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Running title: Oxygen supply or electrical stimulation way to limit hypoxia-induced muscle atrophy

ABSTRACT

In skeletal muscle, chronic oxygen depletion induces a disturbance leading to muscle atrophy. Mechanical stress (physical exercise) and nutritional supplement therapy are commonly used against loss of muscle mass and undernutrition in hypoxia, while oxygenation therapy is preferentially used to counteract muscle fatigue and exercise intolerance. However, the impact of oxygenation on skeletal muscle cells remains poorly understood, in particular on signalling pathways regulating protein balance. Thus, we investigated the effects of each separated treatment (mechanical stress, nutritional supplementation and oxygenation therapy) on intracellular pathways involved in protein synthesis and degradation that are imbalanced in skeletal muscle cells atrophy resulting from hypoxia. Myotubes under hypoxia were treated by electrical stimulation, amino acids supplement or oxygenation period. Signalling pathways involved in protein synthesis (PI3K-Akt-mTOR) and degradation (FoxO1 and FoxO3a) were investigated, so as autophagy, ubiquitin-proteasome system, and myotubes morphology. Electrical stimulation and oxygenation treatment resulted in higher myotubes diameter, myogenic fusion index and myotubes density until 48h post-treatment compared to untreated hypoxic myotubes. Both treatments also induced inhibition of FoxO3a and decreased activity of ubiquitin-proteasome system; however, their impact on protein synthesis pathway was specific for each one. Indeed, electrical stimulation impacted upstream proteins to mTOR (*i.e.*, Akt) while oxygenation treatment activated downstream targets of mTOR (*i.e.*, 4E-BP1 and P70S6K). In contrast, amino acids supplementation had very few effects on myotubes morphology nor on protein homeostasis. This study demonstrated that electrical stimulation or oxygenation period are two effective treatments to fight against hypoxia-induced muscle atrophy, acting through different molecular adaptations.

Keywords: Skeletal muscle hypoxia, electrical stimulation, oxygenation treatment, protein homeostasis, myotubes morphology

BACKGROUND

Impaired skeletal muscle oxygen supply (hypoxia) is observed in various environmental conditions (e.g., exposition at high altitude) or pathological contexts (e.g., chronic obstructive pulmonary disease, COPD), which leads to numerous adaptations in skeletal muscle (Kent et al., 2011; Levett et al., 2012). In this sense, chronic oxygen deprivation of muscle tissue induces an imbalance of

regulatory molecular pathways involved in protein synthesis and degradation of skeletal muscle cells in favour of the degradative pathways (Costes et al., 2015; Langen et al., 2013). A typical example illustrated in literature is the alteration of muscle mass in hypoxemic conditions, in particular a dramatic muscle atrophy in COPD patients (Maltais et al., 2014; Vermeeren et al., 1997). Therefore, there is a pressing need to better understand the signalling pathways involved in the skeletal muscle atrophy resulting from hypoxia.

To limit or even counteract the consecutive muscle atrophy to hypoxia, physical exercise is commonly used as a therapeutic intervention (Maltais et al., 2014). However, at the molecular level, several studies showed that post-translational changes of key proteins involved in protein synthesis or degradation pathways, are weakly modified or remain unchanged in muscles of trained COPD patients (Costes et al., 2015; Vivodtzev et al., 2012). Another way to limit or counteract muscle atrophy is the nutritional supplementation (Paddon-Jones & Rasmussen, 2009). In fact, in normoxia, amino acids are known to be potent modulators of the mTOR pathway (Jewell et al., 2013; Zheng et al., 2019) and the anabolic effects of amino acids through the activation of signalling pathways regulating protein synthesis and degradation are well known (Baptista et al., 2017; Duan et al., 2017). Nutritional supplementation with proteins particularly rich in branched chain amino acids (BCAAs) or with BCAAs alone, has only recently emerged to limit the loss of mass in hypoxic muscles such as cachectic COPD (Dal Negro et al., 2016), but in the context of hypoxic muscle, several questions remain unanswered concerning the intracellular and molecular impact of BCAAs supplementation.

Oxygen therapy is a treatment commonly used against early muscle fatigue in people with chronic hypoxemia, such as moderate and severe COPD (Maltais et al., 2001). Oxygen supplementation in COPD showed an increase in exercise tolerance and physical performance (Maltais et al., 2001), translating an improvement of the muscle function (Emtner et al., 2003). However, origin of these adaptations is poorly understood since studies on intracellular effects of skeletal muscle O_2 supplementation under hypoxia are extremely limited to date. In addition, there is no data concerning the impact of oxygen supplementation on signalling pathways involved in protein homeostasis, strongly dysregulated in hypoxemic muscle.

We hypothesized that the type and nature of each countermeasure to muscle atrophy in hypoxia leads to different intracellular adaptations that could have an impact on the regulation of regulatory pathways for protein homeostasis and morphological adaptation. To test this hypothesis, we carried out our investigations on an *in vitro* model of skeletal muscle cells under hypoxia. This model has the advantage to present atrophy characteristics similar to those observed in animals or humans under hypoxia (Maltais et al., 2001, Martin et al., 2017), but also allows to consider a large panel of experimental conditions and the possibility to perform several measurements over time. In the herein study, we investigated the morphological changes of C2C12 muscle cells differentiated into myotubes under hypoxia after an electrical stimulation, BCAAs supplementation or a short period of oxygenation, as well as the molecular events involved in the protein homeostasis through the key regulators of protein

synthesis and degradation pathways. Morphological analysis showed that electrical stimulation and oxygenation period maintained a high rate of myotubes maturation. Interestingly, the activation pattern of the protein synthesis pathway PI3K-Akt-mTOR is different between both countermeasures. Indeed, electrical stimulation activated Akt at the upstream of the pathway, while oxygenation treatment led to the stimulation of downstream targets of mTOR: 4E-BP1 and P70S6K. Importantly, oxygenation treatment was the single intervention to stimulate these key effectors of protein synthesis.

MATERIALS AND METHODS

C2C12 cell culture

C2C12 mouse myoblasts (ATCC: American Type Culture Collection, Manassas, VA) were grown in proliferation medium (PM), corresponding to Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% foetal calf serum (Gibco) and 1% antibiotics/antimycotics (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/antimycotics. The shifting time to DM was assigned to day-0 of differentiation. The incubation was performed in humidified atmosphere of 20.9% O_2 and 5% CO_2 at 37°C; media were changed every 48h, and myotubes formation was monitored daily.

C2C12 myotubes treatments

For hypoxia induction, oxygen was replaced by nitrogen (N_2) in the hypoxia chamber, placed into incubator through the ProOx P110 oxygen controller (BioSpherix, Lacona, NY). Four days-differentiated myotubes were transferred into the hypoxic chamber maintained at 4% O_2, 5% CO_2 and 91% N_2 as previously described (Bensaid et al., 2019). To assess hypoxia-induced cellular alterations (4% O_2), a viability test by MTT was performed. Hypoxia was maintained until protein extraction of myotubes or May-Grünwald-Giemsa staining. Electrical stimulation (ES), branched chain amino acids supplementation (BCAAs) and oxygenation (O_2) were performed on 5-days differentiated myotubes, which corresponds to an optimal level of maturation of the myotubes for the treatments mentioned above (**Fig. 1**). Before ES, the cells media were changed and the dishes (35 mm) were placed into the hypoxia chamber and were fitted with 6-wells C-Dish electrode and connected to a C-PACE EP Cell Culture Stimulator (Ion Optix, Milton, MA) in order to provide electric pulses (30 V at 1 Hz for 3 ms at 997-ms intervals) for 1h, as described by Miyatake et al (2015). BCAAs was designed with a mix of leucine, isoleucine and valine freshly diluted in differentiation medium at a concentration of 5mM each and applied to hypoxic myotubes. For oxygenation (O_2) protocol, the culture media were changed and the

dishes were removed from hypoxia chamber and placed into the incubator (20.9% O_2, 5% CO_2) for 2 hours. In the literature, it has been established that an oxygenation level above 5% would already induce muscle hypertrophy in cultured C2C12 cells (Sakushima et al., 2020). But the choice of an oxygenation level of 20.9% is that it corresponds to a standard value and that this level is usually used in C2C12 cell culture.

Protein extraction protocol

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 cellular extracts was assayed using Bradford assay (Biorad). 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). This protein extraction was performed for molecular analysis at 2h, 24h and 48h post-treatment (ES, BCAAs, O_2).

Western Blot analysis

Proteins were separated on Mini-PROTEAN TGX Stain-Free 10% precast polyacrylamide gels (Biorad); an internal standard was loaded on each gel. Electrophoretic separation was done at 200V for 35 minutes in migration buffer (25 mM TrisBase; 0.2 M glycine; 1% SDS (p/v)). Stain-Free (SF) technology contains a proprietary trihalo compound which reacts with proteins, rendering them detectable through UV exposure. SF imaging was performed using ChemiDoc MP Imager and Image Lab 4.0.1 software (Biorad) 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 (Biorad). 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; 140mM NaCl; 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated at 4˚C overnight or 2 hours at room temperature with the following primary antibodies: 4E-BP1 (#9452, Cell Signalling); p-4E-BP1 (Ser51; #9451, Cell Signalling); Akt (#9272, Cell Signalling); p-Akt (Ser473; #9271, Cell Signalling); GSK-3β (#9315, Cell Signalling); p-GSK-3β (Ser9; #9336, Cell Signalling); FoxO1 (C29H4; #2880, Cell Signalling); p-FoxO1 (Ser256; #9461, Cell Signalling); FoxO3a (75D8; #2497, Cell Signalling); p-FoxO3a (Ser294; #5538, Cell Signalling); LC3A/B (#4108, Cell Signalling); mTOR (#2972, Cell Signalling); p-mTOR (Ser2441; #2971; Cell Signalling); P70S6K (#9202, Cell Signalling); p-P70S6K (Thr389; #9205, Cell Signalling); Ubiquitin (P4D1; #3936, Cell Signalling). 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 Signalling) in blocking solution for 2 hours at room temperature, and were 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 (Biorad), and images 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, sample control (normoxia 20.9% O_2) and internal standards.

