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Acute normobaric hypoxia blunts contraction-mediated mTORC1- and JNK-signaling in human skeletal muscle

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Abstract
Aim: Hypoxia has been shown to reduce resistance exercise-induced stimulation of protein synthesis and long-term gains in muscle mass. However, the mechanism whereby hypoxia exerts its effect is not clear. Here, we examine the effect of acute hypoxia on the activity of several signalling pathways involved in the regulation of muscle growth following a bout of resistance exercise.

Methods: Eight men performed two sessions of leg resistance exercise in normoxia or hypoxia (12% O2) in a randomized crossover fashion. Muscle biopsies were obtained at rest and 0, 90, 180 minutes after exercise. Muscle analyses included levels of signalling proteins and metabolites associated with energy turnover.

Results: Exercise during normoxia induced a 5-10-fold increase of S6K1Thr389 phosphorylation throughout the recovery period, but hypoxia blunted the increases by ~50%. Phosphorylation of JNKThr183/Tyr185 and the JNK target SMAD2Ser245/250/255 was increased by 30- to 40-fold immediately after the exercise in normoxia, but hypoxia blocked almost 70% of the activation. Throughout recovery, phosphorylation of JNK and SMAD2 remained elevated following the exercise in normoxia, but the effect of hypoxia was lost at 90-180 minutes post-exercise. Hypoxia had no effect on exercise-induced Hippo or autophagy signaling and ubiquitin-proteasome related protein levels. Nor did hypoxia alter the changes induced by exercise in high-energy phosphates, glucose 6-P, lactate or phosphorylation of AMPK or ACC.

Conclusion: We conclude that acute severe hypoxia inhibits resistance exercise-induced mTORC1- and JNK signalling in human skeletal muscle, effects that do not appear to be mediated by changes in the degree of metabolic stress in the muscle.

Keywords
deuterium oxide, FSR, Hippo pathway, muscle metabolites, oxygen
1 | INTRODUCTION

Skeletal muscle mass is vital for locomotor capacity and metabolic health, being a critical factor for both athletic performance and healthy ageing.1 Ageing, physical inactivity, catabolic diseases or injuries negatively impact muscle mass; however, the losses can be diminished or abolished by physical training.2,3 Resistance exercise training is one of the key stimulators of skeletal muscle growth, which is primarily driven by acute increases in the rate of muscle protein synthesis after each exercise bout.4

In addition to contractile activity, muscle mass has also been shown to be sensitive to oxygen levels, where reduced arterial oxygen delivery and tissue oxygenation, i.e., hypoxia, has detrimental effects on muscle mass.5-7 This notion is, eg, also, indirectly, supported by the fact that individuals with anaemia exhibit low muscle mass.8 Moreover, Narici and Keyser9 showed that resistance exercise training for 4 weeks performed at 5050 m resulted in significantly lower muscle hypertrophy compared to training performed at sea level. The high-altitude data might, however, be confounded by the fact that physical activity and food intake normally is reduced in those settings. The observation of an inhibited anabolic response at high altitude was nevertheless in accordance with subsequent results where resistance exercise performed during <1 to 3.5 hours of normobaric hypoxia (FiO2 12%-13.5%) resulted in lower post-exercise rates of muscle protein synthesis compared to normoxia.10,11 In contrast, some studies indicate that acute and non-severe normobaric hypoxia may augment exercise-induced hypertrophy, potentially through increased metabolic stress.12,13 Most published data, however, suggest that long-term and short-term hypoxia reduces muscle mass and its potential to respond to growth stimulus. Regardless, at present, the underlying molecular mechanisms responsible for the observed effects of hypoxia in human skeletal muscle remain unknown.

At the cellular level, it is known that the resistance exercise-induced increases in muscle protein synthesis and skeletal muscle mass are to a large extent dependent on the activation of the mechanistic target of rapamycin complex 1 (mTORC1).14 Since the initial illustration of the pivotal role of mTOR in the control of muscle growth,15 additional molecular pathways have been highlighted as potentially important. The c-Jun N-terminal kinase (JNK) can phosphorylate the transcription factor SMAD2 which then acts in a myostatin inhibitory manner. Lessard et al16 recently showed that muscle-specific JNK knockout mice do not hypertrophy in response to functional overload, and that resistance exercise activated JNK-signalling in human skeletal muscle. Moreover, the Hippo signalling pathway, which includes the proteins, Yes-associated protein (Yap) and transcriptional co-activator with PDZ-binding motif (Taz), has been shown to respond to both mechanical and metabolic stress.17 The Hippo pathway has been illustrated to play a pivotal role in the control of muscle mass during the development, mechanical overload and atrophy in mouse skeletal muscle.18-20

Protein synthesis is considered the critical determinant of muscle hypertrophy following the resistance exercise, but muscle mass is balanced by the rate of protein breakdown which mainly depends on autophagy and the ubiquitin-proteasome system. Current knowledge about the impact of hypoxia on the regulation of proteolysis is limited and somewhat inconclusive. Whereas 14 d of hypobaric hypoxia in rats increased skeletal muscle proteasome activity,21-23 21 d of hypobaric hypoxia did not.22 In contrast, normobaric hypoxia in mice was found to blunt fasting-induced autophagy signalling and reduce MAFbx (FBOX32) and MuRF-1 (TRIM63) gene expression.23 In resting human skeletal muscle, normobaric hypoxia has been shown to stimulate autophagy signalling and alter MAFbx and MuRF-1 gene expression without influencing proteasome activity.24,25

Acute normobaric hypoxia reduces resistance exercise stimulated human muscle protein synthesis10,11 but the mechanism whereby hypoxia exerts its effect is unclear. Accordingly, we investigated the underlying molecular events regulating protein synthesis and breakdown in relation to resistance exercise performed under hypoxia. Specifically, mTORC1-, JNK-., Hippo and autophagy signalling, as well as key proteins levels in the ubiquitin-proteasome pathway, were analysed in a time-dependent manner after resistance exercise performed under normobaric hypoxia, in human skeletal muscle. Considering the negative impact hypoxia has on muscle protein turnover, we hypothesized that mTORC1-signalling would be reduced, autophagy induced and proteins in the ubiquitin-proteasome pathway elevated by hypoxia compared to normoxia.

