THE EFFECTS OF LACTATE ON SKELETAL MUSCLE

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Abbreviations

8-epi-PGF2α	8-epi-prostaglandin F2α
a.u.	arbitrary units (grey values)
AA	ascorbic acid (vitamin C)
act. Casp-3	activated Caspase-3
AMPK	adenosine monophosphate-activated protein kinase
Ash2L	absent, small, or homeotic-like, variant 2
ASK1	apoptosis-stimulating kinase 1
ATF	activating transcription factor
ATP	adenosine triphosphate
BCP	1'-bromo-3'chloropropane
bHLH	basic helix-loop-helix
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumine
CaMK	calcium and calmodulin-acitvated protein kinase
Cat	Catalase
Cdkn1a	cyclin-dependent kinase inhibtor 1a
cDNA	complimentary deoxyribonucleic acid
Ct	cycle threshold
CTL	cytotoxic t-cell
D7F2	myogenic determination protein (MyoD) antibody (trade name)
DAB	diaminobezindine
DAPI	4',6-diamidino-2-phenylindole
DM	differentiation medium
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
Ezh	ehancer of zeste homolog
F5D	myogenin antibody (trade name)
FCS	fetal calf serum
F_{max}	maximum force
G1	gap 1
GLUT	glucose transporter
GPx	glutathione peroxidase
H_2O_2	hydrogen peroxide
H	histone 3

H3K4me3	trimethylated histone 3 lysine 4
H3K9me3	trimethylated histone 3 lysine 9
H3K27me3	trimethylated histone 3 lysine 27
H4K20me3	trimethylated histone 4 lysine 20
HDAC	histone deacetylase complex
HIT	high intensity resistance training
HRP	horse radish peroxidase
HS	horse serum
ICC	immunocytochemical staining
IF	immunofluorescent staining
JMJD	Jumonji domain-containing protein
JNK	c-Jun N-terminal kinase
KDM	lysine demethylase
KMT	lysine methyltransferase
LA	linolenic acid
La	lactate
mTOR	mammalian target of rapamycin
MAPK	mitogen-activated protein kinase
MCT	monocarboxylate transporter
Mef	myocyte enhancer factor
Mf20	myosin heavy chain antibody (trade name)
MHC	myosin heavy chain
MKP	mitogen-activated protein kinase phosphatase
mM	$mmol \cdot L^{-1}$
MOPS	3-(N-morpholino)propanesulfonic acid
MP	dry milk powder
MRF	muscle regulatory factor
MRF4	muscle-specific regulatory factor 4, also known as Myf6
mRNA	messenger ribonucleic acid
Myf5	myogenic factor 5
MyoD	myogenic determination protein
NAc	N-Acetyl-L-cysteine
p-	phosphorylated
p21	Cyclin-dependent kinase inhibitor 1a
p53	transformation related protein 53
PAGE	polyacrylamid gel electrophoresis
Pax7	paired box transcription factor 7
PBS	phosphate buffered saline
PcG	polycomb group protein
PCR	polymerase chain reaction
Pen/Strep	penicillin/streptomycin

PFA	paraformaldehyde
PGC1α	peroxisome proliferator activated-receptor γ coactivator-1α
PKB	protein kinase B
PM	proliferation medium
pRb	retinoblastoma protein
PRC2	polycomb repressor complex 2
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
ROM	range of motion
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse-transcription polymerase chain reaction
SC	satellite cell
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
Ser	serine
SOD	superoxide dismutase
STD	standard resistance training
SWI/SNF	switch/sucrose non-fermentable
TAE	Tris acetate ethylenediaminetetraacetic acid
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween20®
Thr	threonine
T_{melt}	melting temperature
Tris	Tris(hydroxymethyl)aminomethane
Trp53	transformation related protein 53
TSC2	tuberous sclerosis 2
Tx100	TritonX-100
TrxG	trithorax group protein
Tyr	tyrosine
UTX	transcribed tetratricopeptide repeat, X chromosome
WB	Western blot
Wnt	wingless-type MMTV integration site
YY	Ying Yang

Commonly used abbreviations and SI units are not listed separately.

Abstract

Regular exercise and physical activity are cornerstones in the prevention and treatment of numerous chronic conditions, such as type 2 diabetes, coronary heart disease, and age-related sarcopenia. The associated health benefits arise from a number of tissues but due to its high plasticity skeletal muscle plays a pivotal role. The resident stem cells of skeletal muscle tissue, so called Satellite cells (SCs), contribute significantly to skeletal muscle adaptation and hence, maintenance of healthy tissue. The specific stimuli regulating SC development, i.e. activation, proliferation, an differentiation, depend on the form of exercise and consist of hormonal, mechanical, and metabolic signals. While hormonal and mechanical factors have been well documented, the importance of metabolic stimuli such as Lactate (La) remains less clear. La is produced continuously under aerobic conditions, but elevated levels occur during exercise when glycolysis is increased. La is able to induce muscle adaptation, but the underlying molecular mechanisms are not yet understood. Therefore, one aim of this study was to identify the phenotypical effects of high La levels as observed during resistance or high intensity endurance training on the proliferation and differentiation in a model of activated SCs, C2C12 cells. Furthermore, possible signalling targets for La, such as p38 mitogen-activated protein kinase (p38 MAPK), and subsequent histone modifications were investigated. Lastly, to confirm the observed mechanisms *in vivo*, a human intervention study was conducted. Treatment with La (10 mM, 20 mM) increased the serum deprivation-induced withdrawal from the cell cycle and initiated early differentiation in C2C12 cells as analysis of gene expression and protein patterns of cell cycle (Ki67, Trp53, and Cdkn1a) and differentiation markers (Pax7, Myf5, myogenin, and myosin heavy chain) revealed. However, La delays late differentiation in a dose-dependent manner. La-induced production of ROS, marked by high 8-epi-PGF2α levels, might at least be partly responsible as the effects induced by La were reversible by the addition of the antioxidants ascorbic acid, N-acetylcysteine, or linolenic acid. Observed downregulation of p38 MAPK activation and its downstream modifications histone 3 lysine 4 (H3K4) and histone 3 lysine 27 (H3K27) trimethylation suggests that La inhibits late differentiation progress by this mechanism which is crucial for muscle specific gene transcription. Experiments using the p38 specific inhibitor SB203580 add further evidence for this hypothesis. Additionally, it was demonstrated that diminished p38 MAPK activation and subsequent histone modifications are conserved in differentiated muscle tissue *in vivo*. Conclusively, the reported data confirm that La modifies skeletal muscle adaptation via a ROS-sensitive signalling network by delaying late differentiation of SCs, an important mechanism of skeletal muscle adaptation. This conclusion implies reassessment of traditional views on training design and periodisation in order to accelerate skeletal muscle adaptation.

Zusammenfassung

Regelmäßige körperliche Aktivität ist in der Prävention und Therapie vieler chronischer Krankheiten, wie zum Beispiel Typ 2 Diabetes, koronare Herzkrankheiten und altersbedingte Sarkopenie von großer Bedeutung. Die positive Wirkung von körperlicher Aktivität sind u. a. auf die Adaptation der Skelettmuskulatur zurückzuführen, welche eine hohe Plastizität, d.h. eine sehr hohe Anpassungsfähigkeit auf wiederkehrende Reize, besitzt. Die gewebsspezifischen Stammzellen der Skelettmuskulatur, sogenannte Satellitenzellen (SCs), tragen wesentlich zur Skelettmuskelanpassung und damit zur Gesunderhaltung des Gewebes bei. Die Satellitenzellentwicklung, d.h. die Aktivierung, Proliferation und Differenzierung von SCs, wird durch verschiedene Stimuli reguliert, welche von der Art des Trainings abhängen. Diese können hormonellen, mechanischen oder metabolischen Ursprungs sein. Während hormonelle und mechanische Effekte gut beschrieben sind, bleibt die Rolle der metabolischen Reize, wie zum Beispiel Laktat (La), unklar. La wird ständig produziert, vor allem aber bei körperlicher Aktivität und der damit verbundenen erhöhten Glycolyserate. Kürzlich wurde gezeigt, dass La die Genexpression beeinflussen und damit die Muskelanpassung fördern kann. Die zugrunde liegenden molekularen Mechanismen sind aber unbekannt. Daher waren die Ziele dieser Arbeit zum einen, die Aufklärung der phänotypischen Effekte erhöhter Laktatkonzentrationen, wie sie bei Kraft- oder hochintensivem Ausdauertraining auftreten, auf die Proliferation und Differenzierung in einem Modell aktivierter SCs (C2C12-Zellen). Desweiteren sollten Signalmoleküle, die potentiell durch La modifiziert werden könnten, wie z.B. p38 mitogen-activated protein kinase (p38 MAPK) und darauf folgende Histonmodifikationen untersucht werden. Außerdem sollte eine humane Trainingsstudie zeigen, ob die aus der Zellkultur gewonnenen Erkenntnisse auch *in vivo* zutreffen. Genexpressions- und Proteinanalysen von Zellzyklus- (Ki67, Trp53, Cdkn1a) und Differenzierungsmarkern (Pax7, Myf5, Myogenin, Myosinschwerekette) an C2C12-Zellen, die mit physiologisch relevanten La-Konzentrationen (10, 20 mM) inkubiert wurden, zeigten, dass La den durch den Serumentzug verursachten Zellzyklusaustritt verstärkt und die frühe Differenzierung einleitet. Die späte Differenzierung wird jedoch durch La zeitlich und dosisabhängig verzögert. Die Produktion von ROS scheint bei der Vermittlung des La-Effekts eine wichtige Rolle zu spielen. Dieses beruht auf der erhöhten 8-epi-PGF2α-Bildung und der Umkehrbarkeit des Laktateffekts durch Zugabe der antioxidativen Substanzen Ascorbinsäure, N-Acetylcystein oder Linolensäure. Desweiteren konnte eine Reduktion der p38 MAPK-Aktivierung und der Trimethylierung des Histons 3 an den Lysinen 4 und 27 (H3K4me3 und H3K27me3) durch La festgestellt werden. Auch Versuche unter Einsatz des p38-spezifischen Inhibitors SB203580 bestätigen, dass die Differenzierungsverzögerung durch eine Hemmung von p38 MAPK und der damit

zusammenhängenden, für die muskelspezifische Genexpression notwendige H3K4me3 und H3K27me3 zustande kommt. Zudem konnte die Konservierung dieses Mechanismus auch im humanen Modell *in vivo* gezeigt werden. Zusammenfassend hat La einen modifizierenden Effekt auf die Skelettmuskeladaptation. Dieser besteht in der Verzögerung der späten Differenzierung von SCs, welche ROS-vermittelt auftritt. Dieser Schluss impliziert ein Überdenken und Modifikation der bisherigen Trainingsperiodisierung, um die Skelettmuskelanpassung zu beschleunigen.

1 Introduction

1.1 Exercise and physical activity and associated health benefits

Regular exercise and physical activity are cornerstones in the prevention, management, and treatment of numerous chronic conditions, such as hypertension, coronary heart disease, obesity, type 2 diabetes mellitus, and age-related sarcopenia (1, 2). Exercise and physical activity loweres the risk of all cause mortality, coronary heart disease, stroke, bone-related diseases, colon and breast cancer, and depression (3, 4). The associated health benefits arise from a number of tissues. However, skeletal muscle plays a pivotal role as detailed below. It makes up ~40% of body mass and accounts for ~30% of the basal metabolic rate (5). It is the predominant (~80%) disposal site of glucose under insulin-stimulated conditions (6) and a major regulator of glycemic control and metabolic homeostasis (7). One striking physiological characteristic of skeletal muscle tissue is its high plasticity, i.e. it has a high ability to adapt to sustained stimuli. Within the tissue, exercise and physical activity lead to activation of signalling pathways which induce acutely altered gene expression and/or protein synthesis. This is subsequently followed by chronic functional and structural adaptations. Additionally, muscular adaptations not only occur through changes within the existing muscle fibre but also by altering extracellular components. Extracellular matrix remodelling, capillarisation, and especially the addition of skeletal muscle stem cells, so called Satellite cells (SCs), to myofibres contribute further to functional adaptation and remodelling, and hence maintenance of healthy skeletal muscle tissue.

To date it is difficult to recommend specific exercises and activities to achieve a specific outcome as the molecular mechanisms of muscular adaptation in response to different forms of exercise are at large poorly understood. Generally, three forms of exercise can be distinguished: endurance, resistance, and patterned movement exercise (8). While the latter deals with a motor program in the central nervous system resulting in only small biochemical changes within the muscle, the former two have a large impact on muscle phenotype (8). Resistance training which is usually short in duration and high in intensity increases muscle wet mass (9), fibre cross-sectional area (10), RNA and protein content (11), and force generation capacity (12, 13), i.e. hypertrophy. This kind of training elicits health benefits for pathological conditions where muscle weakness comprises function such as sarcopenia and neuromuscular disorders, or following immobilisation, prolonged bed rest or injury (14). In contrast, endurance training which is comprised of low to moderate intensities and high volume induces adaptational changes that differ immensely. Here the main effects observed within skeletal muscle tissue are increased capillarisation (15) and mitochondrial mass (16), decreased glycolytic (17) and increased oxidative enzymes (18), increased slow contractile and regulatory proteins (17, 19), and a decrease in fast fibre area (19). Hence, this kind of

training is recommended in the prevention and treatment of cardiovascular diseases and metabolic abnormalities which involve coronary heart disease or obesity risk factors, such as elevated blood lipids.

The question arising with these findings of distinct adaptations is how skeletal muscle is able to respond differentially to what appears to be the same set of signals. Both forms of exercise elicit changes in intracellular calcium, deplete nutrients, and lead to energy stress (20). Nevertheless, the duration and intensity of those signals vary and might deliver the explanation for exercise form-specific adaptations. A lot of research in this field investigated the distinct signalling pathways activated by each form of exercise. The main conclusions are that [1] resistance training induces adaptation mainly by increasing protein synthesis via the protein kinase B (PKB) and mammalian target of rapamycin (mTOR) pathways; and [2] that in response to endurance training adenosine monophosphate (AMP)-activated protein kinase (AMPK) and calcium and calmodulin-activated protein kinase (CaMKII) are thought to be the key signals inducing gene expression and hence, adaptation (20). AMPK has moreover been shown to activate TSC2, which deactivates mTOR and hence mTOR-initiated protein synthesis (21). This could explain the observation that endurance training impairs hypertrophic effects induced by concurrent resistance training (22). In contrast, strength training improves endurance performance, possibly by improved neuromuscular characteristics and running economy (23). However, underlying molecular mechanisms remain unknown. Regarding health benefits, it is now agreed upon that a combination of both forms of training elicits a favourable additive effect (24), reflected by recent exercise guidelines for health (2). This notion is further supported by the success of training that combines aspects of both classical forms of training, such as high intensity endurance training which uses modes of non-weight bearing endurance exercise (e.g. cycling) combined with high intensities and low volumes usually related to resistance training. This form of training is less time consuming but research shows that is as efficient as traditional endurance-based training regarding physiological adaptations (25). As little as 15 min all out cycle exercise spread out over 2 weeks are sufficient to increase muscular oxidative capacity reflected by protein content and/or maximal activity of mitochondrial enzymes (26, 27). Several weeks of low-volume, high intensity cycling are furthermore associated with increased resting glycogen content (26), increased capacity of whole-body and skeletal muscle lipid oxidation (28), and enhanced peripheral vascular structure and function (29). The underlying molecular mechanisms are only partly understood. However, HIT activates peroxisome proliferator activated-receptor γ coactivator-1α (PGC1α) which is considered the master regulator of mitochondrial biogenesis in skeletal muscle (30). As evidence suggests that the intensity of exercise is the key factor regulating PGC1α activation in human muscle (31) this result is not surprising. Further investigations identified AMPK and p38 mitogen-

activated protein kinase (p38 MAPK) as upstream activators of PGC1α following high intensity endurance training (32), possibly via increased ROS generation (33). Although the beneficial effects of this kind of training have been established, metabolic signals initiating adaptational processes remain to be identified.

1.2 Satellite Cells and skeletal muscle adaptation

Next to altered gene expression and/or protein synthesis in skeletal muscle fibres, another important aspect of skeletal muscle adaptation or regeneration is the activation, proliferation, and differentiation of SCs, i.e. myogenesis. SCs lie quiescent between the basal membrane and the sarcolemma and were first discovered by Alexander Mauro who named them SCs for their localisation surrounding a muscle fibre (34). They originate from the somites and make up the necessary muscle specific stem cell pool needed for embryonic and adult myogenesis. In postnatal myogenesis, as it occurs in response to training or muscle injury, SCs are activated by multiple stimuli to become myoblasts. Myoblasts proliferate and increase in number, but at some point exit the cell cycle to enable differentiation and eventually fuse with existing myofibres to enlarge, to replace or to repair muscle tissue (Figure 1.1).

The role of SCs in muscle regeneration has been well characterised. However, their function in mediating exercise-induced adaptations is lively debated (35). Most emphasis has been placed upon elucidating the contribution of SCs in skeletal muscle adaptation in response to resistance training, where muscle fibre hypertrophy is the main adaptation. It is discussed whether SCs are necessary for hypertrophic events (36). However, the current literature suggests that after an initial phase where muscle hypertrophy mainly occurs through increased protein synthesis by myonuclei within the muscle fibre, the addition of new myonuclei to existing myofibres is required (14) for the maintenance of myonuclear domains, i.e. a theoretical cytoplasmic area associated with a myonucleus, to allow for sufficient supply of genetic machinery and protein synthesis without changing the kinetics of protein production for each nucleus (7). Only a few studies have looked at the role of SCs in response to training regimens that do not induce hypertrophy, e.g. high intensity endurance training as mentioned above. Results from an animal study (37) and two human studies (38, 39) have implied that endurance-based training enhances the SC pool. Furthermore, the literature suggests that this enhancement is influenced by rather the intensity than the duration of exercise (40). This would also explain the results of Snijders et al. who did not find a change the satellite cell content in diabetes type 2 patients following 6 month of a continuous, endurance-type exercise programme (41). Their workload corresponded to about 75% VO_{2max}, whereas the other studies used intermittent protocols with intensities around 75-

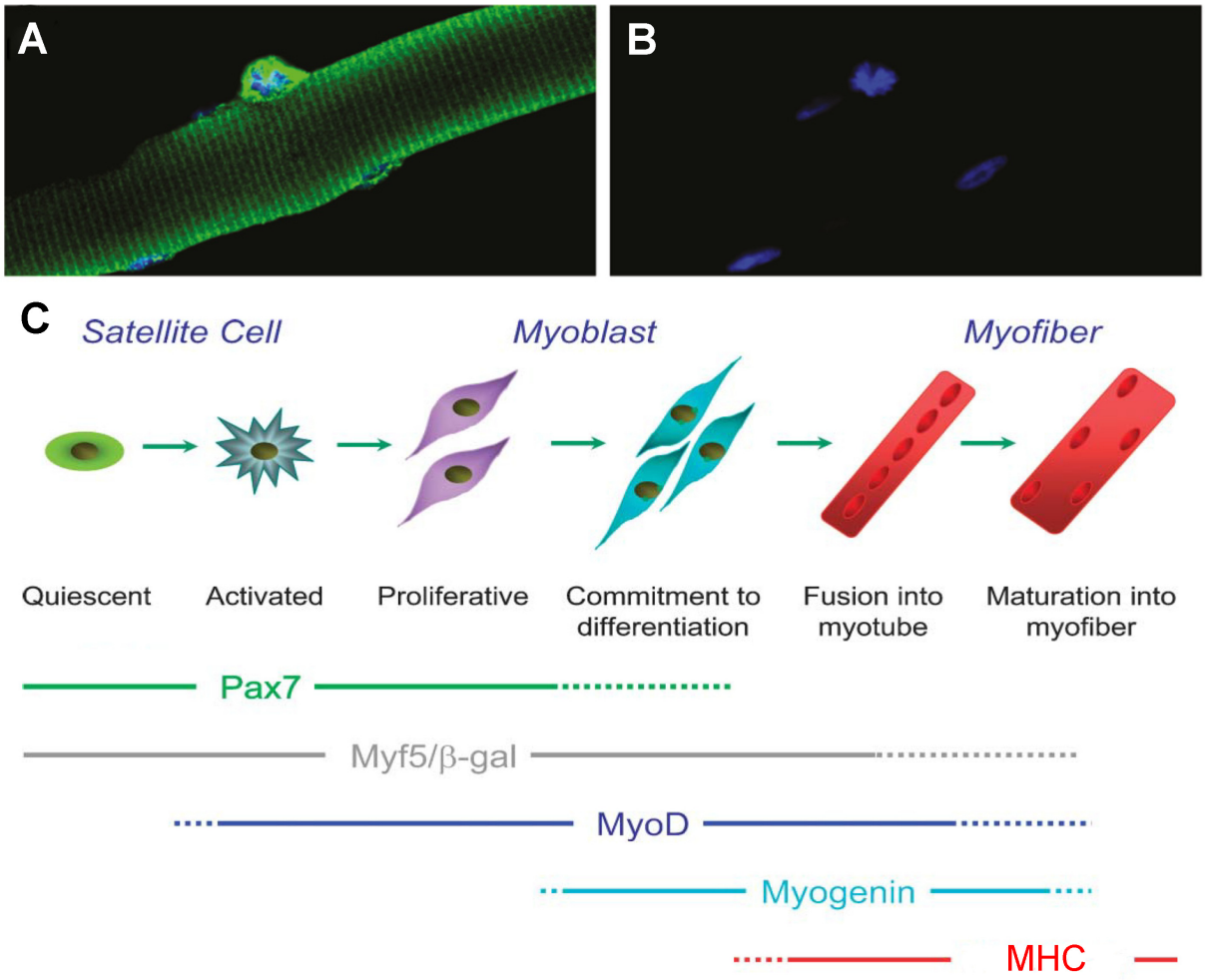

Figure 1.1: Satellite cell development and markers of myogenesis. **A + B** Fluorescent microscopic image of a SC on a mouse muscle fibre (yellow fluorescent protein – SC; blue – DNA). Modified from (52). **C** SC myogenesis scheme and markers typical for each stage. SCs lie quiescent underneath the basal membrane but can be activated by training. Activated SCs are called myoblasts that proliferate and increase in number. At some point they exit the cell cycle and start differentiating and fusing to form myotubes. Pax7 is expressed in quiescent, proliferating and early differentiating cells. Myf5 and MyoD expression increases during the proliferative and early differentiation phase, whereas myogenin marks the commitment to differentiation. During late differentiation, i.e. that of sarcomeric assembly, MHC expression is initiated. SC – Satellite cell; Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; MyoD – myogenic determination protein; MHC – myosin heavy chain. Modified from (167).