Morphological analysis

The maturation and the development status of myotubes were assessed through morphological analysis (Veliça & Bruce, 2011). Myotubes were fixed with 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 x10 magnification. Fifteen fields were chosen randomly for each experimental condition and all the myotubes (≥ 3 nuclei) from each field were measured. The number of images to be analysed was adjusted to include 300 myotubes per condition. 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 (Ren et al., 2008). Morphological measurements were done on 5-days (d5), 6-days (d6) and 7-days (d7) differentiated myotubes.

C2C12 myotubes protein content

Total protein content of C2C12 was determined once cellular protein extraction protocol achieved. 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 then determined, and reported as protein content in mg per dish.

Statistical analyses

All treatments were performed at least in biological triplicates or quadruplicates from 2 or 3 independent cultures. All data were presented as means ± SEM. Data were tested for normality using a Shapiro-Wilk test. The effects of conditions (each condition was compared with each one, *i.e.*, normoxia *vs* hypoxia *vs* electrical stimulation *vs* BCAAs supplementation *vs* oxygenation) and times (d6 *vs* d5 and d7 *vs* d6) 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

Hypoxia induced myotubes atrophy

Representative images of myotubes in normoxia, in hypoxia, and treated-myotubes under hypoxia (*i.e.,* electrical stimulation, BCAAs supplementation or oxygenation) were presented **Fig. 2a**. Morphological analysis of C2C12 myotubes under hypoxia (4% O_2) revealed a decrease in their diameter compared to normoxia-control cells at d6 (6-days of differentiation; -3.29 µm, $P < 0.001$) and d7 (7-days of differentiation; -10.23 µm, $P < 0.001$) (**Fig. 2b**). Exposure to 4% O_2 also showed a significant reduction in the myotubes diameter over time: -2.76 µm from d5 to d6 ($P < 0.001$) and -4.3 µm from d6 to d7 ($P < 0.001$). In contrast, C2C12 myotubes under normoxia indicated an increase in their diameter between d6 and d7 (+2.64 µm, $P < 0.01$) (**Fig. 2b**). The myotubes maturation determined by fusion index was decreased in hypoxia condition between d5 and d6 (44.8 ± 2.3% and 27.1 ± 2%, for d5 and d6, respectively; $P < 0.001$); in addition, myogenic fusion index was significantly lower than normoxic cells at d6 and d7 (-17.3% and -17%, respectively; $P < 0.001$) (**Fig. 2c**). The density of myotubes was also significantly disturbed by hypoxia compared to myotubes under normoxia ($P < 0.001$), as shown by the drastic decrease in the number of myotubes per field, in correlation with the post-treatment duration applied during hypoxia*, i.e*., after 2h (d5: 29.9 ± 1.6 myotubes per field), 24h (d6: 22.6 ± 1.6 myotubes per field; d6 *vs* d5, $P < 0.01$) and 48h (d7: 12.51 ± 1.16 myotubes per field; d7 *vs* d6, $P < 0.001$) of treatments (**Fig. 2d**). Lastly, hypoxic myotubes presented a decrease in the number of nuclei within the myotubes, compared with normoxic myotubes ($P < 0.01$) (**Fig. 2e**).

In parallel of all morphological parameters, the protein content significantly decreased in hypoxic cells (0.16 ± 0.01 and 0.15 ± 0.01 mg/dish at d6 and d7, respectively) compared with normoxic cell (0.43 ± 0.02 and 0.54 ± 0.01 mg/dish at d6 and d7, respectively) ($P < 0.001$). Unlike the hypoxic condition, a normoxic environment allows an increase in protein content between d6 and d7 (+0.11 mg/dish, $P < 0.01$) (**Fig. 3**).

All together, these data clearly showed that hypoxia dramatically led to C2C12 myotubes atrophy, correlated to a decrease of protein content and a decrease of myotubes maturation.

Electrical stimulation and oxygenation treatment reduced hypoxia-induced muscle atrophy

While hypoxia led to a significant decrease of myotubes diameter, the myotubes treated by electrical stimulation (ES) or reoxygenation (O_2) had a greater diameter than hypoxic (H) cells at d6 (+2.86 and +2.32 µm, for ES and O_2, respectively, $P < 0.01$) and d7 (+3.84 and +3.58 µm, for ES and O_2, respectively $P < 0.001$) (**Fig. 2b**); in contrast, the BCAAs supplementation did not present any changes compared with hypoxic cells after d6 or d7. Importantly, both treatments (ES or O_2) allowed to maintain the myotubes diameter (18.1 ± 0.39 µm and 17.56 ± 0.63 µm, for ES and O_2, respectively)

very close to the value under normoxia (18.53 ± 1.04 µm), despite hypoxia after 6-days of differentiation (**Fig. 2b**).

Only the cells treated by O_2 maintained the myogenic fusion index between d5 (45.7 ± 1.85%) and d6 (44.76 ± 1.89%), while all other conditions indicated a reduction in the percentage of fusion (d6 *vs* d5, $P < 0.001$) (**Fig. 2c**). In addition, we observed very few differences between myotubes under normoxia (46.38 ± 1.09%) or treated by O_2 at 6-days of differentiation (d6). ES and O_2 maintained a significantly higher fusion index compared to hypoxia condition at d6, corresponding to 24 hours post-treatment (+9.7 and +17.7 %, ES and O_2, respectively, $P < 0.01$). Furthermore, O_2 condition was the only treatment to get a higher myogenic fusion percentage (+8.12%, $P < 0.05$) compared with hypoxic cells condition at d7 (**Fig. 2c**).

Interestingly, only O_2 allowed C2C12 cells to limit the myotubes consecutive loss to hypoxia between time d5 and d6 (no difference comparing with normoxia but significantly different from hypoxia at d6, $P < 0,01$). Three-days after induction of hypoxia, and 2-days post-treatments (d7), the number of myotubes per field was higher for hypoxic myotubes treated by electrical stimulation ($P < 0.01$) or by oxygenation ($P < 0.001$) (**Fig. 2d**). In the same way, only O_2 treatment observed a same number of nuclei inside myotubes at d6 (4.9 ± 0.18 nuclei per myotube) in comparison to normoxia (5.6 ± 0.32 nuclei per myotube), while hypoxia cells without countermeasure, ES and BCAAs conditions showed a decrease of the nuclei number within myotubes, even if the decrease was minor for ES compared with BCAAs ($P < 0,05$ and $P < 0.001$, for ES and BCAAs, respectively) (**Fig. 2e**). However, at d7, the number of myonuclei was quite similar to hypoxic cells regardless of the used treatment. Lastly, unlike ES and O_2 treatment, we found that hypoxia and BCAAs conditions showed a similar decrease on all morphological analysis parameters and over all observation times (**Fig. 2**).

While we did not measure any morphological differences between normoxia, hypoxia nor the three treatments 2h-post-treatment (d5), we quantified a significant decrease of total protein content in each condition associated with hypoxia (*i.e.,* ES, BCAAs or O_2 treatments) at d5 comparing with normoxia cells (**Fig. 3**). However, at d6, protein content did not significantly decrease across time for cells treated by ES (0.36 ± 0.01 and 0.29 ± 0,01 mg, at d5 and d6, respectively) or O_2 (0.35 ± 0.02 and 0.27 ± 0.01 mg, at d5 and d6, respectively) (**Fig. 3**), whereas supplemented myotubes with BCAAs presented a significant loss of proteins between d5 and d6 ($P < 0.001$), similarly to hypoxia alone. Interestingly, C2C12 cells under hypoxia treated by electrical stimulation or by a period of oxygenation showed a higher protein content than hypoxic cells at d6 (+0.13 mg and +0.11mg for ES and O_2, respectively, $P < 0.05$) and d7 (+0.13 mg and +0.14 mg for ES and O_2, respectively, $P < 0.001$). In contrast, BCAAs supplementation did not prevent the significant loss of protein content since the protein content was quite similar to hypoxia cells (**Fig. 3**).

Countermeasures against hypoxia-induced muscle atrophy impacted the Akt/mTOR signalling pathway.

Analysis of Akt expression and activation status revealed that each treatment applied to C2C12 myotubes at 6-days of differentiation resulted in reduction of Akt expression compared with control cells under normoxia ($P < 0.05$) (**Fig. 4a'**). Only the cells treated by electrical stimulation (ES, 7.31 ± 1.21 a.u., $P < 0.001$) resulted in an increase of Akt phosphorylation on Ser473 in opposition to hypoxia (H, 1.79 ± 1.45 a.u.) and normoxia (N, 1.00 ± 0.06 a.u.) conditions at d5 ($P < 0.001$); furthermore, the effect persisted 24 hours post-stimulation (d6, $P < 0.001$) (**Fig. 4a**). This high level of phosphorylation led to an increase of the ratio p-Akt/Akt (translating thus the activation status of Akt) relative to hypoxia and normoxia conditions over the time (d5, $P < 0.001$; d6, $P < 0.01$) (**Fig. 4a"**). We noted that myotubes under hypoxia, supplemented with BCAAs observed a rise of phosphorylation level of Akt only at d5 (+2.59 a.u.) and the ratio p-Akt/Akt only at d6 (+2.81 a.u.) compared to hypoxia ($P < 0.05$) (**Fig. 4a and 4a"**). In contrast to Akt, the ratio p-mTOR/mTOR, a downstream complex from Akt, didn't show any variation compared to hypoxia alone (**Fig. 4b and 4b'**).

