HYPOXIA- AND EXERCISE-MEDIATED EFFECTS ON THE MUSCLE MASS VIA ENDOGENOUS LOCAL PROGENITORS AND THE HIPPO SIGNALING PATHWAY

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LIST OF ABBREVIATIONS

1-RM	one-repetition maximum
3-MH	3-methyl-histidine
4E-BP1	eukaryotic initiation factor (eIF4E)-binding protein 1
5'TOP	5'terminal oligopyrimidine tract
ACC	acetyl CoA carboxylase
AMBRA1	activating molecule in BECN1 regulated autophagy protein 1
AMOT	angiomotin's protein family
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Atg13	autophagy-related 13
ATP	adenosine triphosphate
Bag3	Bcl2-associated athanogene 3
BCAT2	branched-chain aminotransferase 2
BFR	blood flow restriction
Birc5/2	baculoviral IAP repeat-containing 5 and 2
BMI	body mass index
BMP	bone morphogenetic protein
CAMK	calcium/calmodulin-dependent protein kinase
CASTOR1	cellular arginine sensor of mTORC1
ChIP	chromatin immunoprecipitation
CK	creatine kinase
$CoCl_2$	cobalt dichloride
CSA	cross sectional area

CSAID	cytokine-suppressive anti-inflammatory drug
Ctgf	connective tissue growth factor
Ctrl	Control
D_2O	Deuterium
D-3MH	Deuterated 3-methyl-histidine
eEF2 kinase	eukaryotic elongation factor 2 kinase
eIF4	eukaryotic translation initiation factor 4
ERK	extracellular-regulated kinase
Ex	Exercise
FiO_2	fraction of inspired oxygen
FIP200	family-interacting protein of 200 DA
FoxO	forkhead box O
FSR	fractional protein synthetic rate
GATOR 1/2	GTPase activating protein activity toward Rags 1 and 2
GDF	growth and differentiation factor
GPCRs	G-protein-coupled-receptors
GSK-3β	glycogen synthase kinase 3β
H_2O_2	hydrogen peroxide
HECT	homologous to E6-AP carboxy-terminus
HEK293	human embryonic kidney
HIF-1	hypoxia-inducible factor-1
HR	heart rate
HRE	hypoxia-response element
Hsp70	70-kDa heat shock protein
HYP	Hypoxia

IGF	insulin like growth factor
IL	Interleukin
IRS-1	insulin receptor substrate-1
Jak	Janus kinase
JNK	c-jun NH2-terminal kinases
KLF15	kruppel like factor 15
Lats1/2	large tumor suppressor kinases 1 and 2
Lc3/Atg8	micro-tubule associated protein 1A/1B-light chain 3
LEF	lymphoid-enhancer binding factor
$LKB1^{-/-}$	liver kinase B1-knockout
LPA	lysophosphatidic acid
MAFbx/Atrogin	muscle atrophy F-box
MAPK	mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MEF	mouse embryonic fibroblast
MEF2 :	myocyte-specific enhancer factor 2
MEK/ERK kinase	mitogen-activated protein kinase
MHC	myosin heavy chain
MK2	MAPK-activated protein kinase 2
mLST8	mammalian lethal SEC13 protein 8
MRF	myogenic regulatory factors
MRF-4	myogenic regulatory factor-4
Mst1/2	macrophage stimulating proteins 1 and 2
mTOR	mammalian target of rapamycin
MuRF1	Muscle RING finger 1
Myf-5	myogenic factor-5

MyoD	myogenic differentiation
NF2	neurofibromatosis 2
NF-κB	nuclear factor-kappa B
NICD	Notch intracellular domain
NIRS	near-infrared spectroscopy
NOR	Normoxia
p62/SQSTM1	sequestosome 1
Pax7	paired-box 7
Pcna	proliferating cell nuclear antigen
PCr	Phosphocreatine
PDK-1	phosphoinositide-dependent kinase 1
PDK1	pyruvate dehydrogenase kinase 1
PHD	prolyl hydroxylase domain
Pi	inorganic phosphate
PI3K	phosphatidylinositol 3-kinase
PKB or Akt	protein kinase B
PO_2	oxygen partial pressure
PRAS40	proline-rich Akt substrate of 40kDa
RAPTOR	regulatory associated protein of mTOR
REDD1	regulated in development and DNA damage response 1
Rheb	Ras homologue enriched in brain
RICTOR	rapamycin-insensitive companion of mTOR
ROS	reactive oxygen species
RUNX	runt-related transcription factor
S1P	sphingosine 1-phosphate
S6	ribosomal protein S6

S7K1	protein S6 kinase 1
Ser	Serine
SERCA	sarco-endoplasmic reticulum calcium-ATPase
SIN1	stress-activated protein kinase interacting protein 1
SpO_2	peripheral blood oxygen saturation
SRF	serum response factor
STAT	signal transducers and activators of transcription
Taz	transcriptional co-activator with PDZ binding motif
TCF	T-cell factor
TGF-β	transforming growth factor-β
Thr	Threonine
Tnni1	troponin I1
Tnnt1	troponin T1
TSC1/2	tuberous sclerosis complex 1 and 2
TSI	tissue saturation index
ULK	unc-51-like kinase
v-ATPase	vacuolar H^+ ATPase
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau tumor suppressor gene
Vps34/15	vacuole protein sortin 34 and 15
Yap	Yes-Associated Protein

CHAPTER I: INTRODUCTION

The human body is composed of many compartments, of which, the fat-free mass compartment comprises mainly skeletal muscles and bones. Skeletal muscle compartment represents nearly 40% - 50% of the whole-body mass (1). Skeletal muscle fills two essential functions, a contractile function for locomotion and posture maintenance, and a metabolic function as a protein pool (2). It is considered as dynamic, sensitive to many factors like growth factors, exercise, nutrition and illness. Each factor regulates muscle mass by various intracellular signaling pathways (2) that will be described later on in this introduction.

1. Skeletal muscle

1.1. Structure and properties

1.1.1. Structural description

aSkeletal muscle is composed of muscle fibers arranged in parallel and the fibers are themselves made of myofibers, considered as the basic muscle contractile unit. The muscle fibers are individually surrounded by a cell membrane (sarcolemma) and separated by a connective membrane (endomysium) (3). The individual muscle fibers are pulled together into bundles and surrounded by connective tissue (perimysium) forming skeletal muscle (4). Skeletal muscle is itself surrounded by a connective tissue called epimysium. Myofibers are composed of several interdigitating protein filaments usually called thick and thin filaments forming sarcomeres (5). Thick filaments are made of myosin arrayed in antiparallel fashion while thin filaments are made of series of actin monomers arranged in helical pattern with tropomyosin

regulatory protein inserted along the groove of the helix and troponin at discrete location along the actin filament (6). Other proteins such as titin and nebulin are relevant for both mechanical and physiological properties. Titin links the Z disk of the sarcomere to myosin and stabilizes the thin filament. Nebulin integrates the thin filament (7). All these proteins are relevant for the architecture of skeletal muscle and may be dysfunctional in some myopathies. Besides architectural proteins, muscle fiber sarcoplasm contains organelles essential for contraction. Among these, the T-tubule system is an invagination that conducts the nerve action potential to the cell. Contacting the exterior of the cell, the T-tubule network ensures the spreading of the nerve action uniformly throughout the fiber (6). Calcium release is an important step for muscle contraction and the sarcoplasmic reticulum serves as storage, release and reuptake of calcium. Two proteins in the sarcoplasmic reticulum are essential for calcium homeostasis, the sarco-endoplasmic reticulum calcium-ATPase (SERCA) and calsequestrin (8). SERCA re-uptakes calcium after muscle activation and calsequestrin binds it in the sarcoplasmic reticulum (9). Finally, the mitochondrial network in the muscle fiber provides energy if oxygen is available. Mitochondria are located close to the sarcolemma, reducing diffusion distance of oxygen, but also in the inter-myofibrillar space (6).

Because of their need for essential nutrients, skeletal muscles are highly vascularized. The feeding arteries arise from primary arteries distributed along the principal axis of the muscle (10). These feeding arteries are divided at right angle in secondary arteries, and finally in arteriolar network entering the perimysium. The arteriolar network

gives birth to numerous capillaries embedded in the endomysium and set along the muscle fiber. Numerous capillaries in variable array cross the muscle fiber (11). The distribution of oxygen to the skeletal muscle is non-homogeneous and the diversity and the variable cross-section of each muscle fiber are the evoked explanations (12). The capillaries surrounding the muscle fibers are interconnected and comparison of capillary network anatomy showed greater capillaries interconnections in oxidative muscle (13). The venous circulation is quite similar to the arterial one even though the venous outflow arises from arterioles located at distance (11).

Mature myofibers are contacted by a single motor neuron and express characteristic molecules for contractile function, especially myosin heavy chain (MHC) isoforms and metabolic enzymes (4). Although not fully explained, myofiber contractile properties seem to be determined by both motor neuron type and myoblast origin (4). Muscle contraction needs electrical events known as the excitation-contraction coupling and the generation of an action potential that arises from motor neuron α depolarization, which propagates to the muscle through the neuromuscular joint. Propagation of the action potential along the T-tubule system elicits interaction with the sarcoplasmic reticulum resulting in release of calcium (14). The mechanism of muscle contraction will be detailed in the next section.

1.1.2. Skeletal muscle properties

Skeletal muscle single fibers are made of 70%-80% of actin and MHC, the latter being the principal molecular motor (15). The contractile property

arises from organization of the contractile unit called sarcomere. The sarcomere and the sarcoplasm contain numerous proteins, which contribute to the formation of the cytoskeleton and muscle contraction. Indeed, calcium-dependent troponin and tropomyosin are both associated to actin filaments and contribute to myofilament sliding, thus to contraction (6). Muscle contraction and force generation is the result of sequential events called “the cross-bridge cycle”. Association of calcium to thin filaments troponin allows binding of myosin to actin and release of inorganic phosphate (Pi). The acto-myosin complex induces myosin’s conformation change and sliding of actin filament towards the center of the sarcomere with ADP releasing. The acto-myosin complex detaches upon ATP binding and myosin conformation changes (16).

Muscle contraction can induce two major kinetics known as static and dynamic contractions. Dynamic contraction results in muscle length shortening or stretching while static contraction does not modify muscle length (17). Dynamic muscle activities induce concentric but also eccentric muscular contractions. Concentric muscle contraction results in muscle shortening by a force generation transmitted to the joint via tendon, enabling change in joint angle (18). Eccentric muscle contraction refers to muscle contraction in which the momentary force produced by the muscle is lower than the force applied, resulting in spacing the muscle insertions (19). Based on the force-velocity relationship, eccentric contractions are associated with the highest muscle forces, and this is due to the maximal number of cross-bridges overlap of the myosin and actin filaments that occur at the maximum sarcomere length, but also depends to velocity of shortening (20).

Muscle contraction requires energy that is consumed by molecular motors, myosin heads or cross-bridges, and by ions pumps especially in the sarcoplasmic reticulum. The relative amount of energy needed depends on the type of contraction (maximal vs sub-maximal) and the duration (continuous vs short repeated contractions). As the immediate energy source is adenosine triphosphate (ATP) and the intracellular store of ATP is slight, energy is provided by other metabolic pathways known as aerobic and anaerobic metabolism (21, 22).

Anaerobic metabolism provides energy during short duration of high intensity physical activity. The main pathways required are phosphocreatine (PCr) degradation and muscle glycogen breakdown to lactate and ions (21). PCr degradation needs creatine kinase (CK) to catalyze the reaction PCr + ADP ↔ Cr + ATP. During contraction, skeletal muscle consumes ATP, reducing concentration of PCr, and increasing concentrations of Cr and Pi. In the recovery phase, this trend is reversed and synthesis of PCr is favored (23). Muscular glycogen breakdown and synthesis are regulated by glycogen phosphorylase and glycogen synthase, respectively (23). Glycogen phosphorylase exists in phosphorylated and active form or de-phosphorylated and less active form. Activation of glycogen phosphorylase is catalyzed by phosphorylase kinase at Ser^{14} and de-phosphorylation of glycogen phosphorylase is catalyzed by protein phosphatase 1. Once glycogen phosphorylase is phosphorylated, glycogen is converted into glucose 1-phosphate residues that enter glycolysis and converted in pyruvate. The crucial and limiting step of glycolysis leads to conversion of fructose 6-phosphate, a product of glucose 1-phosphate and glucose 6-phosphate

successively, into fructose 1,6-biphosphate catalyzed by phosphofructokinase considered as the “rate limiting enzyme” (24). Pyruvate, the final product of glycolysis, is converted by lactate dehydrogenase in lactate and NAD^+ (pyruvate + NADH + H^+ ↔ lactate + NAD^+). NAD^+ is consumed in upstream glycolytic reaction to refuel glycolysis (25). Lactate production is related to oxygen availability depending on energy demand and accumulation occurs during heavy exercise and high consumption of ATP. Accumulation of H^+ ions resulting from lactate production induces decrease of the pH up to 6.5 in more extreme situations (26).

Aerobic or oxidative metabolism requires oxygen supply, carbohydrates and lipids for ATP-producing in the mitochondria during prolonged exercise (27, 28). At first, intracellular free fatty acids, glycogen and glucose oxidation contribute to ATP production (22). When the exercise is prolonged, in addition to the intracellular capacity of ATP synthesis, extracellular free fatty acids and carbohydrates are metabolized by β-oxidation and Krebs cycle to produce the amount of ATP needed to supply energy demand. The various substrates are mobilized according to the intensity and duration of exercise (29, 30). Lipid oxidation is the major contributor at rest, about 60% of energy production for non-contracting skeletal muscle, while both lipids and carbohydrates are the main energy sources for moderate intensity exercise (50% of VO_2 max). From 65% to 80% of VO_2 max, the rate of lipid oxidation decreases and carbohydrates are the major energy source providers (31). This is described as the “crossover concept”, which states that substrate utilization in any time point depends on the crossover between exercise

intensity (which promote carbohydrates utilization) and endurance training-induced responses (increased lipids oxidation) (32). The general contribution of branched-chain amino acids and protein to energy provision is limited and ranges from 2% to 10% (22).

1.2. Skeletal muscle fiber types

At first sight, histochemical reactions for myofibrillar actomyosin ATPase identified two major fiber types in mammalian skeletal muscle but biochemical analyses revealed a much greater heterogeneity (33). Skeletal muscle is composed of different types of fibers with different characteristics of which color, force production, velocity of shortening, oxidative capacity, power generation and fatigability (34). Of those characteristics, contractile response (fast vs. slow) and metabolism (aerobic vs. anaerobic) are the most prominent explaining muscle fiber heterogeneity (35). Moreover, early studies observed both red and white colored fibers, and noted the red color is related to myoglobin content, an iron-containing oxygen transporter protein in red blood cells. Thus, muscle fibers are classified based on contractile and metabolic properties (36). Based on this, three major types of muscle fibers are identified: (i) slow, fatigue resistant fibers with low mechanical power output and high aerobic metabolism (ii) fast and quickly fatigable fibers with high mechanical power output and high glycolytic metabolism, and finally (iii) fast intermediate fibers which combine high power output and resistance to fatigue (35, 37). This classification fits with another one considering expression of MHC isoforms type I for slow and fatigue resistant fibers, IIx for fast and fatigable fibers and IIa for fast

intermediate fibers (38). Indeed, each muscle fiber is made of proteins, of which approximately 50% of MHC, the main determinant of contractile performance (35). Although only one type of MHC is mainly expressed in a muscle fiber, hybrid or mixed fibers expressing two or more MHC can be found in skeletal muscle (35). It is commonly accepted that type I and IIa fibers utilize oxidative metabolism as energy source whereas type IIx need anaerobic metabolism for ATP generation (34).

Even though the cross-bridge cycle remains the same, there are three functional signatures for each type of fiber based on MHC (39). First, fast MHC have high myosin isomerization to actin coefficient (K_{CO}>20) leading to fast ADP release while slow MHC isomerization coefficient is weak (K_{CO}<10) and ADP release needs two distinct steps (39). Second, there is different strain sensitivity of ADP release step. Finally, fast MHC has lower duty ratio, defined as the time a myosin motor spends bounded to actin during ATP hydrolysis. Slow MHC has much longer duty ratio. Intermediate MHC types can adjust their duty ratio in response to the load and other cellular signals such as calcium and magnesium levels, the phosphorylation state and concentration levels of actin, amongst others (39).

1.3. Skeletal muscle plasticity

Human body comprises more than 600 skeletal muscles with specific functions (35). They are used in widely various tasks including maintaining a posture, whistling, jumping, breathing and running. The motor control performed by the nervous system and the ability of skeletal muscle to adapt to the functional tasks allow this large range of

motor tasks (40). Skeletal muscle plasticity is referred as the adaptive responses to a prolonged change of functional demand (41). Those responses can be related to the structural and functional properties of distinct fibers populations in the muscle (35). For example, an increase in the cross sectional area (CSA) of type II muscle fibers was obtained after 2 weeks of resistance training with an enhanced effect with protein supplementation, while the same trend was obtained in type I muscle fibers after 4 weeks without protein supplementation (42, 43). Likewise, the functional properties of muscle fibers are modulated by factors such as exercise, micro-gravity and certain diseases (44, 45). Indeed, it is well known that overloading or resistance exercise induces transitions from (fibers type IIx to IIa) fast to slow fiber type, while reduced neuromuscular excitation or unloading drives the transition in the opposite direction (fibers type I to type IIa) (46-48). Other pathological factors can change fiber type composition in muscle such as denervation (49) and hormonal disease, especially dysregulation in thyroid hormone secretion (50).

Although not fully understood, many studies have investigated the underlying mechanisms of the plasticity, mainly using chronic low-frequency stimulation (51-54). Skeletal plasticity seems to be the result of integrated responses of various intracellular proteins-proteins interactions involving both transcription factors and co-activators related to peroxisome proliferator-activated receptor (PPAR)-ϫ coactivator (PGC)-1α (55). AMPK-activated protein kinase (AMPK) pathways, p38 mitogen activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase (CaMK), among others,

have been described upstream although their real contribution to PGC-1α activation remains unsolved (56-58). Exercise induces neuromuscular signals and muscle contractions resulting in calcineurin and CaMK-mediated expression of myocyte-specific enhancer factor 2 (MEF2) and cAMP response-binding element (CREB)-binding protein (59, 60). Once activated, PGC-1α can translocate in the nucleus to interact with activated MEF2 and co-activate the transcription of genes involved in the determination of slow-twitch fiber types and oxidative metabolism (60, 61).

As the transition from slow to fast fibers is mainly driven by pathological states, which are beyond the scope of the present work, the mechanisms will not be described here.

2. Regulation of muscle mass

Skeletal muscle mass gain is important for sport performance while preventing loss of muscle mass is essential for disease recovery or quality of life during ageing (62, 63). It is known to be the result of the balance between skeletal muscle protein synthesis and degradation (64). Both processes are sensitive to exercise, inactivity, nutrition and pathological states (65, 66). If protein synthesis exceeds protein degradation, proteins will accumulate, and muscle mass will increase. Inversely, if protein synthesis is less than degradation, loss of muscle mass will occur. In addition, satellite cell inclusion may contribute to increased muscle mass, while fiber loss results in a reduction of muscle mass.

2.1. Signaling pathways and signals regulating protein synthesis

2.1.1. The mammalian target of rapamycin (mTOR) pathway

Protein synthesis is a highly regulated process in response to many environmental factors such as nutrients, insulin, growth factors and exercise (67). Insulin is one of the most powerful activator of the phosphatidylinositol 3-kinase (PI3K) pathway. By binding to its receptor, insulin induces autophosphorylation of various tyrosine residues and phosphorylation of insulin receptor substrate-1 (IRS-1). Phosphorylation of IRS-1 activates PI3K, which phosphorylates in turn protein kinase B (PKB or Akt) at Thr^{308} via phosphoinositide-dependent kinase 1 (PDK-1) (68). Nevertheless, full Akt activation also requires its phosphorylation at Ser^{473} by rapamycin-insensitive companion of mTOR (RICTOR) a member of mTORC2 complex (69). Phosphorylated Akt activates mammalian target of rapamycin (mTOR) directly and indirectly through tuberous sclerosis complexes 1 and 2 (TSC1/TSC2) at Ser^{924}, Thr^{1518} (70), Ser^{939}, Thr^{1462} (71) and Ras homologue enriched in brain (Rheb) (67, 72, 73). mTOR is part of two distinct multi-protein complexes, mTORC1 and mTORC2 (Fig. 1). mTORC2 is comprised of rapamycin-insensitive companion of mTOR (RICTOR), stress-activated protein kinase interacting protein 1 (SIN1) and mammalian lethal SEC13 protein 8 (mLST8) (74). While mTORC2 is recognized to regulate actin cytoskeleton organization, cells proliferation and survival, mTORC1 is a complex of proteins containing regulatory associated protein of mTOR

(RAPTOR), proline-rich Akt substrate of 40kDa (PRAS40), mammalian lethal SEC13 protein 8 (mLST8) and mediate various aspects of cell growth (75). RAPTOR can directly interact with mTOR, as well as mTOR downstream effectors protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF4E)-binding protein 1 (4E-BP1), while mLST8 only activates the kinase activity of mTOR. PRAS40 inhibits mTORC1 kinase activity by binding to RAPTOR. Insulin phosphorylates PRAS40 and induces dissociation from mTORC1, which can then be activated (76). mTORC1 is implicated in the regulation of translation efficiency, i.e. the rate of mRNA translation, and translation capacity, i.e. the number of ribosomes (76).

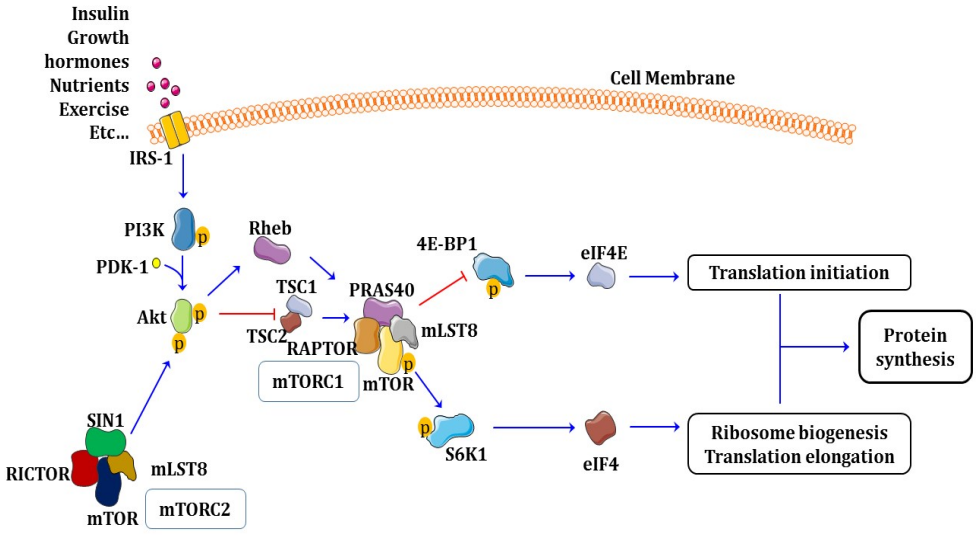

Figure 1. The Akt/mTOR signaling pathway

Although phosphorylation of S6K1 at Thr^{389}, Thr^{229}, Ser^{404} and Ser^{411} residues is inhibited by rapamycin, phosphorylation at Thr^{389} is the most critical for S6K1 loss of activation (77). This is the reason why phosphorylated S6K1 at Thr^{389} is the most commonly used marker of mechanical activation of mTORC1 signaling in skeletal muscle (78). S6K1 phosphorylates the eukaryotic translation initiation factor 4 (eIF4) at Ser^{422} and the eukaryotic elongation factor 2 kinase (eEF2 kinase) at Thr^{56} leading to interaction with eIF3 pre-initiation complex and inactivation of eEF2 kinase, thereby relieving eEF2 kinase-induced inhibition of translation elongation (76). Moreover, S6K1 regulates expression of several genes such as 5'terminal oligopyrimidine tract (5'TOP) mRNAs involved in ribosomes biogenesis, regulating the number of ribosomes and thus translational capacity (79, 80).

Phosphorylation of 4E-BP1 at Thr^{37}, Thr^{46}, Thr^{70} and Ser^{65} residues has been shown to be inhibited by rapamycin (81). When dephosphorylated or hypo-phosphorylated, 4E-BP1 binds to eIF4E, inhibiting interaction of the latter with eIF4G, formation of the eIF4F complex and the recruitment of the 40S ribosomal subunit. In summary, translation initiation is activated when 4E-BP1 is phosphorylated following activation of the PI3K pathway by insulin and growth factors while translation initiation is suppressed when 4E-BP1 is dephosphorylated (76).

2.1.2. Other signaling pathways

Although mTOR is recognized as the master key in protein synthesis regulation, other downstream signaling pathways contribute to the

regulation of skeletal muscle mass such as the glycogen synthase kinase 3β (GSK-3β) and the MAPK signaling pathways (82).

PI3K/Akt/GSK-3β signaling pathway

Upon activation, the PI3K/Akt pathway activates mTORC1 signaling, and in parallel inhibition of GSK-3β when phosphorylated at Ser^9 (83). Once activated GSK-3β normally blocks translation initiation factor eIF2B. Repression of GSK-3β by Akt results in translation initiation and protein synthesis contributing to hypertrophy (84). The PI3K/Akt pathway promotes translation by both mTOR activation and GSK-3β inhibition in an mTOR-independent fashion (84). Muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) or Atrogin are the most cited and studied E3 ubiquitin ligase enzymes involved in muscle atrophy (85-87). They are regulated by GSK-3β-dependent mechanisms as deletion of GSK-3β results in decreased MuRF1 and MAFbx expression at both protein and mRNA levels (88, 89).

Raf/MEK/ERK pathway

IRS-1 activates PI3K/Akt but also Raf/MEK/ERK pathway (where ERK is extracellular-regulated kinase and MEK is mitogen-activated protein kinase or ERK kinase) of the MAPK pathway (90). Although IRS-1 simultaneous activates both PI3K/Akt and Raf/MEK/ERK pathways, these pathways exert opposite effects on muscle protein accretion (91). Indeed, genetically manipulated constitutive activation of Raf, MEK or ERK leads to thinner skeletal muscle fibers contrary to the hypertrophic effect exerted by constitutively active PI3K or Akt (91).

2.1.3. Amino acids

Amino acids are also known to regulate mTOR activation (92-94). Indeed, amino acids restriction in mammalian cells induces decreased protein synthesis rate (94). In contrast, amino acids, and especially leucine, administration induces increased protein synthesis in skeletal muscle through the mTOR signaling (95, 96). It has been shown that mTOR activation requires its recruitment to the lysosomal surface mediated by Rag GTPases multi-subunits complex named Ragulator-Rag complex (97, 98). Actually, mTOR activation seems to be intricate mechanisms involving leucine tRNA synthetase and the vacuolar H^+ ATPase (v-ATPase) located to the lysosome surface, sensitive to leucine and amino acid concentration respectively (99, 100). Indeed, interactions of Rag GTPases with both leucine tRNA synthetase and v-ATPase are required for mTOR activation (100, 101). Moreover, mTOR recruitment to the lysosome surface is regulated by two protein complexes called GTPase activating protein activity toward Rags 1 (GATOR1) and 2 (GATOR2). GATOR1 exerts inhibitory effect on mTOR recruitment to the lysosomial surface repressing Ragulator-Rag complex while GATOR2 favors this mechanism removing GATOR1 inhibition in presence of high amount of amino acids (102). Furthermore, CASTOR1 and Sestrin2 are arginine and leucine cytosolic sensors respectively that can bind GATOR2 inhibiting his function in fasting (103, 104). Supply of leucine and arginine, after a meal, induces GATOR2 released by both CASTOR1 and Sestrin2 leading to mTORC1 activity (105). Glutamine, an abundant amino acid in skeletal muscle (106), is differentially regulated in mouse embryonic fibroblasts (MEFs) (107). Thus, glutamine can

activate mTORC1 independently of Ragulator-Rag complex through both a v-ATPase-dependent mechanism and mTOR recruitment to the lysosomial surface in MEFs (107). Although the regulation of mTOR by amino acids is based on solid evidence and fast evolving, this topic requires further research to better describe the precise mechanism.

2.1.4. Mechanical stress

Physical activity, and resistance exercise in particular, promotes skeletal muscle mass in health, ageing and diseases (108). Since overloading induces hypertrophy through, amongst others, the mTOR signaling pathway (109), various mechanical events have been used to investigate the biomechanical events linking mechanotransduction and this pathway (110-112). Using cytochalasin D, a cytotoxin known to disrupt muscular network and filamentous aggregates, Hornberger et al. (2005) observed blunted mTOR stretch-induced activation, even though insulin-induced mTOR activation was intact (111). This observation revealed the necessary communication of skeletal muscle architectural proteins with mechanical events to induce mTOR activation under physiological conditions. In an attempt to identify the relation between mechanical stimuli and mTOR activation, several studies revealed a role of phospholipase D1 and his second messenger phosphatidic acid, which can bind and activate mTOR at his FK506-binding protein-rapamycin-binding (FRB) domain (113, 114). The rapamycin inhibitory effect on mTOR probably derived from its competition with phosphatidic acid for binding to FRB domain. Finally, the MAPK pathway is suggested to convert contraction into intracellular responses (115). There are four

main components of the MAPK signaling cascade, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, c-jun NH2-terminal kinases (JNK), and ERK5 (116). Among those components, p38 has been implicated in contraction-induced activation in metabolic adaptations to exercise of which mitochondrial biogenesis and slow muscle fibers formation (56). In addition, the hypothesis that MAPK signaling contributes to long-term adaptations arises after elevated p38, JNK and ERK1/2 were observed in skeletal muscle during the early period of recovery after high-power resistance exercise (117). In a synergistic ablation mechanical overload model, muscle hypertrophy and increased protein synthesis were observed as well as elevated ERK1/2 and TSC2 phosphorylation independently of PI3K in early phase of mechanical overload (118).

2.1.5. Cellular energy status

AMPK is a serine-threonine kinase activated by a high variety of cellular triggers increasing AMP and decreasing ATP levels such as muscle contraction (119), fasting (120), hypoxia (121), amongst others. It is considered as a main cellular energy sensor (122). Using high frequency (mimicking resistance training) and low frequency stimulation (mimicking endurance training), Atherton et al. (2005) investigated selective adaptations of skeletal muscle to both endurance and resistance training. They found that endurance training-like stimulation activated AMPK signaling and inhibited TCS2 and downstream regulators of translation initiation while resistance training-like protocol activated Akt/mTOR signaling and downstream translational regulators

(123). In line, calorie restriction led to AMPK activation and mTOR deactivation in murine models (75). Aiming to explain the link between AMPK and mTOR signaling pathways, Inoki et al. (2003) found that under low cellular energy conditions, activated AMPK phosphorylated TSC2 on Thr^{1217} and Ser^{1345} (which corresponds to Thr^{1271} and Ser^{1387}, respectively, in human TSC2) in human embryonic kidney (HEK293) cultured cells (70, 124). Phosphorylation of these TSC2 residues by AMPK enhanced the inhibitory effect on mTOR activity (124). In addition, AMPK can directly phosphorylate RAPTOR on Ser^{722} and Ser^{792} to inhibit mTOR activity through cellular energy stress-induced AMPK activation in HEK293 cultured cells (125). McGee et al. (2008) tested this mechanism studying the hypertrophic response in liver kinase B1-knockout ($LKB1^{-/-}$) mice in comparison with wild type mice. LKB1 is one of the upstream kinases able to induce increased phosphorylation of AMPK on Thr^{172} enhancing its inhibitory effect on mTOR activity in low cellular energy conditions (126). Silencing LKB1 did not impair muscle mass growth that remained comparable to the wild type ones even after 4 weeks of functional overload (127). Analyses revealed α2-AMPK activity was reduced but α1-AMPK was activated in both mice lacking LKB1 and wild type, suggesting α2-AMPK is not the most relevant in the mechanical load signaling to mTOR.

