 h response. **b** Ratio of phosphorylated P70S6K (Thr389) over total P70S6K. **c** Ratio of phosphorylated GSK-3β (Ser9) over total GSK-3β. **d** Representative signals of proteins of interest after 6 or 24 h after induction of hypoxia. The mean values from the quantification of Western blots are represented in arbitrary units (a.u.). Post-hoc analyses for conditions effect significantly from normoxia (basal) values: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Significantly different from hypoxia (4% O_2): $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$. Post-hoc analyses for times effect significantly different from 24 h vs 6 h values: $^{\S}P<0.05$, $^{\S\S\S}P<0.001$ ($n=12$ per condition)

hydroxylase, $CoCl_2$ and DFO lead to the stabilization of HIF-1α (hypoxia-inducible factor-1α, which is the best characterized response to hypoxia in cells) by inhibiting its hydroxylation [5]. Thus, such as physiological hypoxia resulting from oxygen deprivation, the hypoxia-mimetic agents induced the increase of expression of HIF-1α, suggesting that $CoCl_2$ and DFO mimicked a physiological hypoxic condition in regard to the stabilization of HIF-1α. This property and replacement rate of the iron ion of $CoCl_2$ and DFO may explain the overexpression of HIF-1α protein compared to hypoxia condition (4% O_2) after 6 h induction. Indeed, a study by Pisani et al. showed on C2C12 cells treated with $CoCl_2$ (200 μM) an increase of HIF-1α protein expression at 0.5 h and 3 h post-treatment [27], while C2C12 under 5% O_2 showed an increased HIF-1α protein expression only from 72 h of exposure compared to measurements taken at 24 h and 48 h after induction [17].The treatment concentrations range varied from 5 to 500 μM, a 200-μM concentration being widely used for the long-term in vitro treatment (24 h and more) of muscle cells [3, 5]. However, to our knowledge, there are no studies which have precisely determined the similarities and differences between the different methods of induction of hypoxia on signaling pathways involved in neither protein homeostasis nor their repercussion on myotube morphology and on protein content.

The preservation of skeletal muscle mass depends on protein homeostasis, i.e. equilibrium between synthesis and degradation of proteins, their imbalance causing either hypertrophy or on the contrary atrophy of skeletal muscle [13]. Our study shows that skeletal muscle cells deprived of oxygen (4% O_2) or treated with the hypoxia-inducer $CoCl_2$ have a decrease in the activation of Akt and FoxO1 transcription factor. Our data corroborate with those of de Theije and coworkers, who demonstrated a lower p-Akt/Akt ratio and increased of FoxO1 expression in rat skeletal muscle in response to chronic hypoxia, indicating that hypoxia negatively regulates protein synthesis in skeletal muscle [7]. Subsequently, we investigated the consequences of each mode of hypoxia on the activation and/or expression of key proteins in each pathway of protein homeostasis.

Phosphorylation (and so activation) of Akt results in the phosphorylation of multiple direct and indirect downstream