Phosphorylation of GSK-3β showed 2 hours post-treatment (d5) a higher ratio p-GSK-3β/GSK-3β in hypoxic cells treated with ES (+0.37 a.u., $P < 0.05$) or O_2 (+0.51 a.u., $P < 0.001$) compared to untreated hypoxic myotubes, until the ratio p-GSK-3β/GSK-3β returned to basal level at D6 (**Fig. 4c"**). Its expression remained unchanged, no matter the considered condition.

For the mTOR downstream targets 4E-BP1 and P70S6K, 4% O_2 and BCAAs conditions showed a significant decrease of the phosphorylation level of 4E-BP1 on 5-days differentiated myotubes ($P < 0.05$), while O_2 was the only one treatment resulted in a significant increase of p-4E-BP1/4E-BP1 ratio (+0.21 a.u., $P < 0.05$), in opposition to hypoxic myotubes (**Fig. 5a"**). After oxygenation period, P70S6K showed an increase of phosphorylation level (Thr389) (+1.68 a.u.) and ratio p-P70S6K/P70S6K (+1.55 a.u.) compared to hypoxia conditions ($P < 0.001$) (**Fig. 5b and 5b"**). The variation of the phosphorylation level of 4E-BP1 and P70S6K at d5 was not maintained at 24 hours post-treatment (d6), suggesting an acute effect of oxygenation treatment on the regulators of the protein synthesis pathway.

Electrical stimulation and oxygenation treatment decreased protein degradation by FoxOs

In contrast to the anabolic role of Akt in protein homeostasis, the forkhead family (and among them FoxO1 and FoxO3a) plays an important role in proteolytic pathway through its role as transcription factor (Sandri, 2013). We observed that only treatment by ES induced an increase of FoxO1 phosphorylation (+0.28 a.u., $P < 0.05$) compared to hypoxia 2h post-treatment (d5) condition (**Fig. 6a**). All hypoxic cells showed a raise in FoxO1 expression at d5 ($P < 0.01$) while returning to normoxia value at d6. Only BCAAs supplementation showed a greater reduction in FoxO1 protein compared to the normoxia (-0.47 a.u., $P < 0.001$) and hypoxia (-0.32 a.u., $P < 0.01$) conditions (**Fig. 6a'**). Finally, we didn't quantify any changes in the p-FoxO1/FoxO1 ratio aside from the considered conditions (**Fig. 6a"**).

Analysis of Foxo3a revealed an increase of FoxO3a phosphorylation and its expression compared to normoxia and hypoxia condition (d5, $P < 0.01$); however, the ratio of p-FoxO3a/FoxO3a was unmodified at d5 (**Fig. 6b"**). The main changes were observed 24 hours post-treatment (d6) with a reduction of the phosphorylation level on Ser253 and the expression of FoxO3a (**Fig. 6b and 6b'**). Only the hypoxic cells treated by O_2 resulted in an increase of FoxO3a phosphorylation (+0.42 a.u.) and FoxO3a expression (+0.37 a.u.) as opposed to hypoxia condition at d5 ($P < 0.05$). In contrast, the cells under hypoxia treated by BCAAs showed a decrease of FoxO3a (-0.36 a.u.) and total FoxO3a (-0.48 a.u.) phosphorylation, as opposed to hypoxia untreated at d5 ($P < 0.05$). All conditions associated with hypoxia (*i.e.,* untreated and treated hypoxic cells) showed a reduction of FoxO3a phosphorylation and total FoxO3a protein 24 hours post-treatment (d6) ($P < 0.01$) (**Fig. 6b**). Finally, the quantification of p-FoxO3a/FoxO3a ratio showed a high level of phosphorylation for myotubes treated with ES (+0.64 a.u.) and O_2 (+0.61 a.u.) compared to hypoxia condition ($P < 0.05$) (**Fig. 6b"**).

The ratio of LC3 II/I revealed an important increase of autophagy for untreated and treated cells under hypoxia, regardless of the applied treatment, compared to normoxia condition at d5 ($P < 0.05$) and d6 ($P < 0.001$) (**Fig. 7a**). The protein ubiquitination level, commonly used as a marker of muscle atrophy, was increased for all myotubes under hypoxia (untreated- so as treated-myotubes); in contrast with normoxic myotubes at d6 ($P < 0.01$). However, myotubes treated by ES (-0.71 a.u.) and O_2 (-0.8 a.u.) showed a significantly lower ubiquitination level than hypoxia untreated cell at 24h post-treatment (d6) ($P < 0.05$) (**Fig. 7b**).

DISCUSSION

This study demonstrated that electrical stimulation or an oxygenation period applied on hypoxic C2C12 myotubes induced higher myotubes diameter, myogenic fusion index and myotubes density until 48h post-treatment compared to untreated hypoxic myotubes. In addition, electrical stimulation and cells oxygenation had similar effects on different regulatory processes in protein degradation, in particular the inhibition of the transcription factor FoxO3a and the activation of ubiquitin proteasome system in comparison with untreated hypoxic muscle cells. However, we found differences in muscle cell adaptations to electrical stimulation and oxygenation, in particular on the PI3K-Akt-mTOR synthesis pathway that was differentially impacted by the two countermeasures. Indeed, the electrical stimulation treatment sustained the long-lasting activation of Akt while the oxygenation treatment on muscle cells in hypoxia induced an activation of key proteins involved in protein synthesis process such as 4E-BP1 and P70S6K.

More specifically, electrical stimulation increased the level of phosphorylation of key regulating proteins of protein synthesis (Akt and GSK-3β) and degradation pathways (FoxO3a and ubiquitin-protein) (**Fig. 8b**). These findings concerning the activation of PI3K-Akt-mTOR signalling pathway are closely similar to the effects observed *in vivo* (muscle contraction repetitions in normoxia) and *in vitro* (electrical stimulation of muscle cells using an exercise model mimicking the *in vivo* muscle adaptations) (Nedachi et al., 2008). Importantly, our results also showed that the post-translational modifications of proteins were observed as early as 2 hours post-treatment while there was no change at 24 hours following the electrical stimulation, meaning the acute effects of contraction on muscle cells under hypoxia (Krieger, 2010). In skeletal muscle, physical exercise or electrical stimulation activates mechanisms responsible for hypertrophy (Krieger, 2010; Vivodtzev et al., 2012). In addition, *in vitro* studies conducted in normoxia (20.9% O_2) with an electrical stimulation program similar to the one used in our study, showed the activation of several important proteins in the cell growth signaling cascade (mTOR, p70S6K, 4E-BP1, eEF2, ERK1/2, JNK) and increased myotubes size (Nakai et al., 2010; Tarum et al., 2017). Importantly, our data showed that the anabolic effects of electrical stimulation observed in normoxia could be reinvested to limit the myotube atrophy resulting from hypoxia, since we have demonstrated that 48 hours after electrical stimulation, hypoxic muscle cells showed an increased diameter and density of myotubes compared to untreated hypoxic myotubes.

The effect of oxygenation was previously shown on human embryonic kidney cells under hypoxia (Arsham et al., 2003, Tan & Hagen, 2013), but it is the first time to our knowledge that a positive effect was demonstrated on skeletal muscle cells. We have shown that these positive effects of oxygenation on the limitation of hypoxic myotubes atrophy may be related to the decrease of protein's degradative processes (FoxO3a and proteins ubiquitination), but also to the activation of protein synthesis. Indeed, we observed the phosphorylation of mTOR downstream targets. The phosphorylation of P70S6K and 4E-BP1 is known to induce the activation of ribosomal protein S6 and the translation initiation by eIF4E release respectively, which are responsible for the initiation of protein synthesis (Reyes de la Cruz et al., 2004). Importantly, we have demonstrated in the herein study that oxygenation was the only condition leading to the activation of the key protein synthesis initiators 4E-BP1 and P70S6K (**Fig. 8d**) compared to electrical stimulation or branched chain amino acids supplementation, usually used as countermeasures to limit muscle atrophy. The activation of 4E-BP1 and P70S6K proteins by the oxygenation period can be explained by inhibition of AMPK protein on the Akt-mTOR pathway. In hypoxia, AMPK is known to decrease the activation of mTOR through its TSC2 inhibitor (Schneider et al., 2008). Recent research has shown that AMPK protein activity was extremely sensitive to intracellular oxygen level (Sakushima et al., 2020). Reoxygenation used as a treatment for hypoxia was able to momentarily restore a basal level of AMPK. Other intracellular signaling pathways responsible for cell growth are also disrupted under hypoxia, like the Raf-MEK-mitogen–activated protein kinase (MAPK)–extracellular signal-regulated kinase (Erk) cascade (Ren et al., 2010). The p38 MAPK pathway was repressed by hypoxia, while Erk1/2 was more sustained activation under hypoxia

compared to normoxia (Ren et al., 2010). In hypoxia, it may be those treatments by electrical stimulation or reoxygenation would result in variations of MAPK/ERK pathway just as observed on Akt-mTOR pathway (Fig. 8b and 8d)

In addition, our results showed that hypoxic myotubes treated by oxygenation (20.9% O_2) during a short period of 2h, increased diameter, myogenic fusion index and higher myotubes density at 48 hours post-treatment in comparison to the untreated hypoxic cells. Several studies have demonstrated that a reduced oxygen level inhibited the proliferation of myoblasts and the myotube formation, so as the maintenance of the cells in an undifferentiated state compared with standard conditions (Launay et al., 2010; Wang et al., 2015). However, Di Carlo and collaborators observed that hypoxia-mediated inhibition of differentiation was reversible since hypoxia-cultured C2C12 (1% O_2) shifted toward normoxia conditions (20.9% O_2) which were able to differentiate properly for 48h, indicating that muscle cells preserve their ability to proliferate or differentiate by correctly expressing myogenic regulatory factors (Di Carlo et al., 2004). In our study, a return to normoxia for a short period of oxygenation (2 hours) was sufficient to reactivated key proteins of the ~~prosthesis~~ proteosynthesis such as 4E-BP1 or P70S6K, and this independent of transcriptional and translational machinery are essential for the synthesis of a new proteins (Tan & Hagen, 2013). Indeed, the morphological parameters of oxygen-treated cells compared to untreated hypoxic cells indicated the beneficial effects of oxygenation on the differentiation and maturation of myotubes.