95% VO_{2peak} (38) or 75-95% HR_{max} (39) i.e. intensities that were considerably higher. Moreover, one study provides evidence that SCs are not only activated by high intensity endurance training and lead to increase SC availability but also have a role in functional adaptations following non-hypertrophic training such as skeletal muscle fibre remodelling (42). Taken together, the current literature suggests that in response to resistance and high intensity endurance training SCs are activated and play a crucial role in hypertrophic and even possibly non-hypertrophic skeletal muscle adaptations.

1.3 Myogenesis and its regulatory epigenetic modifications

During myogenesis, SCs and myoblasts underlie a strict sequential expression pattern of different transcription factors and structural muscle proteins (43). These can be used as

markers to study the proliferation and differentiation behaviour of this cell population *in vitro*. One of these proteins is the paired-box transcription factor 7 (Pax7). It is expressed during quiescence, activation, and proliferation as well as early differentiation and it is the most commonly used marker to identify SCs as such (44–46). To briefly describe the expression pattern during differentiation which is extensively reviewed in (47), a reduction of Pax7 expression coincides with the withdrawal from the cell cycle and transition from the proliferative to the differentiation phase. This state is also accompanied with the onset of the expression of several myogenic regulatory factors (MRFs). This group of muscle specific transcription factors of the basic helix-loop-helix (bLHL) class of DNA binding proteins consists of four members: myogenic factor 5 (Myf5), myogenic determination protein (MyoD), muscle-specific regulatory factor 4 (MRF4), and myogenin. Whereas MyoD and Myf5 are soon expressed following activation and early differentiation of SCs, myogenin is not expressed until a later stage marking the commitment to differentiation to which it is essential in adult myogenesis (48, 49). The exact expression pattern of MRF4 is less clear. It is expressed before as well as after myogenin expression (50, 51). At an even later stage of differentiation, i.e. that of sarcomeric assembly, the expression and synthesis of myosin heavy chain (MHC) indicates end-terminal differentiation of myotubes and myofibres. A number of stimuli that activate SCs were identified and are comprehensively reviewed in (52) and (53). Among these are transforming growth factor β, myostatin, insulin-like growth factor 1, fibroblast growth factor, hepatocyte growth factor, and the wingless-type MMTV integration site (Wnt) family of proteins. Although a number of SC activators were identified, their mechanisms have not been completely understood. Furthermore, some factors possibly might have not even been identified as yet.

The gene expression pattern during myogenesis is regulated by epigenetic modifications (54, 55), especially histone modifications. Generally, 147 bp of double stranded DNA are wrapped around a histone octamer consisting of two copies of each core histone, namely 2A, 2B, 3, and 4, forming so called nucleosomes which make up the basic units of chromatin. This ensures compactness of the genome to fit into the nuclei but also results in limited accessibility for the transcription machinery and hence hindered gene expression. This structure can however be reversibly modified to allow gene expression if indicated. Histone modifications include acetylation, methylation, phosphorylation, and ubiquitination and regulate gene expression differently. Especially histones 3 and 4 (H3 and H4) are prone to such alterations (56). Histone acetylations, the most common post-translational histone modification, lead to enhanced transcriptional activity. Histone acetyltransferases (HAT) and histone deacetylases (HDAC) add or remove, respectively, acetyl groups from histone lysine residues, converting the charge of the residue and alter DNA-histone binding as well as nucleosome associations (57, 58). Histone methylation however, is more complex as it can

either increase or repress gene expression. It occurs most commonly at lysine and arginine residues where up to three or two methyl groups can be added, respectively (59, 60). Methylated residues provide binding sites for chromodomains (61) and PHD domains (62–64) which are both related to chromatin remodeling. Trimethylation of H3 at lysine 4 (H3K4me3) occurs in as many as 75% of all gene promoters in some cells (65, 66) and it is positively correlated with active gene transcription (67, 68) marking its critical role in gene expression. In contrast, trimethylation of H3 at lysine 9 and 27 (H3K9me3 and H3K27me3) as well as H4 at lysine 20 (H4K20me3) are linked to gene repression. Adding to the complexity compared to histone acetylation is the finding that methylation of different residues appears to be catalysed by different lysine methyltransferases (KMT). They are two forms of KMT: polycomb group (PcG) and trithorax group (TrxG) proteins that work antagonistically. For example, H3K4 methylation is mediated by KMT2, a TrxG member that acts as the subunit of the histone methyltransferase complex absent, small, or homeotic-like, variant 2 (Ash2L), whereas H3K27 is methylated by PcG proteins from the KMT6 family, namely enhancer of zeste homolog 1 and 2 (Ezh1 and Ezh2) that act as the active subunit of the polycomb repressor complex 2 (PRC2), another histone methyltransferase complex (69). The H3K27me3 mark is dominant to H3K4me3 (67). So, in order for gene transcription to occur at gene loci marked with both modifications, H3K27me3 has to be demethylated. This is achieved by the lysine demethylase family 6 (KDM6) members ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) and Jumonji domain-containing protein 3 (JMJD3) (70, 71, 71).

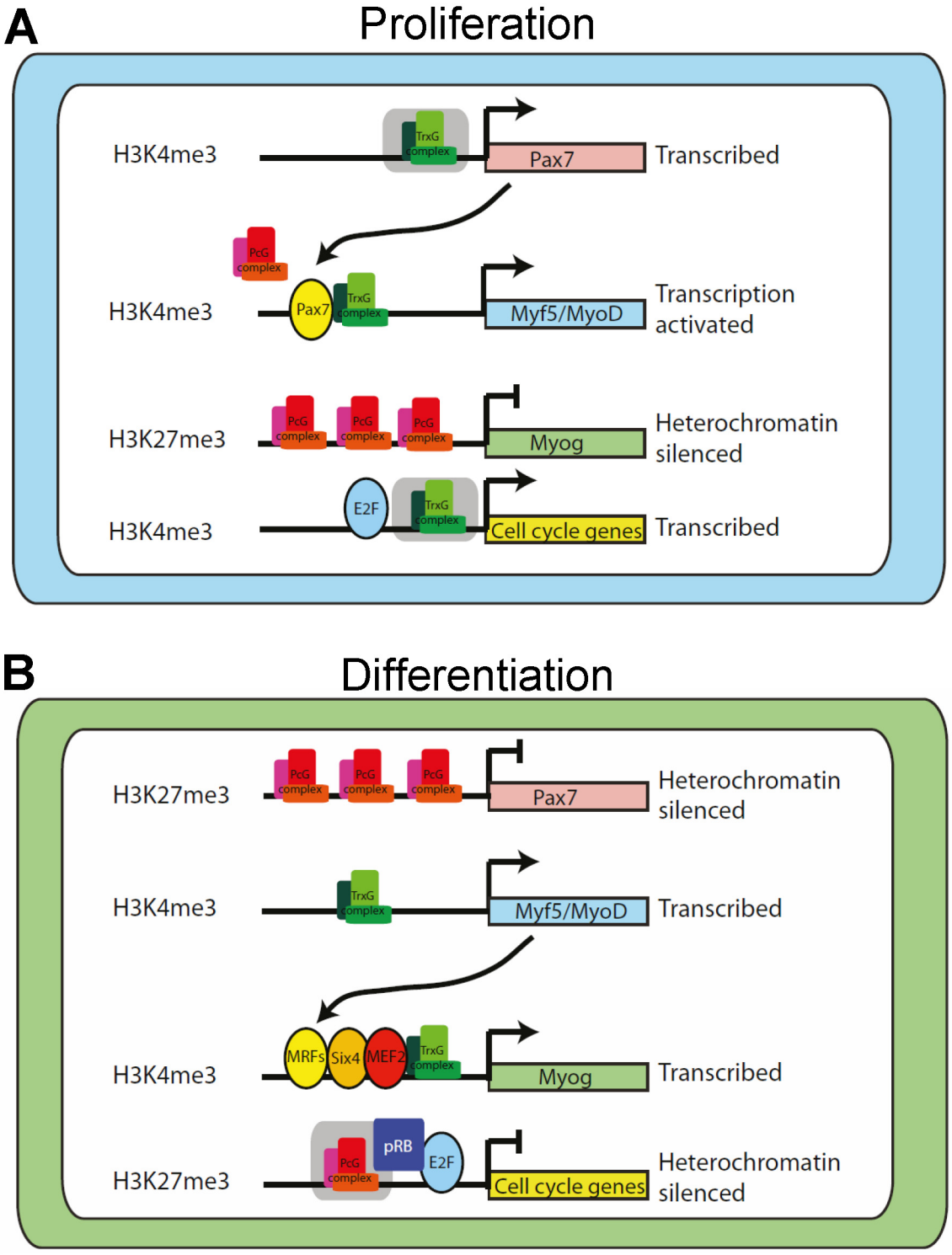

Figure 1.2: Histone modifications H3K4me3 and H3K27me3 during SC proliferation and differentiation. **A** During proliferation and cell cycle progression Pax7 and Myf5 genes are expressed. These genes are known to be marked with H3K4me3. Myf5 is important for the progress from the proliferative to the differentiation stage. The H3K4me mark is established through the recruitment of TrxG proteins by Pax7. **B** In terminally differentiating SCs Pax7 and cell cycle progression genes are silenced. This repression involves PcG mediated H3K27me3 at the promoter regions of the respective genes. At this time point MyoD collaborates with Mef2d and Six4 to establish the H3K4me3 mark at the myogenin promoter, initiating its expression. H3Kxme3 – trimethylated histone 3 at lysine x; SC – Satellite cell; Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; TrxG – trithorax group protein; PcG – polycomb group protein; MyoD – myogenic determination protein; Mef2d – myocyte enhancer factor 2d; Six4 – Six homeobox 4. Modified from (69).

During myogenesis (Figure 1.2), Pax7 expression is switched off in order for differentiation to proceed. p38 MAPK promotes recruitment of PRC2 to the Pax7 promoter via interaction with Ezh2, which is the direct substrate for phosphorylation-mediated association with Ying Yang 1 (YY1). This leads to the enrichment in chromatin occupancy by Ezh2/YY1 which mediates Pax7 repression (72, 73). During proliferation cell cycle genes are enriched with the permissive H3K4me3 mark (74). However, when differentiation is initiated, the repressive H3K27me3 conformation accumulates (75) by a mechanism involving E2F family members and the retinoblastoma protein (pRb) (76). Furthermore, during the proliferative state, Myf5 expression is induced via a Pax7-dependent recruitment of a KMT2 family member and subsequent H3K4me3 (77). Myogenin expression marks the commitment to differentiation in myoblasts. Here, the repressive H3K27me3 is removed during differentiation (78, 79). Furthermore, Rampalli et al. showed that Ash2L is recruited by Mef2d and targets the H3K4me3 mark to specific promoters, such as myogenin (80). The same group further demonstrated that this recruitment is regulated through the p38 MAPK phosphorylation of Mef2d, linking the p38 MAPK signalling pathway with the activation of muscle-specific genes in myogenesis (79, 80). The histone modification H3K9me3 is controversially discussed in the literature. Although it has been thought to repress gene expression (81, 82), it was recently shown to also be able to induce the same (83). The H4K20me3 is another histone methylation known to play a role in myogenesis, but so far it was only shown to affect myogenesis by the repression of myogenin with the further interpretation being elusive (84). Taken collectively, the current literature suggests that epigenetic events, in particular histone trimethylation of H3K4 and H3K27, are fundamental to the regulation of myogenesis. However, apart from histone methylations, other epigenetic modifications might be important during myogenesis.

1.4 The role of p38 MAPK in skeletal muscle adaptation

Interestingly, a number of the above mentioned events are regulated via the p38 MAPK. MAPKs are a family of protein kinases, i.e. proteins that covalently bind a phosphate to a threonine (Thr), serine (Ser), or tyrosine (Tyr) side chain of other proteins and thereby alter their targets activity or interactions with other molecules. This leads to changes in cell cycle machinery, gene expression, protein synthesis, differentiation, metabolism, migration, and cell death (85, 86). 3 subfamilies have been well characterised in the literature: extracellular signal-regulated kinases (ERK1/2), the c-Jun NH2 terminal kinases (JNK1/2/3), and p38 MAPK (p38α, p38β, p38γ, p38δ). During myogenesis, p38 MAPK has been identified as a major regulator (87, 88). Upon differentiation cues, p38 MAPK is phosphorylated by the upstream MAPK kinases 3 and 6 (MKK3 and MKK6) at threonine 180 and tyrosine 182 (p-p38), and activated. The isoforms p38α and p38β were shown to be involved during

myoblasts differentiation (89–91). The role of p38γ in myogenesis is so far not fully understood. Although it is expressed in skeletal muscle and its expression is upregulated during myogenesis (90, 92–94), a differentiation regulatory effect could not be confirmed. p38δ appears to play no important role in skeletal muscle (reviewed in (95)).

Additionally to the histone methylations described above, p38 MAPK has additional pro-myogenic effects (Figure 1.3). Upon differentiation signals, activated p38 MAPK directs switch/sucrose non-fermentable (SWI/SNF) and Ash2L-KMT containing complexes to regulatory muscle genes, promoting their transcription (96–98). Furthermore, p38 is directed to the chromatin of muscle specific genes through interactions with E47 (99), Mef2d (80) and BAF60 (96). CAM-downregulated by oncogenes (CDO) is a transmembrane protein of the Ig-fibronectin III repeat subfamily. It can also activate p38 MAPK which in turn leads to the formation of MyoD-E47 heterodimers that bind to E-box sequences on the regulatory regions of muscle-specific genes (100, 101). It is moreover involved in cell cycle exit. p38 MAPK decreases cyclin D1 expression and pRb phosphorylation, therefore promoting cell cycle withdrawal and differentiation progression (102).

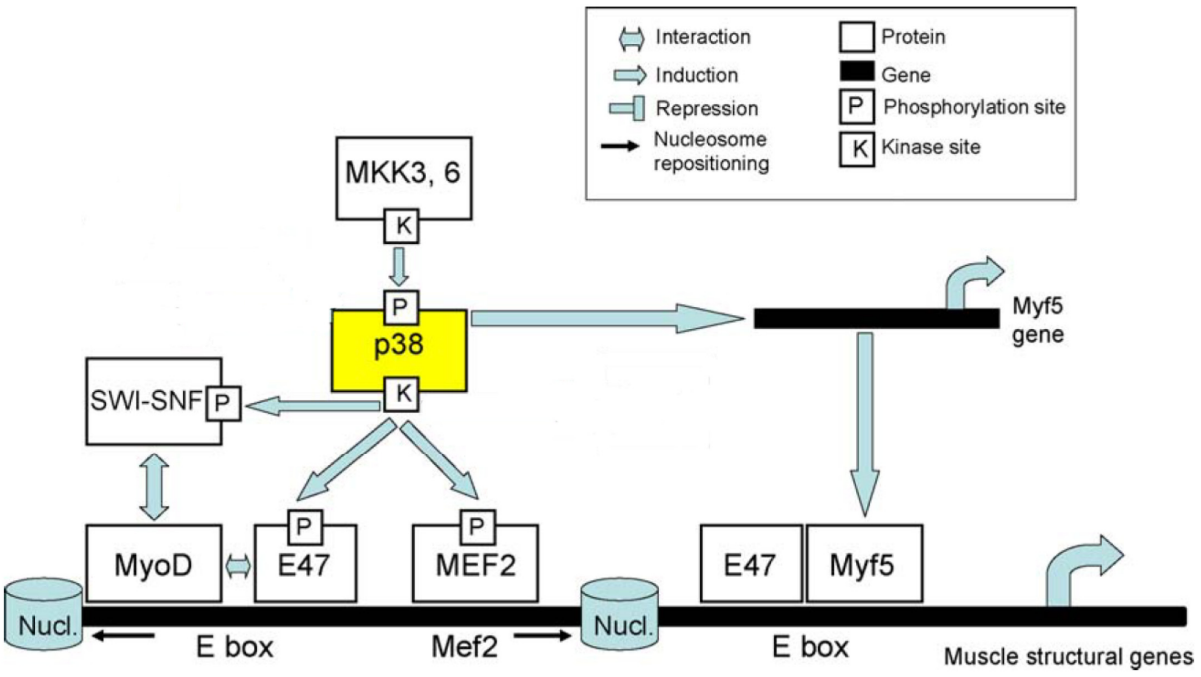

Figure 1.3: A model for the activity of p38 MAPK in myogenesis. p38 MAPK is activated by MKK3/6 in muscle cells and subsequently phosphorylates various substrates contributing to myogenesis. It induces the transcriptional activity of Mef2 transcription factors and promotes the heterodimerisation of E47 with MyoD. Additionally, it induces Myf5 expression. p38 MAPK recruits the SWI/SNF complexes to muscle promoter regions, thereby affecting the epigenetic landscape. p38 MAPK – p38 mitogen-activated protein kinase; MKK3/6 – MAPK kinase 3/6; Mef2 – myocyte enhancer factor 2; MyoD – myogenic determination protein; Myf5 – myogenic factor 5; SWI/SNF - switch/sucrose non-fermentable. Modified from (87).

In addition to its regulatory role during the differentiation of SCs, p38 MAPK has also been shown to possess the ability to induce skeletal muscle adaptation within differentiated muscle tissue in response to exercise or physical activity. The level of phosphorylation and hence activation depends on the exercise status, i.e. p38 MAPK is more phosphorylated in untrained subjects compared to highly trained athletes (103, 104). This in turn leads to the stimulation of activating transcription factor 2 (ATF2) and Mef2 (105) which are upstream transcription factors of the PGC1α gene and induce its expression following exercise (31, 105, 106). These data implies a superior role of p38 MAPK during SC differentiation and regulating gene expression in response to exercise and physical activity making it a key molecule for skeletal muscle adaptation.

1.5 Exercise and physical activity and histone modifications

Epigenetic events are not only important during myogenesis, but also regulate skeletal muscle fibre adaptations in response to exercise and physical activity through exercise-induced alterations of the metabolic state (107–109). This implies that epigenetic responses should also elicit an intensity-dependent effect. This concept was supported by the finding of decreased whole genome methylation in muscle biopsies from sedentary subjects after an acute bout of exercise. Exercise induced dose-dependent expression of PGC1α and Mef2a mRNA accompanied by a pronounced hypomethylation on both promoters, a state associated with gene induction (110). Proposed metabolic stimuli to regulate epigenetic events following exercise are AMPK and CaMKII. Both have a pronounced regulatory effect on class II HDACs. This class consists of HDAC 4, 5, 7, and 9 and are enriched in striated muscle (111). They exert their function by association with the Mef2 family of transcription factors to repress Mef2-dependent transcription (112). Following a single bout of exercise, HDAC5 was found to be less abundant within the nucleus and less HDAC5 was associated with Mef2 (113). These changes were accompanied by increased Mef2-binding activity to the DNA (114) and elevated glucose transporter 4 (GLUT4) mRNA levels (113). The concept that HDAC5 nuclear abundance is reduced following exercise could also be confirmed for HDAC4 (115). AMPK and CaMKII can both phosphorylate HDAC4 and 5, mediate their nuclear export and hence render these enzymes to suppress histone acetylation (115, 116). Although this direct relationship was shown for GLUT4, other oxidative genes are also thought to be regulated by this mechanism (31, 117). The literature also suggests that epigenetic modifications are involved in the differential expression of the MHC genes and therefore determination of muscle phenotype (118). Changes in acetylation and methylation of H3 led to differential expression of MHC1, MHC2x, and MHC2b fibres in response to muscle unloading in skeletal muscle tissue of mice (119, 120). A knockout model of HDAC5 in mice furthermore suggested a regulatory role for slow fibre formation. Following HDAC5

loss, MHC1 fibre type increased (121). Collectively, epigenetic modifications in response to exercise provide evidence for the role of metabolic control of gene expression and the concept that skeletal muscle adaptation via epigenetic mechanisms is therefore exercise-intensity dependent. However, knowledge of epigenetic alterations, especially histone modifications is scarce. Understanding the exercise and physical activity-dependent alterations in skeletal muscle gene expression by altering the epigenetic landscape will add new implications to exercise and physical activity prescriptions in prevention and therapy.

1.6 Lactate as a metabolic signal of gene expression

Muscle phenotype alterations only occur after repeated bouts of exercise. Over time, skeletal muscle demonstrates remarkable plasticity in response to contractile activity by functional remodelling (122, 123). Repeated stimuli triggered in each single session at a sufficient frequency are necessary to induce a new steady state within the muscle (8) including the activation and regulation of the development of SCs necessary for adaptation. These signals include hormonal factors (124), mechanical stimuli (125), and alterations of the metabolic state (126). While the importance of hormonal and mechanical factors has been well established (127, 128), the importance of metabolic influences especially in resistance and high intensity endurance training is indicated (129) but remains elusive.