2 | RESULTS

2.1 | Trial performance, oxygen saturation and pulse rate

Exercise performance (load and repetitions) and time under tension (time per set performed with an active eccentric phase and no rest between repetitions) were virtually identical under both conditions (Table 1). Peripheral capillary oxygen saturation (SpO2) remained at 99%-100% in normoxia but decreased progressively during hypoxia, reaching 77% (range 73%-84%) by
the end of the exercise bout (Figure 1A, \( P < .001 \) for time \( \times \) trial). Pulse rate was on average 14% higher during hypoxia compared to normoxia, with a peak of 112 ± 5 and 103 ± 6 bpm, respectively (Figure 1B, \( P < .05 \) for time and trial).

### 2.2 Blood lactate and glucose

Following exercise, blood glucose levels increased by 7% and 13% in the normoxia- and hypoxia-trial, respectively (Figure 1C, \( P < .05 \) for time). Glucose levels, however, did not differ significantly between trials and had returned to baseline levels after 30 minutes of recovery. Blood levels of lactate changed similarly in both trials (Figure 1D), with a peak of approximately 5.5 mM noted immediately post-exercise.

### 2.3 Muscle metabolites

Muscle levels of lactate increased from 8.6 ± 1.5 at rest to 31.8 ± 6.6 mmol × kg\(^{-1}\) dw after exercise in the normoxia-trial, and 8.1 ± 1.4 at rest to 39.0 ± 5.6 mmol × kg\(^{-1}\) dw after exercise in the hypoxia-trial (Figure 1E, \( P < .05 \) for time). Muscle levels of ATP did not change after the exercise bout in any of the two trials (Figure 1F). Muscle levels of PCr were decreased by 10%-15% after exercise (Figure 1G, \( P < .05 \) for time), with corresponding changes in Cr (Figure 1H, \( P < .05 \) for time), resulting in a 24%-26% decreased PCr/Cr ratio (Figure 1I, \( P < .05 \) for time), with no differences between trials for any of these measures. There was a small, but non statistically significant, increase in muscle levels of Pi in both trials (Figure 1J). Muscle levels of malate (a marker of oxidative metabolism\(^{26}\)) increased from 0.7 µmol × kg\(^{-1}\) dw at rest to 1.8 µmol × kg\(^{-1}\) dw immediately after exercise in both trials (Figure 1K, \( P < .05 \) for time). Glucose-6-P increased approximately 2-fold after exercise in both trials (Figure 1L, \( P < .05 \) for time).

### 2.4 Protein signalling

#### 2.4.1 HIF-1α

The results from the in-depth analysis of HIF-1α protein expression in the hypoxia-trial are shown in Figure 2A. The antibody from Novus Biologicals was able to detect HIF-1α in all the fractions as well as in the IP:HIF-1α, whereas the antibody from Cell Signaling Technology (CST) was only able to detect IP:HIF-1α. Using mixed muscle from all subjects, the analysis showed that HIF-1α levels do not change from pre (resting conditions) to post (immediately after exercise in hypoxia) in any of the fractions. Both antibodies were subsequently also used for blotting of the entire set of muscle biopsy samples (prolonged exposure was required to detect bands with the CST Ab). Muscle levels of HIF-1α protein did not change at any time point following exercise in either of the two trials (Figure 2B,C).

#### 2.4.2 mTORC1-signalling

Resistance exercise-induced robust increases in S6K1\(^{\text{Thr389}}\) phosphorylation during normoxia, ranging from 8- to 14-fold throughout the 3-hour recovery period (Figure 3A). In the hypoxia trial, the increases were attenuated by ~50% at all time points (\( P < .05 \) vs normoxia). In normoxia, the phosphorylation of mTOR\(^{\text{Ser2448}}\) increased ~100% above the level at rest at all time points during recovery (\( P < .05 \), Figure 3B). With hypoxia, the corresponding increase above rest was attenuated by ~40% during early recovery, but the lower response compared to the normoxia-trial did not reach statistical significance (\( P = .09 \)). The phosphorylation of 4E-BP1\(^{\text{Thr37/46}}\) and eEF2\(^{\text{Thr56}}\) responded to the resistance exercise in an expected manner,\(^{27,28}\) without being influenced by hypoxia (Figure 3C,D). The phosphorylation of PRAS40\(^{\text{Thr246}}\) was not influenced by hypoxia, but was reduced to approximately 45% of its initial value after exercise, which was subsequently reversed to a significant increase above baseline at 90 minutes of recovery.

| Repetitions (n) | Set 1 | Set 2 | Set 3 | Set 4 | Set 5 | Set 6 |
|----------------|-------|-------|-------|-------|-------|-------|
| Normoxia       | 9.0 ± 0.4 | 8.7 ± 0.3 | 8.9 ± 0.2 | 8.9 ± 0.1 | 8.6 ± 0.2 | 8.8 ± 0.2 |
| Hypoxia        | 9.0 ± 0.4 | 8.7 ± 0.3 | 8.9 ± 0.2 | 8.9 ± 0.1 | 8.6 ± 0.2 | 8.8 ± 0.2 |
| Load (kg)      |       |       |       |       |       |       |
| Normoxia       | 81 ± 4 | 79 ± 4 | 76 ± 4 | 74 ± 4 | 71 ± 4 | 69 ± 4 |
| Hypoxia        | 81 ± 4 | 79 ± 4 | 76 ± 4 | 74 ± 4 | 71 ± 4 | 69 ± 4 |
| TUT (sec)      |       |       |       |       |       |       |
| Normoxia       | 23 ± 1 | 23 ± 1 | 23 ± 2 | 24 ± 1 | 22 ± 2 | 22 ± 1 |
| Hypoxia        | 23 ± 1 | 22 ± 1 | 23 ± 2 | 22 ± 1 | 22 ± 2 | 21 ± 2 |

**TABLE 1** Exercise performance
Protein levels of REDD1 decreased approximately 10% during early and late recovery in both trials (P < .05, Figure 3F).