We have also considered the BCAAs supplementation because of the anabolic action of BCAAs on muscle cells in normoxia, that lead~~s~~ to the activation of protein synthesis pathways through the activation of the mTOR regulatory complex (Jewell et al., 2013). However, we noted a decreased mTOR activation during BCAAs supplementation in hypoxia. In addition, all of our data showed that in hypoxic condition, the action of BCAAs remained very limited compared to the treatments by electrical stimulation or oxygenation. Since the effect of BCAAs on protein synthesis results from the interaction of mTOR with the activator Rheb at the lysosomal membrane (Jewell et al., 2013), we could hypothesize that the default of BCAAs supplementation effect during hypoxia should result from a defective interaction between mTOR and Rheb and/or a mislocalization of mTOR. In addition, despite several studies on amino acid supplementation in skeletal muscle, it is not known whether cellular uptake and action of amino acids are altered in the hypoxic muscle.

Adversely to BCAAs supplementation, electrical stimulation and oxygenation period increased the ratio p-FoxO3a / FoxO3a 24h post-treatment. FoxO3a had been directly implicated in regulation of target genes involved in protein degradation through autophagy and ubiquitin-proteasome system (Stitt et al., 2004). However, all treatments (ES, BCAAs, O_2) applied in our study didn't cause any beneficial effect on autophagy system regarding to its inhibition; indeed, the autophagy process remains activated in a similar manner than the untreated hypoxic myotubes, whatever the treatment applied to hypoxic myotubes. Overactivation of protein degradation systems (autophagy and ubiquitin) observed in our study associated with a decrease in myogenic markers of differentiation in myotubes such as Myogenin

and MHC type II expression highlighted in other works would explained the decrease in the diameter of the myotubes and protein content especially for the myotubes treated with 4% O_2 (Bensaid et al., 2019; Di carlo et al., 2004). Regarding the ubiquitin-proteasome system, electrical stimulation and oxygenation treatment decreased protein ubiquitination in comparison with untreated hypoxic muscle cells. Interestingly, we also demonstrated herein that a short period of oxygenation may also temporarily inhibits the deleterious effects of some protein degradation systems that are overactivated during hypoxic stress (Arsham et al., 2003).

In summary, our study demonstrated that hypoxia-induced muscle atrophy may be reversed by electrical stimulation or a short period of oxygenation, with a strong impact compared with BCAAs supplementation that had none effect on the limitation of hypoxia-induced muscle atrophy. The protein degradation systems considered in our study were similarly impacted by electrical stimulation and oxygenation of muscle cells subjected to a hypoxic environment, in particular the ubiquitin-proteasome system while the autophagy process was not impacted. In addition, we have demonstrated that the PI3K-Akt-mTOR protein synthesis pathway has been differentially stimulated by the two countermeasures to hypoxia-induced muscle atrophy, *i.e.,* electrical stimulation and oxygenation, while BCAAS didn't have any beneficial effect. Indeed, muscle contraction resulted in the activation of Akt, that is an early regulator of the pathway, while an oxygenation period induced overactivation of downstream targets of mTOR, in particular 4E-BP1 et P70S6K that are regulators at the end of the signalling pathway (**Fig. 8d**). Thus, the adaptation of the proteosynthesis pathway in muscle cells seems to be specific to the nature of the applied treatment. As consequence, electrical stimulation and oxygenation treatment maintained a significantly larger myotubes diameter, myogenic fusion index and density of the myotubes compared to untreated hypoxic muscle cells. In conclusion, we have demonstrated that electrical stimulation and oxygenation are effective countermeasures to limit the hypoxia-induced muscle atrophy, while the BCAAs supplementation was on the contrary ineffective. In addition, our findings highlight the molecular mechanisms and the therapeutic potential of electrical stimulation or oxygen therapy for the patients with hypoxia-induced atrophy muscle.

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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.

Author contributions

Conceived and designed the research: Bensaid S. and Cieniewski-Bernard C

Performed experiments: Bensaid S. and Cieniewski-Bernard C

Analysed data and interpreted results of experiments: Bensaid S, Fabre C, Pawlak-Chaouch M, Cieniewski-Bernard C

Edited and revised of the article: Bensaid S, Fabre C, Cieniewski-Bernard C

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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FIGURE LEGENDS

Fig. 1: Experimental protocol used to explore the effects of treatments on C2C12 myotubes atrophy induced by hypoxia. Myotubes were cultured for 4 days before exposure to 4% oxygen. After 24h of hypoxia (d5, 5-days differentiated myotubes), the C2C12 cells were treated with electrical stimulation (30 V at 1 Hz for 3 ms at 997-ms intervals for 1 hours), branched-chain amino acids supplementation (BCAAs at 5mM) or a short oxygenation period (20.9% O_2 for 2 hours), after which cells were analyzed at 2 hours (d5, 5-days differentiated myotubes), 24 hours (d6, 6-days differentiated myotubes) and 48 hours (d7, 7-days differentiated myotubes) post-treatment. d: day of differentiation of myotubes.

Fig. 2: Hypoxia-induced muscle atrophy was limited by electrical stimulation and oxygenation treatment. a: Representative images of C2C12 myotubes in normoxia (20.9% O_2), physiological hypoxia (4% O_2) or treated by electrical stimulation (Elec.stim), branched-chain amino acids (BCAAs) supplementation at a concentration of 5mM or oxygenation (2 hours, 20.9% O_2). Bright field images were taken at the same magnification (20×) and 48 hours (d7) post-treatment. Scale bar = 100 µm. **b**: Measurement of the diameter of cultured myotubes expressed in µm, 2 hours (d5), 24 hours (d6) and 48 hours (d7) post-treatment. **c**: Analysis of the fusion index of cultured myotubes, expressed in percentage. **d**: Analysis of the number of myotubes per field. **e**: Analysis the number of nuclei per myotubes. At least, 300 myotubes per condition were analysed. Post-hoc analyses for conditions effect significantly from normoxia untreated values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly from hypoxia untreated condition: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$. Significantly different from days-7 *vs* days-6 values: $ $P < 0.05$, $$ $P < 0.01$, $$$ $P < 0.001$ ($n = 6$ per condition).

Fig. 3: Electrical stimulation and oxygenation period maintained a higher protein content compared with untreated hypoxic C2C12 myotubes until 48h post-treatment. Total protein content was determined from normoxic C2C12 myotubes, physiological hypoxia untreated or treated-myotubes by electrical stimulation (Elec.stim), branched-chain amino acids (BCAAs) or oxygenation 2 hours (d5, 5-days differentiated myotubes), 24 hours (d6, 6-days differentiated myotubes) and 48 hours (d7, 7-days differentiated myotubes) post-treatment. Post-hoc analyses for conditions effect significantly from normoxia untreated values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly from hypoxia untreated condition: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: §§§ $P < 0.001$. Significantly different from days-7 *vs* days-6 values: $$ $P < 0.01$, $$$ $P < 0.001$ ($n = 10$ per condition).

Fig. 4: Electrical stimulation activated Akt/mTOR pathway of muscle cells under hypoxia. Activation of key proteins of Akt/mTOR pathway was determined by Western blot in C2C12 normoxic myotubes (n), physiological hypoxia untreated (h) or treated by electrical stimulation (Elec.stim, es), branched-chain amino acids (BCAAs, aa) or oxygenation (O_2) 2 hours (d5, 5-days differentiated myotubes) and 24 hours (d6, 6-days differentiated myotubes) post-treatment. **a'':** Quantification of the ratio of phosphorylated Akt (Ser473) (**a**) over total Akt (**a'**) (n = 10). **b'':** Ratio of phosphorylated mTOR (Ser2448) (**b**) over total mTOR (**b'**) (n = 10).**c'':** Ratio of phosphorylated GSK-3β (Ser9) (**c**) over total GSK-3β (**c'**) (n = 10). **d**: Representative signals of proteins of interest after 2 hours (d5, 5-days differentiated myotubes) or 24 hours (d6, 6-days differentiated myotubes) post-treatment. Post-hoc analyses for conditions effect significantly from normoxia untreated values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly from hypoxia untreated condition: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: § $P < 0.05$, §§§ $P < 0.001$.

Fig. 5: Treatment by oxygenation stimulated proteins downstream of mTOR. Activation level of 4E-BP1 and P70S6K, key regulators of protein synthesis, was determined by Western blot in C2C12 normoxic myotubes (n), physiological hypoxia untreated (h) or treated by electrical stimulation (Elec.stim, es), branched-chain amino acids (BCAAs, aa) or oxygenation (O_2) 2 hours (D5, 5-days differentiated myotubes), 24 hours (D6, 6-days differentiated myotubes) post-treatment. **a'':** Quantification of the ratio of phosphorylated 4E-BP1 (Ser51) (**a**) over total 4E-BP1 (**a'**) (n = 6). **b'':** Ratio of phosphorylated P70S6K (Thr389) (**b**) over total P70S6K (**b'**) (n = 10). **c**: Representative signals of proteins of interest after 2 hours (days-5) or 24 hours (days-6) post-treatment. Post-hoc analyses for conditions effect significantly from normoxia untreated values: * $P < 0.05$, *** $P < 0.001$. Significantly from hypoxia untreated condition: † $P < 0.05$, ††† $P < 0.001$, trends are indicated by the specific P value**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$.