2.1.6. Regulated in development and DNA damage 1 (REDD1)

REDD1 is a stress-induced protein which gene was identified, as well as kruppel like factor 15 (KLF15), as targeted genes involved in

glucocorticoid-mediated muscle atrophy (128). Studies described crosstalk between REDD1 and other signaling pathways such as mTOR and Forkhead box O (FoxO) (129-133). In summary, expression of REDD1 results in TSC1/2 complex activity and inhibition of mTOR with triggering of an atrophy program involving transcription factor KLF15 (128). In coordination with FoxOs, KLF15 induces upregulation of MuRF1 and MAFbx (133). In a feedback, KLF15 is associated to a negative regulation of mTOR and a metabolic process through upregulation of branched-chain aminotransferase 2 (BCAT2) and branched-chain amino acid degradation (128).

2.2. Signaling pathways regulating protein degradation

Proteolytic degradation in the skeletal muscle is mediated by different processes including calcium-dependent calpains, caspases, the autophagy-lysosomal pathway and the ubiquitin-proteasome system (134). These systems are responsive to various factors including mechanical unloading, myostatin, glucocorticoids, inflammatory cytokines (tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6)), oxidative stress (reactive oxygen species and nitric oxide), metabolic stress (ATP levels) and nutrients availability (135).

2.2.1. Calpains

Calpains are calcium-dependent cysteine proteases known to contribute to disassemble myofibrillar proteins such as titin, C-protein, vinculin, nebulin and others, but they cannot degrade actin and myosin (134). Calpains are concentrated in the Z-disks (136) and activated by calcium but also physiological conditions, such as fasting, (137) and pathological

conditions, such as vitamin E deficiency (138) or Duchenne muscular dystrophy (139). Once activated, calpains cleave their substrates generating polypeptide fragments (140), that are ubiquitinated by E3 ubiquitin ligases and addressed to the proteasome (141). There are fourteen calpain members but only three are identified in skeletal muscle cells: μ-calpains (or calpain 1), m-calpains (or calpain 2) and p94 (or calpain 3) (142). μ-calpains and m-calpains are heterodimers composed of 80 and 30 kDa subunits. The 80 kDa subunit, containing the catalytic domain, binds non-covalently to the 30 kDa subunit (often called calpain 4) that contains two regulatory domains, i.e. domain V which is hydrophobic and domain VI which contains the calcium binding site (142). μ-calpains and m-calpains are expressed ubiquitously and require micro and millimolar concentrations of calcium, respectively, for their activation. For activation, p94 requires no or submicromolar calcium concentration and is particular because of its rapid and complete autolysis.

2.2.2. Ubiquitin proteasome system

The ubiquitin proteasome system is one of the catabolic pathways involved in skeletal muscle mass regulation, in conjunction with autophagy and calpains (143). The ubiquitin proteasome system is based on the ability of a short protein of 76 amino acids, known as ubiquitin, to bind covalently to cellular proteins, and the ability of a 26S proteasome complex to recognize ubiquitin chains attached to targeted proteins for ubiquitination and breakdown. Ubiquitination is the first step of degradation by the ubiquitin proteasome system made of enzymatic

cascades events. There are four groups of enzymes named ubiquitin E1 (activating enzyme), ubiquitin E2 (conjugating enzyme), ubiquitin E3 (ligases enzyme) and the uncommon ubiquitin E4, which has the ability to extend ubiquitin chains for some substrates (144, 145). In brief, E1 activating enzyme activates the carboxyl terminal end of ubiquitin forming a thiol-ester bridge with the cysteine residue of the enzyme. Activated ubiquitin is then transferred to the active-site cysteine residue of E2 conjugating enzyme. E2 finally interacts with E3 ligase enzyme, which binds to the substrate and favors transfer of ubiquitin on the substrate. While only one gene encodes E1 in mammalian cells, approximately 30 and 750 genes encode E2 and E3, respectively (146). Each E2 can interact with several E3 ligases containing recognition sites for substrates. The high number of genes encoding these ligases highlights the ability of ubiquitination for the majority of proteins (147). The most cited and studied E3 ubiquitin ligase enzymes are MuRF1 and MAFbx (85-87). Once the proteasome recognizes the substrate, the latter is de-ubiquitinated and ubiquitins are recycled for an upcoming cascade (144). De-ubiquitination enzymes regulate ubiquitin dislocation from the substrate. Approximatively 90 de-ubiquitination enzymes are identified in the mammalian genome, grouped in five families according to their catalytic domain as reviewed elsewhere (148). Recently, the promoter of the E3 ligase UBR5, a member of the homologous to E6-AP carboxy-terminus (HECT) family, was found to be hypomethylated after resistance exercise-induced hypertrophy, which was associated with increased UBR5 mRNA expression (149). Contrary to MuRF1 and MAFbx, UBR5 gene expression seems to be associated with muscle hypertrophy

(149, 150). However, the precise role of UBR5 and other HECT members in skeletal muscle mass management remains unclear (150).

2.2.3. Autophagy

Autophagy is the principal mechanism of degradation of unneeded cellular components such as damaged organelles and proteins, cytoplasmic fractions and proteins complexes. Autophagy releases mostly amino acids and probably lipids and carbohydrates that will be completely degraded or recycled in the cytosol (151). There are three main autophagy pathways: (i) micro-autophagy, which degrades cytosolic fractions by engulfment in lysosomal membranes; (ii) chaperone-mediated autophagy, which identifies targeted proteins by their KFERQ-like motifs using specific chaperones such as heat-shock cognate 70 (Hsc70); and the best-described (iii) macro-autophagy generally called abusively "autophagy" (152). The first step of (macro-)autophagy is the generation of a phagophore that will mature to become an autophagosome surrounding targeted components for degradation (65). The autophagosome fuses with the lysosome forming autolysosomes. Once activated, lysosome proteases will degrade the autolysosome content and provide recycled amino acids (153). Although it is tricky to specifically measure the autophagic flux, several markers are frequently used to assess the different steps of autophagy. AMPK, the unc-51-like kinase (ULK), the Forkhead Box O (FoxO), the micro-tubule associated protein 1A/1B-light chain 3 (Atg8/LC3) and the sequestosome 1 (p62/SQSTM1) are usually used as autophagy markers (151).

AMPK is a cellular energy sensor sensitive to AMP/ATP ratio levels as described previously in this manuscript (119-121, 154). Besides its role in energy homeostasis, AMPK is known as activator of autophagy by phosphorylation of its α chain at Thr^{172} when the AMP/ATP ratio increases, i.e. in energetic stress conditions (122, 154). Then AMPK phosphorylates and activates ULK, mostly ULK1 isoform, presumably at Ser^{317}, ^{465},^{555},^{574} and ^{777}, and Thr^{574} (155, 156). ULK1, in conjunction with autophagy-related 13 (Atg13) and focal adhesion kinase family-interacting protein of 200 DA (FIP200) form a crucial complex for autophagy initiation (157). Activated ULK1 also phosphorylates and activates the beclin-1 protein (encoded by BECN1 gene) on Ser^{15} for complete activation of autophagy (158). Beclin-1 protein association with vacuole protein sortin 34 (Vps34) and 15 (Vps15) forms a complex that, in conjunction with the activating molecule in BECN1 regulated autophagy protein 1 (AMBRA1) interacts with ULK1/Atg13/FIP200 complex to induce nucleation and the phagophore expansion (159). Conversely, phosphorylation of ULK1 at Ser^{757} prevents its activation (156). ULK1 plays a central role in the conversion of LC3-I to LC3-II (156). LC3 can be found in the phagophore, but at first, native LC3 is cleaved by autophagy-related 4 (Atg4) into LC3-I. The latter is conjugated with phosphatidylethanolamine to form a lipidated form called LC3-II. LC3-II is considered as a reliable marker of completed autophagosome formation together with an increased conversion of LC3-I in the lipidated form LC3-II and thereby augmented LC3-II/LC3-I ratio (160). p62, receiving ubiquitinated substrates, is reduced at the later phase of autophagy due to autolysosomal degradation and is then

considered as an autophagy inhibition marker when it accumulates (161). Finally, FoxO acts like ULK1 to support upregulating autophagy-genes expression in catabolic conditions such as endurance exercise (162, 163), resistance exercise (163), nutrient deprivation (164), resulting in energy stress conditions. Indeed, FoxO3 translocates to the nucleus to stimulate autophagy-gene expression when dephosphorylated (165). Akt can induce FoxO3 retention in the cytosol phosphorylating the latter at Thr^{32}, as well as AMPK inhibition by repressing its phosphorylation at Thr^{172} (166).

2.3. Contribution of satellite cells

Satellite cells are skeletal mononuclear stem cells involved in skeletal muscle repair and regeneration due to their self-renewal capabilities (1). Their role in skeletal muscle growth and development is largely described (1, 4, 35) and the literature about their involvement in mature skeletal muscle exercise-dependent regeneration is abundant (6, 167, 168). Located between the myofiber basal lamina and the plasma membrane, they provide myoblasts and myonuclei through sequential steps of activation, proliferation and differentiation (169). Physiological factors such as exercise, mechanical stretches and ageing or pathological factors such as muscular dystrophies can activate satellite cells (170-172). Once activated, satellite cells may proliferate, differentiate and fuse with preexisting myofibers to create new myonuclei or get back to the basal quiescent state (1). These steps are regulated by a family of proteins called myogenic regulatory factors (MRF) comprising myogenic factor-5 (Myf-5), myogenic regulatory factor-4 (MRF-4), myogenic

differentiation (MyoD) and myogenin (173). MyoD together with paired-box 7 (Pax7) are used as satellite cell activation markers, while Myf-5 alone can activate satellite cells in absence of Pax7 (174, 175). Late proliferation is under influence of MyoD while myogenin and MRF-4 favor differentiation (1). Those proteins are sequentially involved in satellite cell activation, proliferation, differentiation and fusion, which are also tightly regulated by signaling pathways such as Wnt, Notch and myostatin amongst others (168).

2.3.1. Wnt signaling

There is a large set of secreted lipid-modified glycoproteins named Wnt, involved in development processes of embryogenesis such as cell-fate specification, progenitor-cell proliferation and the control of cell division, but also in mature tissues to regulate tissue maintenance and remodelling (176). The Wnt pathway is an extremely dynamic, cell-type and development stage-dependent cascade, inducing intracellular responses to binding of Wnt ligands to their specific cell-surface receptors (176, 177). Wnt regulates T-cell factor/lymphoid-enhancer binding factor (TCF/LEF) transcriptional activity through a Wnt/β-catenin-dependent pathway influencing stem-cell determination, proliferation and differentiation. The Wnt/β-catenin-independent pathway is described in cell movement, polarity and various processes such as oncogenesis, gastrulation, dorsoventral patterning, tissue separation and neuronal migration (178, 179). In mature skeletal muscle, the Wnt/β-catenin pathway is implicated in Pax7 expression and

myogenic differentiation through interactions with MyoD, independently of TCF/LEF (180).

2.3.2. Notch Pathway

The Notch signaling pathway comprises core components made of upstream Jagged or Delta-type ligand, a Notch-type receptor and a transcription factor of the CBF1/Su(H)/LAG1 (CSL) family that functions downstream (181). Binding of Delta-ligand to Notch-receptor leads to intracellular domain, named Notch intracellular domain (NICD), cleavage and activation. Activated NICD migrates into the nucleus to remove co-inhibitors and recruit co-activators of CSL transcriptional factors among which hairy/enhancer of split (Hes) and Hey *basic helix loop helix* co-factors (182). In regenerating skeletal muscle, early data postulated that Notch1 activation leads to satellite cells activation and proliferation and differentiation repression (183, 184) but Kitamoto et al. (2010) showed that Notch3 negatively regulates both proliferation and self-renewal of activated satellite cells (185). Thus, Notch signaling pathway contributes to muscle homeostasis during muscle growth and repair process.

2.3.3. Myostatin signaling

Myostatin is a negative regulator of muscle mass, member of the transforming growth factor-β (TGF-β) signaling pathway (186). The TGF-β family consists of over 50 ligands, which are divided in two functional sub-groups: bone morphogenetic protein/growth and differentiation factor (BMP/GDF) and TGF-β/activin (187). TGF-β/activin contains TGF-β1, TGF-β2, TGF-β3 and activins and controls

processes of myotube formation, maintenance of motor neurons and fibrosis development (188, 189). BMP/GDF comprises BMP2, BMP4, osteogenin and myostatin implicated in various processes from cartilage and bone development to organogenesis of heart and kidney and neuronal differentiation (190-192). Briefly, the TGF-β pathway consists of binding of the ligand to type II receptor that associates with corresponding type I receptor forming a heterotetrameric receptor complex. The heterotetrameric complex latent kinase activity is then activated and phosphorylates a receptor-regulated Smad protein, the latter oligomerizes with a co-Smad or Smad4. The Smad oligomere translocates into the nucleus to interact with binding partners and regulate transcription by activation or auto-inhibitory feedback loop (193). The myostatin gene is expressed in skeletal muscle, heart and adipose tissue (194, 195) and homozygous myostatin-null mice show increased muscle mass due to both hyperplasia and hypertrophy and reduced adipose tissue (194, 196). The hypertrophy observed in homozygous myostatin-null mice may be the result of increased activation of satellite cells and fusion of increased number of myonuclei (197) and by increasing translational efficiency (198).

2.3.4. p38 MAPK signaling pathway

p38 is part of the MAPK signaling cascade and exists in four isoforms: p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12) and p38δ (MAPK13) that can be phosphorylated and mainly activated by MAPK kinases MKK6 and MKK3 (199). While p38α, the best characterized (200), is ubiquitously expressed at high levels in most cell types, p38β is

expressed at lower levels in brain whereas p38γ and p38δ have both restricted expression patterns (201). Using SB203580, an inhibitor of p38α/p38β, a pyridinyl imidazole anti-inflammatory drugs of the cytokine-suppressive anti-inflammatory drugs (CSAIDs), studies highlighted the role of p38α and p38β in myoblasts differentiation (202, 203). The subcellular nuclear localization of p38α upon activation seems to be mediated by MAPK-activated protein kinase 2 (MK2) (204). Recently, expression of the stress-inducible 70-kDa heat shock protein (Hsp70) has been shown to increase during differentiation at both transcriptional and translational levels with upregulation in both cytosol and nuclei (205, 206). Therefore, Hsp70 has been proved to interact with MK2 to regulate p38 stability to induce promyogenic factors in nuclei during myogenesis (205).

2.4. Additional pathways implicated in skeletal muscle mass control

Other signaling pathways are associated to skeletal muscle mass regulation but their contribution is not fully elucidated.

2.4.1. Androgens anabolic actions

Androgens are hormones mainly responsible of secondary sex characteristics including muscle mass regulation (207) that are synthesized in adrenal cortex, testes and in lesser extent locally in skeletal muscle from precursor androgens such as Dehydroepiandrosterone (208, 209). It contributes to muscle mass regulation through various mechanisms (210). Directly, androgens bind to androgen receptor that is a ligand-inducible transcription factor able

to bind specific DNA sequences named androgen response elements and recruit coactivators to influence the transcription of target genes in reproductive tissues but this mechanism remains unclear in skeletal muscle (211). Indirect regulation involves crosstalks with IGF-I (212, 213), PI3K/Akt (214) and Myostatin (215). Administration of androgens resulted in muscle fibers hypertrophy mediated, at least in part, by IGF-I (212, 213). In male mice, castration resulted in body weight loss compared to controls. This loss was almost reversed after 42 days of nandrolone administration (207). Moreover, castration was accompanied by downregulation of phosphorylated Akt Ser^{473} and mTOR Ser^{2248} as well as their downstream target S6K1 Thr^{389} and 4E-BP1 $Thr^{37/46}$ (207). Castrated mice treated with nandrolone over a month elicited higher activation of mTOR pathway than controls and castrated ones (207). Finally, in muscle satellite cell-specific androgen receptors knocked out mice, Myostatin gene and protein expressions were downregulated (215). Androgen receptors expression was shown to be expressed mainly in muscle satellite cells and myonuclei (216). Moreover, testosterone administration induced androgen receptors upregulation in satellite cells and myonuclei in human (216). In summary, the effects of androgens on skeletal muscle are mediated by various crosstalks with other signaling pathways, even if mechanisms are still to be elucidated.

2.4.2. Glucocorticoids pathway

Glucocorticoids are steroid hormones physiologically secreted from the adrenal glands in response to physiological and pathological conditions

such as fasting, exercise and illness (217). Glucocorticoids can also be given as drug to treat inflammation (218). Whether locally secreted or given as anti-inflammatory agents, glucocorticoids are well known for their catabolic effects on skeletal muscle (219). This effect is the result of downregulated protein synthesis processes while catabolic pathways are activated (220, 221). To achieve this goal, there are crosstalks between glucocorticoids and other regulatory pathways of protein turnover including PI3K/Akt, mTOR, FoxO, GSK3β and Myostatin among others (128, 222, 223). In brief, glucocorticoids inhibit the transport of amino acids into the muscle, and repress the IGF-I and amino acids stimulating effect on the phosphorylation of mTOR effectors S6K1 and 4E-BP1 (224, 225). Moreover, glucocorticoid-induced activation of REDD1 and KLF15 leads to mTOR and its effectors inactivation (128, 226). The catabolic effect of glucocorticoids goes through the activation of the breakdown pathways of muscle proteins, namely the ubiquitin proteasome system, the calcium-dependent and the autophagy pathways (134). To achieve this effect, glucocorticoids activate KLF15 and FoxO transcription factors that cooperate to upregulate the expression of muscle catabolism genes (128).

2.4.3. Serum response factor (SRF) pathway

Using muscle-specific SRF knockout mice, the transcription factor SRF was found to be critical for muscle growth (227, 228). The effect of SRF seems to be mediated in a paracrine fashion, with release of interleukin 4 and 6 to favor satellite cells proliferation and fusion in response to overload-induced hypertrophy by synergist ablation (229).

2.4.4. Hippo pathway

First described in drosophila (230), the Hippo signaling pathway is evolutionary conserved (231) and described in mammals (232). Some mammalian orthologs of the components of Hippo pathway are thought to be involved in organ size regulation and many researchers investigated the role of Hippo pathway in mammals (233, 234). Macrophage stimulating protein 1 and 2 (Mst1/2) and large tumor suppressor kinases 1 and 2 (Lats1/2), in conjunction with adaptor proteins Salvador (Sav) and Mob1, are activated to phosphorylate and inhibit Yap and Taz, both transcriptional co-activators (235, 236). When dephosphorylated, Yap and Taz bind to TEAD transcription factors in the nucleus and regulate metabolic, proliferative and anabolic genes expression (237, 238). Although blurred, growing evidence suggests a hypothetical implication of this signaling pathway in the regulation of muscle mass in mammals (235, 239, 240). We aimed to describe and discuss available data concerning Hippo pathway involvement in mammals skeletal muscle mass regulation in Chapter II.

3. Hypoxia and skeletal muscle mass regulation

3.1. Hypoxia

Hypoxia is a state of lowered oxygen tension observed in physiological conditions like high altitude (ambient hypoxia) and exercise (241) or in diseases such as chronic obstructive pulmonary disease (COPD), chronic heart failure and obstructive sleep apnea (242). As part of this thesis, we will focus on hypoxia in sport context in healthy subjects. Hypoxia can be normobaric with lowered fraction of oxygen less than 21%, which can be

found in a hypoxic chamber, or hypobaric due to a lowered partial pressure of oxygen as it is the case at altitude. Either normobaric or hypobaric hypoxia eventually results in reduced oxygen availability at tissue and cellular levels (243). Different modalities of exposure to hypoxia exist from single acute and intermittent exposure to chronic and permanent exposure. Three situations of intermittent exposition to hypoxia are described: (i) *live high-train high (LH-TH)* consists of altitude camps where subjects living and training at moderate altitude for several weeks; (ii) *live high-train low (LH-TL)* involves living at moderate altitude with low intensity training at moderate altitude combined with high intensity training at lower altitude or sea level, and finally; (iii) *live low-train high (LL-TH)* implies exposure to hypoxia lasting from seconds to hours that can be repeated over several days with recovery phase in normoxic conditions. This type of hypoxic training modality is also call *intermittent hypoxic training (IHT)* (244). In Chapter IV, the latter hypoxic training modality was chosen.

In practice, hypoxic conditions can be created by several methods divided in two major types: localized hypoxia (245, 246) and systemic hypoxia (247-249). Localized hypoxia uses blood flow restriction (BFR) by inflatable cuff or elastic wraps to limit distal blood flow inducing regional hypoxia limited to a limb during exercise (250). However, it has to be recognized that other physiological responses are created concomitantly to localized hypoxia with this system. Systemic hypoxia can be generated in a room facility in which oxygen fraction or partial pressure can be reduced (247) or using a face mask lowering the oxygen fraction in the inspired air (251). Systemic hypoxia offers the possibility

to train trunk and limbs musculature at the same time, providing a strategy for individuals requiring development of these muscles (247).

In the skeletal muscle, hypoxia definition is less obvious because of lack of consensus threshold in muscular partial pressure (252) knowing that resting intramuscular PO_2 in normoxic conditions is near 27 mmHg (253). However, exercise seems to more efficient to disturb intramuscular PO_2 as exercise can induce greater decrease of PO_2 to near 92% in normoxic conditions (254) while exposure to hypoxia induce decrease of 37%-63% (255, 256).

3.2. HIF pathway

Hypoxia exposure leads to activation of the hypoxia-inducible factor-1 (HIF-1) pathway in many cell types and skeletal muscle (241, 257). Concretely, at the cellular level, hypoxia can be seen as a condition of low O_2 pressure sufficient to induce HIF-1 stabilization and accumulation in the cells (252). HIF-1 is a heterodimeric complex with an hypoxically inducible subunit HIF-1α and a constitutively active subunit HIF-1β (258). Contrary to HIF-1β, HIF-1α has a short half-life (less than 5min) and is regulated by oxygen availability. In normoxic conditions, HIF-1α is constitutively produced but quickly degraded via the ubiquitin-proteasome system involving the von Hippel-Lindau tumor suppressor gene (VHL) (259). In hypoxic conditions, HIF-1α hydroxylation by prolyl hydroxylase domain (PHD) leads to disruption of HIF-1α from VHL, HIF-1α stabilization and translocation to the nucleus where it dimerizes with HIF-1β, forming the transcriptionally active HIF complex (259). By binding to the hypoxia-response element (HRE) in the nucleus (Fig. 2),

the HIF complex, can induce transcription of more than 100 downstream genes with various functions such as erythropoiesis, angiogenesis, glucose metabolism, cell proliferation and apoptosis (260).

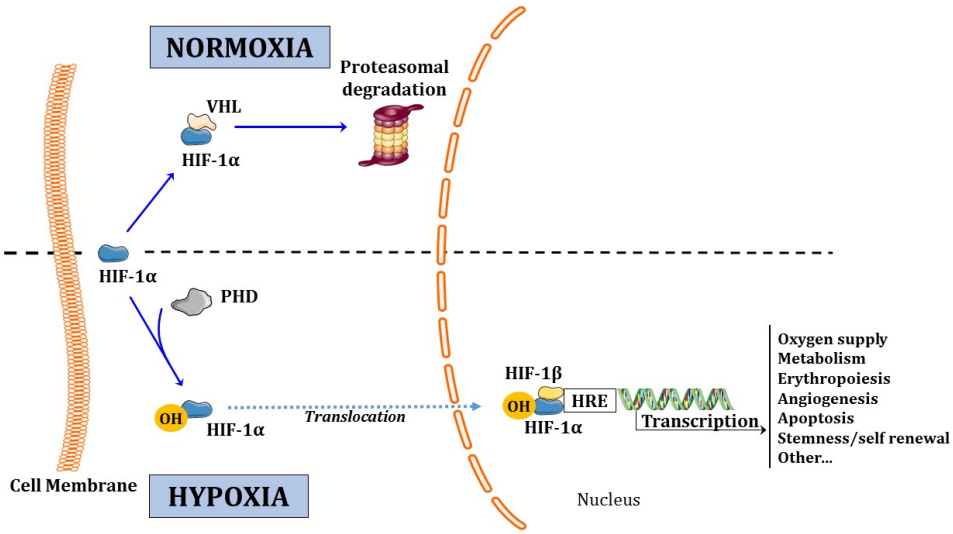

Figure 2. The HIF-1α signaling pathway

Many methods including genome-wide analyses, proteomic analyses and chromatin immunoprecipitation (ChIP) (257, 261, 262) allowed identification of several HIF target genes involved in oxygen supply (263).

HIF-1α is known to promote cell survival by switching from oxidative to glycolytic metabolism, due to regulation of genes providing glucose to pyruvate with decreased pyruvate dehydrogenase kinase 1 (PDK1) mRNA levels and diminished mitochondrial oxygen consumption (264). In order to coordinate efficient use of energy, HIF-1 regulates many genes inducing shift from high oxygen demand toward glycolysis (261,

263, 265-267). Many other genes involved in cellular processes like cell proliferation, stemness/self-renewal, plasma development, apoptotic and anti-apoptotic processes were all shown to be downstream targeted genes of HIF-1 (257, 265). Other genes involved in metastasis, redox homeostasis, inflammation and immunity (see Table 1) are also under the regulation of HIF-1 (268).

Table 1: Compilation of few known HIF-1 targeted genes. The genes mentioned in this list were identified based on both the consensus sequences within HRE and experimental analyses (257, 260-271)

Category	Genes (abbreviations)	Functions
Oxygen supply	*Erythropoietin (EPO)*	Erythropoiesis
	Transferrin (Tf)	Iron transport
	Transferrin receptor (Trf)	Iron transport
	Vascular endothelial growth factor (VEGF)	Angiogenesis
	Ceruloplasmin (CP)	Iron oxidase
	Leptin (LEP)	Angiogenesis
	Plasminogen activator inhibitor-1 (PAI-1)	Blood flow
	Endothelial nitric oxide synthase (eNOS)	Vessel diameter
Metabolism	*Lactate dehydrogenase kinase (LDH)*	Glucose metabolism
	Pyruvate dehydrogenase kinase (PDHK)	Glucose metabolism
	Glucose transporter 1 (GLUT1)	Glucose metabolism
	Phosphofructokinase (PFK)	Glucose metabolism
	Hexokinase 1 (HK1)	Glucose metabolism
	Hexokinase 2 (HK2)	Glucose metabolism
	Aldolase (ALD)	Glucose metabolism
	Glyceraldehyde phosphate dehydrogenase (GAPDH)	Glucose metabolism
	Phosphoglycerate kinase 1 (PGK 1)	Glucose metabolism
	enolase 1 (ENAO-1)	Glucose metabolism
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-3 (PFKFB3)	Glucose metabolism
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-4 (PFKFB4)	Glucose metabolism
	Phosphoglucomutase (PGM)	Glucose metabolism
Cell proliferation	*Insulin-like growth factor-2 (IGF-2)*	Growth factor
	IGF-factor binding protein 1 (IGF-BP1)	Growth factor
	IGF-factor binding protein 2 (IGF-BP2)	Growth factor
	IGF-factor binding protein 3 (IGF-BP3)	Growth factor
	Connective tissue growth factor (CTGF)	Growth factor
Stemness/self-renewal	*Adrenomedullin (ADM)*	Neural stemness
	Endothelin 1 (EDN1)	Cardiac myocytes
	Glycoprotein 1 (GPI)	Differentiation
	Inhibitor of DNA binding 2 (ID2)	Stem cells renewal
	Octamer-binding transcription factor 4 (OCT4)	Stem cells renewal
	Telomerase reverse transcriptase (TERT)	Telomerase
Others	*Transforming growth factor β3 (TGF-β3)*	Plasma development
	Intestinal trefoil factor (ITF)	Intestinal barrier
	Ecto-nucleotidase(CD73)	Intestinal barrier
	Chemokine receptor type 4 (CXCR4)	Cell growth
	c-MET	Proto-oncogene
	Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNIP3)	Pro-apoptotic
	Noxa	Pro-apoptotic
	Protein phosphatase type 5 (PP5)	Anti-apoptotic
	Myeloid cell leukemia 1 (Mcl-1)	Anti-apoptotic
	Nucleophosmin (NPM)	Anti-apoptotic
	Lysis oxidase (LOX)	Metastasis
	Matrix metalloproteinase (MMP1)	Metastasis
	TWIST	Metastasis
	Superoxide dismutase 2 (SOD2)	Development
	Heme oxidase 1 (HMOX1)	Redox homeostasis
	CD18	Inflammation

3.3. Hypoxia and the regulation of skeletal muscle mass

Few studies described the effects of exposure to both acute and chronic hypoxia alone on skeletal muscle mass (272).

3.3.1. Acute exposure to hypoxia

Muscle protein synthesis rates was measured after passive (air group) or active (foot group) ascent from low (550m altitude) to high altitude (4.559m altitude) to study effects of acute hypobaric hypoxia and physical exercise (273). 19-23h after arrival at high altitude, fractional rate of muscle protein synthesis increased in the foot group (35%) while it remained unchanged in the air group (2%), pointing effect of physical exercise in muscle protein synthesis in acute hypobaric hypoxia (273). Comparing hypoxia (12%) to normoxia, Etheridge et al. (2011) investigated effects of hypoxia on myofibrillar protein synthesis at both rest and after resistance exercise (274). Contrary to Imoberdorf et al. (2006) (273), they observed that hypoxia did not affect significantly muscle protein synthesis in the hypoxic group while a significant increase was obtained in the normoxic group (274). Exposure to 4h hypoxic conditions after a meal induced higher responses of both Akt and S6K1 related to increased plasma insulin concentrations compared to normoxic conditions (275). The opposite effects of hypoxia observed could be related to the type of hypoxia (hypobaric *vs* normobaric), the duration of exposure (two days *versus* 3.5 hours) and the type of physical activity (walking *vs* unilateral leg extension).

3.3.2. Chronic hypoxia

Hoppeler et al. (1990) investigated the morphologic adaptations to hypoxia in subjects exposed to 5350m of altitude for 8-10 weeks. A 20% reduction in mean CSA of *m. vastus lateralis* and a 10% reduction in capillary to fiber ratio was measured (276). MacDougall et al. (1991) found concordant results examining *m. vastus lateralis* samples of subjects submitted to 32 days in gradually decompressed chamber to a simulated altitude of near 6700m, where they remained for 4 days. Muscle volume was reduced by 13% in the arm and by 15% in the thigh. The mean fiber area was decreased by 25% in type I fibers and by 26% in type II fibers, although fiber type distribution remained unchanged (277). A trend towards an increase in capillary density was observed as the result of the decrease in muscle fiber area, with no synthesis of new capillaries. Mitochondria density and mitochondria to myofibrillar volume density ratio increased by 8% and 14%, respectively (277). Attempting to highlight the mechanisms underlying the effect of chronic hypoxia on skeletal muscle turnover, Chaudhary et al. (2012) found a decreased skeletal muscle mass of 34% after 14 days of exposure to hypobaric hypoxia (7.620m altitude) in a murine model (278). Both protein synthesis and degradation increased in the hypoxic group but degradation was higher than synthesis resulting in protein depletion with 5-fold more ubiquitinated proteins and a 2.7-fold activation of μ-calpains. At the end of the hypoxic exposure, a reduction of total (22%), myofibrillar (30%) and sarcoplasmic (7%) protein levels was measured in skeletal muscle (278). In human, progressive exposure to hypoxia (up to 3.200m altitude) over 15 days did not modify myofiber CSA or

signaling pathways associated with both protein synthesis and degradation. In addition, a very high interindividual response was noted (279). Cell proliferation markers such as proliferating cell nuclear antigen (Pcna) and Myf5 mRNA were downregulated while other MRFs (myogenin, MRF-4 and MyoD) remained unaffected, indicating no formal changes in protein content (279).