2.4.3 | JNK- and Hippo signalling

In the normoxia trial, the phosphorylation of JNK\textsuperscript{Thr183/Tyr185} increased ~75-fold above the level at rest immediately after exercise (P < .05), but hypoxia blocked the increase by almost 70% (P < .05 vs normoxia, Figure 4A). At 90- and 180-minute post-exercise, JNK\textsuperscript{Thr183/Tyr185} phosphorylation returned to baseline values in both trials. A similar inhibitory effect of hypoxia was noted for SMAD2\textsuperscript{Ser245/250/255} phosphorylation, which increased ~30-fold directly after exercise with normoxia but only 10-fold with hypoxia (P < .05 vs normoxia, Figure 4B). SMAD2\textsuperscript{Ser245/250/255} phosphorylation during
normoxia remained elevated throughout the 3 hours recovery period, but the inhibitory effect of hypoxia was lost during this time. At 90 and 180 minutes of recovery, the level of SMAD2Ser245/250/255 was ~30-fold higher than at baseline in both trials. The phosphorylation of p38Thr180/Tyr182 increased fourfold immediately after exercise in both trials (P < .05) and returned to basal levels at 90 minutes post-exercise, with no differences between trials at any time point (Figure 4C). The phosphorylation of YAPSer127 was not altered after exercise in either of the two trials (Figure 4D). Resistance exercise had a more robust effect on TAZSer89 phosphorylation, which was reduced to 35%-50% of the level at rest in both trials immediately after exercise (P < .05, Figure 4E). The phosphorylation of TAZSer89 remained suppressed below baseline, with no differences between trials, up until 90 minutes post-exercise.

2.4.4 | AMPK- and autophagy-signalling

The phosphorylation of AMPKThr172 increased by 44%-75% immediately after exercise (P < .05, Figure 5A), with no differences between normoxia and hypoxia. Following 90 and 180 minutes of recovery, phosphorylated AMPKThr172 returned to baseline levels in both trials. The same pattern of change, but with greater magnitude, was noted for the phosphorylation of ACCSer79 (Figure 5B) and ULKSer317 (Figure 5C). The phosphorylation of TSC2Ser1387 increased...
by 14%-22% immediately after exercise in both trials (P < .05) and returned to basal levels at 90 minutes post-exercise, with no differences between trials at any time point (data not shown). In accordance with the stimulation of autophagy signalling, the ratio of LC3b-II/I decreased during the recovery in both trials (P < .05, Figure 5D), an effect that was largely driven by a reduced LC3b-II content. The LC3b-II/I ratio was reduced by ~45% immediately after exercise, and then remained significantly reduced throughout 180 minutes of recovery, with no influence of hypoxia at any time point. Protein levels of GABARAP did not change at any time point (Figure 5E), whereas protein levels of BNIP3 were increased by approximately 40% at all time points of the short-term recovery (P < .05 for time), with no influence of hypoxia at any time point (Figure 5F).

2.4.5 Regulation of proteasomal breakdown
Total protein levels of MuRF-1 increased by approximately 25% in both trials after 180 minutes of recovery (P < .05, Figure 6A), with no differences between normoxia and hypoxia.
hypoxia, and returned to basal levels again after 24 hours of recovery. Protein levels of MAFbx were also increased after 180 minutes of recovery, but then reversed to a 15% reduction below baseline after 24 hours ($P < .05$, Figure 6B), with no differences between trials. Total protein levels of UBR5 were unaltered throughout 180 minutes of recovery but were reduced to approximately 75% of initial levels in both trials 24 hours after exercise ($P < .05$, Figure 6C). Representative blots for all proteins are shown in Figure 7.

2.5 | Muscle protein synthesis

We also assessed whether the short-lived effects of acute hypoxia on signalling altered muscle protein synthesis...
over 24 hours. Intracellular enrichment of \(^{2}H\)-alanine (pre-cursor pool) was similar at baseline during both trials and decreased by about 10% after 24 hours recovery during both conditions (Figure 8A, \(P < .05\)). FSR over 24 hours of exercise and recovery were similar between treatments and amounted to 0.065 \(\pm\) 0.008% \(\times\) h\(^{-1}\) in the normoxia trial and 0.078 \(\pm\) 0.012% \(\times\) h\(^{-1}\) in the hypoxia trial (Figure 8B).

### Discussion

In the present study, we investigated the impact of hypoxia during resistance exercise on molecular processes controlling muscle protein turnover in human skeletal muscle. We show, for the first time, that hypoxia inhibits resistance exercise-induced mTORC1-signalling as well as JNK-SMAD signalling in a reversible manner in
human skeletal muscle. However, hypoxia did not affect exercise-induced Hippo signalling, AMPK-and autophagy-signalling, the levels of proteins involved in ubiquitin-proteasome proteolysis or FSR over 24 hours of exercise and recovery. Finally, hypoxia did not affect exercise-induced changes in other key muscle metabolites associated with energy status, demonstrating that the effects on signalling are not evoked by an increase in muscle metabolic stress.