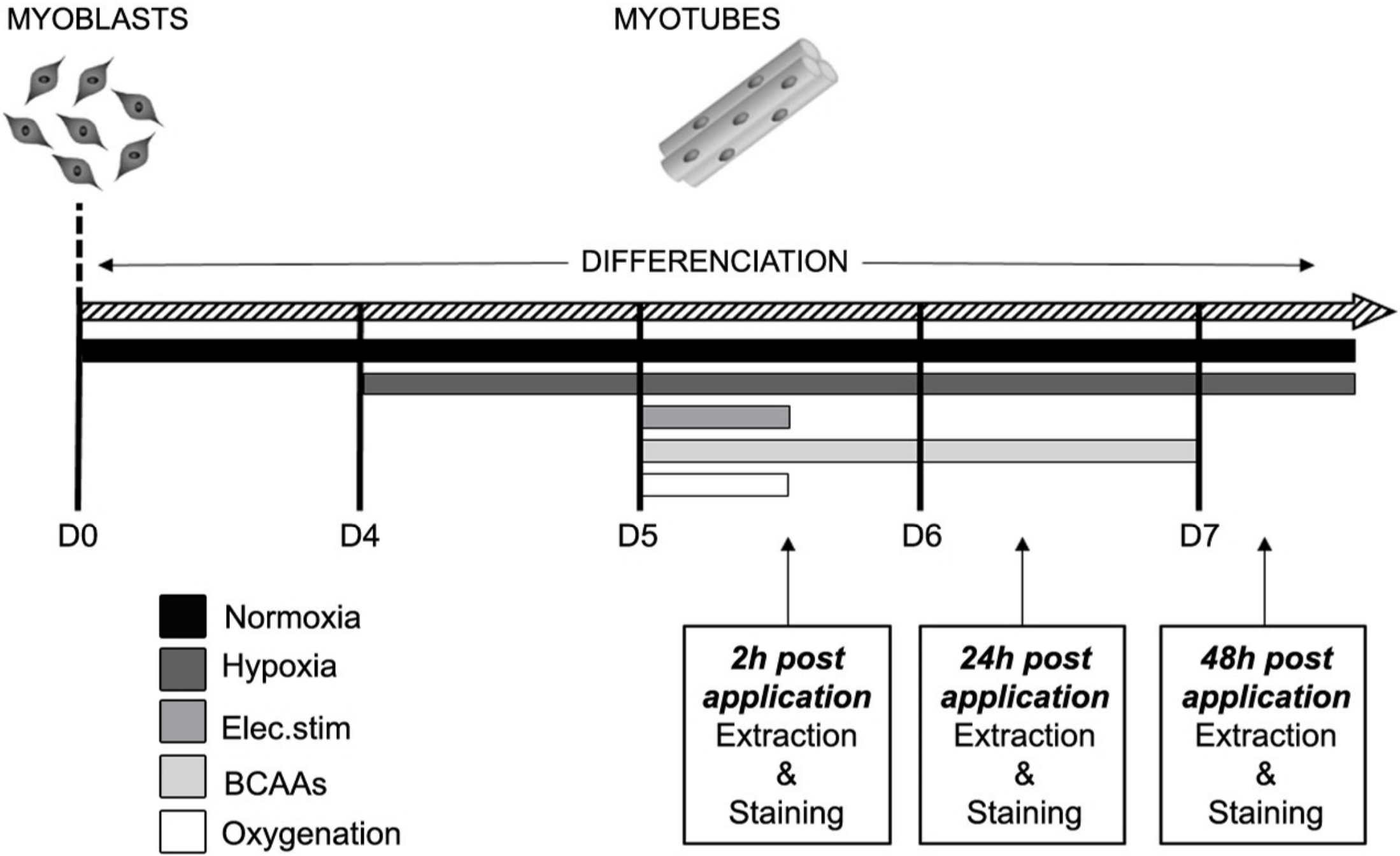
Fig. 6: Electrical stimulation and oxygenation treatment inhibited the protein degradation process through FoxO3A. FoxO1 and FoxO3a, key regulators of protein degradation, were analysed by Western blot in C2C12 normoxic myotubes (n), physiological hypoxia untreated (h) or treated by electrical stimulation (Elec.stim, es), branched-chain amino acids (BCAAs, aa) or oxygenation (O_2) 2 hours (d5, 5-days differentiated myotubes), 24 hours (d6, 6-days differentiated myotubes) post-treatment. **a'':** Quantification of the ratio of phosphorylated FoxO1 (Ser256) (**a**) over total FoxO1 (**a'**) (n = 6). **b'':** Ratio of phosphorylated FoxO3a (Ser253) (**b**) over total FoxO3a (**b'**) (n = 10). **c**: Representative signals of proteins of interest after 2 hours (d5, 5-days differentiated myotubes) or 24 hours (d6, 6-days differentiated myotubes) post-treatment. Post-hoc analyses for conditions effect significantly from normoxia untreated values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, trends are

indicated by the specific *P* value. Significantly from hypoxia untreated condition: † $P < 0.05$, †† $P < 0.01$**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: §§ $P < 0.01$.

Fig. 7: Hypoxia led to an increase in proteolysis systems. Expression level of markers of autophagy and ubiquitin-proteasome systems were determined by Western blot in C2C12 normoxic myotubes (n), physiological hypoxia untreated (h) or treated by electrical stimulation (Elec.stim, es), branched-chain amino acids (BCAAs, aa) or oxygenation (O_2) 2 hours (d5, 5-days differentiated myotubes), 24 hours (d6, 6-days differentiated myotubes) post-treatment. **a**: LC3 II / LC3 I ratio at the protein level ($n = 10$). **a'**: Representative signals of proteins LC3 I and LC3II. **b**: Quantification of ubiquitin-conjugated proteins ($n = 10$). **b'**: 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 untreated values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly from hypoxia untreated condition: † $P < 0.05$, †† $P < 0.01$**.** Post-hoc analyses for times effect significantly different from days-6 *vs* days-5 values: § $P < 0.05$, §§§ $P < 0.001$.

Fig. 8: Recapitulative scheme of the effects of hypoxia (a), electrical stimulation (b), BCAAs supplementation (c) or oxygenation period (d) on signalling pathways regulating protein homeostasis in C2C12 under hypoxia. Large line means "increased", normal continuous line means "basal" and discontinuous line means "decreased".

Figure 1


MYOBLASTS
MYOTUBES
DIFFERENCIATION
D0
D4
D5
D6
D7
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation
2h post application Extraction & Staining
24h post application Extraction & Staining
48h post application Extraction & Staining