4. Resistance exercise/training

4.1. Definition and determinants

Resistance exercise, or strength exercise, is the action of a muscle exerting a force against a load above the aerobic capacity of the muscle; commonly high intensity, short lasting, fatiguing muscle contractions (64). The combination of the weights, repetitions per set, number of sets and duration of the rest period are major determinants for the exercise/training dose and thereby for the training effect (280, 281). Motor units recruitment follows the size principle with the smallest motor units recruited first during a movement and with the largest motor units recruited when force production increases (282). The size principle is the basis of statement according to which heavy loads are necessary for muscle adaptations (282). Mechanical load is then efficient at inducing the hypertrophic response through a phenomenon named mechanotransduction. It is the conversion of mechanical loading into chemical signals by mechano-sensors such as integrins and focal adhesion proteins (283). The mechanical load is determined by the

weight lifted, the repetitions per set, the number of sets and the duration of the rest period.

The chemical signals generated stimulate both anabolic and catabolic pathways leading to skeletal muscle regulation (283). Heavy weights, above 70% of one-repetition maximum (1-RM) were suggested to result in muscle hypertrophy and strength improvement (281, 284, 285).

Another important parameter to take into account is the maximal number of repetitions performed before fatigue occurrence that limits additional repetition and which is inversely proportional to the intensity (% 1-RM) of the exercise (280). Thus, the number of repetitions performed to fatigue is important to design appropriate strength training program and increasing the number of repetitions will lead to lowering the weight lifted. Multiplying the weight lifted by the total number of repetitions (volume) gives the load of a session (280).

In addition to training intensity and training volume, the rest duration between the sets has to be taken into account for inducing a specific training effect. Inter sets rest of 60-120 seconds are usually recommended for novice and intermediate training programs while 120-240 seconds are recommended for advanced training program (281).

The right frequency of training sessions is important to allow rest periods permitting muscle recuperation and development and avoid overtraining of a targeted muscle group (280, 286). It has been shown that training of leg extension muscles for 3 days per week, which corresponds to 48-hour rest between training sessions, was more effective than 1 or 2 days per week at gaining muscle strength (287). A

48-hour rest between training sessions was then recommended to novices with reduced rest for intermediate and advanced training but this recommendation has been recently questioned. Specifically, Barcelos et al. (2018) showed that a protocol of resistance training 5 times a week did not provide additive gains in strength and hypertrophy when compared with protocols of resistance training 2 or 3 times a week (288). In summary, it seems more suitable to consider the training load (volume x intensity) more than the frequency and to take into account intersubject variability, individual training levels and goals (289, 290).

4.2. Resistance exercise/training and the regulation of muscle mass

4.2.1. Resistance exercise/training and protein balance

Muscular adaptations are relative to the specific mode of training and resistance training is recognized to promote both muscle hypertrophy and strength (167, 291).

Acute resistance exercise

Different intensities (ranging from 60% to 90% of 1-RM) of acute bouts of resistance exercise induce protein synthesis and anabolic signaling peak 1h post-exercise in a dose dependent-manner and return to basal values within 3h post-exercise (292). Whilst protein synthesis remains elevated up to 24h post-exercise when resistance exercise is coupled with nutrients (293, 294). However, even performed against low loads (30% of 1-RM), resistance exercise is capable to enhance protein synthesis when performed to failure (294). In fact, resistance exercise performed in fasted state promote slight and non-significant protein

breakdown (295). Ingestion of nutrients and resistance exercise independently can stimulate protein synthesis but this effect is transitory while combination of both has optimizing effect on protein synthesis (296). It appears that anabolic processes remained sensitive to nutrients, particularly to amino acids, for a 90mn open-window post-exercise, the so-called "muscle full" response (297). Input of amino acids in this open-window enhance protein synthesis while protein degradation remain unchanged compared with basal levels achieving a positive net balance and net gain in muscle (298, 299). It therefore undeniable that acute resistance exercise positively alter protein balance favoring protein synthesis more than breakdown and ingestion of nutrients such as amino acids enhances this anabolic effect (300).

Resistance training

Much is known about muscle hypertrophy and fiber cross sectional area increase induced by resistance training but only few tracked protein turnover at multiple times throughout an extended training period (301). Wilkinson et al. (2014) validated efficacy of deuterium (D_2O) to determine the temporal synthesis of muscle protein, at rest and in response to short-term resistance training lasting eight days (302). Applying a protocol made of 4 sessions (4 sets of 8 repetitions against 80% of 1-RM) of unilateral knee extension, they observed increase of 36% of the protein synthetic response above the rested leg at 2-4 days and rapid decrease of 40% at 4-8 days (302). Brook et al. (2015) studied the link between protein synthesis, anabolic signaling and hypertrophy in a 6 weeks unilateral knee extension protocol (3 sessions per week of

6 sets of 8 repetitions against 75% of 1-RM) (303). Muscle protein synthesis was higher at 0-3 weeks than 3-6 weeks and absolute protein synthesis rate increased while absolute rate of protein breakdown remained unchanged in the trained leg (303). In order to evaluate the contribution of protein synthesis to muscle hypertrophy, Damas et al. (2016) measured myofibrillar protein synthesis the days following exercise (304). During 10 weeks-resistance training of bilateral leg-press and leg extension with two training sessions per week, myofibrillar protein synthesis was measured at baseline (T1), at the third week (T2) and the tenth week (T3). Compared to T1, they observed increased CSA of 2.7% and 10.4% at T2 and T3 respectively while myofibrillar protein synthesis was higher at T1 and unchanged at T2 and T3 (304). Finally, a 20 weeks resistance-training program performed 3 times per week showed significant improved muscle protein synthesis of 30% when resistance training is combined to nutrients in young subjects (305).

At the molecular level, it has largely been described that resistance exercise activates protein synthesis and the mTOR pathway concomitantly (109, 306), though with some exceptions (307). For example, a resistance exercise session made of 8 sets of 5 repetitions of leg extension with inter set rest of 180 seconds against 80% of 1-RM induced an increase in Akt phosphorylation by 3-fold at Thr^{308} after 30 minutes and by 2-fold at Ser^{473} after 60 minutes of recovery. Moreover, phosphorylation of S6K1 at Thr^{389} increased by 2.5-fold after 60 minutes of recovery while 4E-BP1 remained unaffected (163). Another protocol consisting of 8 sets of 10 repetitions of leg extensions at 70% 1-RM induced an increase in, Murf-1 mRNA expression 3h post-exercise, which

returned to basal values by 24 hours post-exercise, despite a decrease in FoxO3a phosphorylation at Ser^{253} over time (295). Strength exercise protocol made of 8 repetitions of leg press, extension, curl and calf press in young subjects induces increased phosphorylation of mTOR at Ser^{2448} 1h and 3h post-exercise before returning to baseline 6h later (308). Akt phosphorylation at Ser^{473} follows the same trend but returns back earlier 3h post-exercise to baseline values (308). Temporal analysis of anabolic signaling during a 6 weeks unilateral knee extension protocol (3 sessions per week of 6 sets of 8 repetitions against 75% of 1-RM) revealed significant increase of S6K1 at Thr^{389} and S6 at $Ser^{240/244}$ phosphorylation and an enhanced tendency of mTOR phosphorylation at Ser^{2448}, only after the first exercise bout (303). The reduced anabolic signaling suggests skeletal muscle is efficient in rapid remodeling becoming less sensitive to mechanical and hormonal stress.

4.2.2. Resistance exercise/training and satellite cells

Exercise, and resistance exercise in particular, is known to activate satellite cells (167, 171). Resistance exercise and training were shown to promote increased number and activation of satellite cells in function of the training volume and intensity. The higher the load of the training is, the larger these effects are, suggesting that the larger increase of the CSA of the knee extensor to high training load depends on the higher activation of satellite cells (167, 309).

Acute resistance exercise

Psilander et al. (2003) measured an increase (110%) of MyoD mRNA immediately post-exercise followed by 120% increase of Mrf4 mRNA 2h

post-exercise and finally 440% increase of myogenin mRNA 6h post-exercise (310). Myogenin and Mrf4 mRNA expression levels remained elevated 24h post-exercise (310). Yang et al. (2005) analyzed skeletal muscle samples taken over a 24-hour period after acute exercise composed of 3 sets of 10 repetitions at 70% of 1-RM of bilateral knee extension or 30 minutes of treadmill running at 75% of maximal O_2 uptake (VO_2 max). They observed increased Mrf4 mRNA 2h post-exercise and a peak at 4h, increased myogenin mRNA 8h and 12h post-exercise and finally increased MyoD mRNA 8h post-exercise, while Myf5 mRNA stayed unchanged from pre-exercise in the resistance group (309). In the running group, only MyoD mRNA peaked 8h post-exercise and remained elevated 12h post-exercise (309). Given the differences between both protocols (resistance exercise *versus* running), it was unwise to compare the effects of both protocols on satellite cells regulation. An increased expression of Pax7, a marker for satellite cell activation, was found from baseline to 6h and 24h post-exercise as well as an increased satellite cell number at 6h and 24h post-exercise after acute resistance exercise against 70% of 1-RM (311). Aiming to study the effects of various intensities of acute resistance exercise on MRF mRNA levels, Wilborn et al. (2009) submitted untrained subjects to either moderate-intensity (60%-65% of 1-RM) or high-intensity (80%-85% of 1-RM) leg extension and leg press. MyoD, myogenin, Mrf4 and Myf5 mRNA expression were all upregulated over the course of 6 hours after exercise independently of the exercise intensity. It was then concluded that resistance exercise ranging from 60% to 85% of 1-RM is effective at regulating genes involved in myogenesis in skeletal muscle (312). Lim et al. (2017)

compared activation of satellite cells following acute resistance exercise and combined resistance and plyometric exercises, using Ki67/CD56 expression as satellite cells activity marker. They observed an activation of satellite cells of 232% and 165% in the resistance and the combined groups, respectively (313). The same tendency was observed after both acute resistance exercise and 16 weeks of progressive (from 2 sets at 70% of 1-RM to 4 sets at 80% of 1-RM) resistance training (314). Positive correlations were found between changes in the activation of satellite cells associated with both type I and type II fibers between 24h and 72h after acute exercise and changes in *m. quadriceps* volume (314). Finally, resistance exercise induced concomitant decreased expression of myostatin, a known inhibitor of satellite cells, and activation of satellite cells 24h and 72h post-exercise (314).

Resistance training

Analysis of biopsies taken at pre, after 2 and 11 weeks training from two groups , the 3L-1UB group performing multiple sets leg exercises and a single set upper body exercises while the 1L-3UB group realized a single set leg exercises and multiple sets upper body exercises, showed increased satellite cells in *m. vastus lateralis* of the 3L-1UB group and only a tendency in *m. trapezius* (167). Furthermore, an effect of training volume on satellite cells number in the thigh muscle was detected (167). Attempting to describe early- and later-phase satellite cells responses, Damas et al. (2018) studied changes in fiber types content before and after a 10 week-resistance training program. The training consisted in 3 sets of 45° leg-press followed by 3 sets of leg extensions with 90 seconds

of inter sets rest. The sets were made of 9-12 maximum repetitions performed to volitional fatigue against adjusted loads through training sessions. Similarly to previous results, an activation of satellite cells was observed in both types of skeletal muscle fibers after the first session and an increased content 24h to 72h after (315). There are evidences of satellite cells regulation by resistance exercise but the real implication of activated satellite cells in skeletal muscle mass accretion remains controversial. Indeed, McCarthy et al. (2011) developed skeletal muscle satellite cells-null mice submitted to overload induced-hypertrophy by synergistic ablation. They observed a hypertrophic response that was maintained for 6 weeks after synergistic ablation in the satellite cell null mice (316). It was concluded that satellite cells were not required for muscle hypertrophy, only for new fibers formation or fiber regeneration. In a similar study, Egner et al. (2016) obtained conflicting results as depletion of satellite cells prevented overload hypertrophy. Moreover, overload did not induce changes in the number of myonuclei in satellite cell-null mice contrary to controls (317). In conclusion, the role of satellite cells in muscle hypertrophy is still under debate.

5. Resistance exercise in hypoxia

Originally, resistance training was performed at altitude to preserve muscle mass and strength during long lasting expeditions. Weight loss is often measured after a few days at altitude (249). This loss seems to be partially the result of muscle fiber atrophy, which is independent of physical activity level, even though physical activity and diet can

attenuate this adverse effect of altitude (249). Narici et al. (1995) compared body composition and elbow flexor strength at both altitude (5050m) and sea level after a month of resistance training program made of six series of ten flexions against 75% of 1-RM four times per week (291). They found greater elbow flexor CSA and force gain at sea level than at altitude suggesting either a direct effect on protein balance and/or hormonal changes induced by hypoxia (291).

Nowadays, the use of hypoxic resistance training has evolved as it is now possible to perform resistance training in hypoxic facilities at sea level. This allows athletes to better calibrate the hypoxic dose and duration. It has been found that when performed intermittently with a moderate hypoxic dose, hypoxic resistance training could offer some advantages upon normoxic resistance training, amongst which a higher muscle mass gain (318). While largely used by athletes nowadays, the number of studies having attempted to optimize hypoxic resistance training and to decipher the underlying molecular mechanisms is relatively low.

The following paragraph will present the studies having investigated the effect of hypoxic resistance training on body composition, hormonal responses and muscle adaptations at structural, functional and molecular levels (248, 319). Four weeks of leg extension and flexion resistance training against 30% of 1-RM performed under normobaric hypoxia (FiO_2 of 12%) or normoxia revealed increased strength endurance in both normoxic and hypoxic groups (319). No differences in fiber type distribution or fiber/muscle CSA was found after training in both groups. However, correlations were found between the pre to post

training changes in the mRNA levels of markers of hypoxia (Vegf) and glycolytic enzymes (Pfk and LDH B) in the hypoxic group only (319). Investigating angiogenic response and muscular endurance under systemic hypoxia (FiO_2 of 14.4%), Kon et al. (2014) reported increased lean body mass with improved strength and CSA in both normoxic and hypoxic groups after an 8 week-training program, with no difference between the 2 groups. However, circulating VEGF levels and the capillary-to-fiber ratio in the hypoxic group was higher at week 8, comparatively to the normoxic group (248). Kurobe et al. (2014), obtained greater muscle hypertrophy in the hypoxic group (FiO_2 of 12.7%) compared to the normoxic one, when trained against their respective 10-RM during eight weeks. Moreover, serum growth hormone and cortisol levels significantly increased in the hypoxic group, comparatively to the normoxic one (320). Contrary to the previous results, 6 weeks of back squat training against 10-RM under hypoxic conditions (FiO_2 of 15%) failed to favor both muscle strength and hypertrophy (321). These results show the metabolic and vascular effects of hypoxia while the hypertrophic effects are fuzzy regardless of the workload and the intensity of hypoxia. In addition, the trained body segment appears to be important since these hypertrophic effects have only been observed in the upper limb (320).

Only one study has attempted to determine the molecular adaptations induced by hypoxic resistance exercise. Muscle protein synthesis was increased after one acute bout of resistance exercise in normoxia but not in hypoxia, despite similar S6K1 phosphorylation at Thr^{389} 3h post-exercise in both groups. Interestingly, individual muscle protein

synthesis responses to resistance exercise were correlated to the mean hypoxic SpO_2 during exercise, with the higher SpO_2 correlated with the higher synthetic responses and inversely (274). Using a murine model, Chaillou et al. (2012) observed that hypoxia reduced overload-induced hypertrophy despite no alteration of phosphorylation of Akt at Ser^{473} and mTOR at Ser^{2448} at day 5 of exposure. However, mTOR phosphorylation was highly reduced by hypoxia at day 12. Similarly, S6K1 phosphorylation at Thr^{389} as well as S6K1 total protein expression were impaired at days 5 and 12. Finally, 4E-BP1 phosphorylation at Thr^{70} remained unaffected by hypoxia (322). Looking at protein degradation markers, the mRNA levels of Mafbx and Murf-1 were increased by hypoxia only after 5 days of overload-induced hypertrophy (322).

In conclusion of those 2 studies looking at molecular adaptations to hypertrophic stimuli in hypoxic conditions, it seems that the anabolic response is rather blunted than stimulated. However, neither of those 2 studies reflects the conditions in which athletes train as the volunteers (274) and the rats (322) recovered from the anabolic stimulus in hypoxic conditions while athletes usually recover in normoxic conditions. In addition, Etheridge et al did not care about protein provision after resistance exercise, which is of the upmost importance to favor protein accretion.

6. Aim of the thesis

For the aforementioned reasons, we decided to conduct the present thesis aiming to investigate the molecular mechanisms underlying the regulation of skeletal muscle mass after resistance exercise under hypoxic condition via endogenous local progenitors and the Hippo pathway. We hypothesized that acute hypoxia would potentiate the anabolic response of resistance exercise by: 1) increased protein synthesis and decreased breakdown, 2) enhanced exercise-induced muscle anabolic signaling, and 3) enhanced exercise-induced satellite cells activation with reduced Hippo pathway activation. To achieve this goal, we conducted a two-step process:

In Chapter II, we review the available literature concerning the regulation of skeletal muscle mass by the recently described Hippo pathway in mammals to highlight the regulatory mechanisms. This part was required for a deep analysis of the regulatory mechanisms triggered under hypoxic conditions. Growing evidence shows that the Hippo pathway could contribute to the regulation of muscle mass. Whether this occurs via a regulation of satellite cells or not is still unknown and deserves further investigation.

The Chapter III is devoted to the study the molecular events occurring after a single resistance exercise performed under hypoxia with a particular focus on endogenous progenitor's regulation.

CHAPTER II: HIPPO PATHWAY AND SKELETAL MUSCLE MASS REGULATION IN MAMMALS: A CONTROVERSIAL RELATIONSHIP

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Abstract

Skeletal muscle mass results from the balance between protein synthesis and protein degradation. In addition, satellite cell inclusion may contribute to increase muscle mass while fiber loss results in a reduction of muscle mass. Since 2010, a few studies looked at the involvement of the newly discovered Hippo pathway in the regulation of muscle mass. In line with its roles in other organs, it has been hypothesized that the Hippo pathway could play a role in different regulatory mechanisms in skeletal muscle as well, namely proliferation and renewal of satellite cells, differentiation, death and growth of muscle cells. While the Hippo components have been identified in skeletal muscle cells, their role in muscle mass regulation has been less investigated and conflicting results have been reported. Indeed, the first studies described both atrophic and hypertrophic roles of the Hippo pathway and its effectors Yap/Taz using different biochemical approaches. Further investigation is therefore warranted to determine the role of the Hippo pathway in the regulation of skeletal muscle mass. New components of the pathway will probably emerge and unsuspected roles will likely be discovered due to its numerous interactions with different cellular processes. This mini-review aims to summarize the current literature concerning the roles of the Hippo pathway in the regulation of muscle mass and to develop the hypothesis that this pathway could contribute to muscle mass adaptation after exercise.

Key words:

Yap, Taz, growth, size, mTOR

1. Introduction

Skeletal muscle mass results from the balance between protein synthesis and protein degradation. In addition, satellite cell inclusion may contribute to increase muscle mass while fiber loss results in a reduction of muscle mass. At the molecular level, skeletal muscle mass regulation is the result of coordinated activation/inhibition of numerous signaling pathways. The first identified signaling pathways involved in organ genesis and development were, non-exhaustively, Notch, Wnt, transforming growth factor-beta (TGF-β), Hedgehog (323). The last decades, the mTOR and Hippo pathways were found to be additional regulators of organs size (324). Contrary to the mTOR pathway, which is relatively well-described, numerous mechanisms of regulation of the Hippo pathway remain unclear. Indeed, in Drosophila, after identification of Warts (Wts) in the 1990s, Salvador (Sav) and Hippo in the 2000s, a first description of the Hippo scaffolded pathway was made (323). First identified in mouse, and then in human, some mammalian homologs of the components of the Hippo pathway in Drosophila are thought to be involved in organ size regulation, and many studies began to focus on the Hippo pathway in mammals. The canonical Hippo pathway is now described in mammals but crosstalks and interplays with other signaling pathways as well as its role in organ size regulation are still unclear (234).

Recently, the Hippo pathway has been of great interest for deep comprehension of molecular mechanisms underlying etiology, physiopathology and treatment of pathologic features in chronic

diseases like cancers, diabetes, chronic obstructive pulmonary disease or renal failure. Unfortunately, its involvement in exercise physiology and muscle mass regulation in healthy has been under-investigated up to now (239). Therefore, this mini-review aims to provide a focused highlight on the Hippo pathway organization, regulation and involvement in skeletal muscle mass regulation in mammals and to develop the hypothesis that this pathway could contribute to muscle mass adaptation after exercise.

2. Organization of the Hippo pathway

2.1. Core Components: Mst1/2 and Lats1/2

Warts (Wts), core components of the Hippo pathway identified in Drosophila, and their homologues in mammals, large tumor suppressor kinases 1 and 2 (Lats1/2), are protein kinases of the nuclear Dbf2-related (NDR) family (Fig. 1). Lats1/2 are activated by phosphorylation by macrophage stimulating proteins 1/2 (Mst1/2), members of the Ste20 protein kinase family (325). By binding to Lats1/2, Mob1 enhances the affinity of the latter to Mst1/2. Mst1/2 are themselves phosphorylated by upstream activators, and binding with the adaptor protein Sav enhances their affinity with Lats1/2 (325).

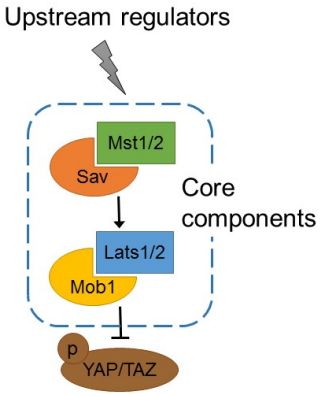

Figure 1. Canonical Hippo pathway

Core components of the Hippo pathway in mammals.

Mst1/2 is phosphorylated by upstream regulators and binding with Sav enhances their affinity with Lats1/2. The latter's affinity is increased by binding with Mob1. See text for abbreviations.

2.2. Yap and Taz are phosphorylated and inhibited by the Hippo pathway

Yes-Associated Protein (Yap) and its ortholog Transcriptional co-activator with PDZ binding motif (Taz) are the main effectors of the Hippo pathway. The critical event is the phosphorylation of those effectors by Lats1/2 (Fig. 2) (326).

– *Phosphorylation and cytoplasmic retention*

Yap contains five HXRXXS sequences that can be phosphorylated by Lats kinases while Taz contains four of them. Phosphorylation of Yap at Ser^{127} and of Taz at Ser^{89} leads to binding with 14-3-3 protein in the cytoplasm, maintaining Yap/Taz in the cytoplasm (327).

– *Non phosphorylation-dependent cytoplasmic retention*

Yap/Taz can also be inhibited and maintained close to cellular membrane by protein-protein bindings with the Angiomotin's (AMOT) protein family (327).

– *Phosphorylation and degradation*

When Yap is phosphorylated at Ser^{381}, and Taz at Ser^{311} by Lats1/2, casein kinase 1 (CK1δ/ε) can in turn phosphorylate Yap at Ser^{400} and Ser^{403} and Taz at Ser^{314} (327). This induces the formation of a phosphodegron, the function of which is to recruit β-transducin repeat-containing protein (β-TRCP) E3 ubiquitin ligase. The formation of phosphodegron, in association with activation of β-TRCP, leads to Yap/Taz degradation by the proteasome in the cytoplasm (238).

In summary, activation of the Hippo pathway induces Yap/Taz phosphorylation and inhibition of the latter complex, which results in its sequestration in the cytoplasm and subsequent degradation by the proteasome.

3. Upstream regulators of the Hippo pathway

Numerous cues, amongst which metabolites, nutrients and mechanical signals, are thought to regulate the Hippo pathway, even if the molecular mechanisms are still unclear (328). In mammals, neurofibromatosis 2 (NF2) mutations induce benign tumors while those tumors are repressed by Yap suppression. It has therefore been postulated that NF2 could be an upstream regulator of Hippo and Yap (329). Recent studies showed that AMOT can bind NF2 directly, promoting interaction and activation of Mst1/2 and sequestration of Yap/Taz in the cytoplasm (330). All together, regulation of Yap activation by NF2 is clearly more complex than it was thought.

G-protein-coupled-receptors (GPCRs), a vast family of membrane receptors, are identified as regulators of the Hippo pathway and Yap/Taz (237). The regulation by GPCRs may activate or inhibit the Hippo pathway and actin filaments and Rho GTPases are thought to mediate the interaction between GPCRs and Yap/Taz (236). The Hippo pathway is also differentially regulated according to the ligands that bind to the GPCRs, amongst which several extracellular metabolites (237). The first metabolite discovered was mevalonate that is responsible for acetyl-CoA conversion in isoprenoides precursors such as cholesterol, biliary acid or steroids, which can all activate the Hippo pathway (237). Similarly, epinephrine and glucagon, mediated by Gαs-coupled signals, activate Lats1/2 and consequently inhibit Yap/Taz. On the contrary, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), mediated by Gα12/13-, Gαq/11- and Gαi/o-coupled signals, repress Lats1/2 (237).

The AMP-activated protein kinase (AMPK) pathway is implicated in cellular homeostasis and can inhibit the Hippo pathway (331). In energy stress conditions, i.e low levels of ATP, AMPK promotes the inhibitory activity of tuberous sclerosis complex (TSC1-TSC2), an upstream component of the mTOR pathway. AMPK also phosphorylates Yap at Ser^{94} and Ser^{61} and maintains the latter in the cytoplasm (331). These two mechanisms trigger catabolic pathways aiming to recover energy homeostasis and basal AMPK activity (237).

Finally, the Hippo pathway is regulated by cell-cell contact and extracellular matrix. Indeed, Yap is phosphorylated and maintained in

the cytoplasm in densely populated cells, while lysis of cell-cell junctions induces Yap/Taz translocation to the nucleus. Therefore, an overexpression of Yap in the nucleus is observed in sparsely populated cells (332).

4. Downstream effectors of the Hippo pathway

Yap/Taz are co-activators and as such, do not possess DNA binding sites. To induce their transcriptional program, Yap/Taz have to bind to transcription factors such as to the TEADs family proteins (333-335). Yap and TEADs together can induce the transcription of specific genes such as Ankrd1, connective tissue growth factor (Ctgf), Gli2, mammalian homologue of Diap1 and baculoviral IAP repeat-containing 5 and 2 (Birc5 and Birc2). Yap can bind an transcription factor that remain unknown to induce transcription of Myc (333). Finally, Yap can bind other proteins like runt-related transcription factor (RUNX), ErbB4 and p73 but none of those complexes seems to regulate organ size or development (327).

Yap/Taz, and more broadly the Hippo pathway, are involved in many interactions and crosstalks with other signaling pathways to regulate cell cycle and cell fate (328). These interactions and crosstalks are described in the next section.

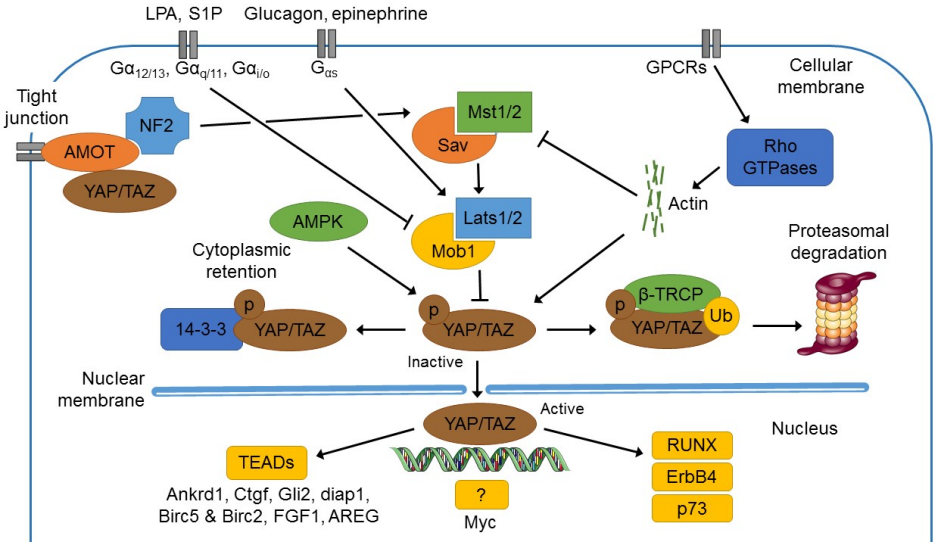

Figure 2. Regulation and functions of the Hippo pathway

Hippo pathway core components are regulated by membrane protein (NF2, AMOT), membrane receptors (GPCR mediated by Rho GTPases), metabolites (mevalonate, epinephrine, glucagon, LPA and S1P), nutrients (in low level ATP conditions) and extracellular matrix.

Phosphorylated Yap/Taz are inactive and sequestered in the cytoplasm: When phosphorylated (1) at Ser^{127} (Ser^{89} for Taz), Yap is bound to 14-3-3 protein for cytoplasmic retention, (2) at Ser^{381} (Ser^{311} for Taz), β-TRCP is recruited and Yap/Taz are ubiquitined for proteasomal degradation.

Dephosphorylated Yap translocate into the nucleus: When Hippo pathway is deactivated, dephosphorylated Yap translocate into the nucleus and bind with TEAD proteins to induce transcription of several genes (Ankrd1, Ctgf, Gli2, diap1, Birc5, Birc2, FGF1 and AREG). Yap can also induce Myc transcription but the binding transcription factor is unknown. Finally Yap can directly bind with proteins like RUNX, ErbB4 and p73. See text for abbreviations.

5. Interplay with other signaling pathways

5.1. Hippo and transforming growth factor-β pathways

TGF-β plays an important role in the proliferation of stem cells and tumors genesis via Smads activation as effectors (336). When phosphorylated, Smad1 affinity to Yap is enhanced, which in turns activates proliferation and renewal of embryonic stem cells (337). Similarly, Taz and Smad2/3 binding induces their nuclear localization, transcription of TGF-β targets genes, and renewal of embryonic stem cells. However, the exact nature of interactions between Yap/Taz and Smads and their contribution to organ size regulation remain unclear (336).

5.2. Hippo and Wnt pathways

Wnt is decisive in stem cell proliferation, differentiation and fate, the aberrant stimulation of which induces tumors genesis (338). Stimulation of Wnt receptors by their ligands elicits a disheveled (Dvl)-dependent inhibition of β-catenin complexes degradation, β-catenin accumulation in the nucleus and interaction with TCF/Lef and finally transcription of Wnt-regulated genes. Contrarily to Wnt, Taz inhibits Dvl1, 2 and 3 by binding and retaining β-catenin in the cytoplasm, preventing the latter to exert its transcriptional activity (339). Seeing that Mst1/2 and Lats1/2 can phosphorylate Taz, they are thought to be able to repress Wnt as well (340). In summary, the Hippo pathway can repress Wnt signaling by blocking Dvl or maintaining β-catenin in the cytoplasm, however, the contribution of the interaction between the Hippo and Wnt pathways to organ size regulation remains unknown (341).

5.3. Hippo and Sonic-Hedgehog pathways

The Sonic-Hedgehog pathway is the main regulator of cell differentiation, proliferation and polarity (342). In medulloblastoma elicited by excessive Sonic-Hedgehog and Wnt activation, an overexpression of Yap and TEAD1 is observed (343). Moreover, Yap and TEAD1 binding may induce expression of Gli2, a Sonic-Hedgehog effector, which re-enforces the hypothesis of an interaction between Hippo and Hedgehog. However, it remains unknown whether other components of the Hippo pathway are involved in the interaction with the Sonic-Hedgehog pathway (342).

5.4. Hippo and Akt-mTOR pathways

The Hippo and the Akt-mTOR pathways are responsible for the regulation of cell numbers and cell size, respectively. mTOR regulates cell size in response to nutrients and growth factors (344). Hippo regulates cells number by phosphorylating Yap/Taz, which in turn will result in a repression of cell proliferation and a stimulation of apoptosis in mammals (324). An overexpression of the effectors of Hippo, i.e. Yap/Taz, increases the phosphorylation of Akt and S6 Kinase1 (S6K1). Inversely, Yap is maintained in the cytoplasm by 14-3-3 protein and its transcription repressed when phosphorylated by Akt (238). In summary, while it is clear that the Hippo and Akt-mTOR pathways interact, the direction and the consequences of the interaction do not seem straightforward.