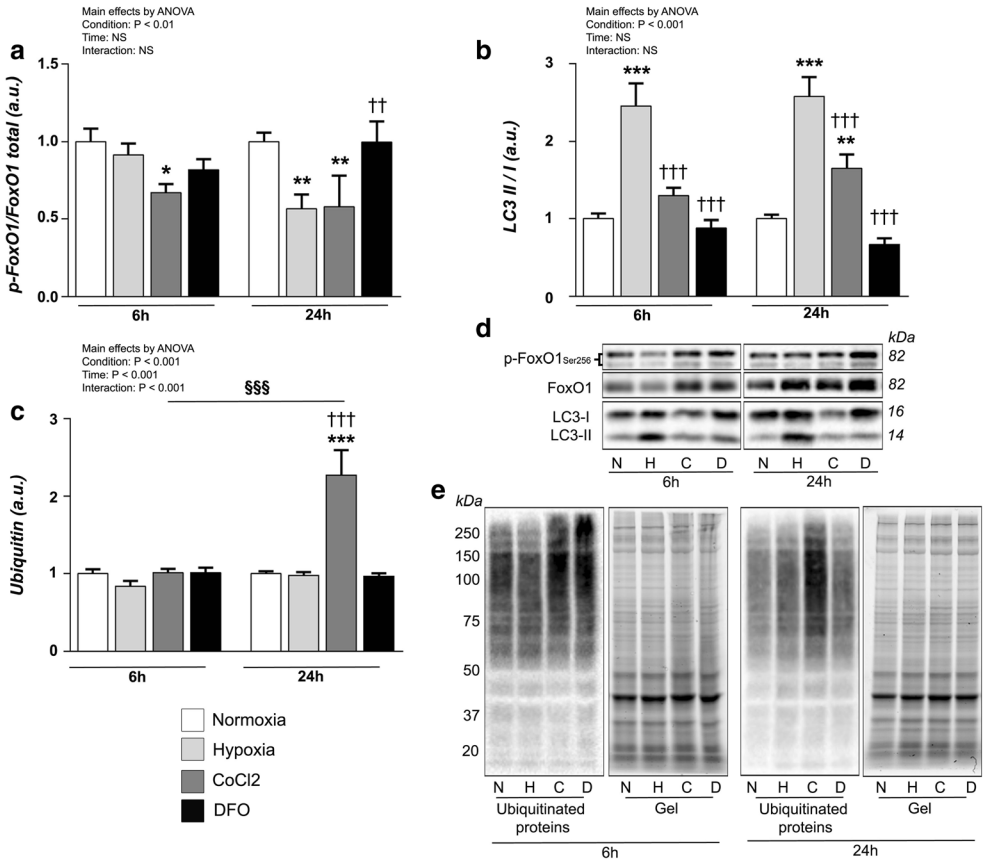

Fig. 6 Effect of different methods of hypoxia induction on protein degradation markers in C2C12 myotubes. Phosphorylation of FoxO1 transcription factor and expression of key proteins involved in autophagy and ubiquitin–proteasome systems determined by Western blot in C2C12 myotubes under normoxia (21% O_2) (N), hypoxia (4% O_2) (H) or treated by hypoxia-mimetic agents: $CoCl_2$ (C) or DFO (D) at a concentration of 200 μM for 6 h or 24 h. **a** Quantification of the ratio of phosphorylated FoxO1 (Ser256) over total FoxO1 after 6 or 24 h response. **b** LC3-II/LC3-I ratio at the protein level. **c** Quantification of ubiquitin-conjugated proteins. **d** Representative signals of proteins of interest after induction of hypoxia. **e** Representative signals of ubiquitinated protein. The mean values from the quantification of Western blots are represented in arbitrary units (a.u.). Post-hoc analyses for conditions effect significantly from normoxia (basal) values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significantly different from hypoxia (4% O_2): $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. Post-hoc analyses for times effect significantly different from 24 h vs 6 h values: $^{\S\S\S}P < 0.001$ ($n = 12$ per condition)

targets. Among them, the glycogen synthase kinase 3 (GSK-3), phosphorylated and inhibited by Akt, is known to suppress protein synthesis by inhibiting eukaryotic transcription factor 2B (eIF2B) [6, 22]. Following the deprivation in O_2, the phosphorylation of GSK-3β decreased, with a consequent reduction of protein synthesis in rats as well as in C2C12 myotubes [1, 7]. Downstream of Akt, the mTOR pathway stimulates ribosomal biogenesis and translation initiation through phosphorylation of P70S6K, which plays a determining role in hypertrophy of muscle cells [24]. Several studies in various models showed that hypoxic stress caused hypophosphorylation of P70S6K on treated C2C12 myotubes, in rodents, in COPD patient's biopsies or in primary cultured cells derived from COPD patient muscle [6, 13, 26]. Data gained from our study revealed a decrease activation of GSK3-β and P70S6K for C2C12 myotubes subjected to oxygen deprivation or treated with $CoCl_2$ as hypoxia mimetic agent, while in contrast, there was no changed for DFO treatment (Fig. 7). Thus, altogether, our data indicated that $CoCl_2$ treatment was quite more similar to oxygen deprivation considering the signaling pathways involved in protein synthesis, in particular the decrease of activation protein signaling pathway.

However, the protein synthesis decrease is not the only cause of the disequilibrium of protein homeostasis leading to muscle atrophy. Indeed, in metabolic regulation of skeletal muscle, protein degradation also plays an important role. This muscle catabolism is a well-identified phenomenon in some COPD patients [8], presenting an important cachexia [29]. Among the degradative processes, the autophagy system, which consists of the capture of organelles and proteins from the cytoplasm for lysosomal degradation, is mainly involved [33]. A study performed from COPD vastus lateralis biopsies shows an increase in LC3-II, translating the activation of autophagy, correlated with a reduction of cross-sectional area of skeletal muscle fibres [14]. In our in vitro