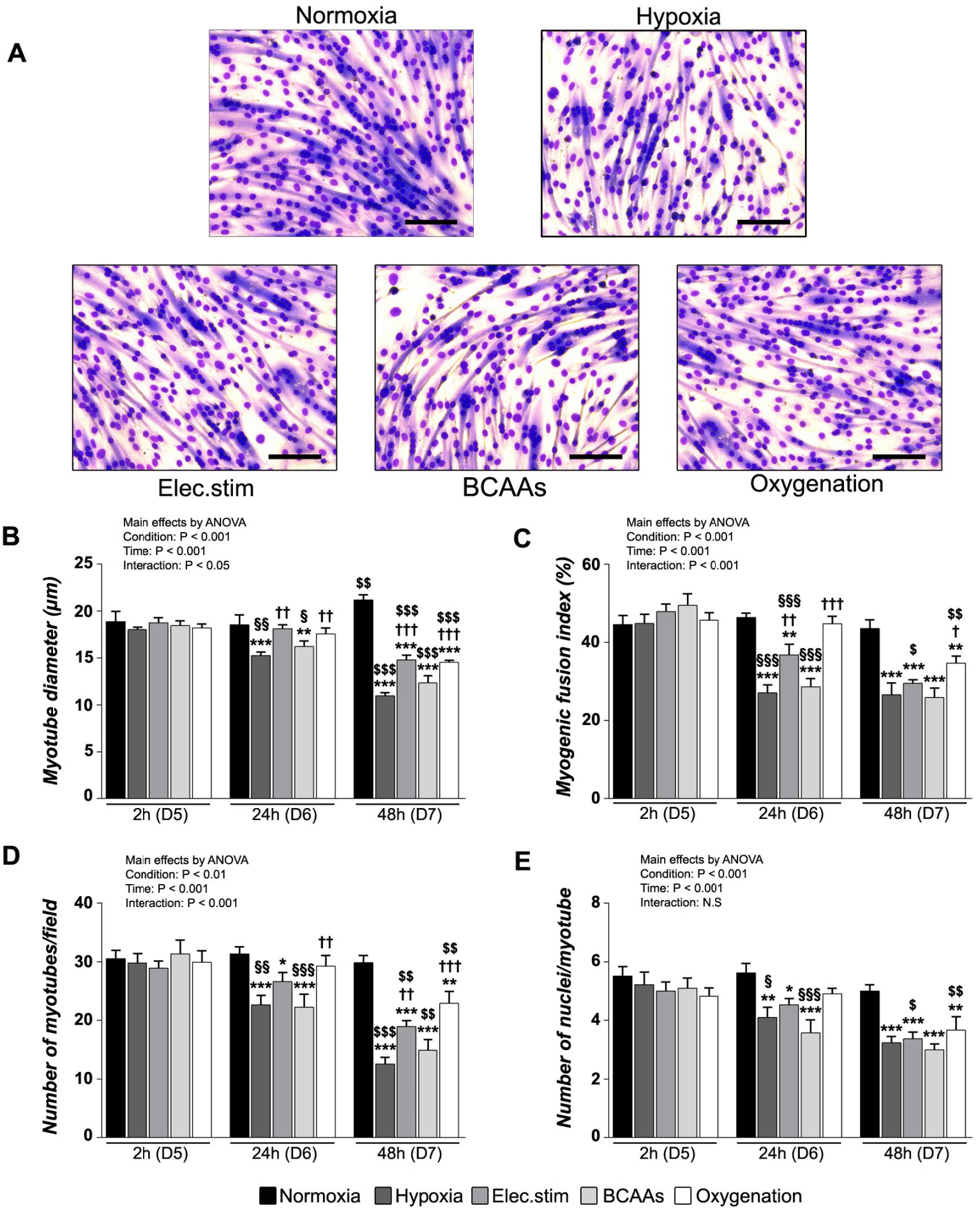
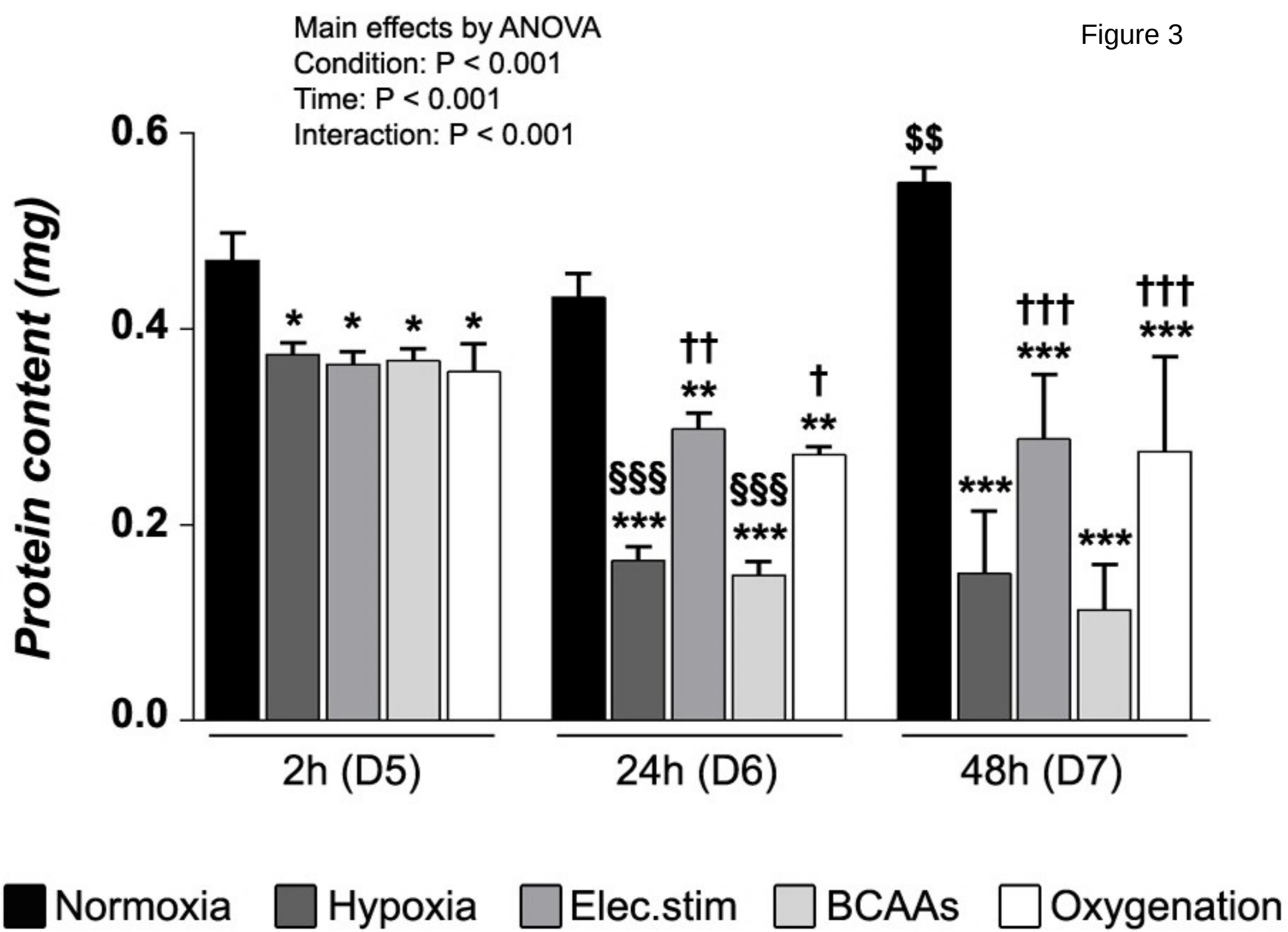

A
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation
B
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: P < 0.05
Myotube diameter (µm)
2h (D5)
24h (D6)
48h (D7)
C
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
Myogenic fusion index (%)
D
Main effects by ANOVA
Condition: P < 0.01
Time: P < 0.001
Interaction: P < 0.001
Number of myotubes/field
E
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: N.S
Number of nuclei/myotube
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

Figure 2

Figure 3


Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
Protein content (mg)
0.6
0.4
0.2
0.0
*
*
*
*
§§§
***
††
**
§§§
***
†
**
$$
***
†††
***
***
†††
***
2h (D5)
24h (D6)
48h (D7)
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

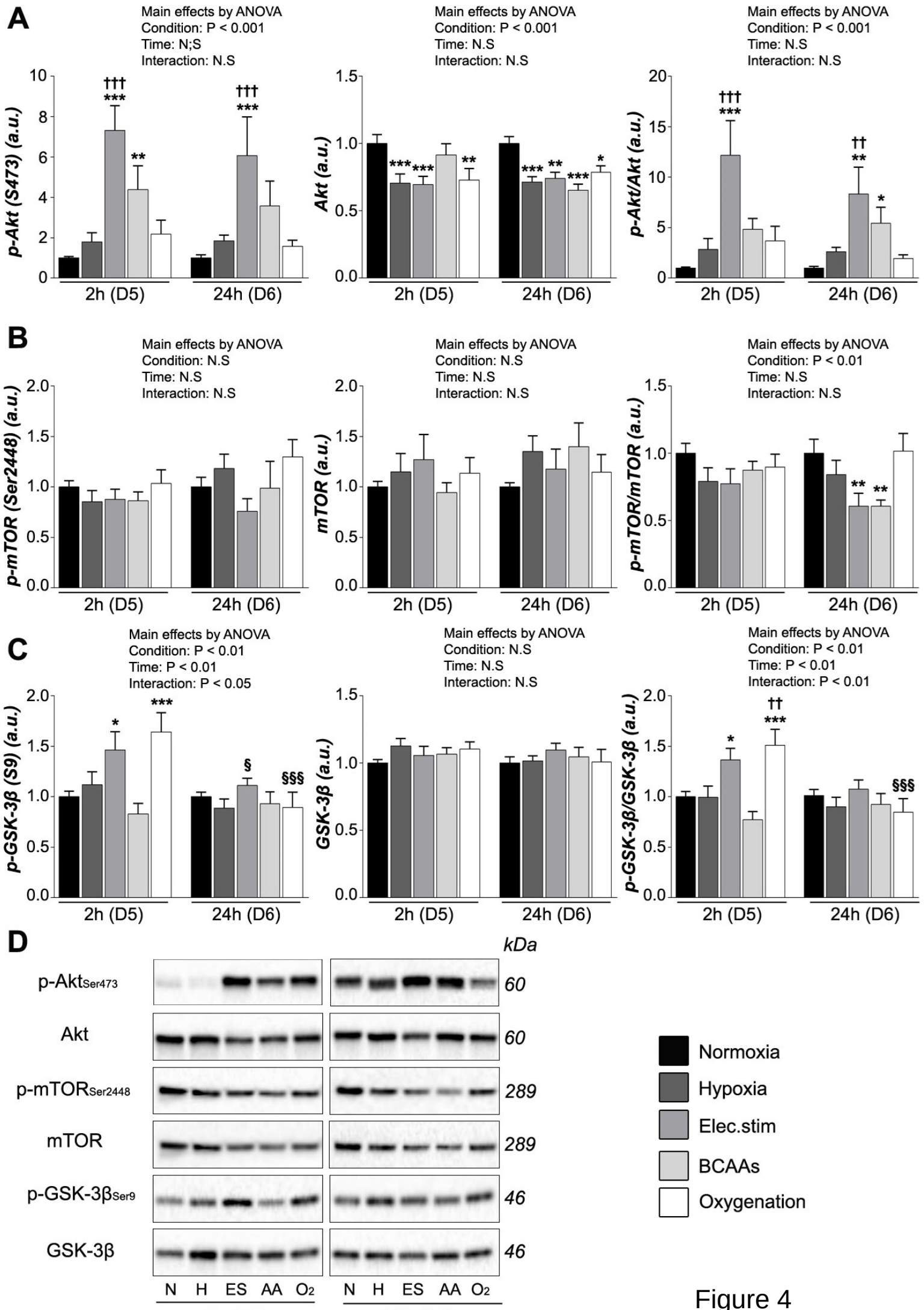
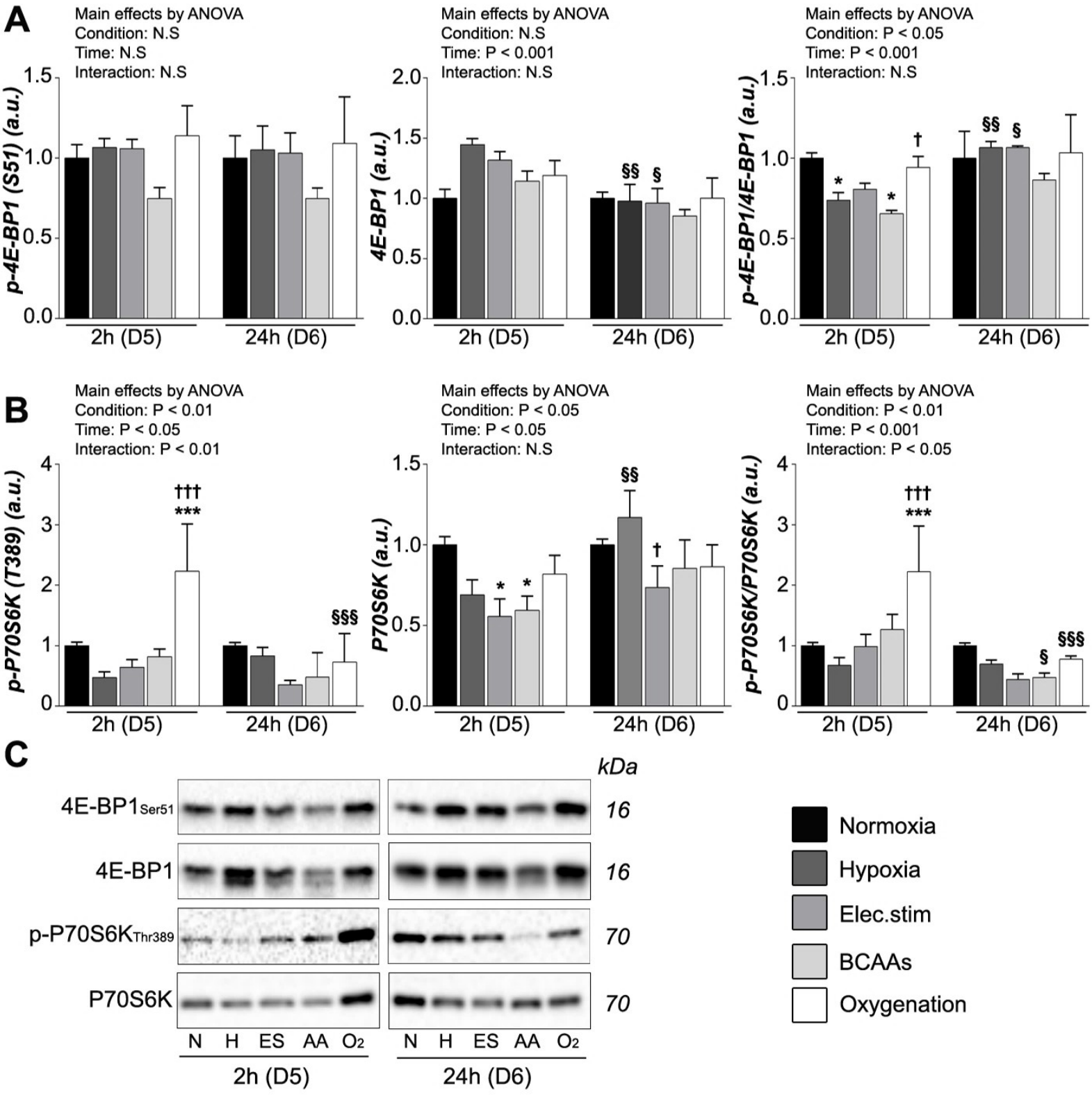