We found a robust and sustained inhibition of S6K\textsuperscript{Thr389} phosphorylation with hypoxia with a tendency for a similar response on mTOR\textsuperscript{Ser2448} phosphorylation, where 71% of data points during recovery were lower with hypoxia. However, there was no influence of hypoxia on 4E-BP1, a finding that is not surprising considering that S6K1 is more sensitive to changes in mTORC1-activity following acute exercise in human skeletal muscle,\textsuperscript{27,29–31} an observation potentially explained by differences in rapamycin sensitivity and raptor binding capacity between the two mTORC1 substrates.\textsuperscript{32–34} The inhibitory effects of hypoxia on mTORC1-signalling are well documented in cell culture studies, but to our knowledge, have not been described in human muscle obtained in vivo. Arsham et al\textsuperscript{35} first showed that hypoxia rapidly (<30 minutes) inhibits insulin and amino acid-stimulated mTORC1-signalling in the HEK293 cell line. Their data suggested that this blunting of mTORC1-signalling by hypoxia does not involve the potential inhibitory action of AMPK,\textsuperscript{36} which has been both confirmed\textsuperscript{37} and opposed\textsuperscript{38} by other cell culture studies. While the in vitro data can be criticized for low physiological relevance, they still provide valuable mechanistic insight considering the paucity of human studies. Our data on muscle taken in vivo now demonstrate an AMPK-independent mTORC1 inhibition induced by hypoxia following resistance exercise. These findings are consistent with previous data showing that exercise-induced AMPK-activation does not inhibit mTORC1 signalling in human skeletal muscle.\textsuperscript{30}

Earlier cell culture studies suggested that hypoxia-induced mTORC1 inhibition involves the protein REDD1\textsuperscript{39} and the TSC1/TSC2 complex.\textsuperscript{30} Hypoxia stimulates REDD1 protein expression through HIF1\textalpha-mediated transcription,\textsuperscript{39} where elevated REDD1 then leads to 14-3-3 sequestering from TSC2 which promotes

![FIGURE 6](image-url)
TSC2/TSC1 interaction that ultimately leads to down-regulation of mTORC1 activity.\textsuperscript{37,41} The involvement of REDD1 for hypoxia-induced mTORC1 inhibition has also been illustrated in the skeletal muscle of rats exposed to long-term hypoxia.\textsuperscript{22} However, we found no effect of hypoxia on REDD1 protein levels to support...
the inhibitory effect noted on S6K1. An involvement of REDD1 in the current study could be questioned, considering that the inhibition of mTORC1 occurred very rapidly after exercise, whereas more time would likely be required to observe an increased transcription and translation of REDD1. Our in-depth analysis of HIF-1α also showed no effect of hypoxia on HIF-1α protein stabilization, which is in line with previous observations in human skeletal muscle, and argues against the involvement of HIF-1α in the noted mTORC1-inhibition. It should, however, be acknowledged that the time delay between the last muscle contraction to biopsy sampling and the rapid blotting of blood from the sample, may have been sufficient for some degree of HIF-1α destabilization in the hypoxia-trial, but the lack of differences in HIF-1α targets speaks against this notion. Moreover, we did not detect any differences between trials with regard to TSC2Ser1387 phosphorylation to support the involvement of the TSC1/TSC2 complex, although it must be recognized that exercise-induced changes in TSC2 phosphorylation do not necessarily reflect TSC2/TSC1 interaction in human muscle. Although we did not assess the Akt-mediated phosphorylation of TSC2Thr1462, hypoxia did not modulate the exercise-induced increases in Akt-mediated PRAS40 phosphorylation. Taken together the data speak against the idea that the acute mTORC1 inhibition induced by hypoxia is mediated by the REDD1-TSC1/TSC2 axis.

We also investigated the role of hypoxia in JNK-signalling, as previous in vitro studies have illustrated its sensitivity to oxygen availability. As with S6K1, the phosphorylation on JNKThr183/Tyr185 and its downstream target SMAD2Ser245/250/255 were robustly downregulated acutely after exercise in hypoxia. JNK (c-Jun N-terminal kinase) is a member of the mitogen-activated protein kinase (MAPK) family that is sensitive to a range of cellular stressors. Numerous in vitro studies, employing hypoxic exposure in the range of hours to days, have demonstrated an induction of JNK-signalling that leads to assembly of the activator protein 1 (AP-1) transcription factors that control the expression of, eg, vascular endothelial growth factor (VEGF). However, we found that hypoxia inhibited resistance exercise-induced JNKThr183/Tyr185 phosphorylation. The explanation for the divergent results is not obvious, given that we are not aware of any human in vivo studies that have analysed JNK-signalling in response to hypoxia, but may involve the duration of hypoxia exposure, experimental condition (resting vs stimulated cells) and/or tissue-specific regulation.

JNK is known to be a mechanosensitive kinase that is activated in human skeletal muscle after an exercise stimulus, particularly after eccentric exercise. Activation of JNK has previously been shown to result in cardiomyocyte hypertrophy, but only recently was JNK shown to be involved in exercise-induced skeletal muscle hypertrophy. Lessard et al demonstrated that JNK is preferentially activated by resistance exercise and results in downstream phosphorylation of a linker region of SMAD2 (Ser 245/250/255) that diminishes TGF-β/myostatin activity. Canonical myostatin activity involves phosphorylation of SMAD2 at the C-terminus (Ser 465/467) leading to nuclear translocation of the protein. Whilst SMADSer245/250/255 is found in the nucleus, this linker region phosphorylation is shown to reduce the presence of SMAD2 in the nucleus leading to altered canonical myostatin induced gene expression. Lessard et al also showed that muscle-specific JNK knockout mice exhibit a blunted hypertrophy response to overload, but an enhanced adaptive response to endurance training. JNK was, therefore, proposed as a molecular switch that could drive specific adaptations to different types of exercise stimulus. Thus, our finding that hypoxia results in diminished JNK signalling appears consistent with the diminished muscle anabolic response during hypoxia shown in human skeletal muscle.

It is well established that short-term (hours) hypoxia results in the downregulation of protein synthesis in skeletal muscle in vitro and in rats and humans in vivo under resting conditions as well as after resistance exercise. Recently, Gnimassou et al also showed complete inhibition of resistance exercise-induced increases in FSR with hypoxia present only during fed state exercise. Here, we chose not to assess FSR during acute recovery, primarily because the acute inhibitory effects of hypoxia have been well documented by others. Also, in a previous study, we were unable to reliably measure acute FSR using deuterium oxide under fasted state conditions because of large exercise-induced changes in muscle alanine production together with the short time for tracer incorporation, which together resulted in large variations in the FSR measurements. In the current study, we, therefore, wanted to expand the scientific literature with a more subacute measure. Regardless, our data argue that the inhibitory effects of hypoxia on signalling pathways (mTORC1 and JNK) are short-lived and do not affect FSR over a 24-hour post-exercise period in normal living conditions. When considering the discrepancy between signalling and FSR in this case it is important to recognize that the signalling assessment reflects a minor part (4 hours) of the FSR period (24 hours) and that non-quantitative and relative changes in protein signalling may not always be directly translated to quantitative changes in FSR.