The metabolic responses to resistance and high intensity endurance exercise are thought to be initiators of skeletal muscle adaptation by influencing motor unit activation, hormones, and muscle damage (130). The challenge arising with this idea is to identify the intracellular signalling molecules that occur at different concentrations depending on the exercise intensity. One possible candidate is La. All muscle contractions require energy in form of adenosine triphosphate (ATP). ATP stores are limited within the muscle fibre and therefore ATP has to be resynthesised at its rate of use during muscular activity in order to maintain contractions. During high intensity exercise the main source of ATP is glycolysis that produces pyruvate. Pyruvate is transferred into mitochondria for further oxidative phosphorylation and ATP resynthesis. However, if exercise intensity and the ATP breakdown rate exceed the rate of oxidative phosphorylation, pyruvate accumulates in the cytosol and is converted to lactic acid by the lactate dehydrogenase enzyme. Lactic acid is almost fully dissociated to La and protons at physiological pH, resulting in La accumulation within the working muscle. La has long been considered a metabolic waste product and its accumulation was thought to be the cause of the decrease in muscle pH and hence, muscle fatigue. This idea has changed massively in the past. La is now recognised as a metabolic stimulus able to induce skeletal muscle adaptation. For example, La has been shown to increase norepinephrine and growth hormone levels following resistance exercise (131–133). Furthermore, it increases recruitment of motor units at a given load (131, 134). La was also

shown to correlate with muscle damage (135). These events lead to enhanced skeletal muscle adaptation and therefore underline the role of La as a metabolic stimulus for altering muscle phenotype.

The aforementioned La effects are indirect and have no immediate impact on muscle gene expression or protein synthesis. However, recently Hashimoto et al. demonstrated that La increases monocarboxylate transporter-1 (MCT-1) and PGC1α mRNA content indicating the induction of mitochondrial biogenesis in L6 rat myoblasts (136). La was termed “Lactormone” as it possesses signalling properties inducing gene expression necessary for skeletal muscle adaptation (137).

With the identification of La as a gene expression-altering molecule in skeletal muscle, the question arises which signalling pathways are involved in the mediation. It was previously shown that in macrophages La concentrations correlated with the induction of angiogenic factor gene expression (138). Later on this effect was shown to be mediated by H_2O_2 (139). H_2O_2 is a member of the reactive oxygen species (ROS) which also include the superoxide anion ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$). Although H_2O_2 is not a free radical and a weaker oxidising agent, it can be oxidised into the extremely active and toxic $\cdot OH$ (140) and with other ROS can affect signalling cascades that affect cell growth, proliferation, migration, and apoptosis (141). ROS are produced at various sites in skeletal muscle. Main sources are Complex I and III of the electron transport chain of mitochondria (142). Furthermore, NADPH oxidases (143) and xanthine oxidase (144) contribute to ROS formation within the cytosol of skeletal muscle. ROS-induced redox signalling regulates numerous transcription factors and alter gene expression and hence skeletal muscle phenotype (145). One known target of ROS are MAPKs (146). Applying the above mentioned mechanism suggested in macrophages to muscle tissue, investigations confirmed that La increases intracellular H_2O_2 and therefore ROS, and subsequently promoted binding of ROS-sensitive transcription factors to DNA (136). In the same study it was furthermore speculated that ROS led to the downstream activation of MAPK (136). However, no data on La and its effect on the MAPKs are available for skeletal muscle and hence the role of these key regulators in La-signal mediation remains to be investigated. Taken together, La is an important signalling molecule for skeletal muscle adaptations. Although a La-activated ROS-sensitive signalling network has been suggested, conclusive data is missing if the La-effects depend on ROS-formation. Furthermore, molecular insights in the mediation of the La effect are not yet provided.

1.7 Aims

1. To identify phenotypical effects of a simulated training microcycle of resistance or high intensity endurance training by intermittent incubation with a potent metabolic signalling molecule, i.e. La, on the proliferation and differentiation in a model for skeletal muscle stem cells.

2. To investigate the role of ROS formation and oxidative stress induced by La and whether these could be responsible for the observed effects.

3. To elucidate whether elevated La levels influence key signalling molecules of skeletal muscle adaptation, i.e. p38 MAPK, and its downstream epigenetic targets H3K4me3 and H3K27me3 to further establish the important role of La as a metabolic signal of gene expression.

4. To confirm that these mechanisms in muscle stem cells are conserved during differentiation and remain active in differentiated muscle tissue exposed to similar La concentrations evoked by exercise *in vivo*.

2 Materials & Methods

2.1 Materials

2.1.1 Lab equipment and disposables

Name	Company
A-Plan 10x/0,25 Ph1 objective	Carl Zeiss AG, Jena Germany
A-Plan 20x/0,4 Korr Ph2 objective (200M)	Carl Zeiss AG, Jena Germany
ApoChromat 63x/1,4 Oil DIC objective	Carl Zeiss AG, Jena Germany
ApoTome	Carl Zeiss AG, Jena Germany
Axiovert 200M microscope	Carl Zeiss AG, Jena Germany
AxioCam MRm	Carl Zeiss AG, Jena, Germany
Biofuge 13	Heraeus, Hanau, Germany
BioMax Exposure Cassette	Eastman Kodak Co, Rochester, NY, USA
BIOSEN S-Line Lactate analyser	EKF-Diagnostic GmbH, Barleben, Germany
Bürker hemocytometer	Karl Hecht, Sondheim, Germany
CCD camera MC-3255	Sony, Tokio, Japan
Cell Culture Flasks and Consumables	BD Biosciences, San Jose, CA, USA
CO_2 Incubator C150	Binder, Tuttlingen, Germany
Criterion™ electrophoresis chamber	Bio-Rad Laboratories, Hercules, CA, USA
Criterion™ XT 4-12% Bis-Tris gels	Bio-Rad Laboratories, Hercules, CA, USA
Filter Set 10 (exc. 450-490 nm; em. 515-565 nm)	Carl Zeiss AG, Jena Germany
Filter Set 14 (exc. 510-560 nm; em. 590 nm)	Carl Zeiss AG, Jena Germany
Filter Set 49 (exc. 365 nm; em. 545-450 nm)	Carl Zeiss AG, Jena Germany
Flexcylcer^2 thermocycler	AnalytikJena AG, Jena, Germany
Glass cover slips	VWR International, Langenfeld, Germany
Hypodermic Needle	Henry Schein, Melville, NY, USA
ISOMED 2000 isokinetic equipment	D & R Ferstl GmbH, Hemau, Germany
Kinetic Microplate Reader	MWG Biotech, Ebersberg, Germany
KS300 microscope	Carl Zeiss AG, Jena Germany
Lamina Air Flow HS12	Heraeus, Hanau, Germany
Leica CM1900 Cryostat	Leica Microsystems, Wetzlar, Germany
Leitz Orthoplan	Leica Microsystems, Wetzlar, Germany
Lode Excalibur cycle ergometer	Lode, Groningen, Netherlands
Micro-dismembrator	Braun, Melsungen, Germany
Microscope slides	VWR International, Langenfeld, Germany
Nalgene™ Cryo 1°C Freezing Container	Thermo Fisher Scientific, Waltham, USA
pH-mV-meter	Mettler Toledo, Gießen, Germany
Plan Neofluar 20x/0.5 objective (KS300)	Carl Zeiss AG, Jena Germany
Poly-L-lysine microscope slides	VWR International, Langenfeld, Germany
PowerPac™ Basic power supply	Bio-Rad Laboratories, Hercules, CA, USA
PVDF membrane	Pall Corporation, Port Washington, NY, USA
Scaltec SBA53 weighing scale	Scaltec Instruments, Heiligenstadt, Germany
Table centrifuge 5417R	Eppendorf, Hamburg, Germany
Thermocycler Mx3005P	Agilent Technologies, Santa Clara, CA, USA
Thermomixer 5436	Eppendorf, Hamburg, Germany

Titramax 101 shaker	Heidolph Instruments, Schwabach, Germany
TransBlot Turbo	Bio-Rad Laboratories, Hercules, CA, USA
Vortex Genie2	Scientific Industries, Bohemia, NY, USA
X-OMAT x-ray films	Eastman Kodak Co, Rochester, NY, USA

2.1.2 Chemical and solutions

Name	Company
100 bp DNA ladder	Invitrogen, Karlsruhe, Germany
Acetic Acid	Merck, Darmstadt, Germany
Agarose	Biozym Scientific, Hessisch Oldendorf, Germany
Aqua-Poly/Mount	Polysciences, Warrington, PA, USA
Ammonium chloride	Merck, Darmstadt, Germany
β-mercaptoethanol	Invitrogen, Karlsruhe, Germany
Bovine Serum Albumine	PAA, Pasching, Austria
BCP	Molecular Research Center, Cincinnati, OH, USA
bromphenol blue	Merck, Darmstadt, Germany
Cell lysis buffer (# 9803)	Cell Signaling Technology, Danvers, MA, USA
Complete Mini® Protease Inhibitor Cocktail	Roche, Mannheim, Germany
D-Glucose	Roth Chemicals, Karlsruge, Germany
DAB	Sigma-Aldrich, Steinheim, Germany
DAPI	Invitrogen, Karlsruhe, Germany
DMSO	Merck, Darmstadt, Germany
DMEM	Invitrogen, Karlsruhe, Germany
DPBS	Invitrogen, Karlsruhe, Germany
Dry milk powder	Bio-Rad Laboratories, Hercules, CA, USA
EDTA	Sigma-Aldrich, Steinheim, Germany
Ethanol	Merck, Darmstadt, Germany
Entellan®	Merck, Darmstadt, Germany
FBS	PAA, Pasching, Austria
Fresubin® protein energy drink	Fresenius Kabi, Bad Homburg, Germany
Gelatine	Sigma-Aldrich, Steinheim, Germany
Glucoseoxidase	Sigma-Aldrich, Steinheim, Germany
Glutamine	Invitrogen, Karlsruhe, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Merck, Darmstadt, Germany
H_2O_2	Merck, Darmstadt, Germany
HALT Phosphatase Inhibitor Cocktail	Pierce Biotechnology, Rockford, IL, USA
HD Green	IntasScience Imaging Instruments, Göttingen, Germany
Hemolyzing solution (ready-to-use)	EKF Diagnostic GmbH, Barleben, Germany
Horse Radish Peroxidase	Dako, Glostrup, Denmark
Isopropanol	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
L-Ascorbic Acid	Sigma-Aldrich, Steinheim, Germany
Linolenic Acid	Sigma-Aldrich, Steinheim, Germany
Methanol	Merck, Darmstadt, Germany
N-Acetyl-L-cysteine	Sigma-Aldrich, Steinheim, Germany

Na_2HPO_4	Merck, Darmstadt, Germany
NiSO4	Merck, Darmstadt, Germany
Paraformaldehyde	Roth Chemicals, Karlsruge, Germany
Penicillin/Streptomycin	Invitrogen, Karlsruhe, Germany
Precision Plus Protein Standard Dual Color	Bio-Rad Laboratories, Hercules, CA, USA
Recovery™ Cell Culture Freezing Medium	Invitrogen, Karlsruhe, Germany
Restore™ Western Blot Stripping Buffer	Thermo Fisher Scientific, Waltham, MA, USA
SB203580	Cell Signaling Technology, Danvers, MA, USA
Sodium bicarboante	Invitrogen, Karlsruhe, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium dodecyl sulfate	Amresco, Karlsfeld, Germany
Sodium-L-Lactate	Sigma-Aldrich, Steinheim, Germany
Sodium pyruvate	Invitrogen, Karlsruhe, Germany
SuperSignal West Dura Luminol/Enhancer	Thermo Fisher Scientific, Waltham, MA, USA
TissueTek® O.C.T.™ Compound	Sakura Finetek, Zoeterwoude, Netherlands
Tris	Merck, Darmstadt, Germany
TritonX-100	Sigma-Aldrich, Steinheim, Germany
TriReagent®	Molecular Research Center, Cincinnati, OH, USA
Trypsin-EDTA	Invitrogen, Karlsruhe, Germany
Tween20	Sigma-Aldrich, Steinheim, Germany
XT MOPS running buffer	Bio-Rad Laboratories, Hercules, CA, USA
Xylol	Alfred Quadflieg, Gelsenkrichen, Germany

All compounds/used chemicals were adjusted for purity.

2.1.3 Buffers and cell culture media

0.1% gelatine	0.1%	gelatine	in DPBS
PM	20%	FCS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	in DMEM
(0 mM La) DM	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	in DMEM
5 mM La DM	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	5 mM	sodium lactate	in DMEM

10 mM La DM	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	10 mM	sodium lactate	in DMEM
20 mM La DM	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	20 mM	sodium lactate	in DMEM
DM + DMSO	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	0.1%	DMSO	in DMEM
DM + SB 203580	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	0.1%	DMSO	
	10 µM	SB203580	in DMEM
20 mM La DM + AA	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	20 mM	sodium lactate	
	100 µm	ascorbic acid	in DMEM
20 mM La DM + NAc	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	20 mM	sodium lactate	
	5 mM	N-acetylcysteine	in DMEM
20 mM La DM + LA	4%	HS	
	1%	pen/strep	
	4 mM	glutamine	
	0.15%	sodium bicarboante	
	1 mM	sodium pyruvate	
	20 mM	sodium lactate	
	5 µM	linolenic acid	in DMEM

TAE	40 mM	Tris		
	20 mM	acetic acid		
	1 mM	EDTA		pH = 8.0
PBS	137 mM	NaCl		
	10 mM	Na_2HPO_4		
	1.8 mM	KH_2PO_4		pH = 7.4
TBS	50 mM	Tris		
	150 mM	NaCl		pH = 7.6
4% PFA	4%	PFA	in PBS	pH = 7.4
Permeabilisation solution	500 mM	NH_4Cl		
	0.25%	Tx-100	in PBS	
DAPI solution	300 nM	DAPI	in PBS	
PB	80 mM	$Na_2HPO_4 \cdot 2\ H_2O$		
	20 mM	$NaH_2PO_4 \cdot H_2O$		pH = 7.4
DAB	2 mM	DAB		
	7.5 mM	NH_4Cl		
	1.5 mM	NiSO4		
	10 mM	D-glucose		
	0.0004%	glucose oxidase	in PB	
Lysis buffer	1%	Tx100	in TBS	pH = 8.0
Laemmli buffer 2x	125 mM	Tris		
	4%	SDS		
	20%	glycerine		
	10%	β-mercaptoethanol		
	0.004%	bromphenol blue		pH = 6.8
TBST	150 mM	NaCl		
	50 mM	Tris		
	0.1%	Tween20®		pH = 7.4
Transfer buffer	25 mM	Tris		
	150 mM	glycine		
	10%	methanol		

2.1.4 Kits

Name	Company
Cell Proliferation ELISA BrdU (colorimetric)	Roche Diagnostics, Mannheim, Germany
RC DC™ Protein Assay	Bio-Rad Laboratories, Hercules, CA, USA

2.1.5 Software

Name	Version	Company
Axiovision	4.6.3	Carl Zeiss AG, Jena, Germany
IBM SPSS Stastistics	19, 20	IBM SPSS Corporation, Chicago, IL, USA
ImageJ	1.46r	National Institue of Health, Bethesda, MD, USA
Metamorph	7.0	Molecular Devices, Sunnyvale, CA, USA
Photoshop	CS5	Adobe Systems, San Jose, CA, USA
Primer3	0.4.0	Whitehead Insitute of Biomedical Research, Cambridge, UK
Microsoft Office	2003	Microsoft Corporation, Redmond, WA, USA

2.2 Methods

2.2.1 Cell Culture

C2C12 mouse myoblasts (147, 148) obtained from the DSZM Braunschweig, Germany, were maintained in proliferation medium (PM) in T75 cell culture flasks. Cells were kept in a humidified incubator at 37°C and 5% CO_2. Only cells not exceeding passage 6 were used in the experiments.

2.2.1.1 Thawing Cells

The freezing vial was taken out of the -150°C freezer and transported on ice. The suspension was thawed carefully in a 37°C water bath and transferred into a 15 mL conical tube. 9 mL of pre-warmed PM were added dropwise. The suspension was then centrifuged (800 g, 5 min, room temperature [RT]) to remove toxic dimethylsulfoxide (DMSO). The pellet was resuspended in fresh PM and cells were plated in T75 cell culture flasks. Before being used in experiments, cells were splitted at least once after thawing.

2.2.1.2 Freezing cells

Cells were detached at 60-70% confluence from the T75 cell culture flasks with 1 mL trypsin and incubated for 3 min at 37°C. The reaction was stopped by addition of 9 mL DPBS and cells were pelleted (800 g, 5 min, RT) followed by resuspension in freezing medium (approximately 1 mL freezing medium per 10^6 cells). Vials were placed in a pre-cooled freezing container and placed at -80°C. The next day vials were transferred to -150°C for long-term storage.

2.2.1.3 Splitting Cells

At 60-70% confluency, cells were splitted to maintain the undifferentiated character. Therefore, the PM was aspired from the T75 cell culture flasks, cells briefly washed with DPBS and trypsinised with 1 mL for 3 min at 37°C. The reaction was stopped by adding 9 mL DPBS and centrifugation (800 g, 5 min, RT). The pellet was resuspended in fresh pre-warmed PM and cells plated in the desired density into a new cell culture flask.

2.2.1.4 Seeding cells

For the experiments, cells were seeded at a density of 10.000 cells per cm^2 onto cell culture petridishes or multi-well plates. If cells were plated on glass cover slips for subsequent microscopic analysis, these were pre-coated with 0.1% gelatine to ensure cell attachment and incubated for at least 45 min at 37°C. Just before use, the remaining gelatine solution was aspired and cover slips air-dried for 1-2 min. The C2C12 cells were briefly washed with DPBS, trypsinised and centrifuged (800 g, 5 min, RT). The pellet was resuspended in fresh PM and cells counted using a hemocytometer. The required resuspension volume was transferred into a conical tube, an adequate amount of PM was added and cells seeded into appropriate cell culture dishes for experimental use.

2.2.1.5 Experimental Design

After plating, cells were kept in PM until 80-90% confluence was reached. Thereafter, medium was switched to differentiation medium (DM) containing 4% horse serum. This serum mitogen withdrawal induces differentiation in C2C12 cells which then start to fuse and form myotubes (147, 149). Sodium L-lactate was added to give various concentrations La DM. The chosen concentrations are based on several reports from the literature. After vigorous exercise, blood plasma concentrations have been reported to rise to up to 25 mM. Intramuscular levels can rise by up to 40 mM (150–155). Usually, levels within the muscle are ~10 mM higher than systemic levels (156). For example, after 400 m sprinting, blood La concentrations are about 19 mM in sprinters, after a 3000 m run La peak was at 12 mM (157). Taken collectively, these data suggest that La concentrations of 10 and 20 mM are physiologically relevant and have therefore been previously applied in the literature (136, 158).

The La concentrations in the different DM were measured before each experiment and did not vary more than 5% from the nominal value. La DM was produced in sufficient amounts for the whole experiment to ensure constant incubation conditions.

La uptake and pH changes

Experiments investigating the La uptake and pH changes were performed using 0, 5, 10, and 20 mM La DM and medium samples were taken before (pre) and after 15, 30 min, 1, and 2 h for La sampling and before (pre) and after 2 h for pH measurement.

Dose-dependency experiments

For the examination of dose-dependent effects of La, cells were incubated intermittently for 2 h at the same time each day with 0, 10, and 20 mM La DM for up to 5 days and fixed or lysed after the incubation phase of the respective day. Intermittent incubation was chosen to create a more realistic training situation where La levels are only elevated for a certain amount of time compared to continuous treatment.

Involvement of oxidative stress

Cells stained for 8-epi-prostaglandin F2α (8-epi-PGF2α) were incubated with 0 or 20 mM La DM as well as 20 mM La DM additionally containing the antioxidants L-ascorbic acid (AA; 100 µm), N-Acetyl-L-cysteine (NAc; 5 mM), or linolenic acid (LA; 5 µM) and fixed immediately after the treatment. Cells for gene expression analysis were treated likewise, but after La incubation C2C12 were incubated with 0 mM La DM in all conditions for another 4 h before RNA was isolated. Furthermore, cells were treated with the same incubation media intermittently for 2 h each day at the same time for 5 days to investigate long-term effects and fixed or lysed immediately after the treatment on day 5.

Involvement p38 MAPK

Cells were treated with 0 mM or 20 mM La DM as well as 0 mM + DMSO and 0 mM + SB203580, a well-described p38α and p38β MAPK inhibitor (159), for 15, 30 min, 1, 4, and 24 h. For the latter two conditions, cells were pre-incubated with SB 203580 or DMSO for 1 h prior to the experiment to ensure inhibition-efficiency and control for the pre-treatment, respectively. SB203580 is insoluble in water and is therefore dissolved in DMSO. To exclude effects being induced by the solvent, 0 mM + DMSO was added as an extra control condition. After each time point cells were lysed. To study long-term effects of this treatment, cells were intermittently incubated with the above mentioned media for 2 h at the same time each day for 5 days. Cells were then fixed or lysed after the last treatment.

Involvement of histone modifications H3K27 me3 and H3K4me3

C2C12 were treated with 0 or 20 mM La DM and fixed before (pre) and after 15 min, 1, and 4 h of treatment. Additionally, long-term effects on the histone modifications were investigated by daily intermittent incubation for 2 h with 0 or 20 mM La DM at the same time each day. After the last treatment, cells were fixed.