6. *Hippo pathway and regulation of organ size*

The ability of the Hippo pathway to regulate cell proliferation and apoptosis as well as stem cells renewal and expansion gives it a role in the management of organ size.

6.1. Hippo and organ size control

The role of the Hippo pathway in organ size management has been convincingly demonstrated by studies in mice. Specific alteration of Mst1/2, Lats1/2 or Sav in liver cells provokes up to a 4-fold growth (327). In another study, the transcriptional activity of Yap was increased during regrowth after partial liver ablation while at the same time, Mst1/2 and Lats1/2 were inhibited (345). This higher activity of Yap was quasi-automatically repressed at the end of regrowth and Mst1/2 and Lats1/2 inhibition released (345). The situation is more complex in the pancreas as Hippo pathway activity seems to be compartment-dependent. Indeed, Mst1/2 inhibition induced hyperplasia and cells death in the exocrine compartment while there was no incidence in the endocrine compartment (346). In the intestine, Yap ablation did not repress development but limited post-lesion regrowth (347). All together, those studies show the role of the Hippo pathway in the regulation of organ size.

6.2. Hippo and stem cells

The Hippo pathway is thought to manage stem cells regulation, renewal and expansion. Indeed, Yap is activated in satellite cells and inhibited during differentiation of embryonic stem cells. Furthermore, embryonic stem cells stay quiescent under regulation by Taz (348). In intestinal

progenitors, hepatic oval cells, neural and epidermal stem cells, Yap is generally required for their stemness, promoting proliferation but inhibiting differentiation (349).

7. Hippo pathway and skeletal muscle mass regulation

Muscle mass results from the balance between protein synthesis and degradation, as well as from satellite cells inclusion and cell death. The Hippo components have been identified in skeletal muscle cells (240) but their role in muscle mass regulation is less investigated. Given its multiple interactions with other signaling pathways, it is postulated that the Hippo pathway could play a role in different regulatory mechanisms, namely proliferation and renewal of satellite cells, differentiation, death and growth of muscle cells (233).

7.1. Role of the Hippo pathway during myogenesis

As observed in other tissues, the mRNA and protein expression of Yap were high during myogenic cells proliferation and low during differentiation (240, 349). In addition, during C2C12 cells proliferation, Yap Ser^{127} phosphorylation was low and Yap localized to nuclei. Upon differentiation, Yap Ser^{127} phosphorylation increased about 20-fold and Yap translocated from the nucleus to the cytosol. During the differentiation phase, overexpression of hYap1 Ser^{127A}, a mutant form of Yap which cannot be phosphorylated at Ser^{127} by Lats1/2, induced a decrease in myogenin and Mef2c expression, two differentiation genes, and a persistent expression of Myf5, a myoblasts proliferation gene (240). Those results are consistent with the hypothesis that

phosphorylation of Yap at Ser^{127} is necessary for myoblasts to withdraw the cell cycle and to differentiate (240). Similar results were obtained in satellite cells isolated from mouse muscle (349). In that study, constitutive Yap activity expanded the pool of activated satellite cells and satellite cell-derived myoblasts but prevented their differentiation. Microarray analyses identified regulators of the cell cycle, ribosomal biogenesis and modulators of myogenic differentiation as genes targeted by Yap. Yap was found to bind TEAD and co-activate muscle-specific cytidine-adenosine-thymidine (MCAT)-elements in myoblasts. In another study in postnatal muscle cells of mice, a high expression of Mst1/2 was found followed by a decrease within the first three weeks of life (235). All together, those results suggest a role of the Hippo pathway in *in vitro* myogenesis as well as in postnatal muscle development *in vivo*.

7.2. Role of the Hippo pathway in mouse skeletal muscle

Due to its regulatory role in the determination of cell size in general, it can logically be expected that the Hippo pathway regulates muscle cell growth as well. To investigate this hypothesis, an inducible skeletal muscle fibre-specific knock-in mouse model (MCK-tTA-hYAP1 Ser^{127A}) was developed to test whether the over expression of constitutively active Yap (hYAP1 Ser^{127A}) is sufficient to stimulate muscle hypertrophy or changes in fibre type composition (350). Unexpectedly, expression of this mutant form of Yap caused, approximately five weeks later, a kind of myopathy together with muscle atrophy, which was followed by a regenerative process comprised of activation, proliferation and differentiation of stem cells. At a molecular level, Myf5, myogenin and

Pax7, markers of satellite cells activation and differentiation, were highly expressed at the mRNA level as well as Mafbx and Murf-1, two markers of protein degradation. Of note, fiber type composition remained unchanged and the processes of atrophy and degeneration remained reversible when preventing the expression of the mutant form of Yap (350).

Contrary to the previous study, overexpressing total Yap, instead of active Yap, could induce hypertrophy via interaction with TEADs and independently of any activation of mTOR (239). It was also found that total Yap muscle expression and phosphorylation at Ser^{112} decreased during maturation of the myofibrils (239). Furthermore, repression of Yap expression caused a reduction in the size of muscle cells, and ultimately a decrease in muscle mass. This reduction in mass was associated with slight and no significant expression of the markers of protein degradation MAFbx, Musa1 and MuRF-1 (239). It was therefore concluded that the expression of Yap would be necessary for maintaining and increasing the size of the myofibrils.

Imposing a significant burden to the plantaris muscle by agonists ablation, a higher expression of Yap was found together with a higher transcriptional activity of TEADs into the plantaris muscle, thereby highlighting Yap sensitivity to mechanical loading on the muscle (351). This phenomenon was accompanied by an increase in Akt phosphorylation in a similar pattern as Yap expression and by hypertrophy that persisted following inhibition of mTOR by rapamycin injection. Corroborating previous results (352), it seems that Yap can

induce hypertrophy independently of mTOR. This hypertrophy was probably due, at least in part, to a low expression of MuRF1 and a high expression of MyoD and c-Myc (351).

It therefore appears that Hippo interacts with other pathways for the management of muscle mass. While some studies suggest that Yap overexpression/activation induces muscle atrophy (350), others found opposite results, i.e. hypertrophy after Yap overexpression (239, 351). Those discrepancies can, at least partially, be explained by the different ways, viral vectors vs gene mutations, used to induce Yap expression and the duration of the treatment (235, 239, 240, 350, 351). But other unknown factors are probably involved as well. This topic definitely deserves further investigation.

8. Perspectives and conclusion

Only one study has investigated the effect of exercise on the Hippo pathway (353). This study was performed in mice and the chosen exercise paradigm was running, which is not expected to increase muscle mass contrary to resistance exercise. Based on its role in muscle cells and satellite cell, we hypothesize that the Hippo pathway could contribute to the accretion of muscle mass after resistance exercise training, which still remains to be tested. In this perspective, the next step is to test whether the results obtained in vitro and in mice can be confirmed in healthy human skeletal muscle. Due to its role in the management of satellite cells, we speculate that the Hippo pathway could play a determinant role in the activation of this specific cell type after resistance

exercise. Finally, it would be interesting to test the involvement of the Hippo pathway after resistance exercise with blood flow restriction. Indeed, this kind of training has proven its efficiency to increase muscle mass by, amongst others, activating satellite cells and stimulating their proliferation (354). Here as well, it is unknown whether the Hippo pathway mediates this effect.

Conflict of Interest

The author declares that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

OG wrote the first draft of the manuscript and approved the final version. MF and LD corrected the draft and wrote the final version.

CHAPTER III: Environmental hypoxia favors myoblast differentiation and fast phenotype but blunts activation of protein synthesis after resistance exercise in human skeletal muscle

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Abstract

We hypothesized that a single session of resistance exercise performed in moderate environmental hypoxic conditions would potentiate the anabolic response during the recovery period spent in normoxia. Twenty subjects performed a one-leg knee extension session in normoxic or hypoxic (FiO_2: 14%) conditions. Muscle biopsies were taken 15min and 4h after exercise in the vastus lateralis of the exercised and the non-exercised legs. Blood and saliva samples were taken at regular intervals before, during and after the exercise session. The muscle fractional protein synthetic rate was determined using deuterium incorporation into proteins and the protein degradation rate by methylhistidine release from skeletal muscle. We found that: 1) hypoxia blunted the activation of protein synthesis after resistance exercise; 2) hypoxia down-regulated the transcriptional program of autophagy; 3) hypoxia regulated the expression of genes involved in glucose metabolism at rest, and genes involved in myoblast differentiation and fusion, and in muscle contraction machinery after exercise; 4) the hypoxia-inducible factor-1α pathway was not activated at the time points studied. Contrary to our hypothesis, environmental hypoxia did not potentiate the short-term anabolic response after resistance exercise, but it initiated transcriptional regulations that could potentially translate into satellite cells incorporation and higher force production on the long-term.

Keywords: hypoxia-inducible factor, deuterium, autophagy, redd1, tissue oxygenation index

1. Introduction

Hypoxia is a state of lowered oxygen tension in tissue that can be created by environmental conditions such as high altitude, or by pathological conditions such as chronic obstructive pulmonary disease (355) and obstructive sleep apnea (356). During exercise, hypoxia can also be generated but, contrary to the previous situations, oxygen restriction is then limited to skeletal muscle (241). Whatever the origin of hypoxia, different tissues will adapt acutely and/or chronically to deal with this reduction in oxygen availability. Interestingly, hypoxia has recently emerged as a particularly efficient stimulus to stimulate muscle cell proliferation and accretion of muscle mass (357). While long lasting hypoxia generally leads to a negative regulation of protein metabolism and a loss of muscle mass (249, 276, 277), repeated intermittent hypoxia seems to rather exert a positive effect on protein balance (357). Thus, hypoxic resistance training has become popular amongst athletes as it is thought to favor muscle accretion. Indeed, previous observations suggest that muscle hypertrophy can be optimized when resistance training is performed in a hypoxic environment (247, 358). However, the molecular mechanisms are largely unknown.

Skeletal muscle mass reflects a dynamic turnover between net protein synthesis and degradation. In addition, satellite cell inclusion may contribute to increase muscle mass, while fiber loss results in a reduction of muscle mass. While controversial (307), protein synthesis is regularly assessed by measuring the activation of the protein kinase B (Akt/PKB)/mammalian target of rapamycin (mTOR) pathway and its

downstream targets ribosomal S6 kinase 1 (S6K1) and ribosomal S6 protein (S6). Muscle protein degradation is mainly regulated by the autophagy-lysosomal and the ubiquitin–proteasome pathways. The latter mechanism is largely regulated by E3 ligases such as MAFbx and MuRF1 (359). The transcriptional regulation of those E3 ligases is partly controlled by the members of the forkhead FoxO family, themselves regulated by Akt (360). Unc51-like kinase 1 (ULK1) plays an essential role in the initiation of autophagy and more specifically in the autophagosome membrane formation (361). The subsequent elongation of the membrane is under the control of several autophagy related-gene (Atg) proteins, including microtubule-associated protein 1 light chain 3 (LC3b). The mature autophagosome, whose membrane includes the lipidated form of LC3 (LC3bII), fuses with lysosomes containing hydrolases. As mentioned earlier, satellite cells may contribute to muscle hypertrophy as well (362). Their activation, proliferation and differentiation are tightly controlled by the so-called myogenic regulatory factors, namely myogenin, MyoD, myf-5 and Mrf4 (362). At a molecular level, a decrease in oxygen tension activates the hypoxia-inducible factor-1α (HIF-1α), which is responsible for many responses to hypoxic conditions such as angiogenesis or increased glucose metabolism, via increased gene expression of vascular endothelial growth factor (Vegf) and phosphofructokinase (Pfk), respectively (363). Whether HIF-1α is directly responsible for the regulation of protein metabolism under hypoxic conditions is unknown.

Due to the lack of data, the purpose of the present study was to determine the rates of protein synthesis and protein degradation as well as the

molecular adaptations after resistance exercise in hypoxia in human skeletal muscle. We hypothesized that one session of resistance exercise performed in moderate environmental hypoxic compared to normoxic conditions would potentiate the anabolic response during the recovery period spent in normoxia.

2. *Materials and methods*

Subjects

Twenty young, physically active, men gave their written informed consent to participate in the study (BMI 22.2 ± 0.3kg/m^2, age 22.2 ± 0.4y). The experiment was approved by the ethical committee of the Université catholique de Louvain and conducted in accordance with the declaration of Helsinki. The exclusion criteria were: cardiovascular or pulmonary disease, chronic medication, any contraindication to resistance training or having been exposed to an altitude above 1500m during the month before the experiment.

Protocol

The subjects were randomly distributed into two groups, hypoxia (HYP, n=10) or normoxia (NOR, n=10) (Fig. 1). Each subject came 3 times to the laboratory.

First visit: A week before the experiment, height and weight were determined. The one-repetition maximum (1-RM) of the right leg was determined on a knee extension device (Pro-Dual Body Solid).

Second visit: The day before the experiment, saliva and blood samples were collected prior to ingestion of deuterium oxide (400ml spread over 2h, Sigma-Aldrich #613428, Overijse, Belgium) and 3D-methyl-histidine (10mg diluted in 100ml water, Cambridge Isotope Laboratories DLM-2949-MPT-PK, Apeldoorn, The Netherlands). A second sample of saliva was collected 90min after ingestion of the last dose of deuterium. Thereafter, a standardized low meat spaghetti bolognese was provided.

Third visit: The day after the second visit, the subjects came to the laboratory in the fasted state. After collection of blood, saliva and urine samples (Fasted), they received a standardized meat-free breakfast complemented with 10g essential amino acids. One hour after meal consumption, the one-leg knee extension exercise session began (8 sets of 8 repetitions at 80% 1-RM, 2-min rest between sets) either in normoxic or hypoxic (FiO_2: 14%) conditions. Each set lasted about 25s to be completed. In total, the whole session, and thereby the exposition to hypoxia or normoxia, lasted for 30min. During exercise, vastus lateralis muscle oxygenation was monitored by near-infrared spectroscopy (NIRS) (Artinis, Elst, The Netherlands), and peripheral blood oxygen saturation (SpO_2) and heart rate (HR) were monitored by pulse oximetry (TCM Tosca, Radiometer, Zoetermeer, The Netherlands). Blood, saliva and urine samples were collected immediately (Post), 2h (2h Post) and 4h after exercise (4h Post). Muscle biopsies were taken from the vastus lateralis of the exercised and non-exercised (control) legs within 15min after exercise (Post) and 4h after exercise (4h Post).

Near-infrared spectroscopy

Changes in muscle oxygenation were measured by NIRS using the difference in absorption characteristics of light between 760nm and 850nm (Portamon, Artinis). Tissue saturation index (TSI, %) was monitored at baseline (30-s averaging before the start of the first set) and during each set. As described in Brocherie et al. (364), the apparatus was fixed with a large bandage to avoid interference by extraneous light and loss of transmitted light out of the field of investigation.

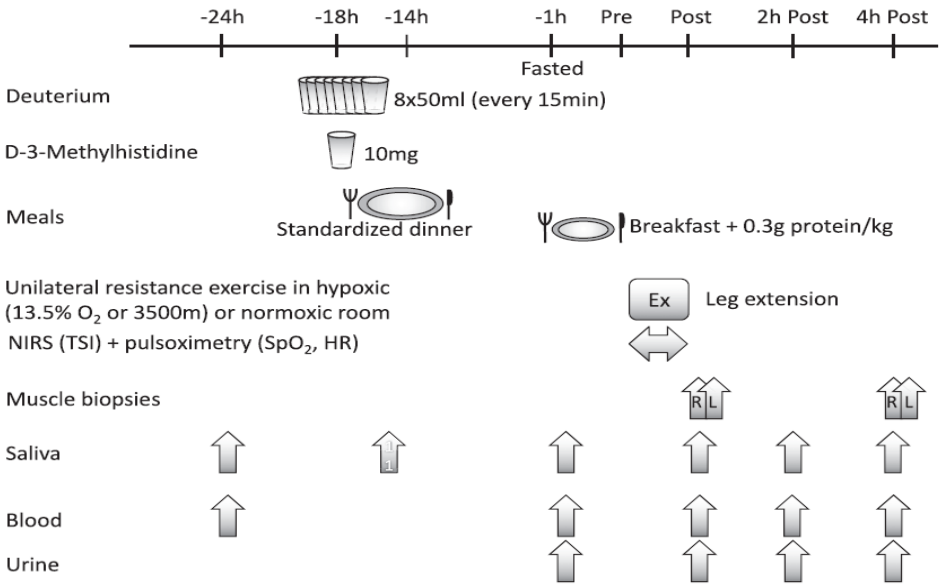

Figure 1. Study protocol

NIRS, near infrared spectroscopy; TSI, tissue saturation index; SpO_2, peripheral blood oxygen saturation; HR, heart rate; Ex, exercise; R, right; L, left.

Samples collection and storage

Blood: Samples were taken in an antecubital vein in two 5-ml tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately put on ice until centrifugation. After centrifugation at 4°C for 10min at 3000g, plasma was transferred to microtubes and stored at -80°C.

Saliva: Samples were collected with a cotton wool, gently chewed during 1 to 2min. The swab was then placed in the suspended insert and the salivette was firmly closed using the stopper. The samples were immediately placed on ice until centrifugation. The salivettes were then

centrifuged at 3°C for 10min at 3000g. The supernatant was aliquoted and stored at -20°C.

Urine: Samples were collected in 2-l bottles, transferred and aliquoted in 50-ml tubes and immediately stored at -20°C.

Muscle biopsies: Vastus lateralis muscle biopsies were taken according to the modified Bergström technique with suction. After local anesthesia (xylocaine 2% without adrenaline, Astra Zeneca, Uccle, Belgium), a 5-mm incision was made with a scalpel and a pressure applied for 10min to reduce blood flow. The Bergström needle was then gently inserted into the incision, at least 1cm beyond the fascia, to reach the muscle and three successive cuts were performed to obtain 150mg per sample. The samples were immediately frozen in liquid nitrogen and stored at -80°C.

Plasma insulin and cortisol concentrations

Plasma concentration of insulin and cortisol were determined by ELISA using the ultrasensitive insulin kit from Mercodia (Uppsala, Sweden) and the human cortisol kit from Abcam (Cambridge, UK), respectively.

Protein extraction

Muscles samples were homogenized with a Polytron mixer in ice-cold lysis buffer (20mM Tris–HCl pH 7.0, 270mM sucrose, 5mM ethylene glycol tetraacetic acid (EGTA), 1mM EDTA, 1mM sodium orthovanadate, 50mM B-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM DTT, 1% Triton-X 100 and a complete protease inhibitor tablet), centrifuged at 10.000g for 10min at 4°C and the supernatant aliquoted and stored at -80°C. The pellet, containing non-soluble

proteins, was stored at -80°C for the determination of the protein synthetic rate. The myofibrillar fraction was isolated from the pellet by the addition of 0.3M NaOH and placed at 37°C for 30min. The non-soluble collagen proteins were removed by centrifugation and the myofibrillar proteins precipitated with 1M PCA. Protein bound amino acids were released using acid hydrolysis in 0.1M HCl and Dowex H+ resin slurry overnight before being eluted from the resin with 2M NH_4OH and evaporated to dryness. The DC protein assay kit was used to determine the protein concentration of each sample using bovine serum albumin as a standard (Bio-Rad, Nazareth, Belgium).

Western Blotting

Thirty to seventy µg proteins were separated by SDS-PAGE (8%-15% gels) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1h and incubated overnight at 4°C with one of the following antibodies: phospho-AMP activated protein kinase (AMPK, Thr^{172}), total-AMPK, phospho-S6K1 (Thr^{389}), total-S6K1, phospho-Akt (Ser^{473}), total-Akt, phospho-FoxO1 (Thr^{24})/FoxO3a (Thr^{32}), total-FoxO1/FoxO3a, phospho-S6 ($Ser^{235/236}$), total S6, phospho-acetyl CoA carboxylase (ACC, Ser^{79}), total-ACC, p62/SQSTM1, HIF-1α (all from Cell Signaling, Leiden, The Netherlands, 1:1000, BSA 1%), regulated in development and DNA damage response 1 (REDD1, ProteinTech, 1:750, BSA 1%), LC3b (Sigma-Aldrich, 1:1000, milk 5%). Secondary anti-mouse (1:10000) or anti-rabbit (1:5000) antibodies conjugated to horseradish peroxidase were used for detection of proteins of interest. Membranes were thereafter scanned and proteins quantified with GeneSnap

software and tools (Syngene, Cambridge, UK). For detection of total protein forms, membranes were stripped and re-probed with the antibody for the total form of the respective protein to ascertain the relative amount of the phosphorylated protein compared with the total form throughout the whole experiment. The results are presented as the ratio phosphorylated/total form of the protein and are expressed in arbitrary units.

Protein synthesis and protein degradation

The muscle fractional synthetic rate was determined using the stable isotope deuterium following the same procedure as in Wilkinson et al (365). The muscle protein degradation rate was determined using the stable isotope tau-methyl-l-histidine based on the procedure described in Sheffield-Moore et al (366). Both rates were determined between 15min and 4h post-exercise.

Immunofluorescence

Cryosections of 6μm thickness were fixed with 3.7% formaldehyde for 10min and incubated in a permeabilization buffer (20mM Tris-HCl pH 8.0, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose, 0.5% Triton X-100) at 37°C for 20min. After blocking nonspecific staining with 1% normal goat serum (NGS) in phosphate buffered saline (PBS) for 30min, sections were incubated with an anti-HIF1α (1:25 in 1% NGS diluted in PBS, Cell Signaling) overnight at 4°C, followed by incubation with a goat anti-rabbit IgG AF 488 (1:200 in 1% NGS diluted in PBS, Invitrogen, Merelbeke, Belgium) for 1h at room temperature. Nuclei were identified with 4',6-diaminido-2-phenylindole (DAPI) provided in the mounting

medium (Vectashield, Vector Laboratories Inc, Brussels, Belgium). Specimens were examined with a Zeiss Axio Vert.A1 fluorescence microscope. HeLa cells and human myotubes treated with 400µM $CoCl_2$ for 24h were used as a positive control.

RNA extraction and real-time PCR

About 20-25mg of muscle biopsy sample was homogenized in 1ml Trizol reagent (Invitrogen, Merelbeke, Belgium) using a Polytron mixer. RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop spectrophotometry. Reverse transcription was performed from 1µg RNA using the iScriptcDNA Synthesis Kit from Bio-Rad, following the manufacturer's instructions. The primers used are presented in Table 1. PCR was run using the following conditions: 3min at 95°C, followed by 35 cycles of 30s at 95°C, 30s at 60°C, and 30s at 72°C. All samples were run in duplicate and each reaction was processed in a 10µl volume containing 4.8µl IQ SybrGreen SuperMix (Bio-Rad), 0.1µl of each primer (100nM final), and 5µl cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. To compensate for variations in input RNA amounts and efficiency of reverse transcription, ribosomal protein L4 (RPL4) and RPL19 mRNA were quantified, and results were normalized to these values. These genes were chosen out of four normalization genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere (367). The results are expressed in arbitrary units.

Microarray

Microarray analyses were performed on biopsies obtained 4h after exercise in the exercised and non-exercised control legs in NOR and in HYP. Bioanalyzer nano 6000 chips (Agilent Technologies, Santa Clara, CA, USA) were used to assess RNA quality. Only samples with an RNA integrity number > 7.7 were used. We thus selected subjects in normoxia (n=8) and hypoxia (n=7), and then pooled their RNA for each condition, i.e. NOR.Rest, NOR.Exercise, HYP.Rest, and HYP.Exercise. GeneChip Human Transcriptome Array 2.0 and GeneChip WT PLUS Reagent Kit were used following manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Affymetrix Transcriptome Analysis Console Software was used to compute gene signals. Functional annotation and pathway analysis for comparisons at rest (HYP.Rest vs. NOR.Rest) and after exercise (HYP.Exercise vs. NOR.Exercise) were performed with DAVID web tool (368). In the DAVID analyses, Fischer exact p-values < 0.05, which are equivalent to $-\log_{10}$(p-value) > 1.3, were considered statistically significant.

Statistics

All values are expressed as means ± SEM. Repeated measures ANOVA was performed considering groups (HYP or NOR) as inter-group factor, and time (15min post-exercise and 4h post-exercise) and condition (rest and exercise) as intra-group factors. Corrected Bonferroni post-hoc analyses were performed when indicated, and statistical significance was fixed at $p<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc version 24.0).

Table 1. Primer sequences

	Sequence: 5' – 3'	
	Forward	**Reverse**
Atg12	AGT AGA GCG AAC ACG AAC CAT C	CCA TCA CTG CCA AAA CAC TCA T
Bnip3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
Bnip3L	CCA AGG AGT TCC ACT TCA GAC	AGT AGG TGC TGG CAG AGG GTG T
Cyclin D1	GAA CAA GCT CAA GTG GAA CC	CAC AGA GGG CAA CGA AGG
Fos	GGC AAG GTG GAA CAG TTA TC	TCT CCG CTT GGA GTG TAT C
Hif-1α	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG
Hif-2α	AAG CTG AAG CGA CAG CTG GAG TAT	GTA CAT TTG CGC TCA GTG GCT TGT
Lc3b	AAT CCC GGT GAT AAT AGA ACG A	GGA GAC GCT GAC CAT GCT GT
Lox	GCA CAG TTG TCA TCA ACA TTA C	GAT GTC CTG TGT AGC GAA TG
Mafbx	CGA CCT CAG CAG TTA CTG CAA	TTT GCT ATC AGC TCC AAC AG
Mrf4	AAT CTT GAG GGT GCG GAT TTC CTG	TGC TCC TCC TTC CTT AGC CGT TAT
Murf-1	AAA CAG GAG TGC TCC AGT CGG	CGC CAC CAG CAT GGA GAT ACA
Myf5	TGA GAG AGC AGG TGG AGA ACT ACT	AGA CAG GAC TGT TAC ATT CGG GCA
Myh1	CAC ACT AGT TTC ACA GCT CTC	GGC ACT CTT GGC CTT TAT C
Myh11	GGA GGA GCA GCT ATC CAT ATT	GCC TGG TCT GTG TTT CTT TC
MyoD	TGC CAC AAC GGA CGA CTT CTA TGA	AAG TGC GAG TGC TCT TCG GGT TT
Myogenin	AAA CTA CCT GCC TGT CCA CCT C	ACA CCG ACT TCC TCT TAC ACA CCT
Pcna	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
Pdk4	CAG CTA CTG GAC TTT GGT TC	CTA ATT GGG TCG GGA GGA TA
Pfk	ATT TGA CGA AGC CCT GAA G	GTG CGA ACC ACT CTT AGA TAC
Plin2	CTC ATG TCC TCA GCC TAT CT	TAG GCA GTC TCT CCT CAA TC
Rpl19	CGC TGT GGC AAG AAG AAG GTC	GGA ATG GAC CGT CAC AGG C
Rpl4	ATA CGC CAT CTG TTC TGC CCT	GCT TCC TTG GTC TTC TTG TAG CCT
Tnni1	CAA CAC CAG GGA GAT TAA GG	ATG GAC ACC TTG TGC TTG
Tnnt1	GAG CTG TCG GAC TGG AT	ACA GCA CGT TGA TCT CAT ATT
Vegf	TTT CTG CTG TCT TGG GTG CAT TGG	ACC ACT TCG TGA TGA TTC TGC CCT

Atg12, autophagy-related protein 12; Atg4b, autophagy-related protein 4b; Bnip3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; Bnip3L, Bnip3 like; Hif-1α, hypoxia-inducible factor 1 alpha; Hif-2α, hypoxia-inducible factor 2 alpha; Lc3b, microtubule-associated proteins 1A/1B light chain 3B; Lox, lipoxygenase; Mafbx, muscle atrophy F box; Mrf4, muscle regulatory factor 4; Murf-1, muscle ring finger

protein-1; Myf5, myogenic factor 5; Myh1, myosin heavy chain IIx/D; Myh11, myosin heavy chain polypeptide 11, smooth muscle; Pcna, proliferating cell nuclear antigen; Pdk4, pyruvate dehydrogenase kinase isoform 4; Pfk, phosphofructokinase; Plin2, perilipin 2; Redd1, protein regulated in development and DNA damage response 1; Rpl19, ribosomal protein L19; Rpl4, ribosomal protein L4; Tnni1, troponin I1, slow skeletal type; Tnnt1, troponin T1, slow skeletal type; Vegf, vascular endothelial growth factor.

3. Results

Blood but not muscle oxygenation was decreased in hypoxia

SpO_2 was about 98% at baseline and during each set in normoxia (Table 2). In hypoxia, SpO_2 decreased to ~93% at baseline, and was lower compared to normoxia during the whole experiment ($p<0.001$). From the 2nd set in hypoxia, SpO_2 increased compared to baseline reaching 94.6 to 95.1% ($p<0.05$). TSI decreased by about 10 to 15% during exercise ($p<0.001$) but was not different between normoxia and hypoxia. Heart rate progressively increased during exercise ($p<0.001$), with no difference between normoxia and hypoxia (+ 30 beats/min in normoxia and + 25 beats/min in hypoxia).

Table 2. Tissue oxygenation and heart rate during exercise

	SpO_2 (%)		TSI (%)		HR (beats/min)	
	NOR	**HYP**	**NOR**	**HYP**	**NOR**	**HYP**
Baseline	98.2 ± 0.1	93.3 ± 0.4***	61.3 ± 0.9	61.4 ± 0.8	73.2 ± 3.1	85.1 ± 4.2
Set 1	98.2 ± 0.2	93.1 ± 0.4***	48.8 ± 1.1$^{\#\#\#}$	50.4 ± 1.5$^{\#\#\#}$	92.6 ± 2.3$^{\#\#\#}$	101.6 ± 3.6$^{\#\#\#}$
Set 2	98.0 ± 0.1	94.6 ± 0.3$^{\#,***}$	51.1 ± 1.1$^{\#\#\#}$	49.4 ± 1.9$^{\#\#\#}$	93.3 ± 3.5$^{\#\#\#}$	100.5 ± 4.6$^{\#\#\#}$
Set 3	98.0 ± 0.1	95.1 ± 0.4$^{\#\#\#,***}$	50.3 ± 1.3$^{\#\#\#}$	50.1 ± 1.8$^{\#\#\#}$	92.5 ± 4.1$^{\#\#\#}$	101.2 ± 5.1$^{\#\#\#}$
Set 4	98.0 ± 0.1	94.9 ± 0.5$^{\#,***}$	51.8 ± 1.4$^{\#\#\#}$	50.5 ± 1.7$^{\#\#\#}$	95.5 ± 3.3$^{\#\#\#}$	101.7 ± 4.7$^{\#\#\#}$
Set 5	98.3 ± 0.1	94.7 ± 0.3$^{\#,***}$	50.9 ± 1.1$^{\#\#\#}$	50.6 ± 1.8$^{\#\#\#}$	98.8 ± 3.9$^{\#\#\#}$	104.6 ± 5.3$^{\#\#\#}$
Set 6	98.3 ± 0.1	95.1 ± 0.4$^{\#\#\#,***}$	52.4 ± 0.9$^{\#\#\#}$	51.4 ± 1.5$^{\#\#\#}$	97.4 ± 4.5$^{\#\#\#}$	106.7 ± 5.1$^{\#\#\#}$
Set 7	98.4 ± 0.1	94.9 ± 0.4$^{\#,***}$	52.5 ± 0.9$^{\#\#\#}$	52.4 ± 1.7$^{\#\#\#}$	102.6 ± 4.8$^{\#\#\#}$	106.7 ± 4.6$^{\#\#\#}$
Set 8	98.4 ± 0.1	94.6 ± 0.5$^{\#,***}$	54.0 ± 0.7$^{\#\#\#}$	52.5 ±1.6$^{\#\#\#}$	106.2 ± 6.1$^{\#\#\#}$	109.3 ± 5.1$^{\#\#\#}$

SpO_2, blood oxygenation; TSI, tissue saturation index; HR, heart rate; NOR, normoxia; HYP, hypoxia. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs Baseline; $^{*}p<0.05$, $^{***}p<0.001$ vs NOR.