Given the negative impact that hypoxia has on muscle mass, we also assessed its acute influence on resistance exercise-induced proteolytic processes. Hypoxia has been shown to stimulate both AMPK-dependent and
as well as HIF-1α-BNIP3 (BCL2 adenovirus E1B 19 kDa protein-interacting protein 3) induced autophagy in vitro, where the latter controls mitophagy. AMPK and mTORC1 exert dual control over ULK1, where AMPK-mediated ULK1 phosphorylation stimulates autophagosome formation, whilst mTORC1-directed ULK1 phosphorylation prevents this action. Masschelein et al. showed that 8 hours of hypoxic exposure at rest induced AMPK Thr172 phosphorylation, increased the LC3b-II/I ratio and lowered p62/SQSTM1 protein levels in human muscle, which suggests increased autophagosome formation and subsequent clearance. We found an exercise-induced increase in AMPK-mediated ULK-Ser317 phosphorylation, increases in BNIP3 protein levels and reduced LC3-II/II ratio that was unaffected by hypoxia despite significant differences in mTORC1-signalling between trials. The lack of modulation by acute hypoxic exposure on AMPK-mediated autophagy is in line with the data of Gnimassou et al. and is consistent with the idea that exercise stimulated autophagy relies primarily on AMPK activation, as indicated by Schwalm et al. Hypoxia has also been shown to alter ubiquitin-proteasome-mediated proteolysis in rat skeletal muscle following several days of hypoxic exposure. This has also been confirmed by changes in MAFbx and MuRF-1 mRNA expression during sustained hypoxia in rats undergoing muscle regeneration or functional overload. In human skeletal muscle, short-term or acute hypoxia at rest or with exercise has shown to have modest or no impact on the gene expression of these ubiquitin ligases.

We found that resistance exercise-induced increases in the protein levels of MAFbx and MuRF-1 that were unresponsive to hypoxia. We also analysed acute changes in protein levels of the novel ubiquitin ligase UBR5, which has been shown to play an important role in loading-induced muscle hypertrophy, but this protein has to our knowledge never been studied in human muscle in response to exercise. Protein levels of UBR5 were reduced by resistance exercise but not responsive to hypoxia. The reduced levels of UBR5 noted 24 hours after exercise might seem controversial given its positive involvement in hypertrophy. However, these findings are consistent with the observation of early reductions followed by later increases in UBR5 protein content during a 14-day functional overload in mouse skeletal muscle. In summary, key proteins in the ubiquitin-proteasome pathway do not seem to be influenced by acute hypoxia.

Earlier studies based on blood lactate levels during and after resistance exercise in acute hypoxia have provided mixed results regarding augmented metabolic stress. In contrast, other studies have consistently demonstrated that respiratory hypoxia during submaximal dynamic exercise results in reduced muscle levels of PCr and marked increases in muscle levels of glucose 6-P and lactate, as well as increased lactate efflux, compared to normoxia. However, to our knowledge, no previous study has determined muscle metabolites after resistance exercise performed in hypoxia, a model that is distinctly different from submaximal dynamic contractions. The trials were composed of work-matched anaerobic exercise at ~70% of 1RM, a load where muscle blood flow is severely or even fully occluded. Accordingly, the addition of extracellular hypoxia to intracellular anoxia is thus not likely to affect energy turnover. Therefore, the lack of difference in both blood and muscle levels of metabolites between trials is not surprising. Potential differences in, eg, glucose 6-phosphate or lactate production would thus likely be an effect of altered metabolism, ie, limited phosphocreatine re-synthesis during recovery. Whilst the PCr recovery could be delayed to some extent in hypoxia, the present three-minute recovery, where reactive hyperemia likely is noticeable, was sufficient to restore PCr levels to the same degree as in normoxia. The lack of differences between resistance exercise during hypoxia and normoxia in high energy phosphates, lactate, as well as AMPK (and its downstream substrate ACC), suggest that hypoxia is not exerting its effects on mTORC1- and JNK- signalling by alterations in muscle energy status. Thus, hypoxia must be exerting its effects on signalling by an analyte that was not measured in the muscle or by a circulating factor.

Whilst our study is strengthened by the cross-over design, the time-course biopsy sampling, the detailed muscle metabolite analysis, and the assessment of a wide range of signalling proteins, there are also limitations. For example, FSR was not determined in resting muscle. However, in the validation of the deuterium oxide approach by Wilkinson et al. the non-exercised leg exhibited a FSR of 1.35%/day under free-living conditions whilst the exercised leg had an increased FSR of 1.79%/day which is similar to the average 1.72%/day noted here. This supports a significant effect of the exercise bout in the present study. Also, as noted earlier, although not applicable with the present methodology, a 3 hours FSR assessment would have strengthened the overall picture. However, that would have required the use of an additional tracer, eg, 13C6-phenylalanine, but unfortunately, that was not feasible here.

In conclusion, these data demonstrate that acute normobaric hypoxia during resistance exercise inhibits contraction-induced mTORC1 and JNK/SMAD signalling, which can explain earlier reports of decreased rates of protein synthesis and diminished muscle growth under short-term and long-term hypoxia, respectively. Moreover, the effects of hypoxia are not mediated by changes in HIF-1α, AMPK- or the degree of metabolic stress in the muscle. Collectively, these data offer important mechanistic
insight into how hypoxia can influence protein synthesis in vivo.