All experiments were conducted in three independent runs except for gene expression analysis which was carried out twice.

2.2.1.6 La and pH measurement

La and pH in the incubation media were measured at the above mentioned time points. For La measurement, 20 µL incubation medium was drawn from the well using an end-to-end plastic capillary and mixed with 1 mL ready-to-use hemolyzing solution buffer. Samples were measured with a Biosen S-Line analyser in duplicates which uses an enzymatic-amperometric test principle. pH was analysed by taking the whole media off the cells and measuring samples with the pH-mV-meter in duplicates.

2.2.1.7 Bromodeoxyuridine(BrdU)-Assay

Proliferation was in one experiment analysed using the colorimetric immunoassay for the quantification of cell proliferation (BrdU-based). Procedures were conducted according to the manufacturer's instructions. Briefly, 1000 cells per well were seeded in a 96-well plate. After 2 days in PM, cells were treated for 2 h with 0, 20 mM La DM, 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, or 20 mM La DM + 5 µM LA. BrdU labelling time was 6 h.

2.2.1.8 Gene expression analysis

2.2.1.8.1 RNA isolation

After treatment cells were washed with DPBS before they were lysed directly in the culture dish with TriReagent® (1 mL per 10^6 cells). Using a cell scraper the cells were detached from the culture dish and transferred to a 1.5 mL reaction tube. The mixture was gently mixed by up-and-down pipetting and incubated for 5 min at RT to allow complete dissociation of nucleoprotein complexes. This step was followed by -80°C storage until isolation of RNA was carried out. For this, samples were thawed on ice and addition of 100 µL bromochloro phenol (BCP) per 1 mL TriReagent® used followed. The samples were vigorously vortexed for 15 s, incubated for 10 min at RT, and centrifuged (12,000 g; 10 min, 4°C). Following the centrifugation, the mixture was separated into three phases: a red phenol-chloroform phase (bottom layer) and interphase (middle layer) containing DNA and protein whereas the aqueous phase (upper layer) contained exclusively the RNA. The aqueous layer was transferred into a new 1.5 mL reaction tube and an equal volume of isopropanol was added to precipitate the RNA which forms a gel-like or white pellet after subsequent centrifugation (12.000 g, 8 min, 4°C). The supernatant was discarded and the pellet washed with pre-cooled 75% ethanol followed by thorough vortexing. The ethanol washing step repeated. After the last centrifugation, the supernatant was completely removed and the pellet air-dried.

If no remaining ethanol could be observed the pellet was dissolved in 20 µL of RNAse free water.

2.2.1.8.2 RNA concentration determination and quality assessment

Absorbance of RNA at 260 nm was measured using the NanoDropTM 1000 spectrophotometer to determine the concentration of the sample. At a wavelength of 260 nm absorption of 1 corresponds to 40 $\mu g \cdot mL^{-1}$ RNA or 50 $\mu g \cdot mL^{-1}$ cDNA. The quality (purity and integrity) was assessed by the additionally measuring the 260/280 nm ratio and 1% agarose gel electrophoresis and HD Green staining. The concentration of samples was adjusted to ~1 $\mu g \cdot \mu L^{-1}$ by adding an adequate amount of RNAse free water.

2.2.1.8.3 Complimentary DNA (cDNA) synthesis

The Qiagen QuantiTect® Reverse Transcription kit was used to eliminate genomic DNA and synthesize cDNA of samples. Procedures were carried out according to the provided instructions. For incubation steps the Flexcycler^2 was used.

2.2.1.8.4 Primer Design

The genomic sequence data available at the University of California at Santa Cruz Genome Bioinformatics site (UCSC Genome Browser) and the web tool Primer3 (160, 161) were used to design primers listed in Table 2.1. Criteria applied to design primers were: primer length (20-25 bp), melting temperature (T_{melt}; 58-60°C), amplicon length (150-250 bp). The National Center for Biotechnology Information's (NCBI's) BLAST, USCS' BLAT, and USCS's *in silico* PCR tool were used to check the specificity of the designed primers.

Table 2.1: Primers used in real-time RT-PCR

Name	Protein	NM		Sequence	Product size [bp]
Pax7		011039	forward	5'GCTACAGTGTGGACCCTGTG3'	229
			reverse	5'GAGACTCAGGGCTTGGGAAG3'	
Myf5		008656	forward	5'TGGTCCCGAAAGAACAGCAG3'	163
			reverse	5'AAGCTGTGTCCTGAAGAGCC3'	
MyoD		010866	forward	5'CGACACCGCCTACTACAGTG3'	214
			reverse	5'GGTCTGGGTTCCCTGTTCTG3'	
Myogenin		031189	forward	5'GTGAATGCAACTCCCACAGC3'	194
			reverse	5'GTTGGGCATGGTTTCGTCTG3'	
MHC1		30679	forward	5'CCAGGACCTTGTGGACAAAC3'	243
			reverse	5'TGGTCACTTTCCTGCACTTG3'	
MHC2		00139545	forward	5'AAGAGACAAGCTGAGGAGGC3'	249
			reverse	5'GAATCACACAGGCGCATGAC3'	
Cdkn1a	p21	001111099	forward	5'TCTGAGCGGCCTGAAGATTC3'	186
			reverse	5'GAAGATGGGGAAGAGGCCTC3'	
Trp53	p53	001127233	forward	5'GCCGGCTCTGAGTATACCAC3'	184
			reverse	5'CTTCTGTACGGCGGTCTCTC3'	
Cyclophilin A		203430	forward	5'GGATTCATGTGCCAGGGTGG3'	212
			reverse	5'CACATGCTTGCCATCCAGCC3'	

All primers were purchased from Invitrogen, Karslruhe, Germany

2.2.1.8.5 Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR was performed on the thermocycler Mx3005P. The cycling conditions used are shown in Table 2.2. Samples were run in triplicates.

Table 2.2: Cycling conditions for real-time RT-PCR

	Cycle length	T	Repititions
PCR initial activation step	15 min	95°C	
Denaturation	15 s	94°C	40
Annealing	30 s	58°C	
Extension	30 s	72°C	

Data was normalized to house keeping gene (cyclophilin A) and the x-fold increase was calculated according to (162), i.e. the following equations:

$$\Delta Ct = Mean\ Ct\ (gene\ of\ interest) - Mean\ Ct\ (cyclophilin\ A)$$

$$\Delta\Delta Ct = \Delta Ct\ (gene\ of\ interest) - \Delta Ct\ (pre\ condition\ of\ gene\ of\ interest)$$

$$x\text{-}fold\ increase = 2^{-\Delta\Delta Ct}$$

Random samples of RT-PCR products of each gene amplified were diluted 1:1 in TAE, run on a 1.5% agarose gel for 20 min at 120 V. A 100 bp DNA ladder was used to assess size of RT-PCR products. Comparison with product sizes predicted by the primer design programme followed. If a single band per gene was observed with the correct amplicon size, primer binding was considered specific and RT-PCR valid.

2.2.2 Human Muscle Biopsies

The human intervention study was co-ordinated by Dr. Sebastian Gehlert and conducted in close co-operation. Biopsies were taken by PD Dr. Dr. Thorsten Schiffer.

2.2.2.1 Subjects

15 healthy male sports students participated in the study. Prior to study participation, subjects were screened for health including clinical history, physical examination, laboratory blood tests, and an exercise electrocardiogram. The study purposely chose trained subjects that were involved in regular exercise and experienced in resistance training (up to twice a week). Participants were informed in oral and written form of the study's purpose and the possible risks involved before giving written informed consent. The study was approved by the Ethic Committee of the German Sport University Cologne in compliance with the Declaration of Helsinki. Subjects were randomly assigned to one of two different exercise regimens: high intensity resistance training (HIT; n = 8), or standard resistance training (STD; n = 7). For anthropometrical data of subjects see Table 2.3.

Table 2.3: Anthropological data of subjects

Exercise group	n	Age [years]	Height [cm]	Weight [kg]
STD	7	23 ± 3	181 ± 6	74 ± 6
HIT	8	23 ± 4	180 ± 7	78 ± 10

2.2.2.2 Experimental design and resistance training protocols

In the 14 days before the start of the study, subjects were asked to refrain from resistance exercise training as well as from any physical activity in the last 48 hours prior to the baseline biopsy, pre-testing and the experimental trial. On the evening (10 pm) before the lab visits all subjects were fed a standardised protein-energy drink (contents: 20 g protein, 24.8 g carbohydrate, 13.4 g fat, providing 1260 kJ) and fasted overnight. The following morning 1 h

prior the baseline biopsy and the experimental trial, subjects were asked to drink a second energy drink in order to ensure the exercise being carried out in the fed state. Subjects drank water ad libitum during all exercise interventions.

7 days before the actual experimental trial subjects reported to the laboratory to undergo pre-testing in order to determine maximum dynamic eccentric and concentric force output during single leg extensions. All subjects were asked to warm-up for 10 min on a Lode Excalibur cycling ergometer with 1 $W \cdot kg^{-1}$ body mass. The testing was performed on an ISOMED 2000 isokinetic machine with visual feedback. Subjects were seated on the isokinetic machine to adjust range of motion (ROM), knee-joint and lever arm axis. All participants performed a warm-up phase consisting of 3 sets of 4 concentric and eccentric repetitions with 90 s resting phase at a subjectively chosen sub-maximal intensity. After 3 min resting, subjects were subjected to a single set of 10 repetitions with concentric and eccentric knee extensions. After the first 5 sub-maximal repetitions, subjects were advised to carry out 3 maximal repetitions during the whole ROM in the concentric and eccentric phase. The last 2 repetitions were carried out passively by the isokinetic machines without actively generated force. Due to force-length and force-angle relationship, maximum force output during concentric and eccentric contractions followed a hyperbolic curve. The highest maximum force (F_{max}) achieved was applied for the experimental trial as the 100% reference curve. As the experimental trial differed in movement speed, pre-tests were carried out at the movement speed corresponding to the specific exercise mode in the experimental trial that each subject was assigned to.

On the day of the actual experimental trial, subjects reported to the laboratory at 7.45 am. Prior to the resistance training session, the same cycle ergometer warm-up as in the pre-testing was carried out. A combined warm-up and main exercise stimulus followed subsequently on the isokinetic leg extension machine. The programme was automatically operated by the control unit of the isokinetic device. ROM and angle velocity were standardized for each subject. The first phase consisted of 3 sets of 4 concentric and eccentric repetitions at 30% F_{max} with 90 s resting in between. The angular velocity was set to $65° \cdot s^{-1}$ movement speed in concentric and eccentric phases for each subject and exercise group. The warm-up was followed by 3 min resting before the actual exercise trial was conducted.

Resistance exercise stimuli:

Both exercise regimens were carried out as unilateral single leg extensions with 70° ROM.

STD: 3 sets of 10 unilateral concentric and eccentric leg extensions with 3 min rest between sets at 75% F_{max}; movement speed 65°·s^{-1} with 66 s time under tension.

HIT: 1 set of 20 unilateral concentric and eccentric leg extensions at 100% F_{max}; movement speed 40°·s^{-1} with 70 s time under tension.

All subjects of the HIT group were verbally encouraged during the exercise session to perform all contractions with maximum voluntary force. A visual feedback of their 100% reference was shown for further encouragement during each contraction.

2.2.2.3 Muscle biopsy

Muscle biopsies were taken from the musculus vastus lateralis of the exercised leg at 15, 30 min, 1, 4, and 24 h post exercise. Biopsies were taken 2 cm apart from each other, so that all 6 biopsies were collected within a range of 8-10 cm. Biopsies were obtained 2.5 cm below the fascia using the percutaneous needle biopsy method. Skeletal muscle biopsies were freed from blood and dissected from any visible fat and connective tissue. Tissue samples for Western blot analysis were immediately placed in cryotubes, frozen in liquid nitrogen and stored at -80°C until further analysis. Muscle samples for immunohistochemical staining were carefully aligned for cross-sectional analysis, embedded in Tissue-Tek®, frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

2.2.3 Protein analysis

2.2.3.1 Western Blot (WB) Analysis

2.2.3.1.1 Cell lysis

Cells were washed with ice-cold DPBS and then lysed with 1 mL lysis buffer containing phosphatase and proteinase inhibitors using a cell scratcher. Lysates were passed through a hypodermic needle (0.4 x 20 mm), vortexed, and kept on ice for 20 min, thereafter centrifuged (10,000 g, 10 min, 4°C), the supernatant frozen in liquid nitrogen and stored at -80°C until further analysis. The pellet contained the cell debris and was therefore disposed of.

2.2.3.1.2 Homogenisation of human muscle biopsies

20 mg of muscle tissue was homogenized in 500 µL of ice-cold ready-to-use cell lysis buffer using a commercially available micro-dismembrator. Homogenates were incubated on a rotor

at 4°C for 60 min. Centrifugation (10.000 g, 10 min, 4°C) followed and the pellet consisting of tissue debris was discarded. Homogenates were pooled for each group and stored at -80°C until further analysis.

2.2.3.1.3 Protein concentration determination and sample preparation

Total protein concentrations were determined using the RC DC™ Protein Assay Kit according to the manufacturer's instructions. 2x Laemmli buffer (163) was added to the samples in an equal volume, incubated for 5 min at 95°C in a thermoblock and briefly centrifuged. In this step, the proteins were denatured and reduced to ensure thorough separation of proteins during gel electrophoresis. Samples were stored at -20°C until used for Western blotting.

2.2.3.1.4 SDS-PAGE and Western Blot

Samples were then loaded in a pre-cast CriterionTM XT 4-12% Bis-Tris gel in MOPS running buffer and gel electrophoresis was run until proteins were thoroughly separated. Afterwards, proteins were transferred onto a polyvinylidene fluoride (PVDF) transfer membrane in a semi-dry blotting setup for 30-40 min, applying 1 A and 25 V. To avoid unspecific binding of antibodies, PVDF membranes were subsequently blocked with 5% BSA or 5% dry milk powder (MP) in TBST, before overnight incubation with the primary antibody diluted (Table 2.4) in 5% BSA or TBST at 4°C. The next day, membranes were thoroughly washed with TBST (4 intervals: 10 min, 5 min, 10 min, 5 min) and incubated with the respective HRP-conjugated secondary antibody (1:2000 in TBST) for 60 min at RT. Another washing step followed (see above). Signals were detected using enhanced chemoluminescence assay exposed on x-ray films. Time of exposure differed between the different antibodies used (2 s – 30 min). It was ensured that bands were properly visible, separated and not overexposed. WB films were analysed and (semi-)quantified using the ImageJ software. More specific, films were scanned in black and white film mode, inverted to give a negative picture and each band was analysed for its integrated density, i.e. the product of area and average grey value. Bands were normalised to actin or tubulin bands.

Table 2.4: Antibodies used for IF/ICC and WB

Name	Species	Dilution		Manufacturer
		IF/ICC	WB	
Pax7	mouse	1:250	1:500	Neuromics, Edina, NM, USA
Myf5	rabbit	1:250	1:500	GeneTex, Irvine, CA, USA
D7F2 (MyoD)	mouse	-	1:500	Developmental Studies Hybridoma Bank, Iowa City, IA, USA
F5D (Myogenin)	mouse	1:250	1:500	Developmental Studies Hybridoma Bank, Iowa City, IA, USA
Mf20 (MHC)	mouse	1:500	1:100	Developmental Studies Hybridoma Bank, Iowa City, IA, USA
MANEX	mouse	1:75	-	Developmental Studies Hybridoma Bank, Iowa City, IA, USA
Ki67	rabbit	1:250	-	Abcam, Cambridge, UK
act. Casp-3	rabbit	1:500	-	BD Pharmingen, Franklin Lakes, NJ, USA
8-epi-PGF2α	goat	1:1500	-	Oxford Biomedical Research, Oxford, MI, USA
p38	rabbit	-	1:200	Abcam, Cambridge, UK
p-p38 (Thr180 + Tyr182)	rabbit	-	1:1000	Epitomics, Burlingame, CA, USA
H3 total	rabbit	1:1000	1:1000	Cell Signaling Technology, Danvers, MA, USA
H3K4me3	rabbit	1:1000	1:1000	Cell Signaling Technology, Danvers, MA, USA
H3K27me3	rabbit	1:1000	-	Cell Signaling Technology, Danvers, MA, USA
Actin	mouse	-	1:4000	Millipore, Billerica, MA, USA
Tubulin	mouse	-	1:2000	GeneTex, Irvine, CA, USA
Alexa488 or Alexa555-conjugated 2° antibodies	goat-anti-rabbit or goat-anti-mouse	1:1000	-	Invitrogen, Karlsruhe, Germany
streptavidin-conjugated 2° antibodies	goat-anti-rabbit or rabbit-anti-goat	1:400	-	Dako, Glostrup, Denmark
HRP-conjugated 2° antibodies	goat-anti-rabbit or rabbit-anti-goat	-	1:2000	Thermo Fisher Scientific, Waltham, MA, USA

2.2.3.2 Immunofluorescent staining (IF)

2.2.3.2.1 Fixation and permeabilisation of cells

IF staining was carried out to detect Pax7, Myf5, F5D (myogenin), Mf20 (MHC), total H3, H3K4me3, and H3K27me3 proteins. Unless otherwise stated all steps were carried out at RT. Cells were fixed by 15 min incubation with 4% paraformaldehyde followed by 3 washing steps with PBS. To make cells permeable for reagents and antibodies, they were treated for 10 min with permeabilisation solution containing TritonX-100 (Tx-100) which disintegrates the cellular membrane due to its detergenic properties. 4 washing steps with PBS followed.

2.2.3.2.2 Fixation of human tissue sections

Frozen, Tissue-Tek® embedded muscle biopsies were cut into 7 µm thin sections with a cryostat and mounted onto Poly-L-lysine coated miscroscope slides, air dried and stored at -80°C until fixation by 8 min incubation with pre-cooled acetone at -80°C followed. The fixation solution was washed off thouroughly with TBS.

2.2.3.2.3 Immunological detection

Unspecific binding sites were blocked by 60 min incubation with 5% BSA in PBS for cells or 5% BSA in TBS for tissue sections. This step was followed by primary antibody incubation at 4°C overnight. All antibodies were diluted in 0.8% BSA in PBS or TBS for cells or tissue sections, respectively. For primary antibody dilutions see Table 2.4. For double staining (H3K4me3 or H3K27me3 and Mf20; H3K4me3 or H3K27me3 and MANEX, i.e. dystrophin) both antibodies were applied in the same step as they were generated in different species and no cross-reactivity was observed. IF controls were carried out with each IF to exclude autofluoroscence or any unspecific binding of the secondary antibody. These samples were incubated overnight at 4°C with 0.8% BSA in the respective buffer. The following day any unbound primary antibody was washed off with 4 buffer washing steps. The specific fluorochrome-conjugated secondary antibody (Table 2.4) recognizes any primary antibody from the respective host species and can therefore be used to label the target protein with a fluorescent signal. For this, cells or tissue sections were incubated for 45-60 min with the appropriate secondary antibody (cells: 1:1000 in PBS; tissue sections: 1:1000 in TBS) or a mixture of two in case of double staining as mentioned above. This was washed off again by 4 buffer washing intervals. The cells/tissue sections were then incubated for 5 min with DAPI solution. DAPI is a fluorescent stain that binds strongly to A-T rich regions of the DNA and therefore allows marking cell nuclei. Again, samples were washed 4 times with the respective buffer before being mounted onto microscopic slides (cells) or covered with glass slips (tissue sections) using Aqua-Poly/Mount.

2.2.3.2.4 Microscopy and analysis

Cover slips were photographed using a Zeiss Axiovert 200M, a MRm camera, and Axiovision software. In case of IF staining, the appropriate filter set for each applied fluorochrome (see 2.1.1) was used. For every condition 20 pictures of cells with a 20x objective were taken and total nuclei number counted using the nucleus counter tool from ImageJ software. Ki-67, Pax7, Myf5, F5D or Mf20 positive nuclei were counted manually. The ratio of Ki67/Pax7/Myf5/F5D/Mf20 positive cells to total number of nuclei in each photograph was calculated. Cells treated to investigate acute and long term effects regarding histone trimethylations were photographed with the exact same settings (exposure time, disabled automatic corrections, etc. to allow for quantification of fluorescent signals) using the 63x objective. 20 pictures for every condition were analysed as described below. Pictures of human tissue sections were taken with 10x and 63x objectives. For each subject, 10 pictures of cross-sectional areas were taken using the 10x objective and exactly identical settings (see above) followed by analysis using the ImageJ software as described below. To take higher quality pictures using the 63x objective, an ApoTome unit was used additionally.

2.2.3.2.5 Quantification of fluorescence

Cover slips were analysed using a Zeiss Axiovert 200M equipped with a MRm AxioCam and Axiovision 4.6 software. Staining intensity was then analysed using Metamorph software. After a series of corrections (e.g. for background), a common threshold was set for each target protein in all conditions. The signal was localized in the nucleus. Therefore, the nuclei of all cells in the photo were (semi-)quantified for staining intensity.

Human tissue sections were analysed using the “Analyze particles...” tool from the ImageJ software. Pictures with the H3K4me3 or H3K27me3 signal were converted to 8-bit, thresholded and inverted, the scale set to 1.6 pixel·μm^{-1} and analysed. The results contained the average area of the fluorescent signal within each nuclei corresponding to the staining intensity and hence, quantification of the signal. In the human study, pre levels were set to 1 and values of subsequent time points are expressed as area change compared to pre.