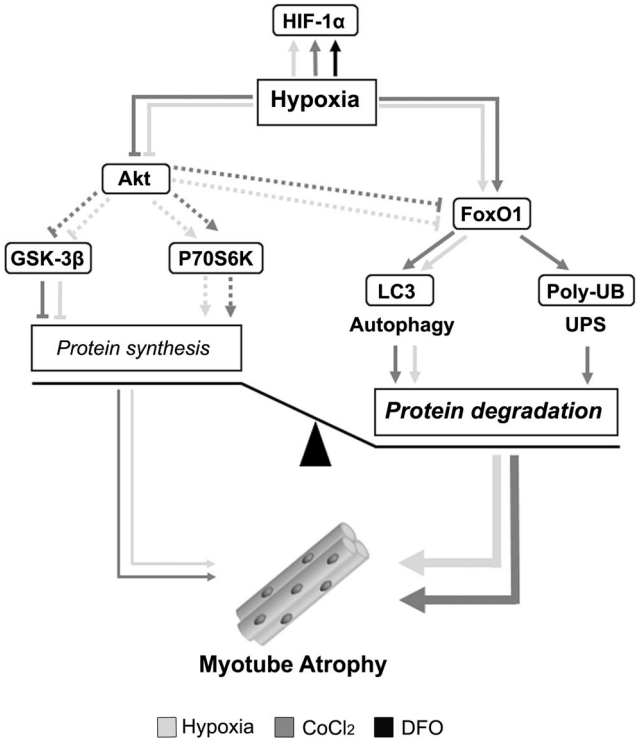

Fig. 7 Schematic representation of the effects of physiological hypoxia (4% O_2) and hypoxia mimetic agents ($CoCl_2$ and DFO) on the regulation of protein homeostasis (i.e. equilibrium between protein synthesis and protein degradation) and myotube morphology. Skeletal muscle cells cultured under hypoxia (4% O_2) or treated with the hypoxia inducer $CoCl_2$ show a decrease in protein synthesis and an increase in protein degradation, resulting in reduction of myotubes diameter. In contrast, DFO treatment has effect neither on the morphology of C2C12 myotubes nor on signaling pathways involved in protein homeostasis. Continuous line means increased and discontinuous line means decreased

study, a convergence between physiological hypoxia and $CoCl_2$ hypoxia induction was also found in activation of autophagic system through increasing the LC3-II/LC3-I ratio after 24 h of exposure. Another degradative process is as important as well: the ubiquitin/proteasome system. In this way, Martin and co-workers showed an increase of MuRF1 mRNA expression in C2C12 myotubes subjected to a drastic reduction of O_2 level (i.e. 1% O_2), while there was no change at 5% O_2 (physiological hypoxia) [26]. Another study revealed a raised expression of E3 ubiquitin ligase (atrogin-1 and MuRF1) of $CoCl_2$ treated C2C12 muscle cells [32], as we observed in our study, while in contrast, DFO has no effect on the ubiquitination of proteins (Fig. 7). These data suggested that ubiquitin/proteasome system is more responsive to $CoCl_2$, while the level hypoxia of 4% O_2 selected in our protocol, which corresponds to "physiological" hypoxia, is too high to stimulate ubiquitination of proteins [7, 26].

Thus, the hypophosphorylation of P70S6K and overexpression of LC3-II clearly showed that skeletal muscle cells were highly sensitive to a reduction of oxygen level [23], leading to an anabolism/catabolism imbalance in favour of muscle atrophy.

Our data about the protein degradation pathways showed that increase expression of FoxO1 and LC3-II associated with a reduction of the Akt/GSK3-β/P70S6K pathway reinforces that hypoxia results in an imbalance of protein homeostasis which can seriously alter the morphology of myotubes. More importantly, our results have demonstrated that a chemical inducer of hypoxia such as $CoCl_2$ had similar effects in terms of protein synthesis, in contrast of DFO which has no effect on the anabolic pathway. In parallel, our data also showed that $CoCl_2$ was quite more similar as physiological hypoxia concerning the autophagy process, while DFO has no effect. But $CoCl_2$ led to an important increase of protein ubiquitination compared to physiological hypoxia (4% O_2) [7, 32]. Although studies performed on skeletal myocyte L6 under DFO treatment observed an increased mRNA expression of atrophy markers (Atrogin-1 and MuRF1) [11, 19], our data showed that DFO has no effects on ubiquitinated protein and morphology of myotubes. Interestingly, this alteration of signaling pathways which govern the protein homeostasis was correlated with changes in myotubes diameter and in protein content of myotubes. Indeed, we measured a decrease in myotubes diameter closely associated to reduction of Myogenin expression and the dramatic loss of protein content, especially for the myotubes treated with 4% O_2 [10] and $CoCl_2$ [3]. This loss of muscle mass was also found in rats following a prolonged period of hypoxia at 8% O_2 [7] and a reduced myotubes diameter in cells derived from COPD muscle [28]. Our data concerning the decrease of phosphorylation of Akt, GSK3-β and P70S6K following hypoxic exposure may explain the morphological analysis after 48 h and 72 h treatment (Fig. 7).

In conclusion, our data demonstrate that physiological hypoxia (4% O_2) and $CoCl_2$ have similar anabolic and catabolic consequences both on signaling pathways responsible for protein homeostasis and morphology of myotubes, while DFO presents several differences in terms of molecular responses of skeletal muscle cells. In addition, this muscle atrophy in response to different methods of induction of hypoxia (oxygen deprivation and chemical induction of hypoxia) is similar at the molecular and phenotypic levels to that can be observed in the skeletal muscle of COPD patients. This study highlights the interest of artificial induction models of hypoxia, which have proved their real efficiency for the investigation and the fine understanding of the physiopathology related to muscle dysfunction associated with hypoxia.

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Author contributions Samir Bensaid: Conceived, designed and performed the experiments, analysed the data and wrote the paper; Julie Fourneau: contributed to acquisition and analysis of the data; Claudine Fabre: conceived the experiments and wrote the paper; Caroline Cieniewski-Bernard: conceived and designed the experiments, performed the experiments, analysed the data and wrote the paper.

Compliance with ethical standards

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Informed consent Not concerned.

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