4 METHODS

4.1 Subjects

Eight healthy male volunteers participated in this study. The number of subjects was based on power calculations set to detect a 25% difference between trials with regard to protein signalling. All subjects were free of injuries, performed resistance exercise involving the legs 1-2 times per week during the last 12 months, and did not consume any drugs or dietary supplements. All subjects were accustomed to training and performed 4-7 training sessions per week involving both resistance and endurance exercise, as well as some team sports activities. Subject characteristics are presented in Table 2. After being fully informed of the purpose of the study, and associated risks, all subjects provided their written consent to participate. The study protocol was approved by the Regional Ethical Review Board in Stockholm (2017/2496-31) and performed in accordance with the principles outlined in the Declaration of Helsinki.

4.2 Study design

In a randomized cross-over fashion, each subject performed two sessions of unilateral leg extension resistance exercise under normoxic (FiO₂ 21%) or hypoxic (FiO₂ 12%) conditions, separated by 7-10 days. The entire study consisted of three preliminary sessions and two experimental trials which were all completed in five to six weeks. The subjects were instructed to maintain their habitual dietary intake and physical activity pattern throughout the entire experimental period, although during the two days before each trial they were told to refrain from physical exercise. A schematic overview of the experimental design is provided in Figure 9.

4.3 Pretests

The study involved three preliminary testing sessions, where the first visit involved a health screening, leg volume and muscle area determination, a maximal single-leg knee-extensor strength test (1-RM) for both legs as well as a 30 second all-out cycling sprint to estimate anaerobic capacity. By surface measurements of participants’ thigh length, together with skinfold at mid-thigh and assessment of epicondyle diameter of the femur, leg volume was calculated according to Tuthill and Stewart, and bone-free leg muscle area was calculated according to Knapik et al.

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**TABLE 2 Subject characteristics**

| Characteristic          | Mean ± SE (n = 8) |
|-------------------------|------------------|
| Weight (kg)             | 88 ± 3           |
| Height (cm)             | 184 ± 3          |
| Age (y)                 | 31 ± 2           |
| 1-RM Left (kg)          | 116 ± 4          |
| 1-RM Right (kg)         | 116 ± 4          |
| Peak power (W/kg)       | 12 ± 0.6         |
| Mean power (W/kg)       | 8 ± 0.4          |
| Type II fibre distribution% | 55 ± 3       |
| Mean fibre area (µm²)   | 6280 ± 300       |
| Leg volume avg. (cm³)   | 10 145 ± 455     |
| Leg muscle area avg. (cm²) | 196 ± 7    |

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**FIGURE 9** Schematic overview of the trial design. Biopsy needles represent muscle biopsy time points. Syringes indicate venous blood sampling time points. Numbers under blocks indicate time (min) after infusion start, set number and time (min) of recovery. Res.Ex stands for knee extensor resistance exercise. WU stands for warm-up, three sets. Inspired fraction of oxygen during a baseline period and the exercise session was set to 21% or 12% in a randomized fashion. After 180 minutes of recovery, subjects were fed a standardized meal (34 g protein, 21 g carbohydrate and 7 g fat) after which they left the laboratory and returned in an overnight fasted state the next morning for 24-hour sampling.
1-RM testing was performed for each leg separately using a leg extension machine (Cybex Eagle, IL, USA) as follows. After a brief warm-up (2 × 10 bilateral extensions at 25 and 50 kg), the load was gradually increased each lift until the subject could no longer perform a single repetition (90-180° knee angle). Five minutes of rest separated each lift for one leg, with each leg alternating between attempts and with the non-dominant leg initiating each round. A 30 second all-out cycling sprint was carried out after 15 minutes of rest on an electronically braked cycle ergometer (SRM Ergometer, SRM, Jülich, Germany). After a 100-W cycling warm-up for 5 minutes, a maximal 30 seconds cycling effort was performed at a fixed cadence of 115 rpm, with the participants being heavily encouraged by the test leaders during the sprint. Power output was sampled every 0.5 seconds to determine peak power and mean power over 30 seconds.

The subjects then visited the laboratory on two occasions for familiarization sessions where they performed the exercise protocol (see below) used during the experimental trials. Here, the desired contraction speed was practised and, if applicable, the load was adjusted to meet the set criteria of the protocol. The first session was performed during normoxic conditions and the second session during moderate hypoxic conditions (FiO₂ 15%). These sessions were scheduled one week apart with the final session being performed 7-10 days prior to the first trial.

### 4.4 Experimental trials

For determination of the rate of muscle protein synthesis, subjects ingested 3.5 mL of 70% deuterium oxide per kg b.w. (Cambridge Isotope Laboratories, Danvers, MA, USA) on the evening prior to the first trial (~12 hours before reporting to the laboratory the next morning). Then again, on the day prior to the second trial, subjects ingested 0.15 mL deuterium oxide per kg b.w. × days between trials to reach the same level of isotope enrichment as in the first trial (eg, 75 kg × 7 days between × 0.15 mL = 79 mL dose). To avoid the known side effects, vertigo and nausea, all doses were divided into smaller (~50 mL) boluses separated with at least 30 minutes (eg, a dose of 225 mL was divided in 5 × 45 mL ingested over a period of 180 minutes).

Subjects reported to the laboratory at 07:00 AM on the day of the trials, after fasting since 10:00 PM, the evening before. Upon arrival, subjects assumed the supine position and a 20G Teflon catheter was inserted into the antecubital vein in a randomized arm, whereafter a baseline blood sample was drawn. Immediately after collection, whole blood was analysed for the levels of lactate and glucose were determined in an automatic analyser (Biosen C-line, EKF Diagnostics, Cardiff, UK). This was followed by a baseline biopsy from the vastus lateralis muscle of the leg randomized for exercise for that trial.