2.2.3.3 Immunocytochemistry (ICC)

2.2.3.3.1 Fixation, permeabilisation and inactivation of endogenous peroxidase of cells

ICC procedures for Ki67, activated Caspase-3 (act. Casp-3), and 8-epi-PGF2α were carried out at RT unless otherwise stated. Cells were fixed and permeabilised as described above (section 2.2.3.2.1). However, for cells undergoing DAB staining an incubation phase with 0.6% hydrogen peroxide (H_2O_2) in 80% methanol to eliminate endogenous peroxidase activity followed.

2.2.3.3.2 Immunological detection and DAB staining

In order to avoid unspecific binding cells were blocked with 5% BSA in PBS for 60 min. Primary antibodies (Table 2.4) were diluted in 0.8% BSA in PBS and cells incubated overnight at 4°C. ICC controls were carried out. These samples were treated with 0.8% BSA in PBS without any primary antibody overnight at 4°C. The next day, cells were washed 4 times with PBS and treated with streptavidin-conjugated secondary antibody (1:400 in PBS) for 60 min. After 4 further washing intervals, the samples were then incubated for 60 min with biotinylated horse radish peroxidase (1:150 in PBS). This solution was washed off by another 4 washing steps with PBS and DAB staining followed. For this, the DAB staining solution was freshly prepared, filtered and pipetted onto the cells. Staining was carried out under the microscope where samples were continuously compared to ICC control samples. As soon as staining was visible (1-15 min), the reaction was stopped by removing the DAB solution and washing the cells 4 times with PBS. Cells were then dehydrated by immersion of cover slips into a graded series of ethanol (70%, 96%, and 100%) and xylol baths and eventually mounted onto microscopic slides with Entellan®.

2.2.3.3.3 Microscopy and densitometry

DAB stained samples (act. Casp-3) were photographed using a Zeiss KS300 microscope equipped with a Sony CCD camera. Pictures at 20x magnifications were taken and at least 200 cells per condition were analysed for staining intensity, i.e. average grey value, using ImageJ software.

2.2.4 Statistical analysis

Data are presented as mean ± standard error of the mean. In case of follow-up statistical analysis, data was tested for the normality of distribution using the Shapiro-Wilk- (small sample size) or the Kolmogorov-Smirnov-test (large sample size). Where appropriate, repeated measure analysis of variance or a one-way analysis of variance was used to test whether the treatment had an effect. To test differences between the groups a post-hoc Bonferroni test was carried out. In case of a significant result for Shapiro-Wilk or Kolmogorov-Smirnov-test, the Kruskal-Wallis- and Mann-Whitney-U-test were used. WB results were not subjected to statistical analysis. Statistical significance was set at $p < 0.05$. Analysis was carried out using SPSS Statistics for Windows.

3 Results

3.1 Changes in La concentration and pH in incubation media

C2C12 cells were treated with 0, 5, 10, or 20 mM La DM for up to 2 h in order to ensure La uptake of the cells and control for any effects possibly induced by associated changes in pH (Figure 3.1). Treatment with 0 or 5 mM La DM resulted in a continuous increase in La concentration over the time course of the experiment. La levels in the 10 and 20 mM La DM decreased within the first hour of treatment indicating La oxidation. After that, incubation media showed increased La levels that rose higher than baseline concentrations (Figure 3.1 A). Given that this phenomenon occurred only after the first hour of incubation, and La levels *in vivo* were shown to return to basal level not until at least 30-45 min independent of active or passive recovery (164), this still represents a realistic situation. The pH only marginally differed between the conditions (Figure 3.1 B). The pH in all incubation media dropped after 2 h of treatment. The differences were within a range of -0.4 (20 mM La DM) to -0.6 (0 mM La DM). However the differences were not statistically significant and are not considered having an impact on the subsequently observed effects.

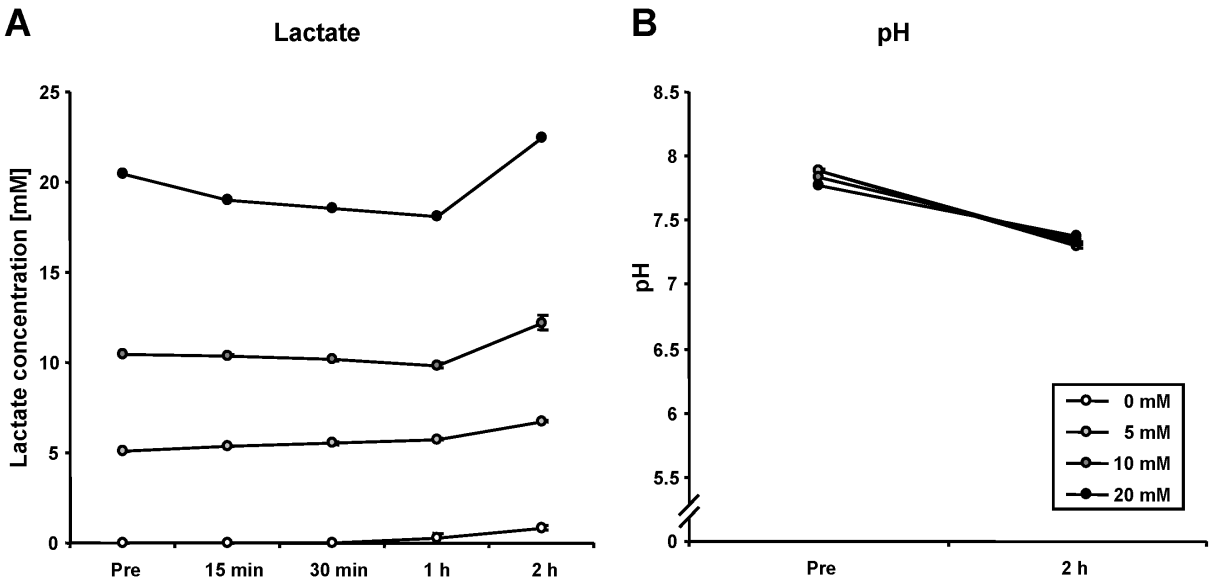

Figure 3.1: Changes in La concentration and pH in incubation media. C2C12 cells were incubated with 0, 5, 10, 20 mM La DM for up to 2 h. **A** La concentration changed over time in the incubation media. In 0 and 5 mM La DM La levels rose continuously over the time period of the experiment. 10 and 20 mM La DM La levels first decreased within the first hour and then rose above baseline values. **B** pH dropped slightly under all conditions. La – lactate; DM – differentiation medium.

3.2 La reduced proliferation

To investigate effects of La on cell proliferation, C2C12 cells were treated with 0, 10, and 20 mM La DM and the ratios of Ki67-positive cells were analysed (Figure 3.2). Ki67 is expressed by cells which are in the G1/S/G2/mitotic phase of the cell cycle, but not in resting (G0) cells (165). Hence, it is a reliable marker of proliferating cells. After the second La

treatment, proliferation ratios in the treated samples decreased independently of the dose about 10-fold (10 mM La DM: 4.5 ± 1.7%; 20 mM La DM: 3 ± 1.5%) compared to control samples (0mM La DM: 48 ± 5.7%; both $p < 0.001$). The following days, proliferation went down to approximately 5% in all conditions and remained around that level until the end of the experiment (Figure 3.2 B).

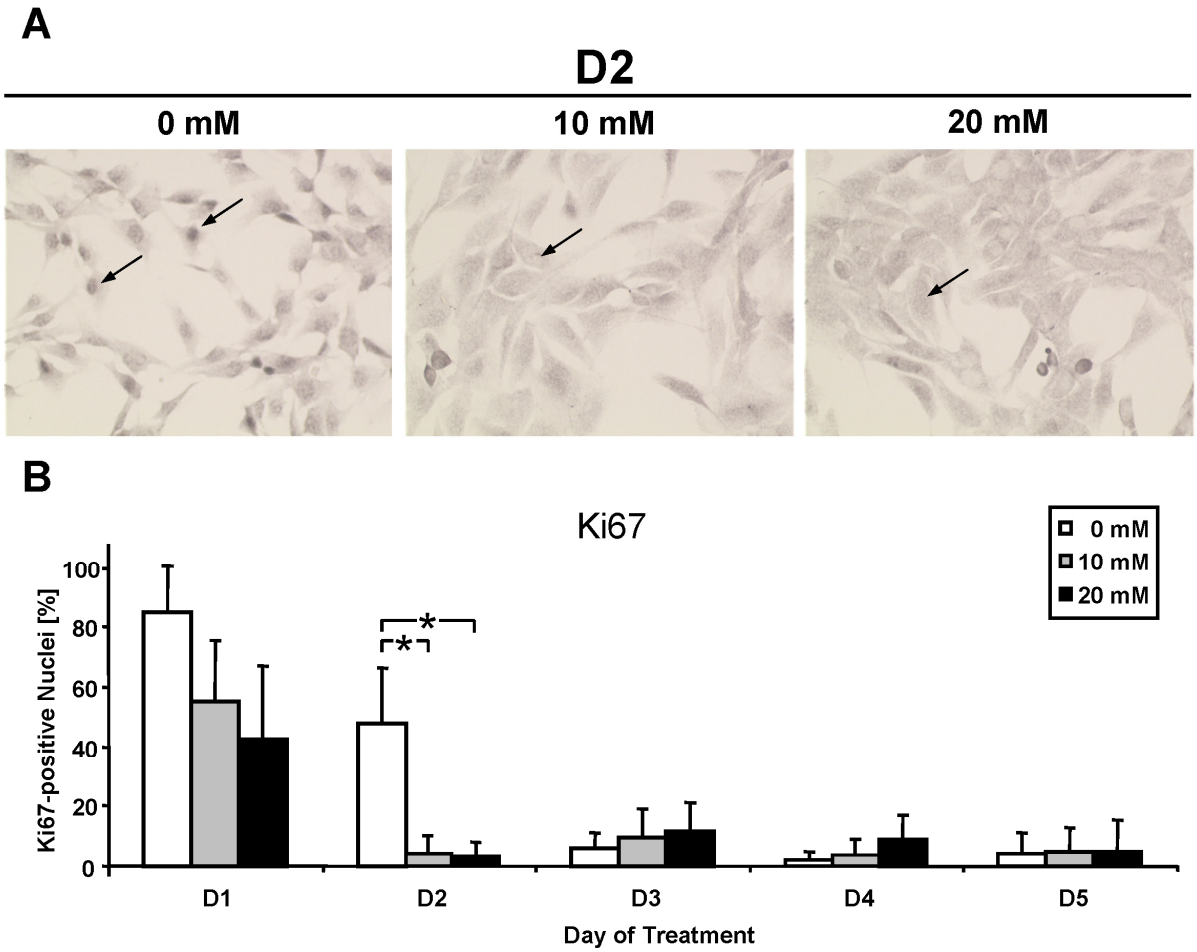

Figure 3.2: La suppressed proliferation of C2C12 cells. C2C12 cells were intermittently incubated with DM containing different La concentrations (0, 10, 20 mM) for 2 h each day for up to 5 days. The ratio of Ki67-positive nuclei was determined by ICC followed by microscopic imaging and manual cell counting. **A** Representative pictures of cells on D2. Arrows indicate positive/negative nuclei. **B** Ratio of Ki67-positive cells. After the second La treatment, proliferation ratios in the La treated samples decreased independently of the dose about 10-fold compared to control samples. The following days, proliferation went down to approximately 5% in all conditions and remained around that level. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; DM – differentiation medium; ICC – immunocytochemistry.

3.3 La increased activation of Caspase-3

Procaspase-3 is cleaved to the executioner act. Casp-3 (reviewed in (166)). It allows identifying cells which are undergoing an apoptotic process (programmed cell death) and can thus be considered a marker of cellular stress. Densitometric analysis of cells stained for act. Casp-3 (Figure 3.3) showed that after the first La incubation, cells treated with 20 mM La DM showed a significant increase in act. Casp-3 compared to control and 10 mM La DM cells (0

vs. 20 mM La DM: p = 0.004; 10 vs. 20 mM La DM: p < 0.001). On day 2, overall activation went down in all conditions. Still, apoptotic induction was significantly higher in both La conditions compared to control DM treated cells (0 vs. 10 mM La DM: p = 0.007; 0 vs. 20 mM La DM: p < 0.001). On day 3, apoptotic induction significantly decreased further in the 0 and 10 mM La DM cells compared to 20 mM La DM treated cells (0 vs. 20 mM La DM: p = 0.003; 10 vs. 20 mM La DM: p = 0.011). As from day 4, cells grew too dense and pronounced myotube formation made it impossible to determine act. Casp-3 levels from that time point on using this method. However, the conclusive result from this experiment is that La induces increased activation of Casp-3 in a dose dependent-manner.

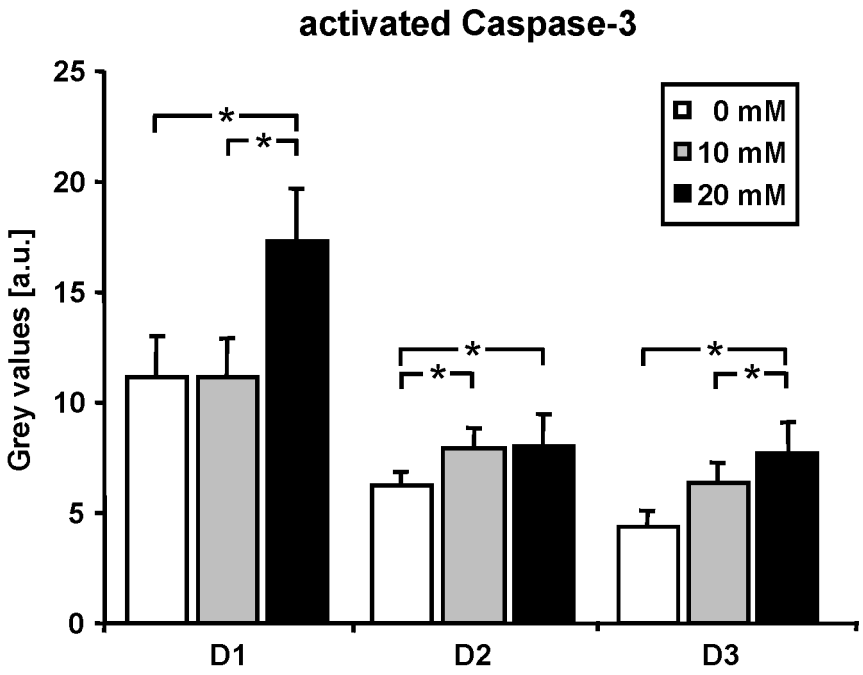

Figure 3.3: La induced cellular stress in C2C12 cells. C2C12 cells were intermittently incubated with DM containing different La concentrations (0, 10, 20 mM) for 2 h each day for up to 3 days. Act. Casp-3 was densitometrically quantified as a protein marker for the induction of apoptotic signalling, i.e. cellular stress. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. p < 0.05. La – lactate; DM – differentiation medium; act. Casp-3 – activated Caspase-3.

3.4 La timely delayed late differentiation in a dose-dependent manner

C2C12 cells were intermittently incubated with DM containing different physiologically relevant La concentrations (10, 20 mM) over a time period of 5 days simulating a microcycle of high intensity endurance training. A screen of samples using WB analysis implied a dose-dependent La effect on several differentiation markers (Figure 3.4). The higher the La concentration in the DM the higher the Pax7 content in the respective samples. No obvious differences could be detected for Myf5 and MyoD. Conversely, markers of late differentiation, i.e. myogenin and MHC, are expressed less the higher the La content in the DM (Figure 3.4 B).

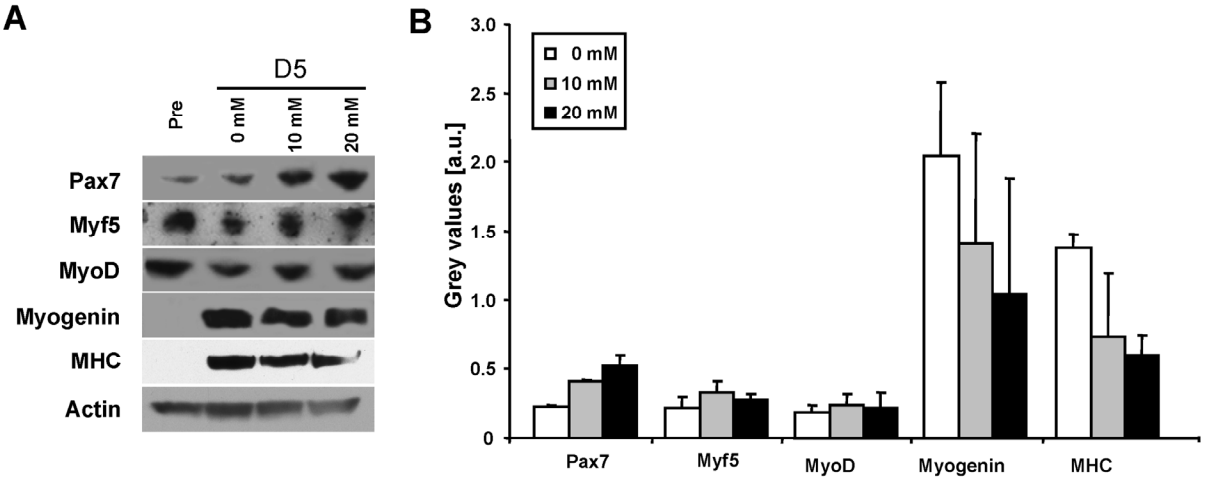

Figure 3.4: La delayed the differentiation process of C2C12 cells in a dose-dependent manner. C2C12 cells were intermittently incubated with DM containing different La concentrations (0, 10, 20 mM) for 2 h each day for 5 days. Western Blot analysis showed that markers of activation and early differentiation (Pax7) are more abundant in La-treated samples, whereas late differentiation markers (myogenin, MHC) are found to be less abundant. Myf5 and MyoD appeared unchanged. **A** Representative blots for Pax7, Myf5, MyoD, Myogenin, and MHC. Actin was used as a loading control. **B** Quantitative analysis of WBs. Data are presented as mean ± S.E.M. La – lactate; DM – differentiation medium; Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; MyoD – myogenic determination protein; MHC – myosin heavy chain; WB – Western Blot.

Myogenin is a marker for the onset of the end-terminal differentiation of myoblasts. The ratio of myogenin-positive nuclei in differentiating C2C12 cells was determined using ICC (Figure 3.5 A + B and appendix Table A.1). Number of myogenin-positive nuclei rose quickest in the 0 mM La DM cells, peaking on day 4 of the experiment. The same level was reached when cells were treated with 10 mM La DM, but not until day 5. Myoblasts treated with 20 mM La did not reach that level at all during the 5 days of the experiment. WB results generally support the findings from the ICC (Figure 3.5 C).

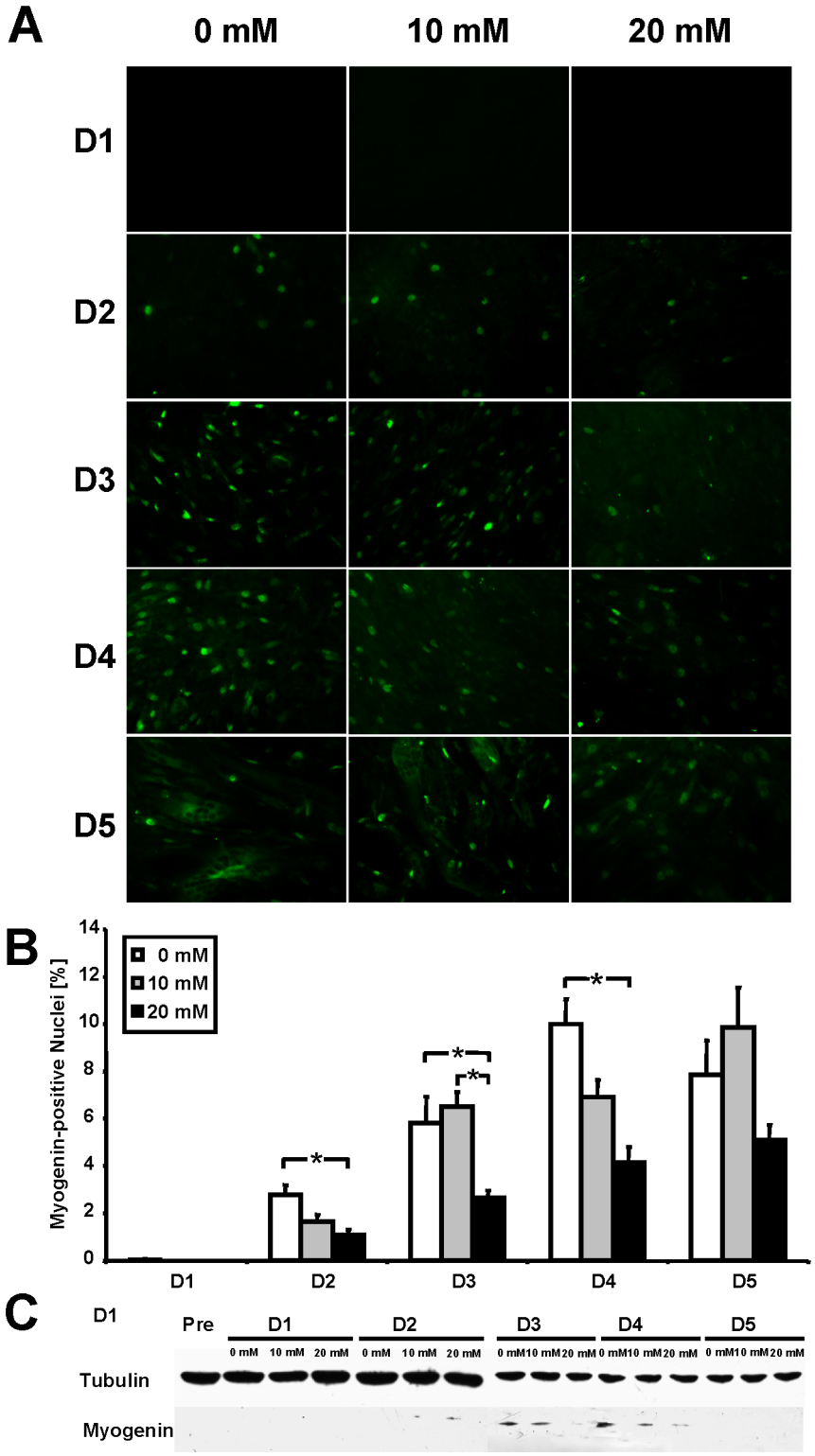

Figure 3.5: La delayed myogenin synthesis in C2C12 cells. C2C12 cells were intermittently incubated with DM containing different La concentrations (0, 10, 20 mM) for 2 h each day for up to 5 days. IF and WB analysis showed that myogenin occured earlier in control than in La-treated samples. Additionally, 10 mM La DM samples showed earlier myogenin occurrence than 20 mM La DM treated cells. **A** Representative photographs of myogenin (green) staining on the respective days. **B** Ratio of myogenin-positive cells on the respective days. **C** Representative WB for myogenin and tubulin. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; DM – differentiation medium; IF – immunofluorescent staining; WB – Western blot.