Plasma insulin levels were higher at Post (after breakfast) compared to Fasted in both normoxia and hypoxia ($p<0.001$, Table 3), as expected. No difference in plasma insulin concentrations was observed between normoxia and hypoxia, although a trend to higher values in hypoxia was measured at 2h Post ($p=0.061$). Compared to Fasted, plasma cortisol concentrations were lower at Post, 2h Post and 4h Post ($p<0.05$), with no difference between normoxia and hypoxia.

Table 3. Plasma insulin and cortisol concentrations

		Fasted	Post	2h Post	4h Post
Insulin	**NOR**	2.7 ± 0.5	12.3 ± 2.2$^{###}$	2.5 ± 0.5	1.9 ± 0.3
(mIU/ml)	**HYP**	2.8 ± 0.4	15.6 ± 1.9$^{###}$	4.9 ± 1.2	2.4 ±0.4
Cortisol	**NOR**	273 ± 33	167 ± 25$^{###}$	120 ± 17$^{##}$	128 ± 26$^{###}$
(ng/ml)	**HYP**	245 ± 29	160 ± 22$^{#}$	138 ± 19$^{###}$	139 ± 19$^{###}$

NOR, normoxia; HYP, hypoxia. $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ vs Fasted

Hypoxia blunts the activation of protein synthesis after resistance exercise

The fractional synthetic rate was increased by about 40% after resistance exercise in normoxia ($p=0.023$, Fig. 2.A), but not in hypoxia. The fractional synthetic rate was not different between normoxia and hypoxia in the control rested leg.

No effect of hypoxia on the mTOR pathway despite decreased AMPK phosphorylation

We next analyzed the phosphorylation state of key mediators of the mTOR pathway. Compared to Post, the phosphorylation of Akt, S6K1 and

S6 were lower at 4h Post in the exercised leg in normoxia and hypoxia ($p<0.001$, Fig. 2B-D). Compared to Post, phospho-Akt ($p=0.004$) and phospho-S6 ($p=0.036$) were lower at 4h Post in the control leg only in hypoxia, while phospho-S6K1 was lower in both normoxia ($p=0.012$) and hypoxia ($p=0.004$). In addition, phospho-S6K1 was higher in the exercised leg compared to the control leg at Post in normoxia ($p=0.016$), and at 4h Post in hypoxia ($p=0.012$). Compared to the control leg, phospho-S6 was higher in the exercised leg at Post in normoxia ($p=0.012$) and hypoxia ($p=0.012$). The expression of the mTOR inhibitor REDD1 was lower at 4h Post in the exercised leg compared to the control leg in both normoxia ($p=0.020$) and hypoxia ($p=0.050$) (Fig. 2E). Hypoxia lowered REDD1 protein expression at 4h Post in the control ($p=0.047$) and in the exercised leg ($p=0.041$). Hypoxia decreased phospho-AMPK at Post in the control leg ($p=0.022$) and at 4h Post in the exercised leg ($p=0.001$) (Fig. 2F). As a readout of AMPK activity, we also tested the phosphorylation of its substrate ACC. Compared to Post in the exercised leg, phospho-ACC was lower at 4h Post in normoxia ($p=0.040$) and in hypoxia ($p=0.004$) (Fig. 2G). Exercise increased phospho-ACC in hypoxia ($p=0.004$) and tended to increase it in normoxia ($p=0.084$).

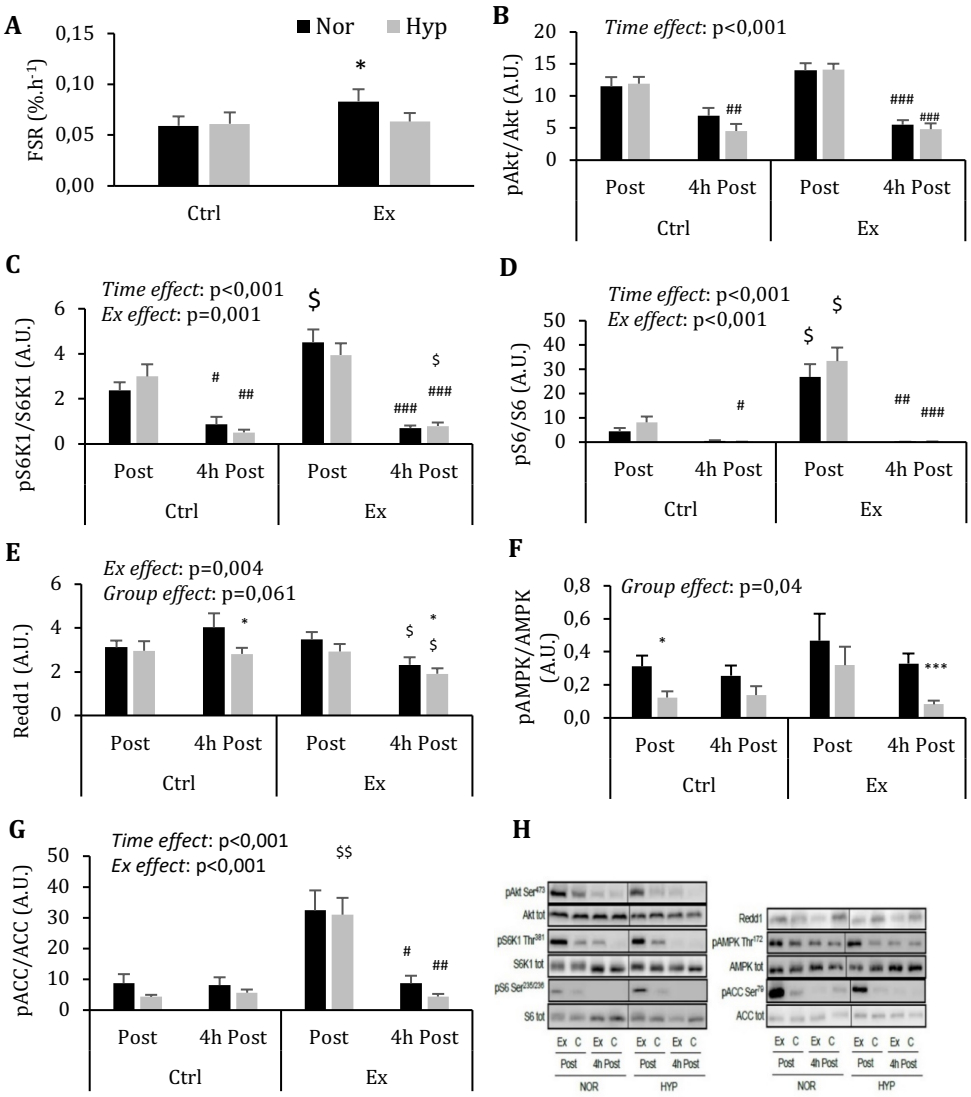

Figure 2. Regulation of the fractional protein synthetic rate and protein synthesis markers by hypoxia and exercise

(A) Fractional protein synthetic rate between 15min and 4h post-exercise in the control and the exercised leg in normoxia and hypoxia. Phosphorylation of (B) Akt Ser^{473}, (C) S6K1 Thr^{389}, (D) S6 Ser$^{235/236}$, (E) protein expression of Redd1, (F) AMPK Thr^{172} and (G) ACC Ser^{79} in the control and in the exercised leg at Post and at 4h

Post in normoxia and hypoxia. (H) Representative western blots. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg; FSR, fractional synthetic rate. *p<0.05, ***p<001 (HYP vs NOR); $p<0.05, $$p<0.01 (Ex vs Ctrl); #p<0.05, ##p<0.01, ###p<0.001 (4h Post vs Post). (n = 10/group).

Hypoxia down-regulated autophagy transcriptional regulation

The muscle protein degradation rate, assessed by the rate of 3-D-methyl-histidine disappearance from the plasma, was similar between normoxia and hypoxia (Fig. 3A). LC3bII/I ratio increased in both legs at 4h Post in normoxia and hypoxia (p<0.01, Fig. 3B). This increase was partially repressed by exercise in normoxia (p=0.012) as well as in hypoxia (p=0.004). A trend to a main exercise effect was observed for p62 (p=0.096, Fig. 3C). Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3, Fig. 3D) and Bnip 3 like (Bnip3L, Fig. 3E) mRNA levels were lower at all time points studied in hypoxia compared to normoxia (p<0.05). Compared to Post, Bnip3 mRNA was lower in the control leg in hypoxia (p=0.036) and tended to be lower in the exercised leg in normoxia at 4h Post (p=0.072). Compared to Post, Bnip3L mRNA was lower in the exercised leg in hypoxia at 4h Post (p=0.024). Similarly, Lc3b mRNA was lower in hypoxia compared to normoxia (p<0.05) except at Post in the control leg (Fig. 3F). Compared to Post, Lc3b mRNA was lower in the control leg in hypoxia at 4h Post (p=0.016). Atg12 mRNA levels were not modified by any condition (Fig. 3G). Compared to normoxia, pFoxO was higher in hypoxia at 4h Post in the exercised leg (p=0.047) and tended to be higher at the same time point in the control leg (p=0.072) (Fig. 3H). Murf-1 mRNA levels were higher at 4h Post in the

exercised leg compared to the control leg ($p=0.024$) (Fig. 3I). Compared to Post in hypoxia, Mafbx mRNA levels were lower at 4h Post in both the control ($p=0.004$) and exercised leg ($p=0.016$) (Fig. 3J).

Hypoxia differentially regulated gene expression at rest and after exercise

Microarray analyses showed that hypoxia up- or down-regulated by at least 1.5-fold a total of 392 genes at rest and 480 genes 4h after exercise (see Fig. 4A for selected genes, and Supplementary Data 1 for the full list). Analyses of functional annotation and signaling pathways revealed that, at rest, hypoxia influenced glucose metabolism and oxygen transport (Fig. 4B and Supplementary Data 1). Similar analyses after exercise revealed that hypoxia influenced myoblast differentiation and fusion, and several components of the muscle contraction machinery (Fig. 4C and Supplementary Data 1). Focusing on hypoxia-induced changes, qPCR analyses confirmed that Myh1 mRNA levels were increased while Tnnt1 mRNA levels were decreased in hypoxia compared to normoxia ($p<0.05$, Supplementary Data 2).

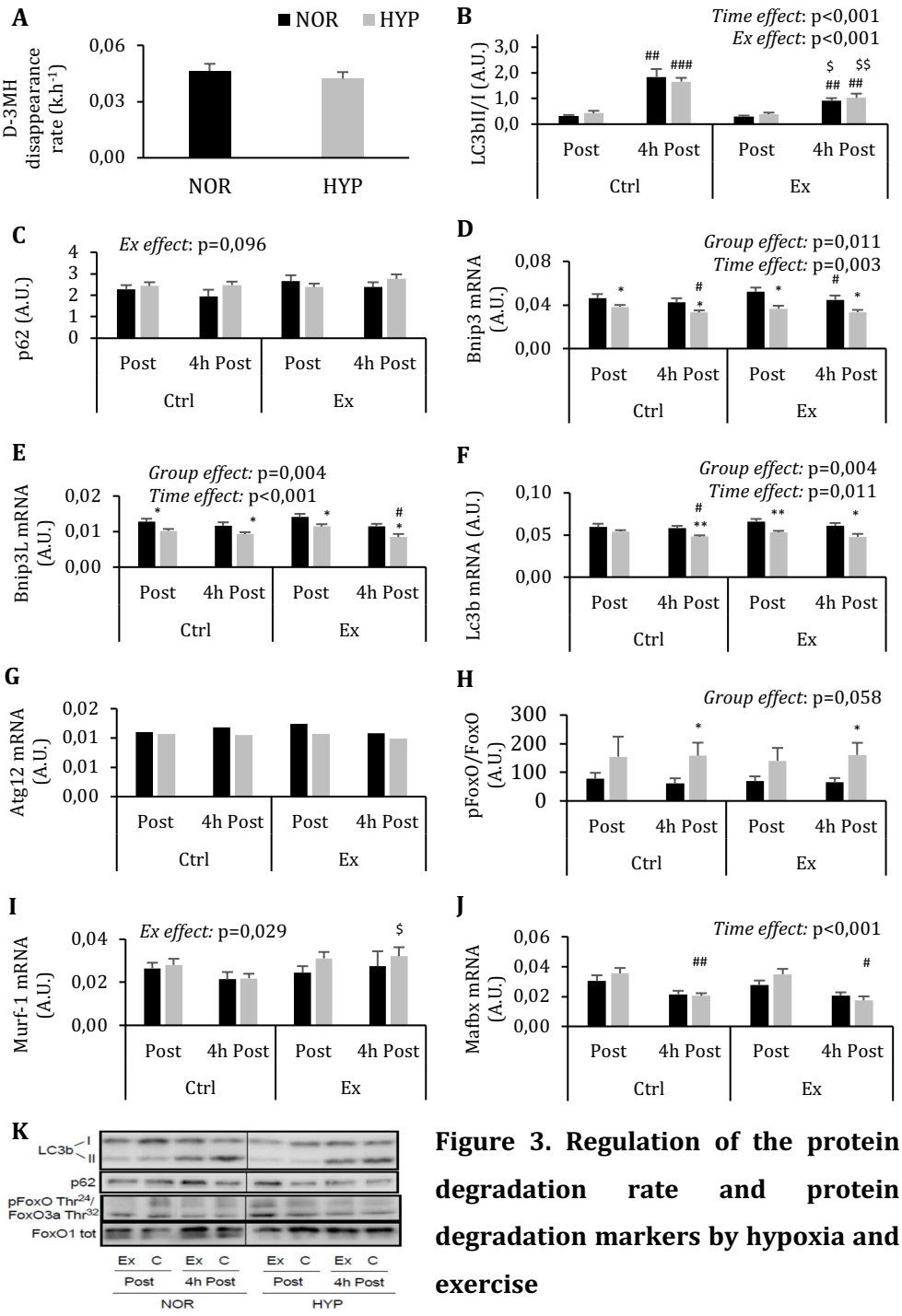

Figure 3. Regulation of the protein degradation rate and protein degradation markers by hypoxia and exercise

(A) Protein degradation rate between 15min and 4h post-exercise in normoxia and hypoxia. (B) LC3bII/I ratio, (C) p62 protein expression, (D) Bnip3 mRNA levels, (E) Bnip3L mRNA levels, (F) Lc3b mRNA levels, (G) Atg12 mRNA levels, (H) FoxO1 Thr^{24}/FoxO3a Thr^{32} phosphorylation, (I) Murf-1 mRNA levels and (J) Mafbx mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. (K) Representative western blots. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg; D-3MH: deuterated 3-methyl-histidine. *p<0.05, **p<0.01 HYP vs NOR; $^{\$}$p<0.05 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01 4h Post vs Post. n = 10/group.

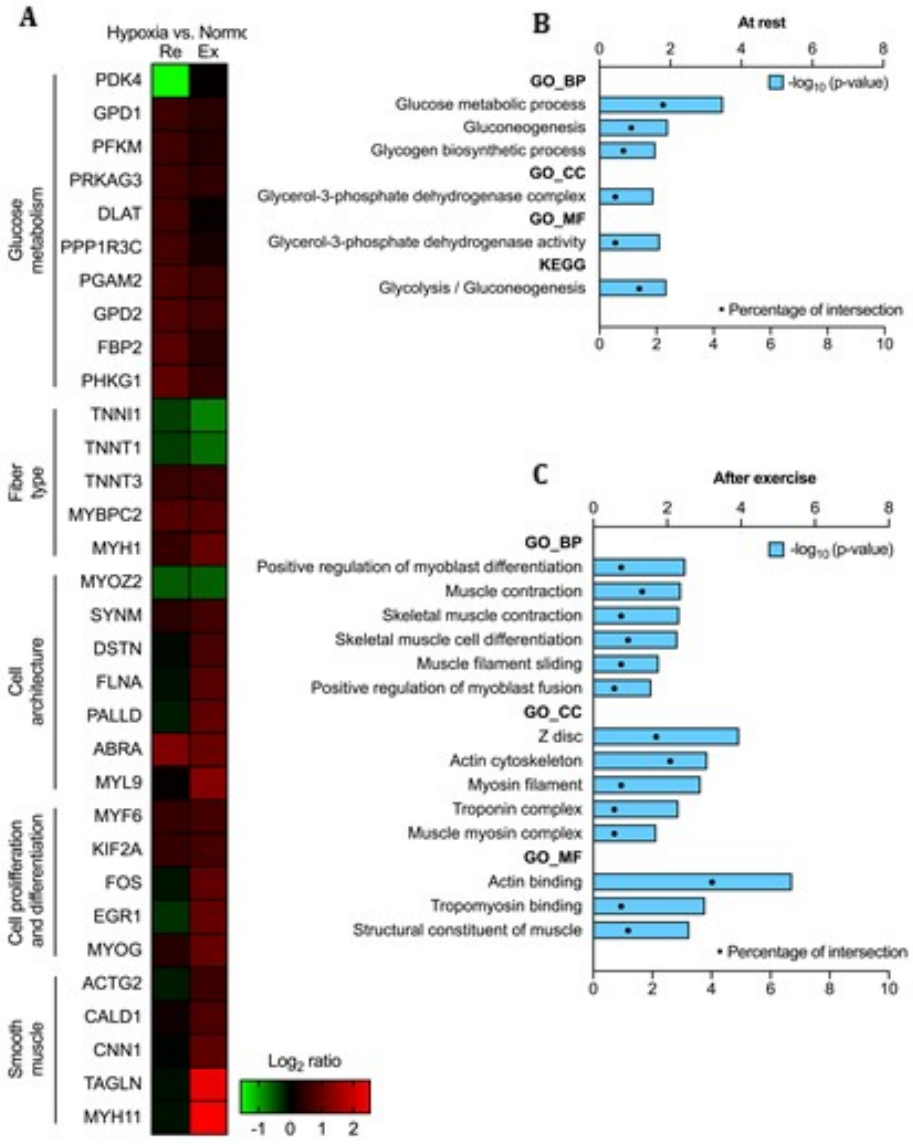

Figure 4. Regulation of gene expression by hypoxia and exercise

(A) Heat map with selected genes >1.5-fold up- or down-regulated in hypoxia vs. normoxia in the control (Re) and the exercised (Ex) legs. Gene ontology by DAVID web tool using the genes >1.5-fold up- or down-regulated in hypoxia vs.

normoxia in the control (B) and in the exercised (C) legs; only significantly modified terms are presented, as −log10(p-value) > 1.3 (Fischer exact p-value). Percentage of intersection represents the percentage of genes regulated in hypoxia vs. normoxia in relation to the genes in the DAVID database. n = 8 in normoxia and n = 7 in hypoxia. BP, biological process; CC, cellular component; GO, gene ontology; MF, molecular function.

Hypoxia regulated markers for cell differentiation but not proliferation

The mRNA levels of Fos were lower at 4h Post compared to Post in all conditions ($p<0.05$, Fig. 5A). At Post, Fos mRNA levels were higher in the exercised leg compared to the control leg in both normoxia and hypoxia ($p<0.01$). In addition, at Post, Fos mRNA levels were higher in hypoxia in the control leg ($p=0.012$) and tended to be higher in the exercised leg ($p=0.098$). The mRNA levels of Cyclin D1 were higher in the exercised leg compared to the control leg at 4h Post in hypoxia ($p=0.028$, Fig. 5B) The mRNA levels of Pcna were lower in the exercised leg compared to the control leg at 4h Post in normoxia ($p=0.020$, Fig. 5C). The mRNA levels of p21 were similarly increased by exercise at 4h Post in normoxia and in hypoxia ($p<0.05$, Fig. 5D). Myf5 mRNA levels were not modified by any condition (Fig. 5E). A time ($p=0.006$) and an exercise ($p=0.046$) effects were found for MyoD (Fig. 5F). The mRNA levels of myogenin were higher in hypoxia compared to normoxia in the control leg at Post ($p=0.009$) and tended to be higher at 4h Post compared to Post in the exercised leg in hypoxia ($p=0.072$). Mrf4 mRNA levels were decreased 4h Post compared to Post in both normoxia and hypoxia in the control leg

as well as in the exercised leg ($p<0.05$, Fig. 5H). In addition, exercise increased the mRNA levels of Mrf4 at 4h Post in both normoxia ($p=0.016$) and hypoxia ($p=0.008$) and at Post in hypoxia only ($p=0.044$).

Hypoxia-inducible factor pathway was not modified by any condition

Neither the localization (Fig. 6A, Supplementary file 3), the protein expression (Fig. 6B), nor the mRNA levels (Fig. 6C) of HIF-1α was modified by any condition. Similar results were found for Hif-2α (Fig. 6D) and Plin2 (Fig. 6G) mRNA levels. Vegf mRNA levels were higher 4h Post in normoxia ($p=0.004$) and tended to be higher in hypoxia ($p=0.064$) compared to Post (Fig. 6E). In addition Vegf mRNA was higher in the exercised leg 4h Post compared to the control leg in both normoxia ($p<0.001$) and hypoxia ($p=0.024$). A main time effect was observed for Lox mRNA levels ($p=0.032$, Fig. 6F), with lower values at 4h Post compared to Post.

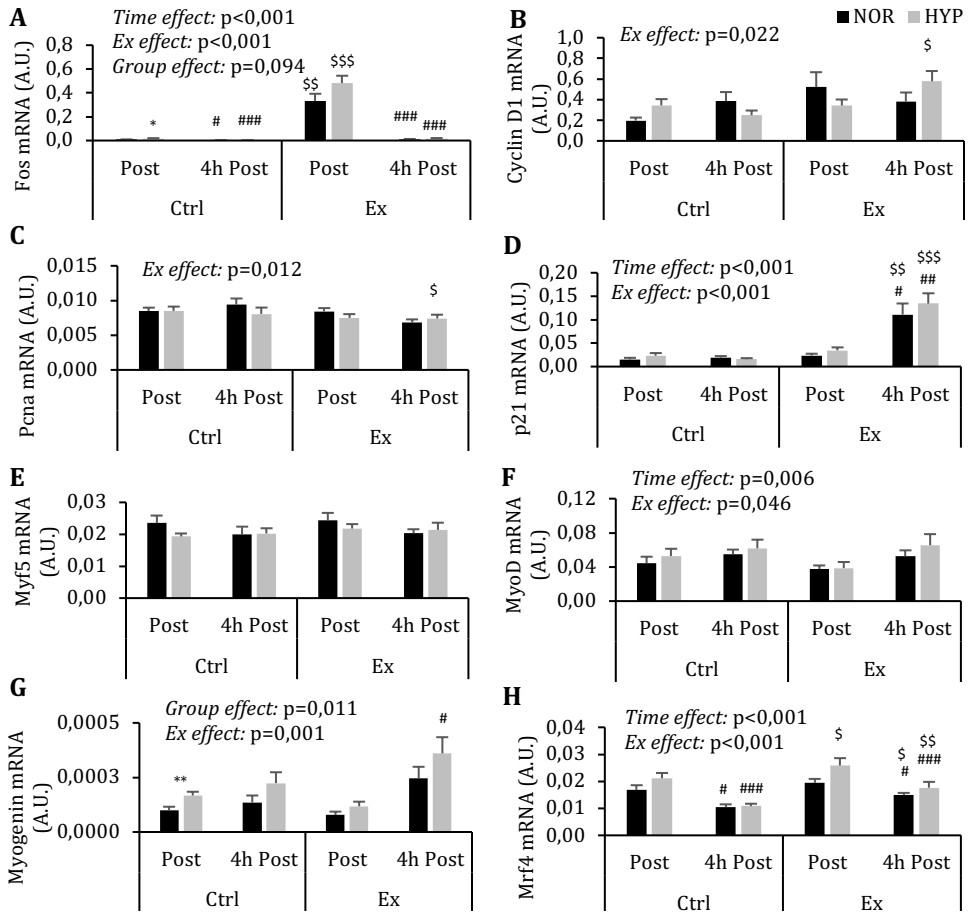

Figure 5. Regulation of the myogenic regulatory factors by hypoxia and exercise

(A) Fos, (B) Cyclin D1, (C) Pcna, (D) p21, (E) Myf5, (F) MyoD, (G) myogenin, (H) Mrf4 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg. **p<0.01 (HYP vs NOR); $p<0.05, $$p<0.01 (Ex vs Ctrl); #p<0.05, ###p<0.001 4h (Post vs Post). (n = 10/group).

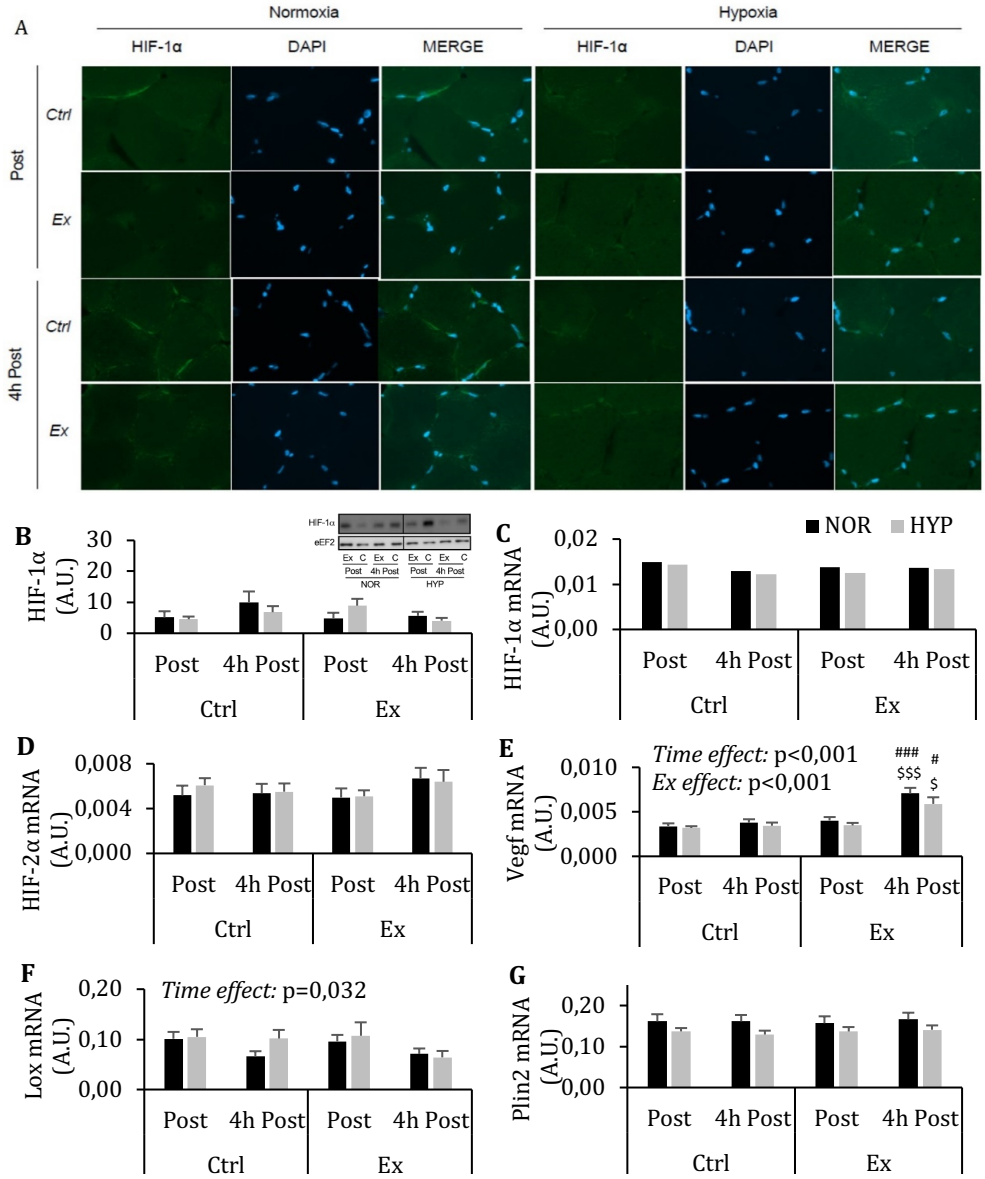

Figure 6. Regulation of the hypoxia-inducible factor 1 alpha pathway by hypoxia and exercise

(A) HIF-1α localization, (B) HIF-1α protein expression, (C) Hif-1α, (D) Hif-2α, (E) Vegf, (F) Lox and (G) Plin2 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP,

hypoxia; Ctrl, control leg; Ex, exercised leg. $^{\$}p<0.05$, $^{\$\$\$}p<0.001$ (Ex vs Ctrl); $^{\#}p<0.05$, $^{\#\#}p<0.01$ (4h Post vs Post). n = 10/group.

4. Discussion

Skeletal muscle adapts to hypoxia and resistance training. Combining both stimuli intermittently seems an effective strategy to favor muscle hypertrophy (306, 320, 369), even if the molecular bases remain unclear. To the best of our knowledge, only one study focused on the regulation of protein metabolism after an acute hypoxic resistance exercise in human (274). Muscle protein synthesis was blunted by hypoxia after resistance training, but no amino acids were provided to favor muscle anabolism and the hypoxic conditions were harsher (12% for 3.5h) than the one typically used by sportsmen (14-15% O_2 for 30min to 1h). As the hypoxic dose is crucial for the regulation of muscle mass (370), we hypothesized that different doses could induce different responses and a resistance exercise session performed in a moderate environmental hypoxia could induce a positive protein balance and favor muscle mass accretion when repeated (371). Contrary to our hypothesis, a lower dose of hypoxia blunted protein synthesis as well. This is surprising because an inverse correlation has been previously found between SpO_2 and the fractional synthetic rate after resistance exercise, with the lowest rates being measured in subjects displaying SpO_2 below 85% (274). Here, SpO_2 averaged 93-94% in the hypoxic group and still protein synthesis did not increase after exercise. As we found no relationship between SpO_2 or TSI and the protein synthetic rate (data not shown), we looked for other possible molecular mechanisms that could have led to the lack of activation of protein synthesis after resistance exercise in hypoxia. We have previously found that the Akt/mTOR pathway at rest (275) and the autophagy pathway after endurance exercise (372) were modulated by

hypoxia. Here, despite a reduced phosphorylation of AMPK and a reduced expression of REDD1, the activation of the Akt/mTOR pathway after exercise was not modified in hypoxia. These results indicate that the activation of AMPK (373) and REDD1 (374) was not sufficient to inhibit the Akt/mTOR pathway or that this inhibitory role was overruled by another undetermined signal. One of those signals could have been insulin, as it has been previously related to an activation of the Akt/mTOR pathway under hypoxic conditions (275). Insulin increased after breakfast in our study, but this increase was not modulated by the environmental conditions in which exercise was performed. Those results suggest that hypoxia did not blunt protein synthesis through a decrease in insulin concentrations and down-regulation of the Akt/mTOR pathway.

For the first time, the muscle protein degradation rate was measured after hypoxic training. We found that the protein degradation rate was similar after normoxic and hypoxic training. Unfortunately, the technique used to determine the muscle degradation rate, i.e. the rate of disappearance of 3-D-methyl-histidine from the plasma (366), only allows comparison between normoxia and hypoxia, not between the rested and the exercised leg. The only study having determined the rate of muscle protein degradation in hypoxic conditions was made in rats exposed to severe hypobaric hypoxia (7620m) for 14 days (375). The degradation rate was higher after 3 days of exposure and increased further after 14 days. As those conditions profoundly differ from ours (species, duration of exposure, hypoxic dose, exercise), no comparison is possible. Though, we ensure the values we obtained were in the range of

those reported previously for both protein synthesis (365) and protein degradation (366) with the same techniques. While calculation of the net protein balance was not possible due to the use of two different isotopes, we can speculate that the exercise session in normoxia induced a more favorable balance than in hypoxia, contrary to our hypothesis.