After completion of baseline sampling, subjects were equipped with a face mask (Hans Rudolph Inc, KS, USA) connected to an altitude generator (MAG-20, HigherPeak, MA, USA) set at normoxia or FiO₂ 12%, in a randomized manner. A LNCS TF-I forehead sensor connected to a Rad-97 pulse oximeter (Masimo, Switzerland) was placed on each subject to monitor blood oxygen saturation (SpO₂) and pulse rate. A baseline period of 20 minutes involving seated rest and 10 minutes of cycling at 50 W was performed and this was subsequently followed by resistance exercise. The exercise trial was performed with one leg starting with three warm-up sets of 10 repetitions at 0%, 25% and 50% of their individual 1-RM, with each set separated by 2 minutes of rest. This was followed by six sets at 70% of their 1-RM, separated by 3 minutes of rest, where the load was gradually decreased if necessary, to ensure that at least 8 but not more than 10 repetitions were completed at fatigue. The individual load, number of repetitions and time under tension was matched between the two trials.

After the completion of the final set, the face mask was removed, and subjects rapidly moved to the supine position for a second muscle biopsy, which was taken 60-120 seconds after the last contraction. Subjects then remained in the supine position for 3 hours and additional biopsies were taken at 90- and 180-minute. Blood samples were drawn as described in Figure 9. Whole blood samples were analysed directly as described above, and the remaining blood was transferred to heparinized tubes. After the 180 minutes recovery period, subjects were provided with a whey protein drink and an energy bar (Enervit, Milan, Italy) that yielded a total of 34 g of protein, 21 g of carbohydrate and 7 g of fat. Subjects then left the laboratory and were instructed to refrain from any type of exercise, maintain a standardized diet and return to the laboratory in the fasted state the following morning for a 24-hour post-exercise muscle biopsy and blood sample. The next trial was performed 7-10 days later with oxygen supply and leg used for exercise and biopsies being randomized in a counterbalanced manner.

Following local anaesthesia, the muscle biopsies were taken with a Bergström needle (Stille, Torshälla, Sweden) with applied suction. The first biopsy was taken approx. 15 cm above the patella and each subsequent biopsy was taken approx. 2 cm proximal to the previous one from a new incision. Tissue samples were immediately blotted free of blood. One piece of muscle was frozen in
isopentane chilled in liquid nitrogen for subsequent cryosectioning and immunohistochemistry, whilst another piece was rapidly frozen in liquid nitrogen and stored at −80°C until further processing.

4.5 | Plasma analysis

Blood samples collected in heparinized tubes were kept on ice until centrifuged at 3000 rpm for 10 minutes to obtain plasma which was subsequently stored at −80°C. Plasma lactate concentrations were analysed spectrophotometrically as described by Bergmeyer82 on a 96-well plate reader (Infinite 200 Pro, Tecan, Switzerland).

4.6 | Muscle tissue processing

After lyophilization, the muscle samples were thoroughly dissected free from blood and connective tissue under a light microscope (Carl Zeiss, Germany). This left only very small fibre bundles intact, which were then carefully mixed and split into homogeneous aliquots for subsequent analyses.

4.7 | Muscle metabolites

Freeze-dried muscle (~2 mg) was extracted in ice-cold 0.5 M perchloric acid, centrifuged, neutralized with 2.2 M KHCO₃ and centrifuged again. The final supernatant was analysed for metabolites (high energy phosphates, creatine, glucose 6-P, lactate, malate and inorganic phosphate) using enzymatic techniques (changes in NAD[P]H adapted for fluorometry83). To correct for variability in solid non-muscle constituents, metabolite values were adjusted for total creatine (sum of phosphocreatine [PCr] and creatine) (TCr). Thus, each metabolite value was divided by the individual TCr value and then multiplied by the mean TCr content for the whole material (145.0 ± 5.5 mmol × kg dry wt⁻¹). TCr was not significantly altered by any condition (data not shown).

4.8 | Immunoblotting sample preparation

For the immunoblotting, 3.5 mg of lyophilized muscle was homogenized in ice-cold buffer (100 µL-mg⁻¹ dry weight) consisting of 40 mM HEPES (pH 7.5), 1 mM EDTA, 120 mM NaCl, 10 mM NaPO₄, 50 mM NaF, 50 mM β-glycerophosphate, 0.3% CHAPS, 1% phosphatase inhibitor cocktail (Sigma P-2850) and 1% (v/v) Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA) using a BulletBlender™ (NextAdvance, New York, USA). After 60-minute rotation of the homogenates at 4°C and subsequent centrifugation at 10 000 g for 10 minutes at 4°C, the resulting supernatants were collected, and the protein concentration was determined using the Pierce™ 660 nm protein assay (Thermo Scientific, Rockford, USA). 4x Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer were used to dilute the samples to a final protein concentration of 1.5 µg µL⁻¹. The samples were then heated at 95°C for 5 minutes to denature the proteins and then stored at −20°C until separation using SDS-PAGE.

For the immunoprecipitation (IP) of HIF-1α, used for an in-depth analysis in the hypoxia trial, the mixed muscle was pooled from all subjects from the PRE as well as the POST biopsy, with both samples being homogenized as described above. From each of the two lysates, 500 µg protein were incubated with 5 µL rabbit anti-HIF-1α antibody (#36169, CST [Beverly, MA, USA]) and 15-µL protein G magnetic beads (Thermo Scientific) overnight at 4°C with rotation. Thereafter, the beads with the bound immunocomplexes were spun down and trapped using a magnetic rack and washed three times in lysis buffer. The magnetic beads with bound HIF-1α were suspended in 50-µL 1x Laemmli sample buffer containing 700 mM 2-mercaptoethanol, boiled for 5 minutes at 95°C and then immunoblotted for HIF-1α, as described below. Additionally, both cytosolic and nuclear extracts were prepared. Mixed and pooled muscle from all subjects from the PRE as well as the POST biopsy, were separately fractionated to cytosolic and nuclear protein fractions as described by Dimauro et al.84 The cytosolic and nuclear fractions were diluted in 4x Laemmli sample buffer and fractionation buffer to obtain a final protein concentration of 1 µg µL⁻¹ and 0.3 µg µL⁻¹, respectively.