A
Main effects by ANOVA
Condition: P < 0.001
Time: N;S
Interaction: N.S
p-Akt (S473) (a.u.)
Main effects by ANOVA
Condition: P < 0.001
Time: N.S
Interaction: N.S
Akt (a.u.)
Main effects by ANOVA
Condition: P < 0.001
Time: N.S
Interaction: N.S
p-Akt/Akt (a.u.)
2h (D5)
24h (D6)
B
Main effects by ANOVA
Condition: N.S
Time: N.S
Interaction: N.S
p-mTOR (Ser2448) (a.u.)
Main effects by ANOVA
Condition: N.S
Time: N.S
Interaction: N.S
mTOR (a.u.)
Main effects by ANOVA
Condition: P < 0.01
Time: N.S
Interaction: N.S
p-mTOR/mTOR (a.u.)
C
Main effects by ANOVA
Condition: P < 0.01
Time: P < 0.01
Interaction: P < 0.05
p-GSK-3β (S9) (a.u.)
Main effects by ANOVA
Condition: N.S
Time: N.S
Interaction: N.S
GSK-3β (a.u.)
Main effects by ANOVA
Condition: P < 0.01
Time: P < 0.01
Interaction: P < 0.01
p-GSK-3β/GSK-3β (a.u.)
D
kDa
p-Akt Ser473
60
Akt
60
p-mTOR Ser2448
289
mTOR
289
p-GSK-3β Ser9
46
GSK-3β
46
N H ES AA O2
N H ES AA O2
2h (D5)
24h (D6)
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

Figure 4


A
Main effects by ANOVA
Condition: N.S
Time: N.S
Interaction: N.S
p-4E-BP1 (S51) (a.u.)
Main effects by ANOVA
Condition: N.S
Time: P < 0.001
Interaction: N.S
4E-BP1 (a.u.)
§§
§
Main effects by ANOVA
Condition: P < 0.05
Time: P < 0.001
Interaction: N.S
p-4E-BP1/4E-BP1 (a.u.)
*
*
†
§§
§
2h (D5)
24h (D6)
B
Main effects by ANOVA
Condition: P < 0.01
Time: P < 0.05
Interaction: P < 0.01
p-P70S6K (T389) (a.u.)
†††
***
§§§
Main effects by ANOVA
Condition: P < 0.05
Time: P < 0.05
Interaction: N.S
P70S6K (a.u.)
§§
*
*
†
Main effects by ANOVA
Condition: P < 0.01
Time: P < 0.001
Interaction: P < 0.05
p-P70S6K/P70S6K (a.u.)
†††
***
§
§§§
2h (D5)
24h (D6)
C
kDa
4E-BP1Ser51
4E-BP1
p-P70S6KThr389
P70S6K
16
16
70
70
N H ES AA O2
N H ES AA O2
2h (D5)
24h (D6)
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

Fig

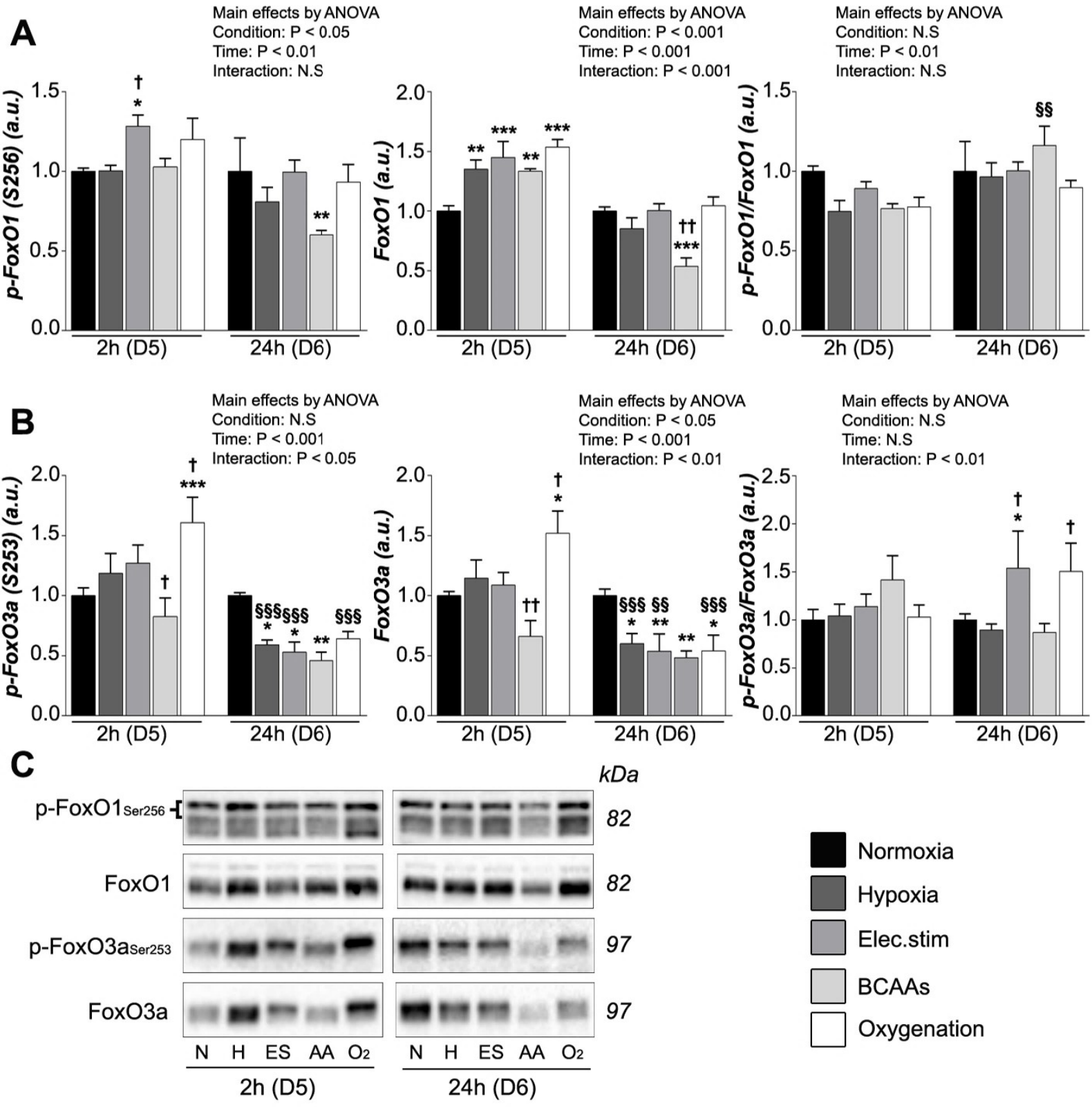

A
Main effects by ANOVA
Condition: P < 0.05
Time: P < 0.01
Interaction: N.S
p-FoxO1 (S256) (a.u.)
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
FoxO1 (a.u.)
Main effects by ANOVA
Condition: N.S
Time: P < 0.01
Interaction: N.S
p-FoxO1/FoxO1 (a.u.)
2h (D5)
24h (D6)
B
Main effects by ANOVA
Condition: N.S
Time: P < 0.001
Interaction: P < 0.05
p-FoxO3a (S253) (a.u.)
Main effects by ANOVA
Condition: P < 0.05
Time: P < 0.001
Interaction: P < 0.01
FoxO3a (a.u.)
Main effects by ANOVA
Condition: N.S
Time: N.S
Interaction: P < 0.01
p-FoxO3a/FoxO3a (a.u.)
C
kDa
p-FoxO1Ser256
FoxO1
p-FoxO3aSer253
FoxO3a
82
82
97
97
N H ES AA O2
N H ES AA O2
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