MHC is part of the sarcomeric structure of skeletal muscle and occurs with the end-terminal differentiation of myoblasts into functional myotubes (167). Here, a pronounced concentration-dependent effect was observed (Figure 3.6 A + B and appendix Table A.2). MHC was detected on D2 in the control and 10 mM La DM incubated cells, but not in the 20 mM La DM treated samples. Furthermore, 10 mM La DM resulted in significantly less nuclei lying within MHC-positive myotubes compared to the 0 mM La DM condition. The same pattern was found on D3. As from D4 on, no differences between the 0 and 10 mM La DM treated samples could be observed. 20 mM La DM samples showed significantly less nuclei within myotubes compared to 0 and 10 mM La DM on all days of the experiment. Again, results from the protein analysis via WB generally underline these results (Figure 3.6 C). After 10 days of incubation with 20 mM La DM cells were differentiated to the same extent as 0 and 10 mM La DM cells on D5 (data not shown as 0 and 10 mM La DM treated cells started to dedifferentiate and detach). Summarizing these data, La treated-samples exhibited timely delayed late differentiation in a dose-dependent manner.

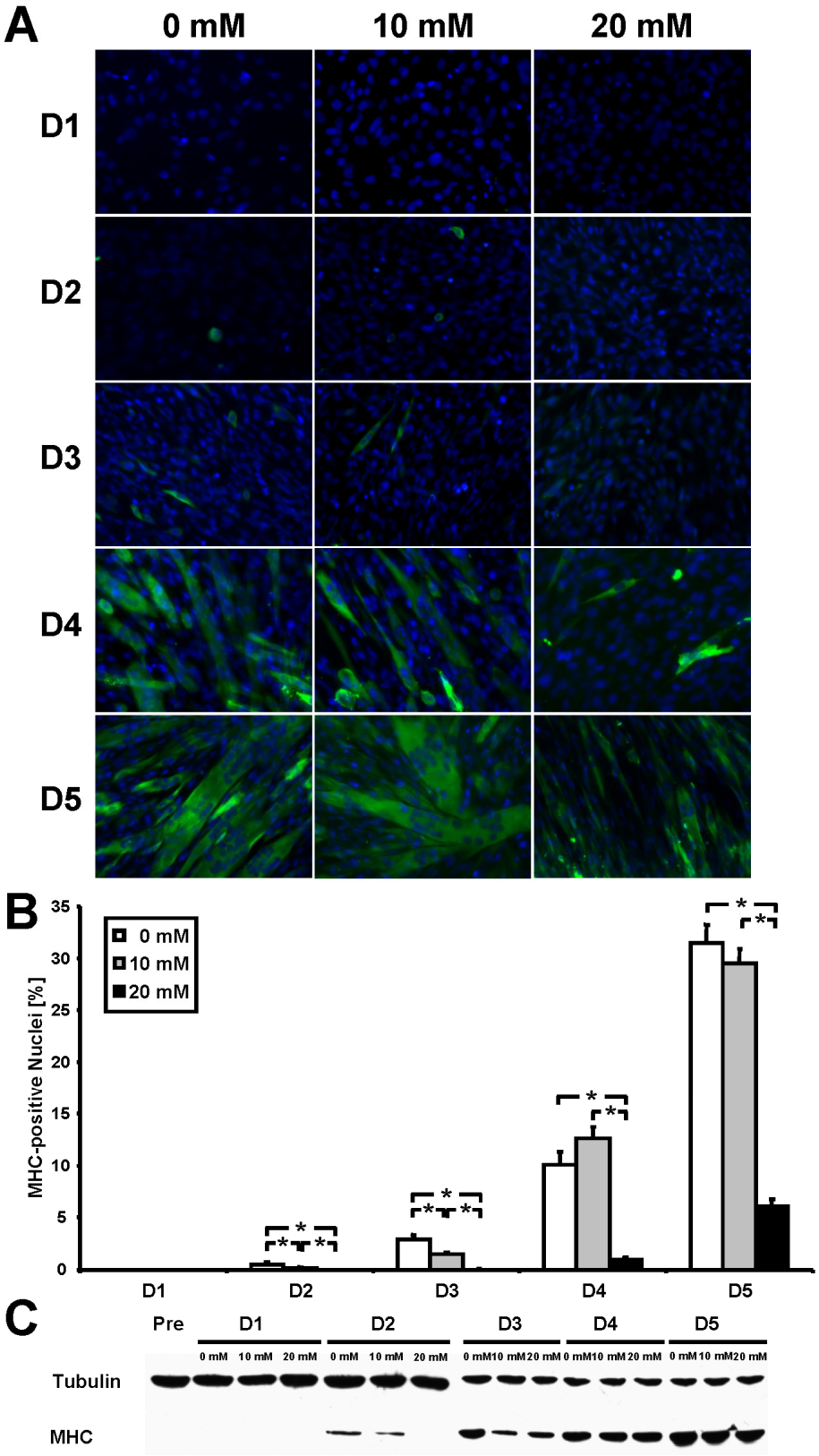

Figure 3.6: La delayed MHC synthesis in C2C12 cells in a dose-dependent manner. C2C12 cells were intermittently incubated with DM containing different La concentrations (0, 10, 20 mM) for 2 h each day for up to 5 days. IF and WB analysis showed that MHC occurs earlier in control than in La-treated samples. A dose-dependent effect could also be observed. **A** Representative photographs of MHC (green) and DAPI (blue) staining on the respective days. **B** Ratio of MHC-positive myonuclei on the respective days. **C** Representative WB for MHC and tubulin. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; DM – differentiation medium; IF – immunofluorescent staining; WB – Western blot; MHC – myosin heavy chain.

3.5 Lactate increased oxidative stress

A previous study suggested that the La effects are at least partly transduced within the cell by the shift of the redox potential, increased ROS production, and subsequent activation of MAPKs (136). To further elucidate this assumption cells were treated with 0, 20 mM La DM, and 20 mM La DM additionally containing different antioxidants and stained for 8-epi-PGF2α. This molecule is formed by a free radical-mediated, non-enzymatic peroxidation of arachidonic acid in membrane phospholipids and therefore a reliable marker to assess oxidative stress (168). 8-epi-PGF2α was formed under all experimental conditions (Figure 3.7). However, 20 mM La DM showed by far the highest staining intensity whereas the addition of antioxidant agents led to control (LA) or lower (AA, NAc) levels of 8-epi-PGF2α.

If the La effects are induced by the generation of ROS, the decrease in proliferation and the delaying effect on the late differentiation of myoblasts should be at least reduced, if not annihilated by the addition of antioxidants to the La DM. To test this hypothesis, BrdU incorporation, gene expression and protein synthesis were examined.

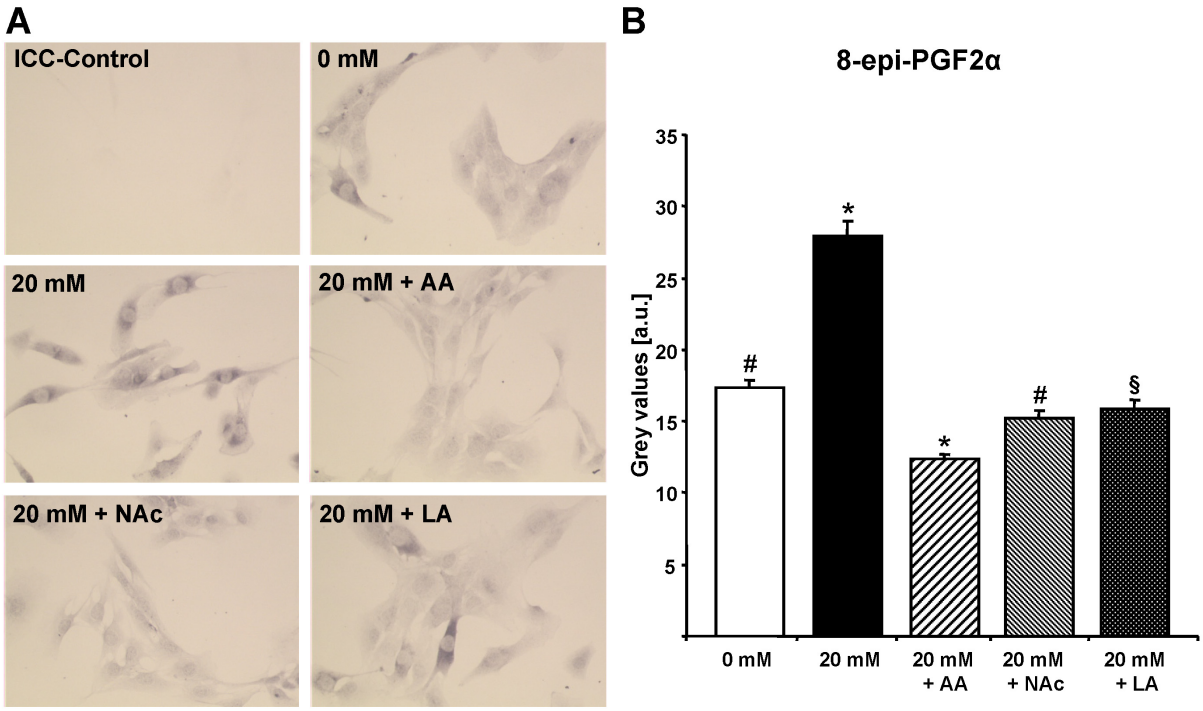

Figure 3.7: La increased oxidative stress. C2C12 cells were treated with 0, 20 mM La DM, 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, or 20 mM La + 5 µM LA for 2 h. Immediate fixation and follow up ICC staining of treated cells for 8-epi-PGF2α as a marker of oxidative stress revealed that cells treated with 20 mM La DM were exposed to high levels of oxidative stress. This effect was negated when antioxidants were added to the 20 mM La DM. **A** Representative photographs of stained cells. **B** Staining intensity of 8-epi-PGF2α-stained cells. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$, compared to all other conditions. # indicates statistical significance compared to all other conditions except 20 mM La + 5 µM LA. § indicates statistical significance compared to 20 mM La DM and 20 mM La DM + 100 µM AA. La – lactate; DM – differentiation medium; AA – ascorbic acid; NAc – N-Acetyl-L-cysteine; LA – linolenic acid; ICC – immunocytochemistry; 8-epi-PGF2α – 8-epi-prostaglandine F2α.

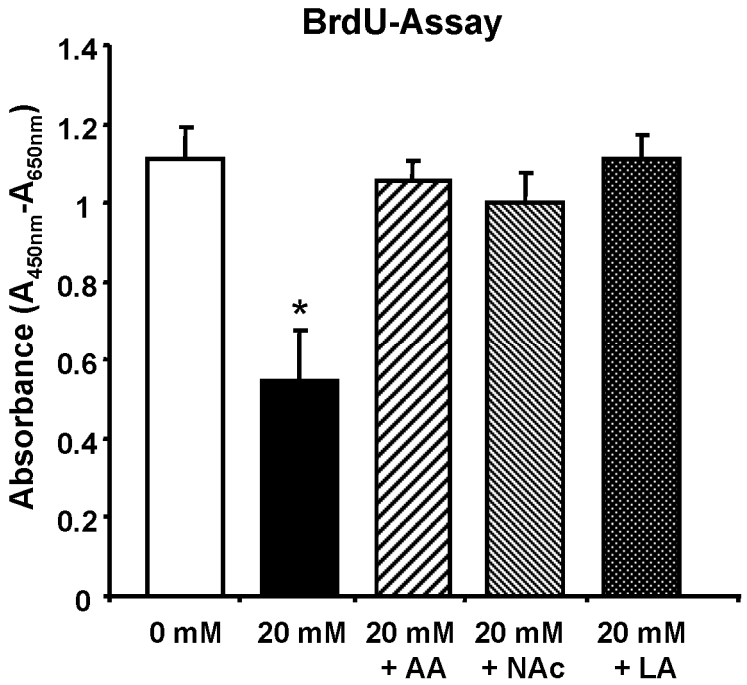

Figure 3.8: La-induced cell cycle withdrawal was reversible by different antioxidants. C2C12 cells were treated with 0, 20 mM La DM, 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, or 20 mM La + 5 µM LA for 2 h. A BrdU assay was performed and results showed a drop in BrdU-positive cells which was reversed by the addition of different antioxidants to the 20 mM La DM. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; DM – differentiation medium; AA – ascorbic acid; NAc – N-Acetyl-L-cysteine; LA – linolenic acid; BrdU – bromodeoxyuridine.

A BrdU assay was performed to investigate the reversibility of the La-induced decrease in proliferation by different antioxidants. Results clearly show that a single 20 mM La DM administration significantly reduces proliferation rate of myoblasts (Figure 3.8) compared to all other conditions (0 vs. 20 mM La DM: $p = 0.02$; 20 mM La DM vs. 20 mM La DM + AA: $p = 0.018$; 20 mM La DM vs. 20 mM La DM + NAc: $p = 0.031$; 20 mM La DM vs. 20 mM La DM+ LA: $p = 0.015$) confirming the results from Ki67 analysis and demonstrating that AA, NAc, and LA were indeed able to reverse the La effect on proliferation behaviour. These results were furthermore supported by the gene expression analysis of cell cycle control genes transformation related protein 53 (Trp53; Figure 3.9 A and appendix table A.3) and the cyclin-dependent kinase inhibitor 1a (Cdkn1a; Figure 3.9 B and appendix table A.3). Trp53 encodes the p53 protein, which is generally a regulator of cell development and differentiation. Specifically in myoblasts, p53 increases retinoblastoma (pRb) gene expression. pRb can co-operate with MyoD to transcribe later markers of differentiation (169) but it does not affect cell cycle withdrawal (170, 171). The Cdkn1a gene encodes the p21 protein. p21 is an important regulator of cell cycle withdrawal during myogenesis. It leads to pRb hypophosphorylation which is associated with cell quiescence or differentiation compared to the hyperphophorylated state which is more abundant in proliferating cells (172, 173). Trp53 expression decreased in all conditions compared to pre. However, La treatment

did not reveal an effect on its expression, whereas AA and LA induced elevated expression. In contrast, Cdkn1a expression was significantly increased by 20 mM La DM compared to pre and 0 mM La DM. This effect was however not reversible by the addition of antioxidants.

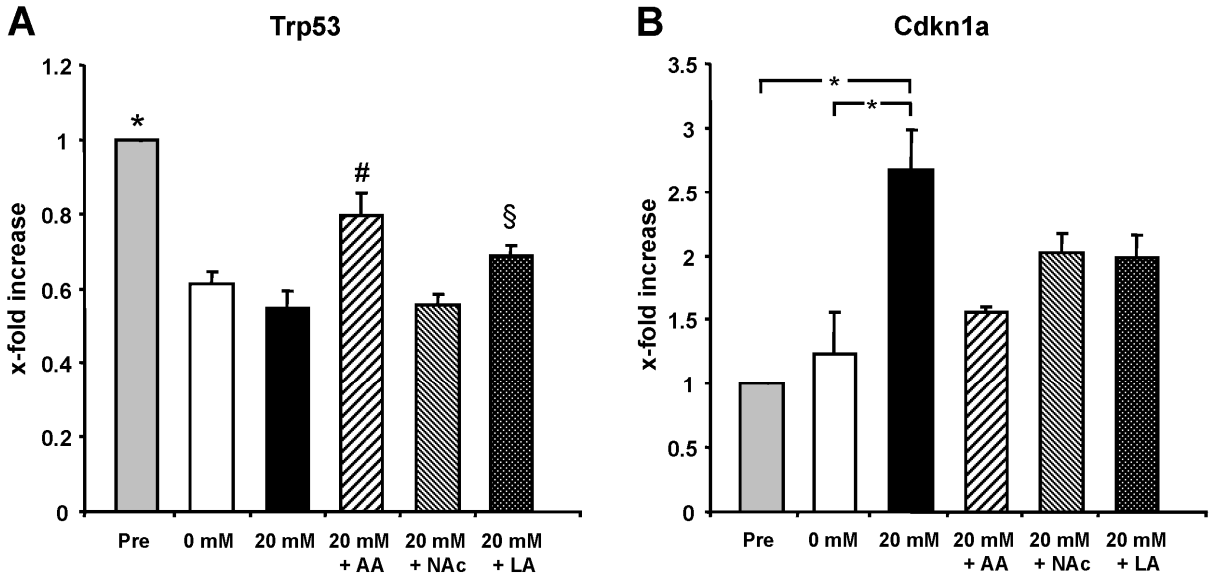

Figure 3.9: La altered gene expression of cell cycle control genes Cdkn1a and Trp53. C2C12 cells were treated with 0 mM, 20 mM La DM, 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, or 20 mM La + 5 µM LA for 2 h. After further 4 h incubation with 0 mM La DM RNA was isolated. Results from quantitative real-time RT-PCR showed **A** decreased Trp53 expression in all samples independent of treatment compared to the pre situation, whereas **B** Cdkn1a expression increased in the La-treated samples. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; Cdkn1a – cyclin-dependent kinase inhibitor 1a; Trp53 – transformation related protein 53; DM – differentiation medium; AA – ascorbic acid; NAc – N-Acetyl-L-cysteine; LA – linolenic acid; RNA – ribonucleic acid; RT-PCR – reverse transcription polymerase chain reaction.

Gene expression of Pax7, Myf5, myogenin and MHC following a single La incubation bout were also examined (Figure 3.10 and appendix Table A.4). Expression levels before (pre) start of the experiment were considered baseline levels and were used as the control in the calculation of $\Delta\Delta$Ct. Pax7 expression (Figure 3.10 A) was significantly down-regulated by La treatment compared to all other conditions. AA increased Pax7 mRNA levels significantly compared to pre, 0, and 20 mM La DM. Myf5 expression (Figure 3.10 B) was also significantly decreased by 20 mM La DM, but only compared to 20 mM La + antioxidative treatment. The increase of myogenin expression (Figure 3.10 C) in 0 mM La DM cells was attenuated by the addition of 20 mM La DM. Further addition of antioxidant substances restored myogenin expression to 0 mM La DM levels. MHC1 expression appeared to be lower under La conditions (Figure 3.10 D), differences were nevertheless only significant for 0, 20 mM La DM + NAc, and 20 mM La DM + LA. The same observation was made for MHC2 (Figure 3.10 E). Although mRNA levels seemed to increase after DM treatment, differences were only significant for 20 mM La DM + AA if compared to pre and 20 mM La DM. Taken collectively, the addition of La to the DM significantly decreases mRNA levels of muscle-specific genes.