4.9 | Immunoblotting

For the immunoblotting, 22.5 µg of protein from each sample was loaded on 26-well Criterion TGX gradient gels (4%-20% acrylamide; Bio-Rad Laboratories), and electrophoresis was then performed on the ice at 300 V for 30 minutes. All samples from each subject were loaded onto the same gel. After the protein separation, gel equilibration was performed in transfer buffer (25 mM Tris base, 192 mM glycine and 10% methanol) for 30 minutes at 4°C. Protein transfer was performed using polyvinylidene fluoride membranes (Bio-Rad Laboratories) with a constant current of 300 mA for 3 hours at 4°C. To confirm equal loading and transfer the membranes were stained.
with MemCode™ Reversible Protein Stain Kit (Thermo Scientific).85

Following imaging of the membranes, they were destained and subsequently blocked for 1 hour at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% non-fat dry milk. Subsequently, the membranes from each gel were cut in strips for each target protein and then assembled, so that all membranes and accordingly all samples would be exposed to the same blotting conditions. The membranes were then incubated overnight with primary antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% non-fat dry milk (TBS-TM). After primary antibody incubation, membranes were washed with TBS-TM and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. Following washes with TBS-TM (2 × 1 min, 3 × 10 min) followed by 4 × 5 min with TBS, proteins were visualized by the application of Super Signal™ West Femto Chemiluminescent Substrate (Thermo Scientific) to the membranes followed by detection in the molecular imager ChemiDoc™ MP. Band density was quantified by using the Image Lab™ software (Bio-Rad Laboratories). Following visualization, the membranes were stripped of the phosphospecific antibodies, using Restore western blot stripping buffer (Thermo Scientific), for 30 minutes using the Image Lab™ software (Bio-Rad Laboratories).

Antibodies

For immunoblotting, primary antibodies against S6K1 (Thr389, #9234; total #2708), mTOR (Ser2448, #2971; total, #2983), 4E-BP1 (Thr37/46, #2855; total, #9644), eEF2 (Thr56, #2331; total, #2332), JNK (Thr183/Tyr185, #4668), SMAD2 (Ser450/505/550, #3104; total, #5339), TAZ (Ser89, #3175; total, #8690), LC3b (#2775), GABARAP (#13733), BNIP3 (#44060) TSC2 (Ser1387, #5584; total, #3635) PRAS40 (Thr246, #13175; total, #2691) and p38 (Thr180/Thr182, #4511; total, #8690), were purchased from CST (Beverly, MA, USA). Primary antibody for total YAP (sc-101199), total TAZ (sc-293183), total JNK (sc-7345), MuRF-1 (#sc-396808), MAFbx (#sc-16806) and UBR5 (#sc-515494) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Primary antibody for YAP (Ser127, #PA5-17481) was purchased from Thermo Scientific. Primary antibody for REDD1 (#63059) was purchased from Abcam (Cambridge, UK). Primary antibody for HIF-1α (#NB100-134) was purchased from Novus Biologicals (Abingdon, UK).

All primary antibodies were diluted 1:1000 except for the total eEF2 and total 4E-BP1, which were diluted 1:2000, and total JNK, total YAP, total TAZ, UBR5, MuRF-1 and MAFbx which were diluted 1:500. Secondary anti-rabbit (#7074; 1:10 000) and secondary anti-mouse (#7076; 1:10 000) were purchased from CST.

4.11 Fibre-type composition and area

Muscle fibre-type composition and area was determined as described in Horwath et al86 on all 10 biopsies per subject, with individual fibre-type composition and mixed fibre area thus being an average of the 10 biopsies. The number of fibres of each type was counted on the whole muscle section to accurately determine the fibre-type composition, including an average of 770 ± 130 fibres per biopsy. Mixed fibre area was determined as the average of type I and type II fibre area, which was determined on 102 ± 10 and 105 ± 6 fibres respectively.

4.12 Muscle protein fractional synthetic rate (FSR)

For the quantification of mixed muscle protein FSR, intracellular free levels and muscle protein-bound levels of 3H-alanine were analysed. Using a glass rod and 2% perchloric acid, 3.5 mg of lyophilized muscle tissue was pelleted and extracted twice. These two extracts were combined and analysed for intracellular enrichment of free 3H-alanine using LC-MS/MS as described by Bornø and van Hall.87 The remaining pellet was washed twice with 70% ethanol, hydrolyzed overnight in 6 M HCl at 110°C, and dissolved in 50% acetic acid after which the solution was passed through a cation exchange resin column. The amino acids derived from the purified pellet were converted to their N-acetyl-n-propyl esters and analysed by gas chromatography–pyrolysis–isotope ratio mass spectrometry (GC-P-IRMS, Delta V, Thermo Scientific, Bremen, Germany), to determine enrichment of protein-bound 3H-alanine.

The protein fractional synthesis rate was determined using the precursor–product approach: FSR = ΔE_p/ (Eic × t) × 100, where ΔE_p is the difference in protein-bound 3H-alanine enrichment between biopsies taken at rest and after 24 hours of recovery; Eic is the average intracellular free 3H-alanine enrichment in the two biopsies and t is the time between biopsies in hours multiplied by 100 to express FSR in percentage per hour (%·h⁻¹).
4.13 | Statistical analyses

Data were analysed using TIBCO Statistica 13 for Windows (TIBCO Software Inc, Palo Alto, CA, USA). Data are presented as mean ± standard error of mean (SEM) unless otherwise noted. Normal distribution of variables was explored prior to execution of tests with histograms and Shapiro-Wilks test of normality. For the immunoblotting data, some variables were skewed and after log-transformations, all data were deemed acceptable for parametric statistical tests. A two-way repeated-measures analysis of variance (ANOVA) trial × time was used for data analysis on heart rate, blood lactate and glucose, muscle metabolites, protein signalling and total protein content. Bonferroni corrected multiple comparisons were performed if significant main effects or interaction effects appeared. Differences in FSR were determined with the Student’s paired t test. Statistical significance was set at $P < .05$. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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