Figure 6

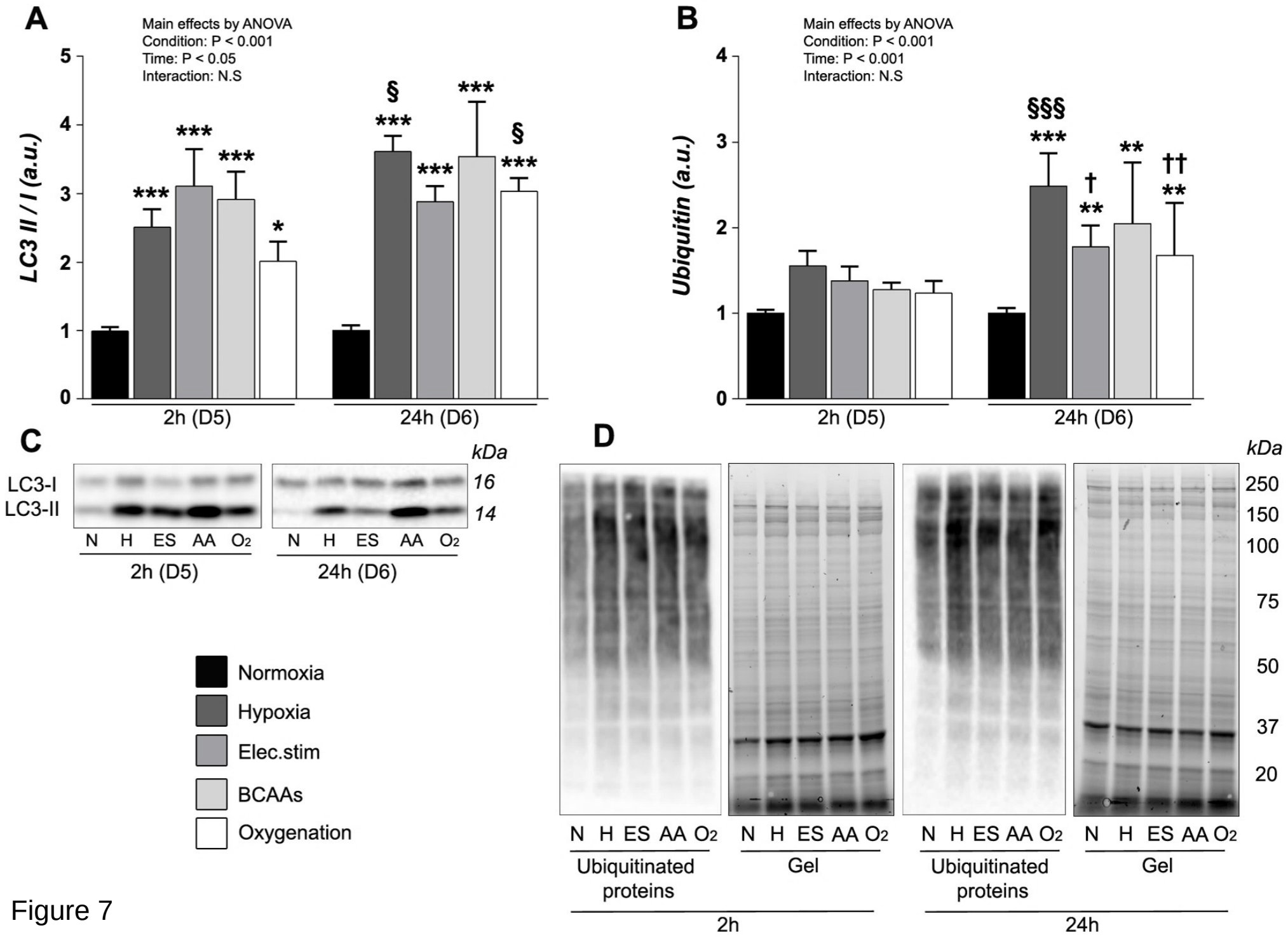
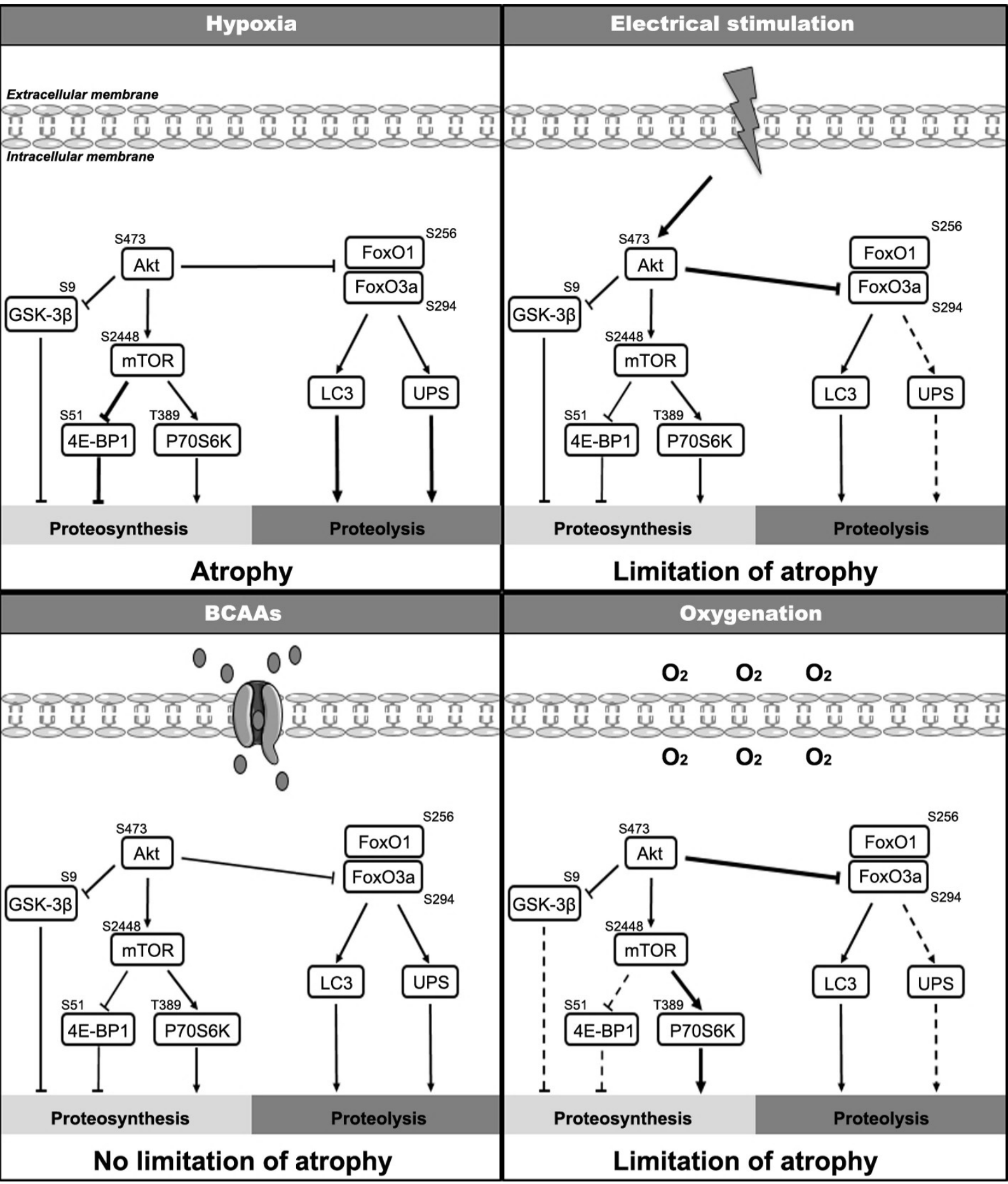

A
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.05
Interaction: N.S
LC3 II / I (a.u.)
2h (D5)
24h (D6)
B
Main effects by ANOVA
Condition: P < 0.001
Time: P < 0.001
Interaction: N.S
Ubiquitin (a.u.)
2h (D5)
24h (D6)
C
kDa
LC3-I
LC3-II
16
14
N H ES AA O2
2h (D5)
24h (D6)
D
kDa
250
150
100
75
50
37
20
N H ES AA O2
Ubiquitinated proteins
Gel
2h
24h
Normoxia
Hypoxia
Elec.stim
BCAAs
Oxygenation

Figure 7


Hypoxia
Extracellular membrane
Intracellular membrane
S473
Akt
S9
GSK-3β
S2448
mTOR
S51
4E-BP1
T389
P70S6K
S256
FoxO1
FoxO3a
S294
LC3
UPS
Proteosynthesis
Proteolysis
Atrophy
Electrical stimulation
S473
Akt
S9
GSK-3β
S2448
mTOR
S51
4E-BP1
T389
P70S6K
S256
FoxO1
FoxO3a
S294
LC3
UPS
Proteosynthesis
Proteolysis
Limitation of atrophy
BCAAs
S473
Akt
S9
GSK-3β
S2448
mTOR
S51
4E-BP1
T389
P70S6K
S256
FoxO1
FoxO3a
S294
LC3
UPS
Proteosynthesis
Proteolysis
No limitation of atrophy
Oxygenation
O2 O2 O2
O2 O2 O2
S473
Akt
S9
GSK-3β
S2448
mTOR
S51
4E-BP1
T389
P70S6K
S256
FoxO1
FoxO3a
S294
LC3
UPS
Proteosynthesis
Proteolysis
Limitation of atrophy