A similar global protein degradation rate did not exclude that specific proteolytic processes were regulated by hypoxia or that protein degradation was differently regulated in the exercised and in the control leg. Therefore, as we have previously found that hypoxia could activate autophagy after endurance exercise (372), we investigated whether the same occurred after resistance exercise. Initiation of autophagy through LC3b lipidation was highly activated by the nutritional state, inversely following insulin levels, and partially reversed by resistance exercise. However, hypoxia had no influence on those regulations on LC3bII/I, nor on p62 expression, which remained unaltered by any condition. Those results contrast with our previous study (372), probably because the hypoxic conditions were less severe here. The level of oxygen was higher here (14% vs. 11%) and the duration much shorter (30min vs. 8h), indicating that moderate hypoxia does not influence the post-translational regulation of autophagy. Unexpectedly after such short exposure duration, hypoxia down-regulated the expression of a series of genes that compose the so-called transcriptional response of autophagy, amongst which Lc3b and Bnip3. Usually, the transcriptional response is activated when autophagy lasts for hours or days to replenish the components of the machinery (376). The lower mRNA levels of Lc3b and Bnip3 in hypoxia are therefore surprising and opposite to the regulation

of FoxO. The latter activates autophagy at both the post-translational and transcriptional levels (376). Here, FoxO phosphorylation was higher in hypoxia but did not translate into an activation of autophagy at any level. It still remains to determine why Lc3b and Bnip3 mRNA levels were lower, and except a regulation of their stability by hypoxia, no other explanation can be put forward. In summary, moderate hypoxia down-regulated the expression of a series of genes involved in the transcriptional response of autophagy without impacting the initiation of the process as measured by the unchanged LC3bII/I ratio in hypoxic compared to normoxic conditions.

The microarray analyses revealed that hypoxia regulated the expression of genes involved in glucose metabolism at rest, and genes involved in myoblast differentiation and fusion, and in muscle contraction machinery after exercise. This is the first microarray analysis reporting the transcriptional regulation induced by a single exposure to environmental hypoxia in human skeletal muscle. The higher expression of genes involved in glucose metabolism at rest is not surprising, as a better glucose handling (377) and higher dependence on blood glucose (378, 379) have been shown in people living at moderate to high altitude. Here, we show that a single exposure of 30min is enough to initiate those adaptations at a transcriptional level, confirming previous results from our lab showing a higher abundance of GLUT4 at the sarcolemmal membrane after 4h of hypoxic exposure (380). After exercise, genes involved in myoblast differentiation and fusion, and in muscle contraction machinery were differently regulated in hypoxia compared to normoxia. Globally, genes related to the slow phenotype were down-

regulated, such as troponin I1 and troponin T1, whereas those related to the fast phenotype were-upregulated, such as troponin T3 and Myh1. Studies having looked at fiber typing before and after hypoxic exposure in human are scarce and data are not straightforward. In addition, the role of physical activity in those adaptations is unclear. One study found no change in myosin heavy chain type I and type IIx mRNA levels after 6 weeks of endurance training (381), while another study found an increase in type I and a decrease in type IIx protein expression after a 43-day Himalayan expedition (382). However, the higher mRNA level we observed for Myh1, which encodes for myosin heavy chain type IIx, is a positive molecular response to resistance exercise, which requires explosive and powerful muscle contractions. Whether this will translate into higher protein content and better performance needs to be investigated with a training study.

Environmental hypoxia regulates myogenesis during embryonic developement, myoblasts and satellite cell proliferation and differentiation (243). Here, we were particularly interested in the effect of hypoxia on the proliferation of satellite cells and their differentiation into myoblasts. Both processes are tightly regulated by the myogenic regulatory factors. Myf5 and MyoD are expressed upon activation and proliferation of satellite cells (383). MyoD remains expressed at early stages of differentiation and myogenin, followed by Mrf4, at a later stage (383). In vitro, low oxygen levels (3-6%) seem to favor myoblast proliferation, whereas very low oxygen levels (<1%) tend to alter myogenic differentiation and myotube formation (384). Murine myoblasts cultured in a severe hypoxic environment (1% O_2) have been

shown to up-regulate the expression of genes related to cell cycle and metabolic processes and to down-regulate the expression of genes associated with catabolic processes and muscle development (385). Our results contrast with the aforementioned studies as no effect of hypoxia was found on specific markers for cell proliferation such as Cyclin D1, Pcna or p21 whereas hypoxia increased the expression of some markers for cell differentiation. Both the microarray and the qPCR analyses showed that the mRNA levels of myogenin were higher in hypoxia compared to normoxia. In addition, a trend to an increase was observed for MyoD and Mrf4 mRNA. If this translates at the protein level, a well-controlled dose of hypoxia would favor satellite cell incorporation and differentiation into existing myofibres, which could be a mechanism contributing to the increased muscle mass after intermittent and moderate hypoxia previously reported in human (247).

Whether HIF-1α is responsible for the regulation of protein metabolism and satellite cells under hypoxic conditions is currently unknown. In vitro, HIF-1α and the mTORC1 pathway have been shown to regulate each other (386-388), but this has not been confirmed in vivo. Data on intramuscular PO_2 are sparse, but a critical threshold of 7-8 mmHg has been suggested below which intramuscular HIF-1α accumulates (389). When humans are passively exposed to environmental hypoxia, the drop in intramuscular PO_2 does not surpass this threshold (255) and HIF-1α does not stabilize as we previously showed (275). Contrarily, HIF-1α can be stabilized during repeated muscle contractions in humans, which are known to largely decrease intramuscular PO_2 (255), coupled or not to blood flow restriction (241). Here, the tissue saturation index decreased

by about 10% during resistance exercise, but no additional effect of hypoxia was observed. It seems therefore that repeated contractions or endurance exercise is more potent to stabilize HIF-1α than resistance exercise. Of note, no decrease in tissue oxygenation index was detected under hypoxia, probably because 14% O_2 was not severe enough as skeletal muscle is a potent tissue to face a lack of oxygen. Therefore, the molecular regulations observed in the present study after resistance exercise and/or hypoxia were probably independent of the HIF-1α pathway as HIF-1α was not stabilized and the expression of downstream target genes (Vegf, Redd1, Lox and Plin2 mRNA) was not increased at the two time points analyzed here.

The strength of the present study is the direct measurement of protein synthesis and degradation together with the quantification of commonly used markers to assess these processes. The microarray data provide new perspectives in terms of further investigation and more particularly at the level of satellite cells. The main limitation of the present study is the absence of muscle biopsy before the exercise session, as the protocol already comprised four muscle biopsies within a period of four hours. Thus, we cannot exclude a systemic effect from the exercised to the rested leg since both biopsies were taken at the same time. This said, we were mainly interested in the effect of hypoxia, which could be properly studied by comparison to the normoxic condition.

All together, we conclude that, contrary to our hypothesis, environmental hypoxia did not potentiate the short-term anabolic response after resistance exercise, but it initiated transcriptional

regulations that could potentially translate into satellite cells incorporation and higher force production on the long-term, which remains to be investigated.

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Author contributions

L.D., M.F., K.S. and P.J.A. designed the research; O.G., H.N., D.N. and L.D. conducted the research; A. D. and J-B. D participated to the development of analytical techniques; O.G., R.F-V., M.B., E.B., D.N., M.S. and J.C. analyzed and interpreted data; O.G. and L.D. wrote the manuscript; all authors critically revised and contributed to the manuscript; all authors approved the final version of the manuscript; L.D. has primary responsibility for final content. Data collection took place at Université catholique de Louvain. Data analyses took place at Université catholique de Louvain and the University of Nottingham.

Additional information

The authors declare no competing financial interests.

Supplemental Data 1

Table 1 : DAVID analysis of genes regulated at rest

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment
GOTERM_BP_DIRECT	GO:0006006~glucose metabolic process	8	2.23	3.27E-04	PPP1R3C, TNF, PHKG1, PDK4, PGAM2, DLAT, FBP2, PFKM	166	129	16412	6.13
GOTERM_BP_DIRECT	GO:2001179~regulation of interleukin-10 secretion	3	0.84	5.95E-04	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	4	16412	74.15
GOTERM_BP_DIRECT	GO:0032673~regulation of interleukin-4 production	3	0.84	9.85E-04	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	5	16412	59.32
GOTERM_BP_DIRECT	GO:0002381~immunoglobulin production involved in immunoglobulin mediated immune response	3	0.84	2.04E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	7	16412	42.37
GOTERM_BP_DIRECT	GO:0002437~inflammatory response to antigenic stimulus	4	1.11	2.44E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, RBPJ	166	27	16412	14.65
GOTERM_BP_DIRECT	GO:0002455~humoral immune response mediated by circulating immunoglobulin	3	0.84	2.70E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	8	16412	37.08

GOTERM_BP_DIRECT	GO:0006959~humoral immune response	5	1.39	2.74E-03	TNF, CCL2, IFNA7, IFNA8, RBPJ	166	58	16412	8.52
GOTERM_BP_DIRECT	GO:0042088~T-helper 1 type immune response	3	0.84	6.21E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	12	16412	24.72
GOTERM_BP_DIRECT	GO:0044281~small molecule metabolic process	29	8.08	6.28E-03	CYP1B1, ADH1B, PGAM2, ANKRD1, AGMAT, CDS2, PPP1R3C, SLC2A5, GADL1, SLC22A3, HBB, IP6K2, GPD2, GPD1, SLC25A5, PHKG1, PDK4, GSTT1, HBA2, FBP2, HBA1, DLAT, PFKM, MT1X, DHFR, UCP3, FABP4, SLC25A16, OAT	166	1706	16412	1.68
GOTERM_BP_DIRECT	GO:0015671~oxygen transport	3	0.84	9.68E-03	HBA2, HBA1, HBB	166	15	16412	19.77
GOTERM_BP_DIRECT	GO:0016045~detection of bacterium	3	0.84	9.68E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	15	16412	19.77
GOTERM_BP_DIRECT	GO:0006094~gluconeogenesis	4	1.11	1.17E-02	GPD2, GPD1, PGAM2, FBP2	166	47	16412	8.41
GOTERM_BP_DIRECT	GO:0043403~skeletal muscle tissue regeneration	3	0.84	1.24E-02	MTPN, PAX7, MSTN	166	17	16412	17.45

GOTERM_BP_DIRECT	GO:0010880~regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	3	0.84	1.24E-02	ASPH, DHRS7C, CASQ2	166	17	16412	17.45
GOTERM_BP_DIRECT	GO:0042744~hydrogen peroxide catabolic process	3	0.84	1.70E-02	HBA2, HBA1, HBB	166	20	16412	14.83
GOTERM_BP_DIRECT	GO:0001708~cell fate specification	3	0.84	1.86E-02	PSEN1, CDON, C8ORF22	166	21	16412	14.12
GOTERM_BP_DIRECT	GO:0071407~cellular response to organic cyclic compound	4	1.11	1.96E-02	TNF, CCL2, CYP1B1, ANKRD1	166	57	16412	6.94
GOTERM_BP_DIRECT	GO:0002286~T cell activation involved in immune response	3	0.84	2.21E-02	IFNA7, PSEN1, IFNA8	166	23	16412	12.90
GOTERM_BP_DIRECT	GO:0005978~glycogen biosynthetic process	3	0.84	2.59E-02	PRKAG3, PPP1R3C, PHKG1	166	25	16412	11.86
GOTERM_BP_DIRECT	GO:0010942~positive regulation of cell death	3	0.84	2.79E-02	HBA2, HBA1, HBB	166	26	16412	11.41
GOTERM_BP_DIRECT	GO:0019221~cytokine-mediated signaling pathway	8	2.23	2.79E-02	CCL2, IFNA7, HLA-DRB1, HLA-DRB4, LRRC66, HLA-DRB5, IFNA8, IP6K2	166	291	16412	2.72
GOTERM_BP_DIRECT	GO:0045471~response to ethanol	5	1.39	2.80E-02	ACTC1, CCL2, TNC, MSTN, SPARC	166	114	16412	4.34
GOTERM_BP_DIRECT	GO:0030183~B cell differentiation	4	1.11	2.88E-02	IFNA7, IFNA8, RBPJ, MYB	166	66	16412	5.99

GOTERM_BP_DIRECT	GO:0035774~positive regulation of insulin secretion involved in cellular response to glucose stimulus	3	0.84	3.42E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	29	16412	10.23
GOTERM_BP_DIRECT	GO:0001656~metanephros development	3	0.84	3.64E-02	IRX3, GDNF, CTSH	166	30	16412	9.89
GOTERM_BP_DIRECT	GO:0032689~negative regulation of interferon-gamma production	3	0.84	3.64E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	30	16412	9.89
GOTERM_BP_DIRECT	GO:2001240~negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	3	0.84	4.09E-02	TNF, HSPA1B, GDNF	166	32	16412	9.27
GOTERM_BP_DIRECT	GO:0006919~activation of cysteine-type endopeptidase activity involved in apoptotic process	4	1.11	4.53E-02	TNFSF10, TNF, VCP, CTSH	166	79	16412	5.01
GOTERM_BP_DIRECT	GO:0042130~negative regulation of T cell proliferation	3	0.84	4.57E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	34	16412	8.72
GOTERM_BP_DIRECT	GO:0045597~positive regulation of cell differentiation	3	0.84	4.81E-02	RPS6KA3, GDNF, JUNB	166	35	16412	8.47
GOTERM_BP_DIRECT	GO:0002503~peptide antigen assembly with MHC class II protein complex	2	0.56	4.93E-02	HLA-DRB1, HLA-DRB4	166	5	16412	39.55
GOTERM_CC_DIRECT	GO:0071682~endocytic vesicle lumen	4	1.11	5.25E-04	HBA2, HBA1, SPARC, HBB	181	16	17701	24.45
GOTERM_CC_DIRECT	GO:0031838~haptoglobin-hemoglobin complex	3	0.84	6.09E-04	HBA2, HBA1, HBB	181	4	17701	73.35

GOTERM_CC_DIRECT	GO:0033017~sarcoplasmic reticulum membrane	4	1.11	4.51E-03	MRLN, ASPH, DHRS7C, CASQ2	181	33	17701	11.85
GOTERM_CC_DIRECT	GO:0005833~hemoglobin complex	3	0.84	6.35E-03	HBA2, HBA1, HBB	181	12	17701	24.45
GOTERM_CC_DIRECT	GO:0042613~MHC class II protein complex	3	0.84	1.12E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	181	16	17701	18.34
GOTERM_CC_DIRECT	GO:0016020~membrane	30	8.36	1.70E-02	CAV2, HLA-DRB1, TNC, BROX, ARF6, ART4, CDS2, MED28, EXOC4, SLC22A3, HLA-DRB5, LMOD1, HIST1H4D, FAM129A, NPHP1, ACTC1, NCEH1, SLC12A2, SLC25A5, HBA2, HBA1, FRZB, ATP6V1D, RAB40AL, OGFRL1, PSEN1, SLC7A2, ANXA11, SYTL2, DNAJB6	181	1901	17701	1.54
GOTERM_CC_DIRECT	GO:0030018~Z disc	5	1.39	2.38E-02	IGFN1, FBP2, HOMER1, CASQ2, DNAJB6	181	107	17701	4.57
GOTERM_CC_DIRECT	GO:0009331~glycerol-3-phosphate dehydrogenase complex	2	0.56	3.02E-02	GPD2, GPD1	181	3	17701	65.20
GOTERM_CC_DIRECT	GO:0071556~integral component of lumenal side of endoplasmic reticulum membrane	3	0.84	3.27E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	181	28	17701	10.48

GOTERM_CC_DIRECT	GO:0030658~transport vesicle membrane	3	0.84	4.66E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	181	34	17701	8.63
GOTERM_MF_DIRECT	GO:0031720~haptoglobin binding	3	0.84	2.97E-04	HBA2, HBA1, HBB	166	3	16483	99.30
GOTERM_MF_DIRECT	GO:0019825~oxygen binding	4	1.11	4.31E-03	CYP1B1, HBA2, HBA1, HBB	166	33	16483	12.04
GOTERM_MF_DIRECT	GO:0005344~oxygen transporter activity	3	0.84	8.38E-03	HBA2, HBA1, HBB	166	14	16483	21.28
GOTERM_MF_DIRECT	GO:0004601~peroxidase activity	3	0.84	1.68E-02	HBA2, HBA1, HBB	166	20	16483	14.89
GOTERM_MF_DIRECT	GO:0004368~glycerol-3-phosphate dehydrogenase activity	2	0.56	1.99E-02	GPD2, GPD1	166	2	16483	99.30
GOTERM_MF_DIRECT	GO:0035256~G-protein coupled glutamate receptor binding	2	0.56	2.97E-02	HOMER3, HOMER1	166	3	16483	66.20
GOTERM_MF_DIRECT	GO:0005125~cytokine activity	6	1.67	3.01E-02	TNFSF10, TNF, IFNA7, MSTN, IFNA8, LTB	166	173	16483	3.44
GOTERM_MF_DIRECT	GO:0005164~tumor necrosis factor receptor binding	3	0.84	3.61E-02	TNFSF10, TNF, LTB	166	30	16483	9.93
GOTERM_MF_DIRECT	GO:0042605~peptide antigen binding	3	0.84	3.83E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	166	31	16483	9.61

GOTERM_MF_DIRECT	GO:0070061~fructose binding	2	0.56	4.91E-02	SLC2A5, PFKM	166	5	16483	39.72
KEGG_PATHWAY	hsa05164:Influenza A	10	2.79	5.71E-04	TNFSF10, TNF, CCL2, IFNA7, HLA-DRB1, HLA-DRB4, HLA-DRB5, HSPA1B, IFNA8, MAPK10	95	174	6891	4.17
KEGG_PATHWAY	hsa05323:Rheumatoid arthritis	7	1.95	1.07E-03	TNF, CCL2, HLA-DRB1, HLA-DRB4, HLA-DRB5, LTB, ATP6V1D	95	86	6891	5.90
KEGG_PATHWAY	hsa05168:Herpes simplex infection	9	2.51	3.26E-03	TNF, CCL2, IFNA7, HLA-DRB1, TAF5, HLA-DRB4, HLA-DRB5, IFNA8, MAPK10	95	182	6891	3.59
KEGG_PATHWAY	hsa05144:Malaria	5	1.39	4.30E-03	TNF, CCL2, HBA2, HBA1, HBB	95	49	6891	7.40
KEGG_PATHWAY	hsa05320:Autoimmune thyroid disease	5	1.39	4.97E-03	IFNA7, HLA-DRB1, HLA-DRB4, HLA-DRB5, IFNA8	95	51	6891	7.11
KEGG_PATHWAY	hsa05206:MicroRNAs in cancer	11	3.06	5.23E-03	MIR26A2, MIR335, CYP1B1, MIR16-2, TNC, MIRLET7F1, MIR29B1, ZEB2, CDK6, CCNG1, MIR181B2	95	285	6891	2.80
KEGG_PATHWAY	hsa05310:Asthma	4	1.11	6.95E-03	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5	95	29	6891	10.01

KEGG_PATHWAY	hsa05143:African trypanosomiasis	4	1.11	9.98E-03	TNF, HBA2, HBA1, HBB	95	33	6891	8.79
KEGG_PATHWAY	hsa05332:Graft-versus-host disease	4	1.11	9.98E-03	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5	95	33	6891	8.79
KEGG_PATHWAY	hsa00010:Glycolysis / Gluconeogenesis	5	1.39	1.29E-02	ADH1B, PGAM2, DLAT, FBP2, PFKM	95	67	6891	5.41
KEGG_PATHWAY	hsa05330:Allograft rejection	4	1.11	1.37E-02	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5	95	37	6891	7.84
KEGG_PATHWAY	hsa04940:Type I diabetes mellitus	4	1.11	1.92E-02	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5	95	42	6891	6.91
KEGG_PATHWAY	hsa04612:Antigen processing and presentation	5	1.39	1.97E-02	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5, HSPA1B	95	76	6891	4.77
KEGG_PATHWAY	hsa05145:Toxoplasmosis	6	1.67	2.22E-02	TNF, HLA-DRB1, HLA-DRB4, HLA-DRB5, HSPA1B, MAPK10	95	118	6891	3.69
KEGG_PATHWAY	hsa05152:Tuberculosis	7	1.95	3.35E-02	TNF, IFNA7, HLA-DRB1, HLA-DRB4, HLA-DRB5, IFNA8, MAPK10	95	177	6891	2.87

Table 2: DAVID analysis of genes regulated after exercise

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment
GOTERM_BP_DIRECT	GO:0010628~positive regulation of gene expression	12	2.84	5.49E-04	NTRK3, ACTG2, HFE, SMAD3, TLR3, CDK6, HSPA1A, HSPA1B, MSN, EIF2AK3, CTSH, HNRNPU	237	232	16412	3.58
GOTERM_BP_DIRECT	GO:2001179~regulation of interleukin-10 secretion	3	0.71	1.21E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	4	16412	51.94
GOTERM_BP_DIRECT	GO:0032673~regulation of interleukin-4 production	3	0.71	2.00E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	5	16412	41.55
GOTERM_BP_DIRECT	GO:0031397~negative regulation of protein ubiquitination	5	1.18	3.04E-03	KLHL40, SOX4, HSPA1A, HSPA1B, TNFAIP3	237	42	16412	8.24
GOTERM_BP_DIRECT	GO:0045663~positive regulation of myoblast differentiation	4	0.95	3.22E-03	MYF6, CXCL9, MYOG, NR2C2	237	21	16412	13.19
GOTERM_BP_DIRECT	GO:0098779~activation of mitophagy in response to mitochondrial depolarization	8	1.89	3.26E-03	HAPLN1, CD93, COX8A, LMCD1, MYH11, SLC22A3, TXLNA, NR2C2	237	134	16412	4.13

GOTERM_BP_DIRECT	GO:0043086~negative regulation of catalytic activity	6	1.42	4.00E-03	PINLYP, PPP1R2P3, PDE6H, HNRNPR, ANXA2, ANXA2P2	237	73	16412	5.69
GOTERM_BP_DIRECT	GO:0002381~immunoglobulin production involved in immunoglobulin mediated immune response	3	0.71	4.12E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	7	16412	29.68
GOTERM_BP_DIRECT	GO:0006936~muscle contraction	7	1.65	4.28E-03	ACTG2, CALD1, MYH11, LMOD1, TRIM63, ANXA2, MYL9	237	106	16412	4.57
GOTERM_BP_DIRECT	GO:0003009~skeletal muscle contraction	4	0.95	4.75E-03	TNNT3, TNNT1, CHRNA1, TNNI1	237	24	16412	11.54
GOTERM_BP_DIRECT	GO:0035914~skeletal muscle cell differentiation	5	1.18	5.32E-03	MYF6, EGR1, FOS, ATF3, MYOG	237	49	16412	7.07
GOTERM_BP_DIRECT	GO:0034198~cellular response to amino acid starvation	4	0.95	5.35E-03	ATF3, SLC38A2, UCP2, EIF2AK3	237	25	16412	11.08
GOTERM_BP_DIRECT	GO:0002455~humoral immune response mediated by circulating immunoglobulin	3	0.71	5.45E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	8	16412	25.97
GOTERM_BP_DIRECT	GO:0002437~inflammatory response to antigenic stimulus	4	0.95	6.66E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, RBPJ	237	27	16412	10.26
GOTERM_BP_DIRECT	GO:0010941~regulation of cell death	3	0.71	8.59E-03	JUND, HSPA1A, HSPA1B	237	10	16412	20.77
GOTERM_BP_DIRECT	GO:0006955~immune response	12	2.84	1.17E-02	HLA-DRB1, TENM1, HLA-DRB4, IGHA1, CXCL9, HFE,	237	346	16412	2.40

					HLA-DRB5, SMAD3, IGKC, TNFAIP3, LTB, HLA-DQA1				
GOTERM_BP_DIRECT	GO:0042088~T-helper 1 type immune response	3	0.71	1.24E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	12	16412	17.31
GOTERM_BP_DIRECT	GO:0030049~muscle filament sliding	4	0.95	1.71E-02	TNNT3, TNNT1, MYBPC2, TNNI1	237	38	16412	7.29
GOTERM_BP_DIRECT	GO:0045184~establishment of protein localization	4	0.95	1.83E-02	ZDHHC15, CHMP1B, ITGA8, FLNA	237	39	16412	7.10
GOTERM_BP_DIRECT	GO:0016045~detection of bacterium	3	0.71	1.91E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5	237	15	16412	13.85
GOTERM_BP_DIRECT	GO:0010467~gene expression	23	5.44	2.05E-02	TAF3, HIST1H4K, RPL13, LCMT2, COX8A, SMAD3, HSPA1A, RORA, TRMT10C, NR4A3, RPS2, HNRNPR, NR2C2, HNRNPU, PPA1, PRPF19, EIF4A2, HIST1H4C, NEDD4L, SAP30L, SPCS2, RBPJ, LTB	237	958	16412	1.66
GOTERM_BP_DIRECT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	23	5.44	2.22E-02	ABLIM1, MYF6, EGR1, ABLIM2, SMAD3, TLR3, SOX4, FHL5, RORA, NR4A3, GDNF, SOHLH1, NR2C2, FOS, ATF3, ZNF148, ETS2, JUND, ABRA, MYOG, MAFA, RBPJ, PBX3	237	966	16412	1.65

GOTERM_BP_DIRECT	GO:0007608~sensory perception of smell	7	1.65	2.40E-02	OR2T29, OR5V1, OR2J2, OR2T34, OR10A7, OR2W1, OR51J1	237	154	16412	3.15
GOTERM_BP_DIRECT	GO:1901741~positive regulation of myoblast fusion	3	0.71	2.71E-02	MYF6, CXCL9, MYOG	237	18	16412	11.54
GOTERM_BP_DIRECT	GO:0009416~response to light stimulus	3	0.71	3.00E-02	FOS, JUND, CLU	237	19	16412	10.93
GOTERM_BP_DIRECT	GO:0006629~lipid metabolic process	6	1.42	3.06E-02	UCP3, TECRL, CLU, ATP5A1, ACAT2, THRSP	237	121	16412	3.43
GOTERM_BP_DIRECT	GO:0050821~protein stabilization	6	1.42	3.65E-02	CLU, SMAD3, SOX4, HSPA1A, HSPA1B, FLNA	237	127	16412	3.27
GOTERM_BP_DIRECT	GO:0060548~negative regulation of cell death	4	0.95	4.06E-02	NTRK3, SOX4, HSPA1A, HSPA1B	237	53	16412	5.23
GOTERM_BP_DIRECT	GO:0070434~positive regulation of nucleotide-binding oligomerization domain containing 2 signaling pathway	2	0.47	4.25E-02	HSPA1A, HSPA1B	237	3	16412	46.17
GOTERM_BP_DIRECT	GO:0006089~lactate metabolic process	2	0.47	4.25E-02	LDHB, PFKFB2	237	3	16412	46.17
GOTERM_BP_DIRECT	GO:0050911~detection of chemical stimulus involved in sensory perception of smell	12	2.84	4.40E-02	OR4K5, OR9K2, OR2T29, OR5H14, OR2A7, OR5V1, OR6C1, OR2J2, OR2T34, OR10A7, OR2W1, OR51J1	237	425	16412	1.96

GOTERM_BP_DIRECT	GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	5	1.18	4.52E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1, KIF2A	237	93	16412	3.72
GOTERM_BP_DIRECT	GO:0007565~female pregnancy	5	1.18	4.97E-02	FOS, THBD, SLC38A2, UCP2, HFE	237	96	16412	3.61
GOTERM_CC_DIRECT	GO:0030018~Z disc	9	2.13	1.14E-04	IGFN1, PGM5, SORBS2, FBXO32, SYNPO2, MYOZ2, TRIM63, PALLD, MYL9	244	107	17701	6.10
GOTERM_CC_DIRECT	GO:0072562~blood microparticle	10	2.36	1.45E-04	ACTG2, CLU, ITIH4, IGHA1, HBA2, HSPA1A, HSPA1B, MSN, IGKC, CIB2	244	141	17701	5.15
GOTERM_CC_DIRECT	GO:0005925~focal adhesion	16	3.78	3.47E-04	HACD3, PDLIM1, SYNPO2, HSPA1A, HSPA1B, CSRP1, PALLD, RPS2, DDR2, FLNA, PGM5, SORBS2, ITGA8, GIT2, MSN, CNN1	244	391	17701	2.97
GOTERM_CC_DIRECT	GO:0015629~actin cytoskeleton	11	2.60	8.29E-04	ABLIM1, ABLIM2, SORBS2, CALD1, ABRA, SYNPO2, MYOZ2, PALLD, FLNA, LRRC7, DSTN	244	216	17701	3.69
GOTERM_CC_DIRECT	GO:0032982~myosin filament	4	0.95	1.25E-03	ACTG2, MYH1, MYBPC2, MYH11	244	16	17701	18.14
GOTERM_CC_DIRECT	GO:0042613~MHC class II protein complex	4	0.95	1.25E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	16	17701	18.14
GOTERM_CC_DIRECT	GO:0005667~transcription factor complex	9	2.13	3.86E-03	FOS, JUND, SMAD3, PDLIM1, MYOG, NR4A3, RBPJ, PBX3, HDAC9	244	183	17701	3.57

GOTERM_CC_DIRECT	GO:0009897~external side of plasma membrane	10	2.36	4.36E-03	KCNJ5, HLA-DRB1, SERPINA5, HLA-DRB4, IGHA1, CXCL9, HFE, HLA-DRB5, MFGE8, IGKC	244	228	17701	3.18
GOTERM_CC_DIRECT	GO:0005861~troponin complex	3	0.71	4.98E-03	TNNT3, TNNT1, TNNI1	244	8	17701	27.20
GOTERM_CC_DIRECT	GO:0071556~integral component of lumenal side of endoplasmic reticulum membrane	4	0.95	6.50E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	28	17701	10.36
GOTERM_CC_DIRECT	GO:0030658~transport vesicle membrane	4	0.95	1.12E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	34	17701	8.53
GOTERM_CC_DIRECT	GO:0016020~membrane	39	9.22	1.14E-02	LDHB, HIST1H4K, PANX1, HLA-DRB1, CYP51A1, RPL13, LRRC8C, TLR3, SGMS1, TXLNA, ARHGAP15, RPS2, PRPF19, FOS, P4HA1, SERPINA5, EXOC4, HLA-DRB5, SLC22A3, HIST1H4C, LMOD1, FAM129A, KIF2A, NCEH1, RRBP1, LRRC1, MFGE8, HBA2, FRZB, LPCAT3, HLA-DQA1, FLNA, HNRNPU, ANXA2, MUC4, KDSR, SYNM, ATP5A1, EIF2AK3	244	1901	17701	1.49
GOTERM_CC_DIRECT	GO:0030669~clathrin-coated endocytic vesicle membrane	4	0.95	1.86E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	41	17701	7.08

GOTERM_CC_DIRECT	GO:0005859~muscle myosin complex	3	0.71	1.98E-02	MYH1, MYH11, MYL9	244	16	17701	13.60
GOTERM_CC_DIRECT	GO:0012507~ER to Golgi transport vesicle membrane	4	0.95	2.96E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	49	17701	5.92
GOTERM_CC_DIRECT	GO:0032588~trans-Golgi network membrane	4	0.95	3.12E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	244	50	17701	5.80
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	67	15.84	3.34E-02	LRRC8C, RRAD, OR2J2, SGMS1, DDR2, OR10A7, OR9K2, CD93, SLC2A3, ROBO1, OR6C1, TAS2R9, ITIH4, SLC22A3, ABRA, MAS1L, ERAS, MSN, FAM129A, CHRNA1, LTB, NSF, PALLD, HLA-DQA1, FLNA, OR2W1, TAS2R14, THBD, OR5H14, OR5V1, AKAP5, PMP22, SLC38A2, HLA-DRB1, PANX1, LITAF, CYP51A1, CALD1, GPR148, HFE, HLA-DRB4, EXOC4, SCN9A, HLA-DRB5, SLC4A7, RHOBTB1, NEDD4L, OR2T34, IGKC, CRIM1, OR51J1, OR4K5, OR2A7, MAP1B, SMAD3, ANXA2, GPR33, KCNJ5, OR2T29, TENM1, ITGA8, ETS2,	244	3900	17701	1.25