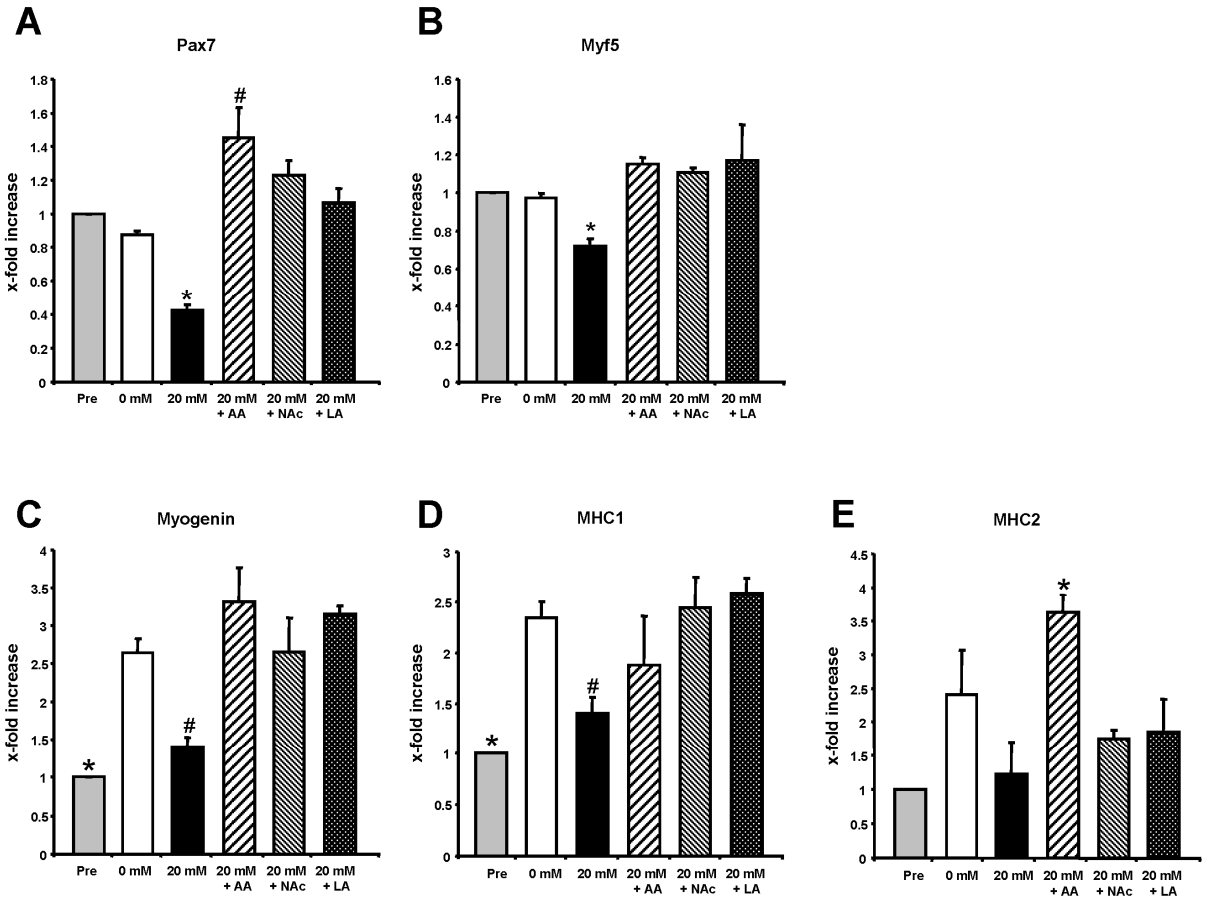

Figure 3.10: La influenced gene expression of Pax7, Myf5, myogenin, MHC1, and MHC2. C2C12 cells were treated with 0, 20 mM La DM, 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, or 20 mM La + 5 µM LA for 2 h. After another 4 h in 0 mM La DM RNA was isolated. Results from quantitative real-time RT-PCR showed altered gene expression of **A** Pax7 * indicates statistical significance, i.e. $p < 0.05$ compared to all other conditions. # indicates statistical significance, i.e. $p < 0.05$, compared to all other conditions except 20 mM La DM + 5 mM NAc. **B** Myf5 * indicates statistical significance, i.e. $p < 0.05$, compared to 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, and 20 mM La + 5 µM LA. **C** Myogenin * indicates statistical significance, i.e. $p < 0.05$, compared to all other conditions except 20 mM La DM. # indicates statistical significance, i.e. $p < 0.05$, compared to 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, and 20 mM La + 5 µM LA. **D** MHC1 * indicates statistical significance, i.e. $p < 0.05$, compared to all other conditions except 20 mM La DM. # indicates statistical significance, i.e. $p < 0.05$, compared to 20 mM La DM + NAc and 20 mM La DM + LA. **E** MHC2 * indicates statistical significance, i.e. $p < 0.05$, compared to pre and 20 mM La DM. La – lactate; Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; MHC – myosin heavy chain; DM – differentiation medium; AA – ascorbic acid; NAc – N-acetylcysteine; LA – linolenic acid; RNA – ribonucleic acid; RT-PCR – reverse transcription polymerase chain reaction.

Furthermore, results from the experiments investigating the differentiation effects on protein level (Figure 3.11 and appendix table 5) show that C2C12 cells treated with 20 mM La DM elicited a significantly higher ratio of Pax7-positive nuclei than control treatment (Figure 3.11 C). This effect was annihilated by the addition of AA to the 20 mM La DM. However, the lower ratio of Pax7-positive cells was not significant for 20 mM La DM + NAc and 20 mM La DM + LA. Overall, these findings were confirmed using WB analysis (Figure 3.11 D). Less clear are the results of Myf5 investigation. Overall, a tendency for a La effect can be seen in ICC and WB (Figure 3.11 E + F), but the differences were only significant between 20 mM La DM and 20 mM La DM + AA treatment in the ICC. The most distinct results were attained

from the myogenin and MHC staining. Here, the La effect was clearly observed in the 20 mM La DM incubated cells where compared to control conditions the number of myogenin- and MHC-positive cells was significantly reduced. This effect was completely reversed by all of the applied antioxidants in both the ICC (Figure 3.11 G + I) and the WB (Figure 3.11 H + J).

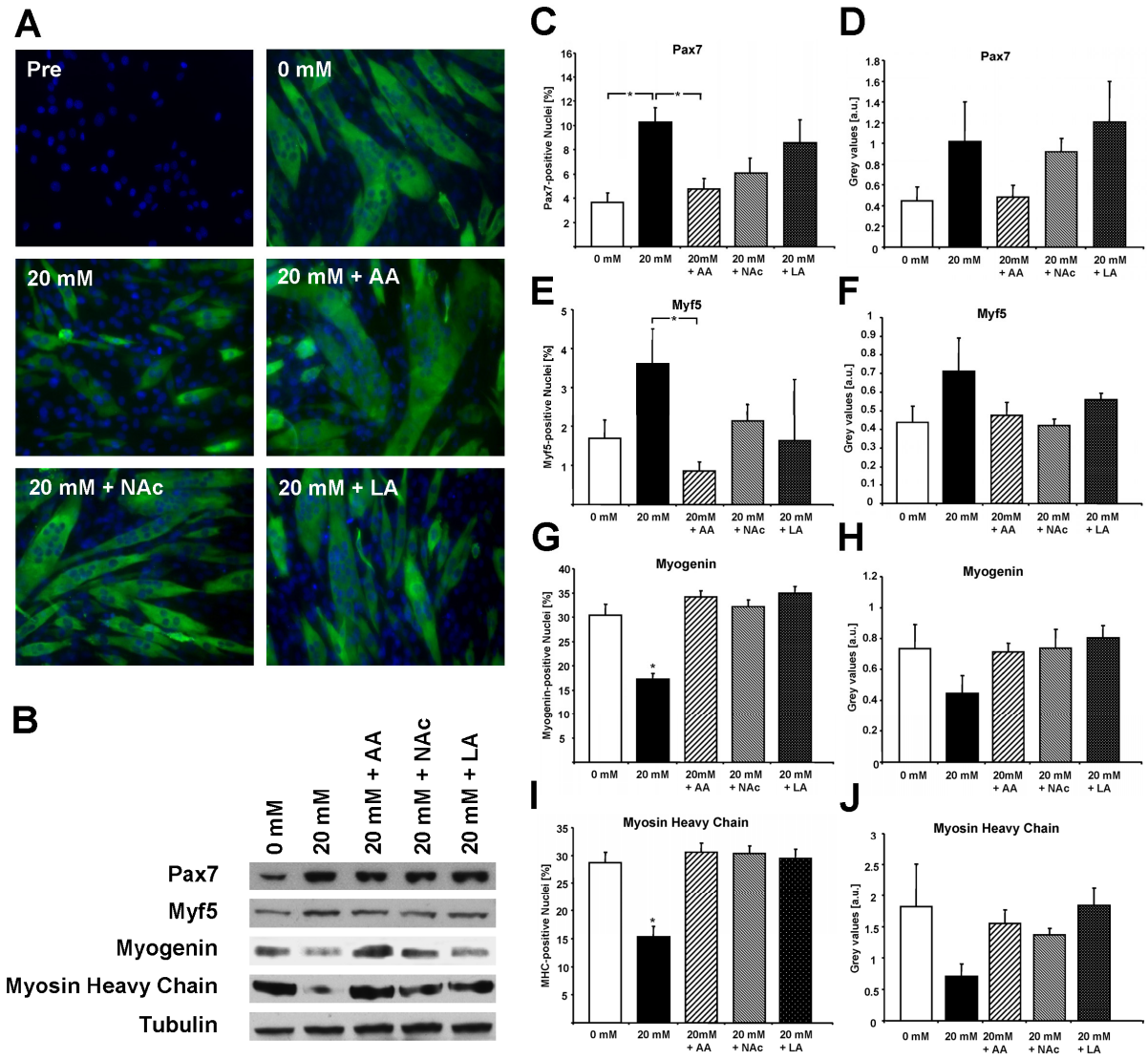

Figure 3.11: La-induced delayed differentiation in C2C12 cells was reversible by the use of antioxidants. C2C12 cells were treated with 0 or 20 mM La DM or 20 mM La DM + 100 µM AA, 20 mM La DM + 5 mM NAc, 20 mM La + 5 µM LA for 5 days followed by IF (ratios of marker-positive nuclei) or WB analysis. Briefly, 20 mM La DM treated samples contained more Pax7 and Myf5 (early differentiation markers) and less myogenin and MHC (late differentiation markers). These effects were mostly reversible by the addition of different antioxidants to the cell culture medium. **A** MHC- (green) and DAPI-staining (blue) in C2C12 myoblasts before (pre) and after 5 days of incubation with the respective DM. **B** Representative WBs for Pax7, Myf5, myogenin, and MHC. Tubulin was used as a loading control. **C-J**: Ratios of positive nuclei as analysed from IF (**C, E, G, I**) and quantification of WBs (**D, F, H, J**) for Pax7 (**C, D**), Myf5 (**E, F**), myogenin (**G, H**), and MHC (**I, J**). Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$, compared to the indicated experimental group (bars). If no bars are shown then significance is given to all other experimental groups. La – lactate; DM – differentiation medium; IF – immunofluorescent staining; WB – Western blot, Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; MHC – myosin heavy chain; AA - ascorbic acid, NAc - N-Acetyl-L-cysteine, LA - linolenic acid.

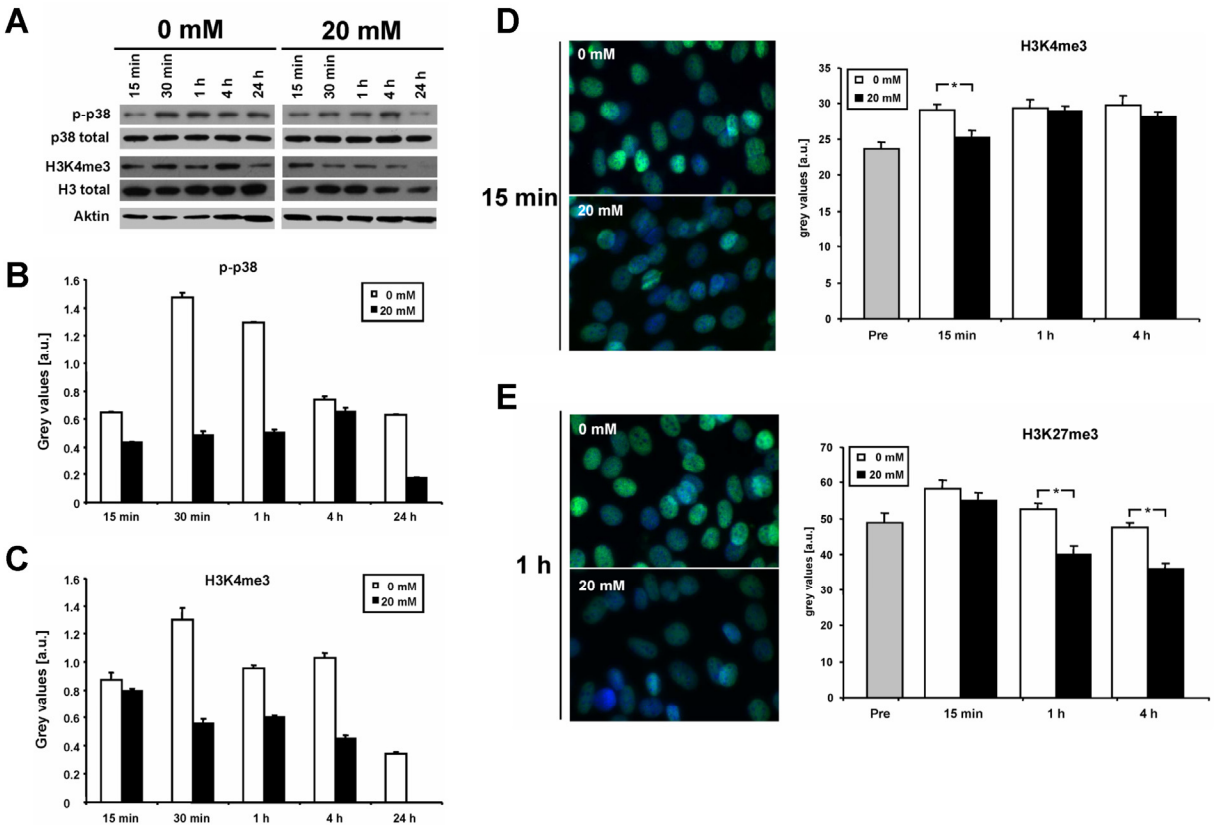

Figure 3.12: La has acute effects on p38 MAPK phosphorylation and H3K4 and H3K27 trimethylation. C2C12 cells were incubated with 0 and 20 mM La DM for up to 24 h. Cells were lysed and analysed for p-p38 and H3K4me3 using WB techniques or fixed and stained for H3K4me3 or H3K27me3 (green) using IF. Nuclei were counterstained with DAPI (blue). Fluorescence was quantified using Metamorph Software. **A** Representative WBs for p-p38, total p38, H3K4me3, total H3, and actin as a loading control. **B** Quantification of WB results showed decreased p-p38 levels with La-treatment. **C** Quantification of WBs results revealed diminished HK4me3 with la-incubation. **D** Representative pictures and staining intensity of H3K4me3. H3K4me3 staining intensity was significantly reduced by La-incubation after 15 min compared to control cells. **E** Representative pictures and staining intensity of H3K27me3. H3K27me3 staining intensity was significantly reduced at 1 and 4 h of La-incubation compared to control cells. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; DM – differentiation medium; H3Kxme3 – trimethylated histone 3 at lysine x; IF – immunofluorescent staining; WB – Western blot; p-p38 – phosphorylated p38 mitogen-activated protein kinase.

3.6 Involvement of p38 MAPK and histone modifications in vitro

Western Blot analysis showed a pronounced phosphorylation of p38 MAPK at Threonin 180 and Tyrosin 182 (p-p38) after switching from PM to 0 mM La DM. Interestingly, this effect was abolished by the addition of 20 mM La to the DM (Figure 3.12 A + B). Therefore, an experiment was designed to elucidate the question whether the La effect is p38-dependent and effects are the same if p38 is inhibited by SB203580 added to 0 mM La DM. For this, cells were incubated intermittently for 5 days with 0 mM, 0 mM + DMSO, 0 mM + 10 µM SB203580, and 20 mM La DM (Figure 3.13 and appendix Table 6). Results revealed no effect of DMSO addition to the DM compared to 0 mM La DM. In contrast, IF analysis showed that addition of SB203580 or 20 mM La lead to increased number of Pax7 positive cells (Figure 3.13 C). This result was confirmed using WB (Figure 3.13 B + D) which showed increased Pax7 content in cell lysates of 0 mM La DM + SB203580 and 20 mM La DM cells.

The same observations were made for Myf5 protein content. IF showed a pronounced increase of Myf5 positive cell number and WB analysis of cell lysates also showed increased Myf5 content when cells were treated with SB203580 or 20 mM La DM (Figure 3.13 E + F). For later differentiation proteins myogenin and MHC higher contents of protein were found in 0 mM La DM and 0 mM La DM + DMSO by IF and WB (Figure 3.13 A, B, G – J). Taken collectively, La treatment led to an increase in early differentiation proteins and decrease of late differentiation markers. DMSO had no effect, whereas SB203580 exhibited the same results as obtained from 20 mM La DM incubation. These results imply that La inhibits p38 and its pro-myogenic effect.

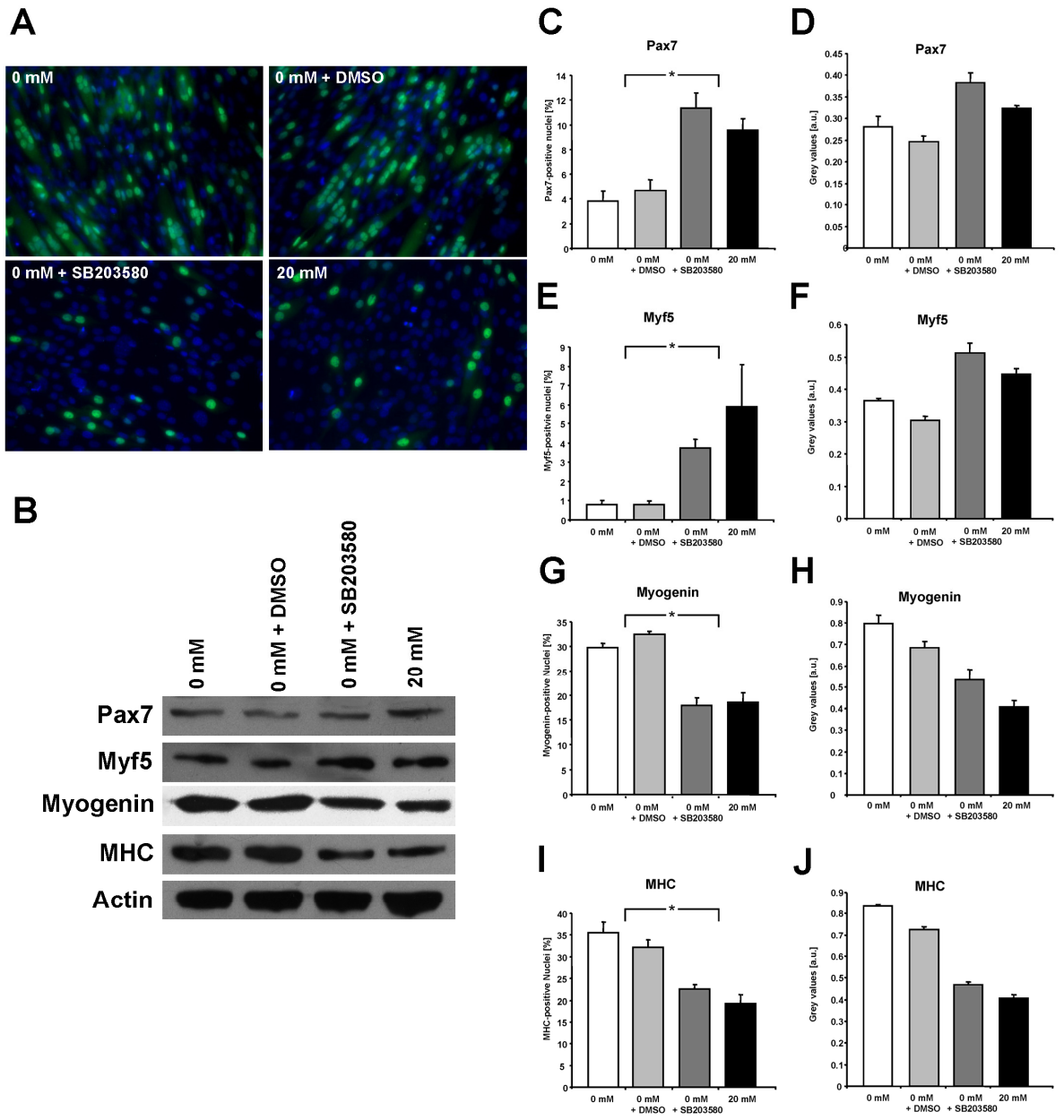

Figure 3.13: La-induced delayed late differentiation in C2C12 cells is similar to p38 MAPK-inhibition in cells treated with 0 mM La DM + SB203580. C2C12 cells were treated intermittently with 0 or 20 mM La DM or 20 mM La DM + 0.1% DMSO, 0 mM La DM + 10 µM SB203580 or 20 mM La DM for 5 days followed by IF (ratios of

marker-positive nuclei) or WB analysis. Briefly, 0 mM La DM + SB 203580 and 20 mM La DM treated samples contained more Pax7 and Myf5 (early differentiation markers) and less myogenin and MHC (late differentiation markers) compared to control samples **A** Myogenin- (green) and DAPI-staining (blue) in C2C12 myoblasts before (pre) and after 5 days of incubation with the respective DM. **B** Representative WBs for Pax7, Myf5, myogenin, and MHC. Actin was used as a loading control. **C, E, G, I** Ratio of positive nuclei from analysis of the IF. **D, F, H, J** quantification of WBs for Pax7 (**C, D**), Myf5 (**E, F**), myogenin (**G, H**), and MHC (**I, J**). Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. For every marker analysed, differences between 0 mM La DM/0 mM La DM + DMSO and 0 mM La DM + SB203580/20 mM La DM were always significant. The former two were never statistically different from each other, nor were the later two. La – lactate; p38 MAPK – p38 mitogen-activated protein kinase; DM – differentiation medium; IF – immunofluorescent staining; WB – Western blot, Pax7 – paired box transcription factor 7; Myf5 – myogenic factor 5; MHC – myosin heavy chain.