					ADAM20, RGS8, ATP5A1, MERTK, FAM126A				
GOTERM_CC_DIRECT	GO:0031902~late endosome membrane	5	1.18	3.67E-02	CHMP1B, HLA-DRB1, HLA-DRB4, HLA-DRB5, ANXA2	244	91	17701	3.99
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	46	10.87	4.65E-02	NKAP, HIST1H4K, LITAF, SOX4, HSPA1A, SKIV2L2, HSPA1B, TRMT10C, RORA, RPS2, PTMA, NR2C2, PRPF19, FOS, ZNF148, GIT2, DHX16, MAS1L, MYOG, HIST1H4C, NEDD4L, SAP30L, THRSP, ZNF281, MYF6, EGR1, TAF3, ALPK2, RYBP, LMCD1, TMEM70, SMC6, SMAD3, CDK6, NR4A3, HNRNPR, HNRNPU, ATM, ATF3, DHFR, ETS2, WHSC1L1, SH3RF2, FBXO32, RBPJ, HDAC9	244	2561	17701	1.30
GOTERM_MF_DIRECT	GO:0003779~actin binding	17	4.02	4.22E-06	ABLIM1, ABLIM2, MYBPC2, MYH1, CALD1, SYNPO2, MYOZ2, PALLD, TNNI1, DSTN, TNNT3, TAGLN, AKAP5, ABRA, CNN1, LMOD1, MSN	238	287	16483	4.10
GOTERM_MF_DIRECT	GO:0005523~tropomyosin binding	4	0.95	9.50E-04	TNNT3, TNNT1, CALD1, LMOD1	238	14	16483	19.79

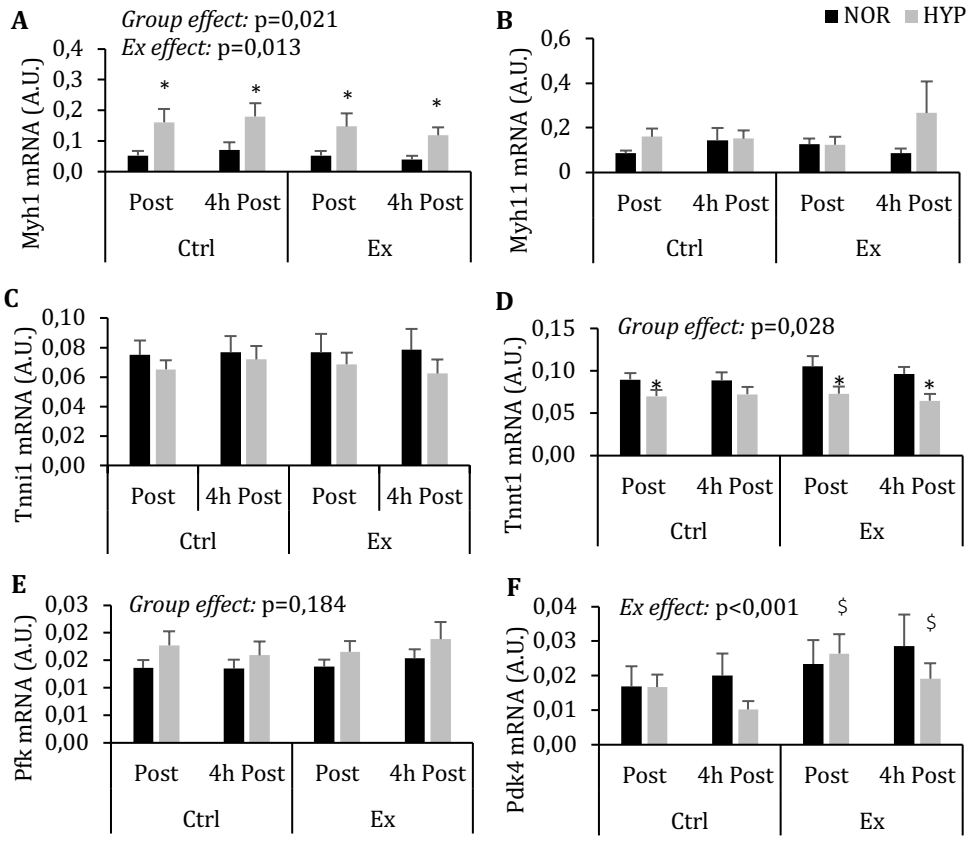
GOTERM_MF_DIRECT	GO:0042605~peptide antigen binding	5	1.18	9.66E-04	HLA-DRB1, HLA-DRB4, HFE, HLA-DRB5, HLA-DQA1	238	31	16483	11.17
GOTERM_MF_DIRECT	GO:0008307~structural constituent of muscle	5	1.18	2.54E-03	MYBPC2, SORBS2, MYH11, SYNM, MYL9	238	40	16483	8.66
GOTERM_MF_DIRECT	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	12	2.84	1.17E-02	MYF6, ZNF281, FOS, ATF3, ZNF148, ETS2, JUND, SMAD3, MYOG, NR4A3, MAFA, RBPJ	238	346	16483	2.40
GOTERM_MF_DIRECT	GO:0032395~MHC class II receptor activity	3	0.71	1.24E-02	HLA-DRB1, HLA-DRB4, HLA-DQA1	238	12	16483	17.31
GOTERM_MF_DIRECT	GO:0042623~ATPase activity, coupled	3	0.71	1.24E-02	HSPA1A, HSPA1B, NSF	238	12	16483	17.31
GOTERM_MF_DIRECT	GO:0005516~calmodulin binding	8	1.89	1.46E-02	ARPP21, MYH1, CALD1, RYR3, AKAP5, MYH11, RRAD, CNN1	238	178	16483	3.11
GOTERM_MF_DIRECT	GO:0001046~core promoter sequence-specific DNA binding	4	0.95	2.37E-02	SOX4, RORA, NR4A3, SOHLH1	238	43	16483	6.44
GOTERM_MF_DIRECT	GO:0016887~ATPase activity	7	1.65	2.54E-02	EIF4A2, CLU, HSPA1A, HSPA1B, ATP5A1, NSF, KIF2A	238	156	16483	3.11
GOTERM_MF_DIRECT	GO:0001228~transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	5	1.18	4.08E-02	MYF6, ATF3, MYOG, MAFA, SOHLH1	238	90	16483	3.85

GOTERM_MF_DIRECT	GO:0046982~protein heterodimerization activity	13	3.07	4.20E-02	MYF6, HIST1H4K, PANX1, SOX4, SMAD3, NR2C2, FOS, ATF3, TENM1, HIST1H4C, MYOG, MAFA, SIM1	238	474	16483	1.90
GOTERM_MF_DIRECT	GO:0004984~olfactory receptor activity	12	2.84	4.41E-02	OR4K5, OR9K2, OR2T29, OR5H14, OR2A7, OR5V1, OR6C1, OR2J2, OR2T34, OR10A7, OR2W1, OR51J1	238	425	16483	1.96
GOTERM_MF_DIRECT	GO:0001664~G-protein coupled receptor binding	4	0.95	4.46E-02	AKAP5, HSPA1A, HSPA1B, FLNA	238	55	16483	5.04
GOTERM_MF_DIRECT	GO:0004930~G-protein coupled receptor activity	16	3.78	4.58E-02	GPR33, OR4K5, OR9K2, OR2T29, OR5H14, OR2A7, OR5V1, GPR148, OR6C1, OR2J2, MAS1L, OR2T34, FRZB, OR10A7, OR2W1, OR51J1	238	644	16483	1.72
KEGG_PATHWAY	hsa05323:Rheumatoid arthritis	7	1.65	3.28E-03	FOS, HLA-DRB1, HLA-DRB4, HLA-DRB5, LTB, HLA-DQA1, MMP1	118	86	6891	4.75
KEGG_PATHWAY	hsa05321:Inflammatory bowel disease (IBD)	6	1.42	4.45E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, SMAD3, RORA, HLA-DQA1	118	64	6891	5.47
KEGG_PATHWAY	hsa04612:Antigen processing and presentation	6	1.42	9.18E-03	HLA-DRB1, HLA-DRB4, HLA-DRB5, HSPA1A, HSPA1B, HLA-DQA1	118	76	6891	4.61
KEGG_PATHWAY	hsa05310:Asthma	4	0.95	1.26E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	29	6891	8.05

KEGG_PATHWAY	hsa05332:Graft-versus-host disease	4	0.95	1.80E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	33	6891	7.08
KEGG_PATHWAY	hsa05330:Allograft rejection	4	0.95	2.44E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	37	6891	6.31
KEGG_PATHWAY	hsa05164:Influenza A	8	1.89	2.82E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, TLR3, HSPA1A, HSPA1B, EIF2AK3, HLA-DQA1	118	174	6891	2.68
KEGG_PATHWAY	hsa05166:HTLV-I infection	10	2.36	2.89E-02	EGR1, FOS, ATF3, HLA-DRB1, ETS2, HLA-DRB4, HLA-DRB5, SMAD3, HLA-DQA1, ATM	118	255	6891	2.29
KEGG_PATHWAY	hsa05140:Leishmaniasis	5	1.18	3.21E-02	FOS, HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	71	6891	4.11
KEGG_PATHWAY	hsa04940:Type I diabetes mellitus	4	0.95	3.39E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	42	6891	5.56
KEGG_PATHWAY	hsa05168:Herpes simplex infection	8	1.89	3.48E-02	FOS, TAF3, HLA-DRB1, HLA-DRB4, HLA-DRB5, TLR3, EIF2AK3, HLA-DQA1	118	182	6891	2.57
KEGG_PATHWAY	hsa04672:Intestinal immune network for IgA production	4	0.95	4.51E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HLA-DQA1	118	47	6891	4.97
KEGG_PATHWAY	hsa05145:Toxoplasmosis	6	1.42	4.99E-02	HLA-DRB1, HLA-DRB4, HLA-DRB5, HSPA1A, HSPA1B, HLA-DQA1	118	118	6891	2.97

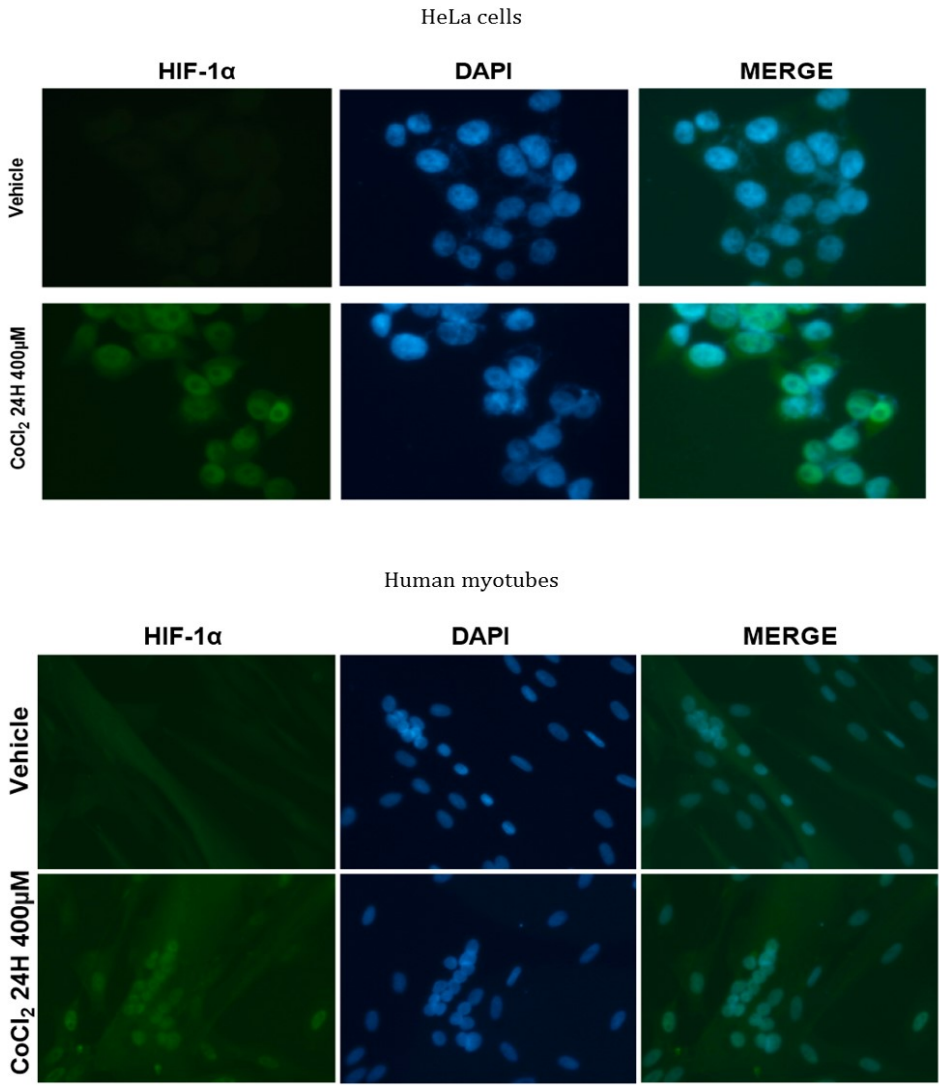
KEGG_PATHWAY	hsa00982:Drug metabolism - cytochrome P450	4	0.95	5.00E-02	GSTM3, ADH1B, GSTT1, ADH7	118	49	6891	4.77

Supplemental Data 2



(A) Myh1, (B) Myh11, (C) Tnni1, (D) Tnnt1, (E) Pfk and (F) Pdk4 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg. *p<0.05 HYP vs NOR; $p<0.05 Ex vs Ctrl. n = 10/group.

Supplemental Data 3



Cobalt dicloride (CoCl2) 400 μM for 24h was used as a positive control to validate the antibody used to localize HIF-1α as well as the technique for immunofluorescence in (A) HeLa cells and (B) human myotubes

CHAPTER IV: GENERAL DISCUSSION, LIMITATIONS, PERSPECTIVES, AND CONCLUSIONS

Muscle mass management is one of the basis to improve performance in many sports. The advent of hypoxic strength training modalities even with low loads to improve both muscle mass and strength represents an opportunity for many sports practitioners, even if the molecular and cellular mechanisms remain unsolved.

Systemic hypoxia results in lowered fraction of available O_2 to sustain active muscles with activation of many imbricate signaling pathways with HIF-1α as central pathway inducible. Using the ability of resistance exercise to induce greater hypertrophy, we aimed to investigate the molecular and cellular mechanisms underlying skeletal muscle mass regulation, precisely protein synthesis and degradation under hypoxic conditions. We also aimed to study the potential involvement of satellite cells in these mechanisms. Therefore, we will first discuss the activation of the central pathway HIF-1α induced under hypoxic conditions and the related expressed genes. Since muscle mass results from the balance between protein synthesis and degradation, these processes will be addressed. At last, we will discuss mechanism involving satellite cells and hypothetically the contribution of the Hippo pathway.

1. General discussion and perspectives

1.1. Acute hypoxia effects

1.1.1. HIF-1 signaling activation

Usually, the physiological effects induced by hypoxia in skeletal muscle are mediated by the transcription factor HIF-1α in response to changes

in O_2 concentration (390). Our results show HIF-1α localization and expression remained unaltered at both protein and mRNA levels after exposure to moderate hypoxia. It may be that HIF-1α has not been activated in order to translocate in the nucleus and express target genes. On the other hand, it is possible that HIF-1α was rapidly degraded in the cytoplasm due to its short half-life of about 1 minute (391) whereas post-exercise biopsies in our study were taken 15 minutes after resistance exercise. In addition, we cannot exclude that the way proteins were isolated was not optimal for HIF-1 preservation. We cannot exclude that HIF-1 was degraded during the isolation procedure. This is consistent with what has been found in a previous study, which revealed that HIF-1α expression at both protein and mRNA levels remained unaltered while only Vegf mRNA increased after 4h of exposure to moderate hypoxia alone (275). Those results are conflicting with the reference study of Ameln et al. (2005), which showed higher expression of HIF-1α protein post-exercise and sustained until 360 minutes, and an increase in Vegf mRNA 120 minutes after 45 minutes of cycling (241). However, it noteworthy the type of exercise (endurance) was different and hypoxia was induced by BFR. Using the same strategy to induce hypoxia (BFR) with low-intensity (20% of 1-RM) resistance exercise in older people, Fry et al (2010) observed unchanged expression of HIF-1α in both the BFR and normoxia groups (392).

In cultured muscle cells, it appears that stabilization and activation of HIF-1α is more evident because of the possibility of using very low oxygen fractions (0-3%) of O_2. In electro-stimulated mice skeletal muscle cultured C2C12 cells exposed to 1% of O_2, Baker et al. (2016) obtained

50% and 20% increased expression of HIF-1α and HIF-2α, respectively (393). In cultured human myoblasts cells, whether hypoxia was induced with low-fraction (1%) of oxygen or mimicked chemically with Cobalt dichloride ($CoCl_2$), HIF-1α peak expression was observed after 4 to 6h of exposure (394). Furthermore, several studies succeeded to observe HIF-1α nuclear localization induced by low oxygen fractions in murine and human cultured cells (395-397).

Oxygen saturation and technical considerations

In the present work, we reported the results of TSI measured by spectroscopy and SpO_2. Our results indicate that exposure to moderate hypoxia (FiO_2 of 14%) could not disturb TSI despite decreased SpO_2. SpO_2 during exercise decreased to 93% in hypoxic conditions, thereby attesting the hypoxic conditions since SpO_2 remained near to 99% in the normoxic group. Conversely, TSI decreased from 61% to nearly 49% in both groups during exercise with no differences between hypoxia and normoxia. This result confirms that in the present study, the hypoxic condition did not induce tissue hypoxia and therefore activation of HIF-1. Studies observed SpO_2 decrease of about 20%-25% after exposure to hypoxic conditions (11%-12% of FiO_2) at rest during 4h (275, 398) and decrease of SpO_2 of near to 15% when moderate hypoxia (12%-14% of FiO_2) is coupled with single resistance exercise (274, 399). The lower level of decrease in SpO_2 at exercise (274, 399) can be attributed to the increased blood flow providing nutrients and oxygen to fulfill the growing demand (400). This suggested that skeletal muscle was well protected against deoxygenation probably using myoglobin as oxygen

storage at rest and "buffer" at exercise (401). When oxygen demand increases, as it is the case during exercise, myoglobin releases oxygen in order to fill the deficit but also to maintain a stable oxygen saturation within the muscle cell (401, 402). NIRS measurements allowed detection of myoglobin desaturation during perfusion with hemoglobin-free buffer, indicating a significant contribution (about 50%) of signals derived from myoglobin to the NIRS signals and progressive desaturation of myoglobin with work intensity (403). Under hypoxic conditions, intracellular O_2 tension of myoglobin decreases expanding the O_2 gradient that supports the rising of muscle O_2 consumption during muscle contraction (404). In the next paragraph, we will discuss how this drop in O_2 levels can be sensed in the cell.

Oxygen sensors

PHD have been proposed to be "oxygen sensors" serving as the link between O_2 levels and HIF-1α pathway activation (405). In skeletal muscle, PHD2 and PHD3 are the most abundant prolyl hydroxylases and PHD2 contains a HRE that is recognized by HIF-1α while PHD3 contains a HRE recognized by HIF-2α (406, 407). Indeed PHD2 expression in cultured cells is low under normoxic condition but increased in hypoxia or when treated with NO

The activation of HIF-1α is not only due to the lowered tension of O_2 but also to the generation of reactive oxygen species (ROS) (252). Exposure to hypoxia induced oxidative stress with production of free radicals in proportion to the duration of exposure with strong relation between HIF-1α protein content and free radical production (252, 278, 408).

Previous studies revealed that contracting muscle also generates heat that induces production of ROS and would be favorable to strength production at lower concentrations but would induce muscle fatigue at high concentrations, the so called “hormesis effect” (409-412). In fact, initially thought to be produced only by mitochondria, ROS are recognized to be also produced by sarcoplasmic reticulum, plasma membrane and T-tubule system in response to mechanical and metabolic stimuli in the muscle (413-415). Endogenous ROS production caused by nutrient deprivation, rapamycin treatment or deprivation of leucine was shown to promote autophagy, as measured by the numerous autophagosomes in myotubes (416). Knowing that autophagy is regulated by mTOR in coordination with the FoxOs and AMPK pathways (417) and that induction of ROS by TGF-β is also an upstream event leading to atrophy (418), the regulatory mechanisms deserve further research. Altogether, these results suggest that hypoxia can promote either hypertrophy or atrophy depending on its intensity but the underlying mechanisms remain unclear.

1.1.2. Genes regulated by hypoxia and exercise

The microarray analyses highlighted numerous genes that were differentially regulated at rest and after resistance exercise in hypoxic conditions. Our results revealed that hypoxia could influence myoblasts differentiation and fusion, and the phenotype of components of the muscle contraction machinery. We also found an upregulation of oxygen transport and glucose metabolism genes under hypoxia whether at rest or after exercise but this has already been reported and discussed by

others (419-421). Recently, using transcriptomics, Gan et al. (2017) investigated changes in genes expression in mice skeletal muscles at rest exposed to 8% of O_2 for 2 and 6h (422). They found that after 2h of exposure, 385 genes were upregulated and 383 were downregulated, and by 6h, 481 genes were upregulated and 302 downregulated with only 20% of genes common to both time points (422). Approximately 330-450 genes were classified into each of the 4 groups including rapidly (2h) or slowly (6h) upregulated genes and those rapidly or slowly downregulated. Thereby, genes implicated in extracellular matrix organization were fastly downregulated while genes associated to TGF-β receptor and to regulation of transcription were slowly downregulated (422). Although the hypoxic conditions used were very low and beyond physiological limits in human, this study demonstrated that acute hypoxia induces dynamic changes in transcriptional expression level of many signaling pathways including those involved in skeletal muscle mass regulation (422).

Development of mature skeletal muscle vascularization matches with expression patterns of VEGF, an endothelial cell mitogen important in angiogenesis (423) and regulated by several factors such as hypoxia (424, 425), oxidative stress (426), muscle contraction (427), growth factors and cytokines (428, 429). Acute resistance exercise alone has been shown to increase expression of muscle Vegf mRNA and protein until 4h post-exercise (427, 430). Acute leg extension for 30 minutes at 50% of maximum work rate led to increased Vegf mRNA levels in both normoxia and hypoxia 1h post-exercise without marked difference between conditions (431). A significant decrease of intracellular PO_2 was

observed during resistance exercise in both normoxic and hypoxic groups, but hypoxia induced greater decrease (431). The fall of PO_2 was not correlated to Vegf mRNA expression in both hypoxic and normoxic groups (431). This is surprising given the upregulation of oxygen transport under hypoxic conditions (420, 421). Muscle contraction, induced by electric pulse stimulation, in combination with hypoxia induced higher HIF-1α protein level than hypoxia alone indicating that those factors exerted an additive effect on HIF-1α stabilization (421). Actually, Glut4 mRNA expression was upregulated when hypoxia was combined with electric pulse stimulation in cultured human skeletal muscle cells. Moreover, silencing HIF-1α (siHIF1A) repressed insulin action and contraction-stimulated glucose uptake while HIF-1α stabilization using $CoCl_2$ treatment led to insulin-mediated Akt activation, confirming the role of hypoxia in glucose metabolism (421). Early studies revealed that glucose metabolism was increased at rest and exercise under hypoxic conditions (432, 433), probably to compensate downregulation of oxidative metabolism with both acute and chronic hypoxia, providing ATP to fulfill the increased demand for exercise (434, 435).

Skeletal muscle is made of three types of muscle fibers named fibers type I, type IIa and type IIx corresponding to three isoforms of MHC found in human skeletal muscle (35, 36, 38). However, skeletal muscle is able to adapt its size as well as its metabolic and contractile properties to a wide variety of stimuli (41, 436). Our micro-arrays analysis revealed a trend to increased expression of fast phenotype contrary to the slow phenotype that was rather downregulated. In fact, exposure to hypoxia

induced upregulation of Myh1 mRNA, and downregulation of Tnnt1 mRNA in both exercised and non-exercised muscle. Myh1 mRNA is known to encode MHC type IIx (437), while Tnnt1 mRNA encodes muscular slow phenotype (438). Clearly, our results suggest an adaptation toward a fast phenotype, but these regulations needed to be confirmed at the post-translational level after a resistance-training program investigating the expression of the different MHC isoforms. In rodents, exposure to chronic hypoxia induced either a shift of muscle fibre-types from type IIa to I and from MHC IIa toward MHC I (439, 440) or, on the contrary, a shift of muscle fibre-type from type I to IIa and from MHC I to MHC IIa (441). It appeared that fibre-type shift was more evident in young rodents than in aged one (440) and that exercise was a major determinant to guide the direction of this shift (441). In human, a 4 weeks resistance-training program performed in hypoxia (FiO_2 of 12%) failed to promote significant effects on both the fibre CSA and the percentages of the different fibre types. In fact, transcriptional analysis of mRNAs showed increased mRNA level of MHC IIx ranging from 16% to 427% in 75% of subjects in hypoxia while the increase varied from 32% to 634% in 66.66% of subjects in normoxia (319). Decreased expression level of MHC IIx from 68% to 78% was observed in 25% of subjects in hypoxia while 92% decreased expression was observed in 33.33% of subjects in normoxia (319). The fibre type IIa percentages increased from 33.1% to 38% in normoxia and from 28.4% to 35.2% in hypoxia while fibre type I percentages remained constant and fluctuated from 50% to 50.5% in normoxia and from 47.5% to 45.1% in hypoxic condition (319). In human, the direction of the fibre-type shift is blurring

when hypoxia is combined to exercise because, amongst others, of inter-personal high variability (319).

1.1.3. Special case of localized hypoxia: BFR and its effects

The advent of BFR or *Kaatsu* sparked interest for ancient resistance Japanese training inducing hypertrophy with low loads in sport physiology (245). Many studies established increased skeletal muscle mass after exercise or training sessions using devices creating pressure by inflation in the proximal portion of the limbs (245-247, 250, 251). Low intensity strength exercise performed with BFR resulted in a rapid increase of growth hormone and serum cortisol from 10 minutes to 30 minutes at least, while they remained unchanged in the control group (369). At the protein level, although no differences were found in phosphorylated Akt and mTOR, greater phosphorylation of S6K1 at Ser^{389} in the BFR group was observed 3 hours post-exercise (369). In a similar designed study, low-intensity resistance exercise realized with BFR induced changes at the mRNA levels of anabolic, catabolic and hypoxia related genes that were, however, not different when compared to the same protocol without BFR (442). Resistance exercise with BFR against low intensity loads are described to induce a rapid increase of plasma concentrations of lactate and growth hormone while inflammatory cytokines such as IL-6 gradually increase within 90 minutes after exercise (246). Lactate accumulation and growth hormones are known to activate the mTOR pathway through Akt/mTOR and MAPK pathways whereas inflammatory cytokines are shown to regulate both anabolic and catabolic processes and related pathways

(443-447). Although defined as a proinflammatory cytokine, IL-6 also acts as regulator of metabolic pathways during exercise (448) and as a growth factor, depending on the cell type (449). In skeletal muscle, hypertrophic effects through activation of proliferative capacity of muscle stem cells has been described (450). Using an overloading model in mice lacking IL-6 gene in skeletal muscle, Serrano et al. observed blunted hypertrophic growth with impaired satellite cells proliferation and myonuclear accretion by reducing STAT3 activation and its target gene cyclin D1 (450). IL-6 deleterious effects, such as atrophy and muscle wasting, have also been described (451). Indeed, local infusion of high doses of IL-6 that mimics the IL-6 plasma concentration after intense prolonged exercise in human resulted in about 50% decreased muscle protein turnover with more prominent decreased protein synthesis (448). Although not fully elucidated, mechanisms of the regulation of skeletal muscle mass by BFR are, at least, related to the mTOR signaling pathway (306, 442).

BFR associated to resistance exercise seems relevant to increase post-exercise expression of HIF-1α target genes related to angiogenesis, satellite cells functions and protein turnover (354, 452-454). Combination of BFR with low-intensity resistance exercise lasting 5 to 8 minutes elicited higher mRNA expression of Vegf 2h and 4h post-exercise while HIF-1α mRNA only showed a trend of increasing, when compared to controls without BFR (453). An 8h post-exercise analysis of expressed mRNA after BFR with low-intensity resistance exercise showed downregulation of FoxO3a, Mafbx and Murf-1 mRNA expression (454). A significant increased protein synthesis was also reported in BFR-induced

hypoxia 3h after resistance exercise while it remained unaffected in controls (369, 392). Indeed, using BFR combined to low-intensity resistance exercise, made of a total of 4 sets and 75 repetitions against 20% of 1-RM, led to 46% and 56% of increased fractional synthetic rate in young (369) and older people (392) respectively. Furthermore, compared to resistance exercise alone, resistance exercise performed to failure with BFR led to increased satellite cells number per muscle fibre 1h post-exercise (452). However, not all studies are consistent on the effects of BFR on the regulation of muscle proteins catabolism. Bilateral knee-extension against low resistance associated to BFR lasting 4 to 5 minutes induced increased mRNA levels of HIF-1α, p21, MyoD and MurF1 at 3h post-exercise without difference between BFR group and control (442). All together, these results suggested fostered protein synthesis, downregulation of the proteolysis machinery and satellite cells activation after low-intensity RE coupled with BFR (454).

Despite activation of muscular adaptations by hypoxia, localized-induced hypoxia differs from systemic hypoxia by many aspects. Firstly, hemodynamic responses such as ischemia/reperfusion and hypoxia/re-oxygenation result in different transcriptional responses (455). It is suggested that BFR creates greater hyperemic responses than systemic hypoxia, inducing greater cellular swelling and anabolic responses (456). Second, as exposure to hypoxia seems to favor compensatory vasodilatation to oxygen demand at muscular level (457), this compensatory vasodilatation might be exacerbated in BFR in the localized hypoxic muscle although reactive hyperemia seems to not to be

relevant in increased protein synthesis after resistance exercise with BFR (456).

1.2. Muscular protein turnover under hypoxic condition

1.2.1. Protein synthesis rate

We hypothesized that an acute resistance exercise performed under moderate hypoxic conditions would potentiate the anabolic response during the recovery phase in normoxia. In contrast, the fractional synthetic rate of muscle protein was unchanged after exposure to hypoxia combined with resistance exercise comparatively to normoxic conditions, in which the fractional synthetic rate was increased. These results suggest that hypoxia blunts the activation of protein synthesis after resistance exercise. Previous data have shown a 215% increase in fractional synthetic rate in normoxia and 81% in hypoxia (FiO_2 of 12%) 3.5h after an acute resistance exercise resulting in reduced protein synthesis in hypoxia (274). Although the protocol consisted in a total of 8 sets and 48 repetitions at 70% of 1-RM was quite similar to ours (8 sets and 64 repetitions at 80% of 1-RM), post-exercise recovery was done in hypoxic conditions contrary to our protocol (274). Thus, the blunting effect was observed whether the recovery phase was under hypoxic conditions or not (274). Knowing that mTOR is the major actor of adaptive hypertrophy in skeletal muscle, this effect suggests mTOR downregulation by hypoxia. Although the mechanism through which hypoxia disrupts the mTOR signaling remains poorly understood, REDD1 (458) and AMPK activation were shown to repress mTOR activation in HEK293 cells under hypoxic conditions (70, 124). In vivo,

acute exposure to hypoxia induced upregulation of phosphorylated AMPK on Thr^{172} and REDD1 expression at rest that failed to repress Akt phosphorylation at Ser^{473} (372). Our analysis of mTOR pathway reveals higher phosphorylated Akt, S6K1 and rpS6 in the exercised-leg immediately post-exercise in both normoxia and hypoxia. This is consistent with previous studies as resistance exercise is known to stimulate mTOR activity and enhanced MPS through activation of S6K1 and S6 promoting ribosomal linkage to mRNA for protein synthesis (459-461) and transcription of the translational machinery (79). The increased protein synthesis rate observed in normoxic group probably required both mTOR-dependent and independent mechanisms. It could be thought that blunted protein synthesis observed in hypoxic group was offset by an increase in protein breakdown, which was not the case. Furthermore, phosphorylated AMPK Thr^{172} and REDD1 expression were downregulated under hypoxia. Protein synthesis regulation under hypoxic condition definitively require other mechanisms poorly known to date.

Technical considerations about the deuterium method

Over decades, skeletal muscle protein turnover has been investigated using flooding stable isotopically amino acids with either heavy carbon (^{13}C), hydrogen (^{2}H) or nitrogen (^{15}N) (462-464). Unfortunately, these techniques are invasive and require a complex experimental set-up such as arterial and venous cannulation, alongside multiple biopsies and are limited to acute (within 8-12 hours) protein turnover in a controlled laboratory setting (465). Thanks to the recent advances of mass-

spectroscopy systems for high–precision measurement of hydrogen and oxygen stable isotopes (466, 467), deuterium oxide (D_2O or "heavy water") has been reintroduced in metabolic research (468, 469). Comparisons of labeled amino acids flooding and deuterium enriched-water absorption showed similar fractional synthetic rate in both rats and humans (365, 468) while D_2O demonstrates a few advantages over labeled amino acids. Indeed, use of D_2O reduces the invasiveness of the study, clinical inputs and study cost and reveals safer for vulnerable populations (frail elderly, adolescents, clinical populations) (365). Moreover, this technique presents the advantage to be suitable for long-term studies lasting months with administration of regular boluses that enable long-term measurement of muscle protein synthesis (470). Muscle mass balance also depends on catabolic processes that will be discussed in the next section.

1.2.2. Protein degradation processes

The present work brings on data about skeletal muscle protein breakdown rate, in conjunction with protein synthesis rate to analyze muscle protein turnover. Exposure to acute hypobaric hypoxia (simulated altitude of 9144 m for 6h) led to higher muscle protein degradation directly impacting protein turnover and resulting in muscle mass loss in rats (471). Likewise, chronic exposure to hypoxia is also known to increase muscle protein degradation (278, 472) presumably due in part to physiological effect toward eating with a decreased appetite and reduced food intake in both human and murine models (473, 474). In the present study, the muscle protein degradation rate was

similar between normoxia and hypoxia after a single resistance exercise session. Focusing on the transcriptional regulation of proteolysis, we found that Bnip3, Bnip3L and Lc3b mRNA expression were lowered post-exercise and 4h post-exercise in hypoxia compared with normoxia despite increased phosphorylation of FoxO. Furthermore, differences were observed between the rested and the exercised leg in hypoxia. "Post-hoc analysis revealed that hypoxia reduced Bnip3, Bnip3L and Lc3b mRNAs expression levels post-exercise and 4h post-exercise in the exerted leg and Bnip3, and Lc3b mRNA expression 4h post-exercise in the rested leg. A single resistance exercise alone failed to regulate protein breakdown despite increased Murf-1 mRNA levels (295). In human, exposure to hypoxic conditions (FiO_2 of 10.7%) during 8h increased Mafbx and Bnip3 mRNA expression at rest that was lowered by endurance exercise while Murf-1 mRNA remained unaltered from pre to post-exercise (372). The marker for autophagosome initiation, i.e. LC3-II/LC3-I, was positively regulated under hypoxic conditions at rest while p62, a marker for autophagosome degradation, was lowered. Together those results indicated that the autophagic flux was increased as more autophagosomes were formed and discarded as shown by the decreased expression of the membrane-bound p62. Endurance exercise lowered the LC3-II/LC3-I ratio and p62 expression suggesting an acceleration of the degradation of the autophagosomes by exercise (372). All together, these observations suggest that exercise under hypoxic conditions could repress, at least partially, the catabolic processes.

The muscle protein degradation rate has only been very scarcely determined under hypoxic exposure, certainly in human. Direct

measurement methods are comprised of free amino-acid tracer-dilution approach and the use of 3-methyhistidine (3MH), inspired from the first method (475). These methods provide the total rate of protein breakdown indifferently of the different pathways responsible although the use of 3MH allow distinction between myofibril and collagen fractions (475, 476). An early study estimated protein catabolism using 3MH excretion although they acknowledged that it would have been more suitable to study the subjects on a creatine free diet to exclude the dietary contribution to 3-methylhistidine release into the circulation (477). Actually, following degradation of myofibrillar proteins, 3MH cannot be reutilized nor metabolized and is therefore excreted in the urine (478). Using this isotope required caution as refraining from eating meat 3 days before administration of 3-MH, as it can be provided in meat, and needs 24-hour urine collection that can be hindrances for compliance with this technique (478, 479). To avoid these limitations, Sheffield et al. (2014) used stable deuterated-3MH (D-3MH) and observed that enrichments was similar following meat containing or a meatless meals from 12h to 22h post-ingestion (366). Furthermore, isotope decay constants were quite similar in both plasma and urine regardless of diet, and plasma or urine samples distributed over 5-6h yield decay constants were similar to those collected over 10h (366). This technique reduce the need for 3 days abstention from meat to 1 with permission of low-meat meal, and reduced the requirement of 24-hour urine collection to 5-6 hours the day after ingestion of the isotope (366). This led us to provide standardized low-meat meal to our subjects the day before experiment. These results make this technique effective and

reliable for measuring the muscle protein breakdown rate while reducing the obstacles.

1.2.3. Intermittent hypoxic resistance training

In the previous section, our acute microarray results suggested a shift toward a fast phenotype but these adaptations needed to be confirmed at the post-translational level after a resistance-training program investigating the expression of the different MHC isoforms. To confirm those results, we performed a long-term study, aiming to investigate skeletal muscle mass regulation by resistance training performed under either normoxia or moderate intermittent hypoxia (FiO_2 of 13%) and molecular adaptations. Therefore, young healthy subjects were submitted to a 4-week resistance-training program made of 6 sets of 8 repetitions of one-leg extension against 80% of 1-RM repeated 3 times a week. Although not published yet, histochemical analysis and comparison of post-training muscle biopsies with pre ones revealed no hypoxia effect on fibre-types proportions (Fig. 1).

Taking advantage of the deuterium technique, we aimed to compare protein synthesis rates in hypoxic and normoxic groups after the 4-week resistance training program. At the time of writing this manuscript, the results are not available yet. The preliminary results show an increase in the vastus lateralis thickness (+6.5%, p= 0.04) and the 1-RM (+25.2%, p<0,001) in the normoxic group after 4 weeks training, while the m. vastus lateralis thickness did not change (-0. 8%, NS) despite a higher increase of the 1-RM in the hypoxic group (+35.6%, p<0.0001) compared to the normoxic group (Fig. 2). Increased muscle mass and strength has

previously been observed after 3 weeks of resistance training similar to ours under normoxic conditions, that was, at least partially, due to increased myofibrillar protein synthesis (303). Based on those results and our preliminary results, we hypothesize that hypoxia also blunts muscle protein accretion after a whole training protocol as we observed after one acute bout of resistance exercise. The results yet to come of the fractional synthetic rate using the deuterium method are required to confirm this hypothesis.

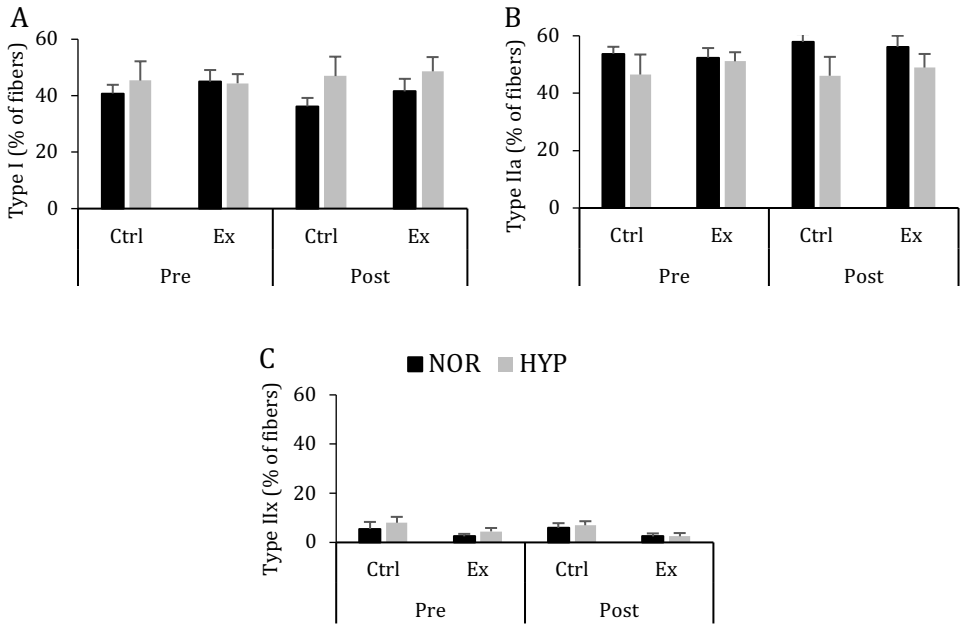


Figure 1. Distribution of fiber type percentages pre and post-training in both exercised leg and controls in normoxia and hypoxia groups

A) Type I fibers percentages (B) Type IIa fibers percentages (C) Type IIx fibers percentages (No significant change).

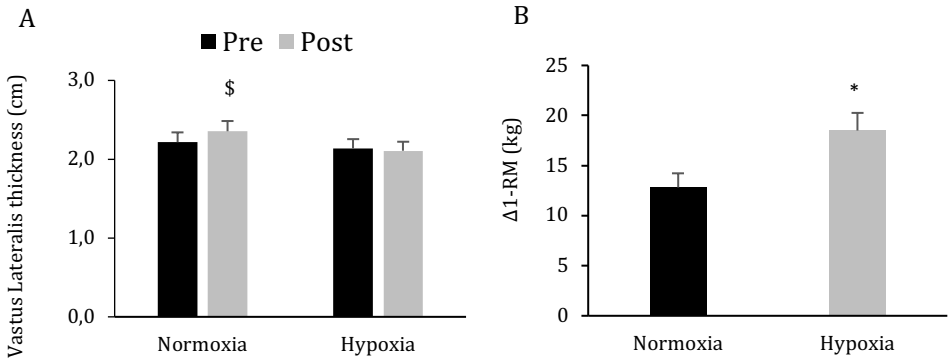


Figure 2. Evolution of physiological measures pre and post-training in normoxia and hypoxia groups

A) Vastus lateralis thickness (B) Variation of 1-RM (Δ1-RM). $ $P < 0.05$ (Pre *vs.* Post), * $P < 0.05$ (NOR *vs.* HYP)

The anabolic and catabolic signaling are not the only processes involved in skeletal muscle mass regulation as satellite cells are thought to contribute to its regulation (1). Their contribution will be discussed in the next section.

1.3. Satellite cells

1.3.1. Contribution of satellite cells

The role of satellite cells activation, differentiation and fusion with pre-existing fibers during muscle resistance exercise-induced hypertrophy remains under debate (1), which led us to investigate their regulation. Markers of cell cycle activation were differentially regulated post-exercise and 4h post-exercise. Fos mRNA was higher in both normoxia and hypoxia at Post in the exercised compared with the control leg and

decreased 4h Post while p21 mRNA was upregulated in both groups by exercise 4h post-exercise. Cyclin D1 and Pcna mRNA were higher in the exercised leg 4h Post in hypoxia and normoxia respectively. As well, differentiation markers were differentially regulated. Mrf4 mRNA was downregulated in hypoxia and normoxia 4h Post in both hypoxia and normoxia indifferently from the condition. Finally, we observed that myogenin mRNA, a gene involved in differentiation and fusion of satellite cells was differentially regulated by hypoxia at Post.

Muscle contractions have been shown to induce satellite cells activation lasting 24h to 120h post-exercise after a single bout (480-482). Resistance exercise against 60% of 1-RM performed in normoxic conditions succeeded to promote higher activation of satellite cells testified by increased expression of Ki67/CD56 (313), two reliable markers of satellite cells proliferation (483). Indeed, resistance exercise induced an increase of Ki67/CD56 by 232% 3h post-exercise concomitant to higher phosphorylation of Akt and 4E-BP1 (313). The PI3K/Akt pathway has been identified as one of the key regulator of myoblast differentiation since Akt inhibition resulted in MyoD-mediated muscle differentiation (484). Rat skeletal myoblasts exposed to 1% O_2 induced reduction of phosphorylated Akt at T^{308} compared to those exposed to 5% and 21% O_2 (485). Moreover, when treated with insulin, cells exposed to 5% and 21% O_2 exhibited higher amount of phosphorylated Akt T^{308} and enhanced fusion indexes when compared to the untreated ones while those exposed to 1% O_2 failed to fuse (485).

Few studies performed in both younger and older men highlighted the inhibitory effect of myostatin, a well-known repressor of mTOR pathway, on satellite cells activation and proliferation (482, 486). An acute bout of resistance exercise performed in both young and older men resulted in increased satellite cells in both groups, however satellite cells remained 34% and 21% lower in the old than in the young group 24h and 48h post-exercise, respectively (486). This was accompanied by an increased expression of myostatin in older men that remained higher from baseline until 24h post-exercise while it remained unaffected in young men (486). Furthermore, myostatin expression in satellite cells decreased in both younger and older men but remained significantly higher in the older group at 24h and 42h suggesting myostatin may inhibit activation and proliferation of satellite cells (486). A single resistance exercise in both younger and older men induced increased activation of satellite cells in both young and older men that was delayed in older (482). Moreover, MyoD significantly increased in the younger group from 12h post-exercise and at all time points while it significantly increased in older men 24h pre-exercise (482). On the other hand, exposure to both chronic and intermittent hypoxia was shown to promote activation of satellite cells (487). Skeletal muscle of both young and old mice exposed to chronic (continuous) and intermittent (1 hour intervals for 12 hours and 12 hours in normoxia) moderate hypoxia with a FiO_2 of 16% and lasting for 5 days showed higher satellite cells to muscle fibers ratios than those exposed to normoxia (487). Indeed, 24h and beyond of exposure to hypoxia has been shown to increase MyoD localization in the nuclei and inhibit the canonical Wnt pathway by reducing nuclear translocation of

β-catenin (488). Cells cultured in physiologic normoxic conditions (3-9% O_2) increased the potential of satellite cells proliferation compared to those cultured in hyperoxic conditons (20-21% O_2) (485, 489-491) while more severe hypoxia (1% O_2) harshly slowed proliferation (485, 492). Opposite results were found in mice cloned cells with either low proliferative or high proliferative potential cultured in hypoxia (2% O_2) or hyperoxia (20% O_2), which resulted in enhanced proliferation under hypoxic conditions (493). In fact, mice satellite cells have been shown to be heterogeneous and composed of two sub-populations expressing or not Myf5 (494). They were therefore divided into Pax7+/Myf5+ and Pax7+/Myf5- myogenic progenitors sub-groups representing 90% and 10% of the total progenitors pool, respectively (494). Rossi et al (2010) distinguished these two subgroups using phase-contrast microscopy and observed that only around 25% of satellite cells were able to proliferate at high rate at the clonal level (495). High proliferative clones were then distinguished from low proliferative ones by their glycolytic phenotype and higher ROS generation (495). It remains unknown if these differences are identical in humans and if they affect differentiation.

The role of hypoxia in satellite cells differentiation and involvement in skeletal muscle mass regulation is fuzzy. Indeed, while it has been found that knocking down HIF-1α in normoxic conditions led to myoblasts differentiation inhibition (496), other studies observed impaired differentiation under hypoxic conditions (488, 492, 497). Furthermore, alteration of myoblasts differentiation by hypoxia was found to be inversely related to the hypoxic level, as cells differentiation was better in cells cultured at 2% O_2 than those at 0.5% O_2 and 0.01% O_2 (498).

Drastic decreased expression of MyoD, and myogenin mRNA and extinction of E2A mRNA inversely related to O_2 level confirmed the gradual inhibitory effect of hypoxia on cells differentiation although MyoD mRNA remained functional under severe hypoxia (498). It was previously shown in cultured cells that severe hypoxia (0.5% of O_2) repressed differentiation of cultured myoblasts since both mRNA and protein expression levels of MyoD and myogenin protein expression were drastically lower than those in normoxia (499). Knocking down HIF-1α in those cells was unable to reverse the enhanced mRNA and protein expression of MyoD and myogenin, and led to 1.5-fold increase in myotubes formation in normoxia and restored myotubes formation under hypoxia to 58% of normoxic control levels (499). Deleting HIF-1α/HIF-2α in cultured satellite cells under normoxia (21% O_2) and hypoxia (1% O_2), Yang et al. (2017) found reduced self-renewal but increased differentiation without affecting proliferation under hypoxic conditions (500). On the other hand, hypoxia induced upregulated Bhlhe40 mRNA, a member of basic helix-loop-helix family transcriptional factors, whose overexpression induced reduction of myogenin mRNA and terminal differentiation without MyoD mRNA alteration (385). On the contrary, myogenin mRNA expression increased five to seven time when Bhlhe40 mRNA was knocked down and differentiation was improved (385). Thus, satellite cells differentiation is probably regulated by both HIF-1α-dependent and independent mechanisms (499). In summary, O_2 availability appears to be more relevant than HIF-1α itself to modulate muscle progenitor

differentiation. In addition, the degree of inhibition of myogenesis seems to be proportional to the severity of hypoxia (485, 499).

1.3.2. Hypothetically contribution of the Hippo pathway

Hippo pathway involvement in organ size regulation mainly arose from its tumor-suppressor genes Mst1/2, Sav, Lats1/2 and Mob1's mutations role in organ size regulation (501, 502). Briefly, upon activation of the Hippo pathway by upstream regulators such as nutrients, metabolites, energy stress and mechanical cues, Mst1/2 is phosphorylated, and once linked to Sav their affinity with downstream Lats1/2 is reinforced (503). Activated Mst1/2 phosphorylate Lats1/2 whose sensitivity increase with Mob1 linkage (503, 504). Finally, activated Lats1/2 phosphorylate their effectors Yap/Taz which are either degraded by the proteasome or inhibited in the cytoplasm by binding to the 14-3-3 or AMOT proteins (326). When dephosphorylated in case of inactivation of Hippo pathway, Yap/Taz migrate in the nucleus where they interact with TEADs protein family amongst others to induce specific genes (505, 506). Of note, although less investigated, Taz share with Yap 60% of high protein sequence similarity (507) and seems to have lower impact on cell size than Yap (508).

Recently, there has been increasing evidence for the involvement of the Hippo pathway in the regulation of muscle mass in response to both exercise and hypoxia (351, 353, 509). However, to the best of our knowledge, none of those studies has investigated skeletal muscle mass regulation by exercise under hypoxic conditions and the contribution of the Hippo pathway. Of note, in the present study resistance exercise was

chosen, as it is the best mode of exercise promoting protein synthesis and muscular hypertrophy (109, 301). To fill that gap, we investigated the regulation of the Hippo pathway effectors Yap/Taz at post-exercise after acute exposure to hypoxia (14% of O_2) combined with resistance exercise. We observed decreased phosphorylated Yap at Ser^{127} 4h after resistance exercise in the hypoxic group (Fig. 3.A) and early and transient increased of Yap mRNA expression in the normoxic group (unpublished data). At mRNA level, Ctgf and Cyr61, two Yap responsive genes were higher 15min post-exercise in both hypoxia and normoxia and returned to basal levels at 4h post-exercise (Fig. 3C-D). Hypoxia favored increased expression of a target gene of Taz named peripheral myelin protein-22 (pmp22) as well as phosphorylation of signal transducer and activator of transcription 3 (STAT3), a transcription factor linked to many signaling including inflammatory and TGF-β signaling (unpublished data) (510). The regulation of pmp22 mRNA could be due to activation of the inflammation pathway rather than the Hippo pathway as pmp22 is a known target of STAT3. Furthermore, besides the classic upstream activators of the Hippo pathway, signaling molecules and crosstalk with other pathways have been shown to regulate the Hippo pathway with implications on skeletal muscle mass regulation (511, 512).

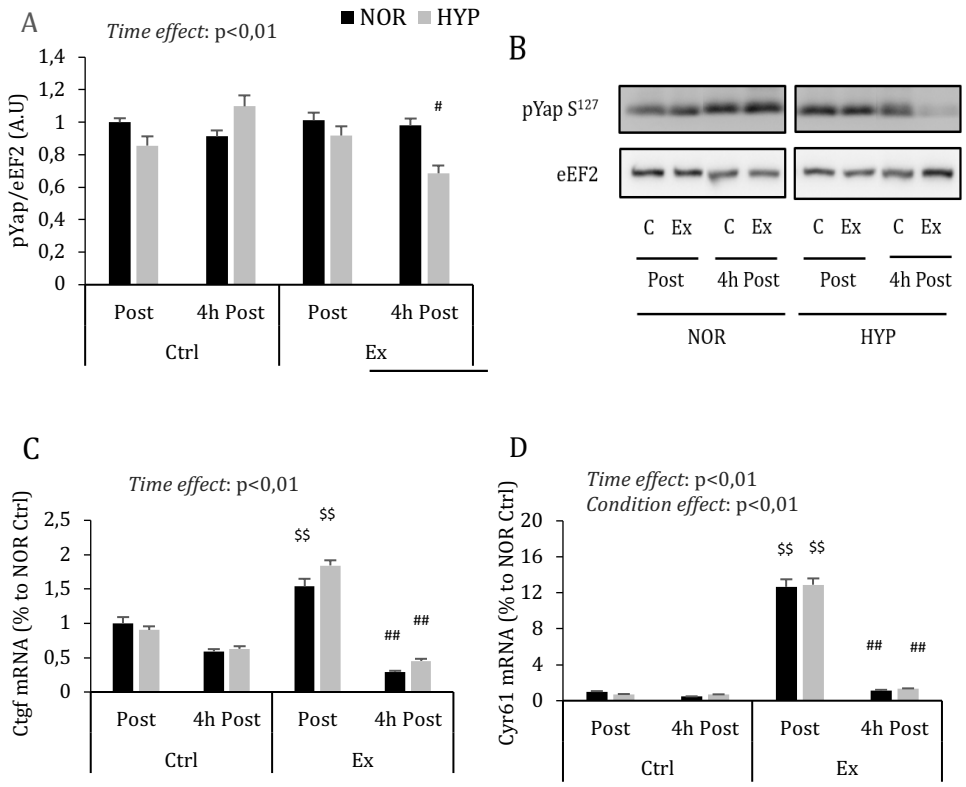


Figure 3: Hippo pathway effector and related genes levels pre and post-training in normoxic and hypoxic groups.

A) Yap Ser^{127} phosphorylation (B) Representative Western blot images of p-Yap Ser^{127} (C) Ctgf mRNA levels (D) Cyr61 mRNA levels. $$ $P < 0.01$ (Post *vs*. 4h Post), ## $P < 0.01$ (Ctrl *vs*. Ex).

Both exercise and blocking of myostatin/activin induced increased phosphorylation of Yap at Ser^{127} acutely without additive effect when combined (353). Lowered expression levels of downstream genes Mst1, Lats1 and Taz of the Hippo pathway reflected this inhibitory effect (353).

Conversely, overexpression of Yap reduced by about 85% Smad binding element activity indicating decreased Smad2/3 transcriptional activity (351). Knowing that myostatin negatively regulates mTOR and favors activation of catabolic signaling pathways (513, 514), it appears that a potential crosstalk could exist between the Hippo and mTOR pathways. Previous studies showed Yap overexpression could regulate muscle fibres size positively through both mTOR-dependent and independent mechanisms (76, 239, 515). On the contrary, Judson et al. (2013) observed that overexpression of Yap induced loss of muscle mass and activation of proteolytic pathways (350). Although conflicting, these studies clearly stated critical regulation of skeletal muscle mass by Hippo pathway even though the mechanisms remain unclear.

Energy stress has also been shown to regulate Yap phosphorylation and activation in HEK293 cultured cells (352, 516). Indeed, AMPK was shown to bind directly Yap on several residues including at Ser^{61} (352) or indirectly through phosphorylation and activation of Amotl1 Ser^{793} (516) although phosphorylation of Yap at Ser^{127} remained the main inhibitory core components. In skeletal muscle, AMPK is known to repress mTOR activity by inhibiting the complex TSC1/TSC2 or directly RAPTOR (124). Resistance exercise upregulated AMPK activity immediately after with progressive decrease within 1 and 2h post-exercise while protein synthesis progressively increased from immediate post-exercise to 1 and 2h post-exercise (517). Our results objectify a trend of decreased phosphorylation of AMPK in both normoxic and hypoxic conditions post-exercise and significant lowered phosphorylation of AMPK at post-exercise in the rested leg and at 4h

post-exercise in the exercised leg under hypoxic condition. It can therefore be hypothesized that regulation of AMPK phosphorylation in skeletal muscle by resistance exercise under hypoxic conditions reduce Yap phosphorylation and cytoplasmic retention. The decreased phosphorylation of Yap at Ser^{127} 4h post-exercise in the hypoxic group corroborate this hypothesis with the caution that its translocation and the nuclear fraction were not analyzed (Fig. 3.A).

Bcl2-associated athanogene 3 (Bag3) and filamin-C are two proteins localized to the Z-disk in muscle, important for muscle function as their mutations cause severe myopathies (518, 519). While filamin-C is mechanosensitive, Bag3 is recognized as the sensor of mechanical loading of filamin once they bind together and seems to play a role in chaperone-assisted selective autophagy (520). Indeed contraction of actin network leads to mechanical unfolding of protein domains within the filamin, recognition by chaperone-assisted selective autophagy and formation of the chaperone-assisted selective autophagy complex initiated by Bag3 (520). Resistance exercise initiated binding of Bag3 with TSC1, thereby relieving its inhibition on mTOR and stimulated ribosomal protein S6 phosphorylation, thereby favoring protein translation (521). Under mechanical tension within the muscle, Bag3 sequesters Lats1 and Amotl1, releasing Yap inhibition, nuclear translocation and expression of Hippo pathway target genes (520). Moreover, the muscle submitted to mechanical stresses induces myonuclei distortion (522), the latter can cause Yap translocation to the nucleus and expression of Hippo target genes (523). Altogether, filamin-C-Bag3-mTOR and Yap activity in response to mechanical cues are

possible mechanisms of muscle hypertrophy regulation by both mTOR-dependent and independent mechanisms.

As reminded above, PHDs are known to hydroxylate HIF-1α leading to its proteasomal degradation in the cytoplasm in normoxia (259). Hypoxic conditions induced Siah2 that possesses potent E3 ubiquitin ligase activities and led to PHDs degradation and consequently increased stability of HIF-1α (524-526). Moreover, in HEK293 cells, Siah2 induction by hypoxia has been shown to antagonize Hippo and initiate Lats2 degradation through the proteasome pathway and phosphorylated Yap Ser^{127} reduction (527). Concomitantly, Siah2-induced downregulation of Lats2 was accompanied with enhanced Yap nuclear translocation, indicating Siah2 antagonizes Hippo signaling and activates Yap (527). Furthermore, loss of function of Yap promoted degradation of HIF-1α and reduced expression of HIF-1α target genes. A positive feedback loop may be established to enhance HIF-1α and Yap/Taz activity under hypoxic conditions (527). Given the involvement of Yap whose activation induces atrophy (350) or hypertrophy (239, 351), the Hippo pathway might play a role in the regulation of muscle mass following resistive exercise under hypoxic conditions. This could be induced by Siah2 activation due to hypoxia followed by Yap activation and transcription of target genes from the Hippo pathway.

In cultured cells, Yap expression was low in quiescent satellite cells and increased upon activation and proliferation, indicating Yap expression remained elevated during proliferation and decreased during differentiation or self-renewal (349). Indeed, Yap phosphorylation at

Ser^{127} increased more than 20-fold after 24h in differentiation medium than in myoblasts and this increase was accompanied by Yap translocation in the nucleus of myotubes (240). Moreover, when transfected with hYap1 Ser^{127}, a mutant Yap1 that cannot be phosphorylated at the residue Ser^{127}, cells did not differentiate and most of them remained within the cell cycle (240). This suggested that phosphorylation of Yap1 at Ser^{127} and impaired Yap activity is crucial for cell cycle withdrawal and differentiation of myoblasts in myotubes (240, 349). However, looking for the distinct functions of Hippo effectors, Sun et al. (2017) observed that Yap and Taz had similar proliferative functions whereas Taz plays a dominant role in myogenic differentiation and fusion (528). SiRNA-mediated knockdown of Taz resulted in suppressed expression of Myf5 and myogenin mRNA and enhanced Pax7 mRNA expression (528). Consistent with the SiRNA results, satellite cells isolated from Taz-knockout mice fused less into post-mitotic myotubes when compared with controls (528). Although Yap and Taz have common functions during activation and proliferation of satellite cells, Taz appears to be more active during late differentiation and fusion of satellite cells with pre-existing myofibrils (528).

In summary, the Hippo pathway seems to be related by many ways to skeletal muscle mass regulation. Indeed, the Hippo pathway regulates mTOR through crosstalks with myostatin/TGF-β/Smad, AMPK/TSC2 and Bag3/TSC1 pathways while hypoxia seems to promote Yap accumulation that can regulate mTOR activity. Finally, Hippo activation regulates satellites proliferation, self-renewal and differentiation.

2. Limitations and perspectives

Our goal was to investigate the effects of a single resistance exercise in hypoxic conditions on anabolic and catabolic signaling with measure of protein synthesis and breakdown rates with particular attention on endogenous myogenic factors. Given the complexity of the mechanisms involved in the tight regulation of muscle protein balance, thorough analysis of endogenous and systemic adaptations such as cytokines (e.g. MCP-1, IL-6, IL-8 and TNF-α) expression should have been taken into account as plasma insulin dosage was not significantly affected by hypoxia. Moreover, basal biopsies should have been collected to highlight acute exercise and hypoxia effects comparatively to baseline. Moreover, the duration (25 minutes) and the intensity (FiO_2 of 14%) of the exposure to hypoxia where relatively low, resulting in a low hypoxic dose whereas many mechanisms relating to hypoxia are dependent on the intensity and the duration of exposure. It seems that the higher the hypoxic dose, the higher the responses, at least up to a certain level (370). This would favor activation of HIF-1α and its interaction with other pathways. Future studies should take into account these limitations for better comprehension of hypoxia molecular mechanisms and interactions.

A planned program of resistance training over a few weeks can achieve muscle hypertrophy. It would be relevant to investigate muscle mass regulation and molecular events induced by resistance training using the various modalities of exposure to hypoxia known as *LH-TH*, *LL-TH* and

LH-TL to clearly describe the role of hypoxia. In addition, variables such as intensity, duration of exposure to hypoxia, and recovery conditions (hypoxia vs normoxia) should be managed to investigate the "hypoxic dose" effect. The comparison of the differential regulation of skeletal muscle mass and the molecular processes induced by the various hypoxic modalities could be of interest. Secondly, the precise implications of myogenic progenitors and the potential contribution of the Hippo pathway in mature muscle hypertrophy could be investigated.

Coming back to our aim to favor muscle mass accretion in athletes, we have to acknowledge that our work failed to support the anabolic effect of hypoxia exposure on skeletal muscle. Though, intermittent hypoxic resistance training resulted in increased strength, which eventually is what matters to athletes. We can therefore recommend to athletes to train 2x/week in hypoxia to improve muscle strength but to keep at least 1 resistance session a week in normoxia to maintain muscle mass.

3. Conclusion

This work aimed to study the molecular and cellular mechanisms of skeletal muscle mass regulation by resistance exercise under acute hypoxia. We hypothesized that acute hypoxia would potentiate the anabolic response of resistance exercise. Contrary to our hypothesis, acute hypoxia blunted anabolic signals toward muscle mass accretion by resistance exercise. Analysis of master proteins of signaling pathways involved in skeletal muscle mass did not show marked differences between normoxic and hypoxic conditions. Using transcriptional

investigations we observed regulation of several mechanisms by hypoxia at rest and after resistance exercise involving differentiation of satellite cells but the mechanism remained unclear. Furthermore, many studies mark increasing interest for the Hippo pathway and its contribution to skeletal muscle mass regulation by Yap and Taz, both key Hippo signaling effectors. This research area certainly deserves further interest and research.

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