Histone modifications play an important role in gene expression regulation. Therefore, samples were analysed for the relevant histone modifications H3K27me3 and H3K4me3 (Figure 3.12). The quantification of IF stained cells showed a significant lower H3K27me3 signal in 20 mM La DM treated samples after 1 and 4 h (both $p < 0.001$) compared to 0 mM La DM (Figure 3.12 E). Notably, the H3K4me3 signal, an important downstream target of p38 activation, follows the same timely pattern as the p38 phosphorylation (Figure 3.12 A + C). Results showed an increase in H3K4me3 in control samples after 30 min and reduction to baseline levels after 24 hours. The La-treated cells did not exhibit an increase, but the signal decreased over time and was not detectable at all after 24 h using WB technique (Figure 3.12 C). Closer investigation of the trimethylated H3K4 by IF and quantification revealed an increase in the H3K4me3 signal after 15 min in the control but not in La samples ($p = 0.002$; Figure 3.12 D + E). Although control cells showed higher staining intensity after 1 and 4 h compared to 20 mM La DM cells, the differences were not statistically significant. Hence, the staining intensity does not differ between control and La-treated samples. Given the results from the WB where a decrease of the H3K4me3 signal was observed, it has to be concluded that the number of nuclei with the modification decreases following La incubation but not the intensity, and therefore the abundance of the epigenetic mark within a single nuclei. Collectively, these data show that a single 20 mM La DM incubation bout decreases H3K27me3 and H3K4me3 observed after 0 mM La DM exposure.

Repeated exposition to 20 mM La for 2 h daily for 5 days did not elicit any differences in H3K27me3 compared to 0 mM La DM (Figure 3.14 A). However, overall trimethylation went down in myotubes compared to myoblasts independent of La. In contrast, 20 mM La DM significantly decreased H3K4me3 in myoblasts ($p = 0.002$) and in myotubes ($p < 0.001$) compared to 0 mM La DM treated cells (Figure 3.14 B). Therefore, the results indicate a strong influence of La on H3K4me3 by repeated exposure during differentiation, but not on H3K27me3.

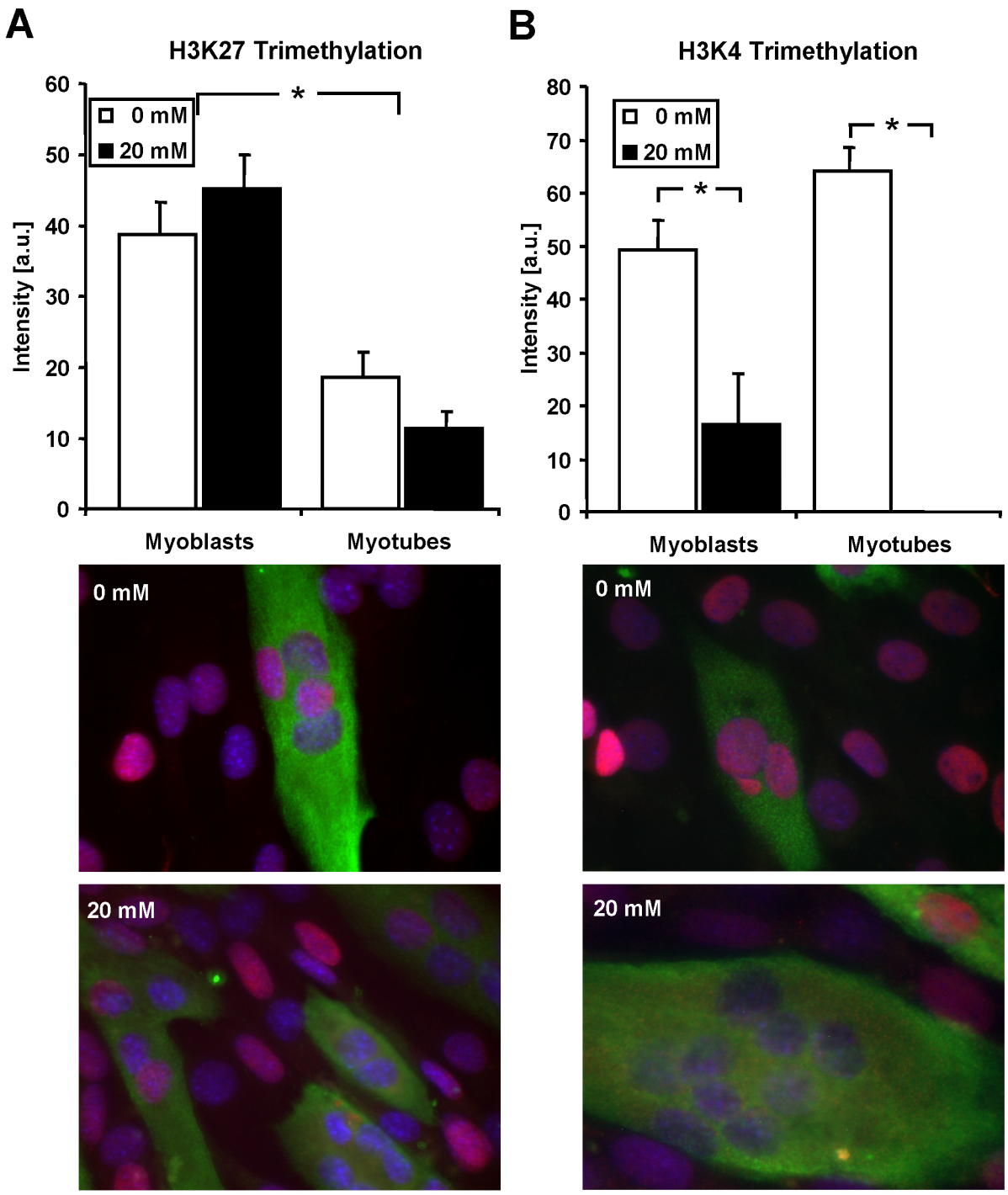

Figure 3.14: La did not affect H3K27me3, but decreased H3K4me3 during differentiation. C2C12 cells were intermittently incubated with 0 and 20 mM La DM for 2 h each day for 5 days. IF for H3K27me3 (**A**; red) or H3K4me3 (**B**; red), MHC (green); and DAPI (blue) followed. **A** Overall H3K27me3 was down-regulated in myotubes compared to myoblasts, but there was no difference between the control (0 mM La DM) and 20 mM La DM treated cells. **B** H3K4me3 remained unchanged under control (0 mM La DM) conditions between myoblasts and myotubes, whereas 20 mM La DM decreased H3K4me3 substantially. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. La – lactate; H3Kxme3 – trimethylated histone 3 at lysine x; DM – differentiation medium; IF – immunofluorescent staining; MHC – myosin heavy chain.

3.7 p38 MAPK and histone modifications *in vivo*

Obtained cell culture results were confirmed using a human exercise model where HIT was compared to STD training (Figure 3.15). WB analysis of muscle biopsy homogenates showed a slightly increasing phosphorylation of p-p38 at 15, 30 min and 1 h post STD training compared to the pre-situation (Figure 3.15 D). After 4 h the signal decreased, before reaching baseline levels at 24 h post exercise. The HIT group displayed a prominent dephosphorylation of p38 MAPK up to 30 min after the intervention. Then the phosphorylation of p38 MAPK increased at 1, 4, and 24 h post exercise, respectively. Furthermore, muscle homogenates were analysed for H3K4me3 (Figure 3.15 D). In the STD group H3K4me3 content peaked 30 min post exercise, but was not detectable at 4 and 24 h post exercise. In contrast, in the HIT group the trimethylation of H3K4 was diminished at 15 min after exercise.

Analysis of fixed muscle sections stained for H3K27me3 showed a strong decrease in H3K27me3 following STD whereas HIT exhibited a pronounced increase of this trimethylation, resulting in a significant difference 4 h post intervention ($p < 0.001$; Figure 3.15 B). The opposite results were obtained for H3K4me3 (Figure 3.15 A + C). Here, H3K4me3 increased after the STD bout and decreased following HIT. The difference here was also significant ($p = 0.004$). Conclusively, these data show a strong influence of training intensity on p38 MAPK activation and subsequent H3K4me3 and H3K27me3 in human skeletal muscle.

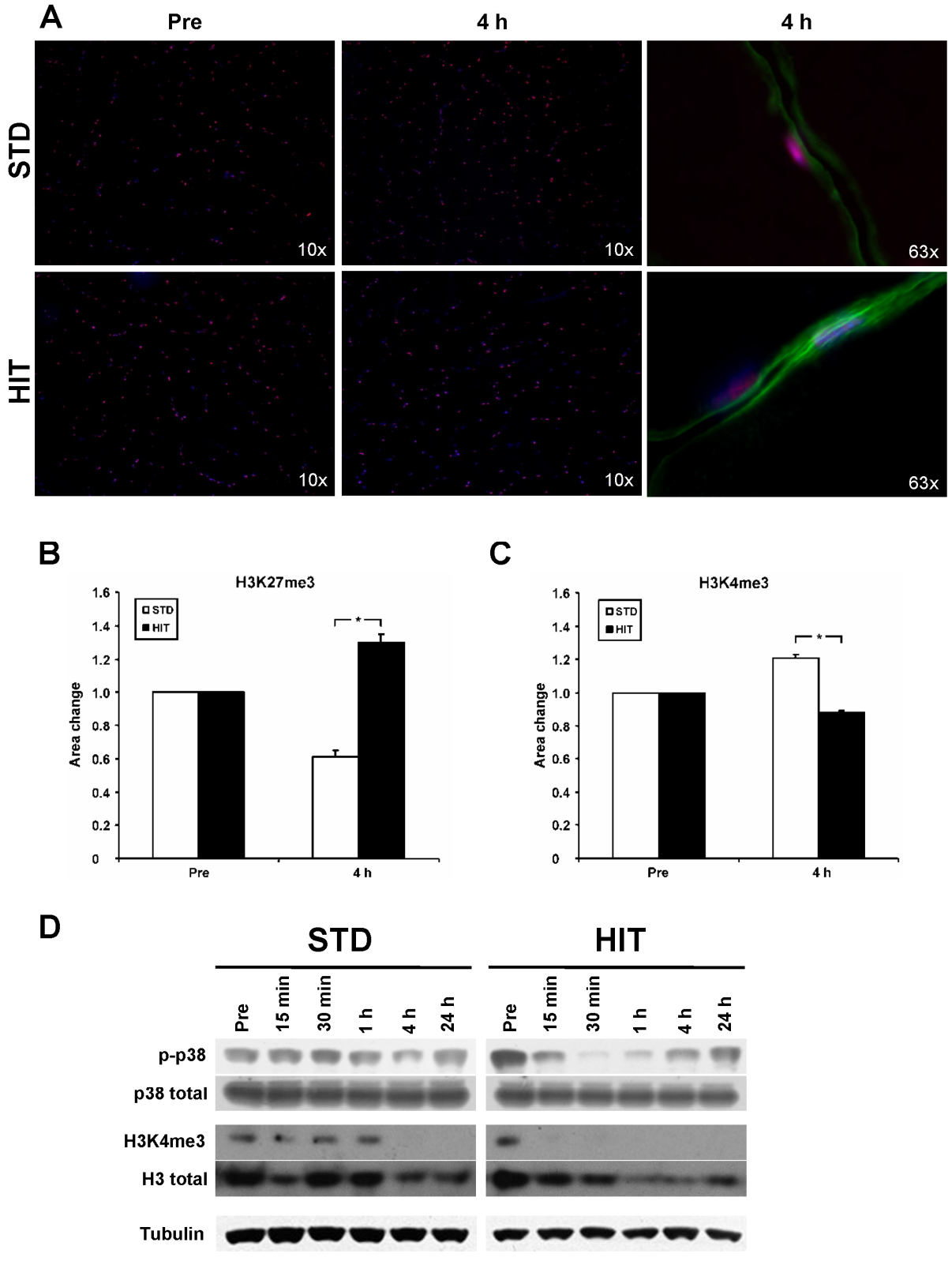

Figure 3.15: HIT dephosphorylates p38 MAPK and diminishes H3K4me3 and H3K27me3 compared to STD. Subjects conducted either a STD or HIT followed by muscle biopsies at 15, 30 min, 1, 4, and 24 h post exercise. Human skeletal muscle tissue was homogenized and pooled for each group for WB analysis for p-p38 and H3K4me3. Furthermore, one part of the sample was cut into sections followed by IF for H3K4me3 and H3K27me3. **A** Representative photographs of human muscle biopsy sections stained for H3K4me3 (red), dystrophin (MANEX; green) and DAPI (blue) before (pre) and after (4 h post) STD or HIT exercise intervention. **B** Quantification of the average area of the H3K27me3 signal in nuclei in human muscle biopsies pre and 4 h post STD or HIT training. C Quantification of the average area of the H3K4me3 signal in nuclei in human muscle biopsies pre and 4 h post STD or HIT training D Representative Western Blots for p-p38, total p38, H3K4me3 of

human muscle homogenates pre and after up to 24 h post exercise. Data presented as mean ± S.E.M. * indicates statistical significance, i.e. $p < 0.05$. HIT – high intensity resistance training; p38 MAPK – p38 mitogen-activated protein kinase, p-p38 – phosphorylated p38 MAPK; H3Kxme3 – trimethylated histone 3 at lysine x; STD – standard resistance training.

4 Discussion

The first aim of this study was to establish the phenotypical changes of intermittent La incubation, thereby simulating a training microcycle of resistance or high intensity endurance training, on the proliferation and differentiation behaviour in a strong model of skeletal muscle stem cells (174), namely C2C12 cells, for their essential contribution to hypertrophy (175, 176) and regeneration (for a review, see (177)).

Serum deprivation in cell culture experiments leads to changes in cell cycle regulators (178) and eventually to permanent withdrawal from the cell cycle (149, 179), an event necessary to induce early differentiation. Data from the Ki67-analysis and the BrdU assay show that proliferation in La DM-treated cells is much lower. This finding is further in line with the observation that Cdkn1a mRNA was more abundant in La-samples. The Cdkn1a gene encodes p21 which is an important regulator of cell cycle withdrawal during myogenesis. It leads to pRb hypophosphorylation, a state associated with differentiation rather than proliferation (172, 173). Trp53 encodes for the p53 protein, another important cell cycle regulator. Trp53 expression was not altered by La treatment. Although p53 has been shown to regulate p21, this is not contradictory to increased Cdkn1a expression. pRb hypophosphorylation by p21 was shown to be p53-independent (180, 181). and p53 was demonstrated to not necessarily contribute to cell cycle withdrawal in myoblasts (170, 171). p53 can be involved in differentiation progress. It induces pRb expression which subsequently associates with MyoD and activates expression of markers of late differentiation (169). Here, all treatments led to a decrease in Trp53 transcripts and in some samples late differentiation markers were nevertheless expressed. This might be explained by the finding that p53-requirement can be overcome by cell-cell contacts (169). In La-treated samples proliferation went down. Possibly cell density was hence not sufficient to overcome p53-requirement. Conclusively, these data reveal that La enhances the serum-deprivation induced cell cycle withdrawal. The indicated mechanism is increased Cdkn1a expression, and possibly successively higher p21 content. However, analysis of p21 and p53 protein abundance and phosphorylation status of pRb is indispensable to confirm this notion.

During myogenesis, differentiation progression is marked by cell cycle withdrawal and decrease of Pax7 expression. Another prerequisite is low Myf5 and elevated MyoD levels (149) when cells withdraw from the cell cycle during the gap 1 (G1) phase to undergo differentiation (182). These requirements are met by cells treated with La indicating their early differentiation. However, expression of late differentiation markers myogenin, MHC1 and MHC2 was significantly decreased by La treatment indicating blocked differentiation progression. Furthermore, after 5 days of intermittent incubation Pax7 and Myf5 protein levels were increased. Pax7 protein represses myogenic progression in C2C12 myoblasts

(77, 167, 183, 184) and other models of muscle stem cells (184–186). Additionally, Myf5 is expressed in cells that remain undifferentiated although differentiation was induced (149). Myogenin is required to proceed to end-terminal differentiation and expression of muscle specific genes, such as MHC (48, 49). These findings conclusively suggest that La-treated cells are arrested in a quiescent state with retained potential to undergo proliferation or differentiation dependent on further cues. A limitation of this study is the missing data on MyoD. Myf5 and MyoD have contrasting transient levels during cell cycle progression. This includes their phosphorylation and subsequent degradation during the mitotic (Myf5) or G1/synthesis (MyoD) phase of the cell cycle (149, 174, 187). Analysis of MyoD might deliver conclusive information of the state and/or cell cycle phase that cells are arrested in and whether MyoD levels are also affected by La. Taken together, La inhibits one or more events which are essential for progression to late differentiation. This suggests that these events are regulated by the metabolic environment.

The results from experiments investigating the dose-effect relationship of La on differentiation progress suggests that its effect is dose-dependent for at least some observations, i.e. apoptotic signalling induction and late differentiation, but not necessarily for cell cycle withdrawal. This is not surprising as these phenomena are regulated by several independent pathways. More experiments are nonetheless necessary to shed light on this topic and investigate especially how exactly the expression of late markers like myogenin and MHC is inhibited by La to explain the dose-dependent phenomena.

The second aim of the study was to investigate whether the La effects are induced by the formation of ROS and hence, oxidative stress. The results clearly demonstrate increased 8-epi-PGF2α levels following 20 mM La DM incubation, indicating ROS production. Furthermore, the observations from the experiments reveal that La-induced ROS formation can be reversed by the use of antioxidant substances. Notably, the areas around the nuclei were mostly affected by oxidative stress. This coincides with the localisation of highest mitochondrial density. It is therefore additionally argued that La leads to increased ROS formation within the mitochondrial membranes. This notion is further supported by the finding that AA and NAc showed the highest efficacy of ROS scavenging. Both antioxidative substances in contrast to LA unfold their direct antioxidative capacity mainly in the mitochondria (188–192). AA and NAc were both able to reduce oxidative stress even below 0 mM La DM, and hence control levels. The mechanism by which LA deploys its antioxidative capacity remains elusive, but it has been described to act via the upregulation of the antioxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat) (193) which suggests a time-delayed and therefore weakened effect for the time period observed in this experiment. Nevertheless, further experiments are necessary to

provide clear evidence of the idea that mitochondrial ROS generation is essentially elevated by La.

The formation of ROS induced by La is controversially discussed in the literature. On the one hand, it was shown to be capable of ROS scavenging in the absence of cells (194–196). Whereas this finding was shown to be also present in cultured hepatocytes (195), a protective effect was not established in neuronal precursor cells (196). In contrast, other studies demonstrated that La increases the formation of ROS. One report describes the La-dependent enhancement of ·OH generation by the Fenton-reaction in a cell-free model (197). Another study found an increased production of H_2O_2 in L6 myoblasts by 20 mM La incubation indicating increased oxidative stress which is in agreement with our findings (136). Additionally, the results for act. Casp-3 imply that La is able to induce apoptotic events, i.e. cellular stress. Whereas no data on the ability of La to induce the apoptotic pathway in C2C12 exists, ROS are able to initiate apoptotic signalling and programmed cell death in this type of cells (198–200). The obtained results unambiguously reveal that with La exposure the oxidative stress increases within C2C12 cells. The rise in cleaved act. Casp-3 is therefore not surprising and provides further evidence for the conclusion that La induces cellular stress, marked by the increased generation of ROS and apoptotic signalling induction.

ROS can modulate numerous processes. For example, cell cycle control is sensitive to oxidative stress. In several tissues and cell lines p21 mRNA increases in response to oxidative stress (201–203). Further research revealed that this elicits a cytoprotective effect via the induction of cell cycle arrest and allowing time for DNA repair in response to oxidative damage (204). However, one study found decreased levels of p21 following increased ROS. The experimental model applied was electron transportchain deficient HeLa cells that furthermore lack normal p53 signalling, a main regulator of p21 (205) indicating that these results are neglectable in the here reported context. 20 mM La DM treatment increased gene expression of Cdkn1a. This effect was reversible by the addition of antioxidants, further supporting the notion of ROS-induced p21 gene expression induction.

As many cellular processes were shown to be ROS-sensitive, if La treatment acutely increases ROS formation and chronically to the initiation of early but a delay in late differentiation, one might consider a link between these events. In order to investigate this hypothesis, different antioxidants were added to the incubation medium. Results provide strong evidence that the La-effects depend on the formation of ROS as the outcomes were reversible by the use of AA, NAc, and LA. Although no data is available on the effects of La on C2C12 differentiation, numerous studies exist elucidating the question of how ROS influence this process. One report implies that certain endogenous ROS concentrations are essential in differentiating myoblasts and that the addition of antioxidants to the cells resulted in differentiation inhibition. In contrast, Cyclosporin A-induced ROS generation has been

shown to block myoblast differentiation in early myoblasts. The conclusion was that the addition of Cyclosporin A to differentiating cells led to toxic levels of ROS, blocking muscle differentiation even when antioxidants were added (206). Another report from the same group shows that the ROS-induced activation of NF-κB/iNOS pathway is necessary for differentiation (207). Other studies provide evidence for a negative effect of ROS on myoblast differentiation. Langen et al. described that oxidative stress per se is sufficient to block myogenic late differentiation (208). H_2O_2 administered in different concentration (20-200 µM) reduced myogenin and MHC protein content, creatine kinase activity as well as troponin I gene transcription all in a dose-dependent manner and that the inhibition of myotube formation was reversible when NAc was added to the culture medium (208). Furthermore, it was demonstrated that 25 µM H_2O_2 markedly reduced Myf5 and MRF4 gene expression 2-3 fold, whereas myogenin was even 60-fold down-regulated (209). Applying 1 mM H_2O_2 to C2C12 cells decreases Myf5 and MHC gene expression (210). These data clearly support the idea that expression of differentiation progression markers, such as myogenin and MHC are very susceptible to oxidative stress, and are thus La-sensitive. As the literature reports contrasting outcomes of ROS-exposure to myoblasts, the notion that ROS can have different outcomes depending on the dose (140, 145). Regarding the results in this thesis it can be implied that La highly elevates ROS levels that result in inhibition of late differentiation.

Regarding the data attained in this study and the information available from the literature for late differentiation markers as well as the findings on the reversibility of the La-effect by the addition of antioxidants, it can be conclusively argued that the La-induced effects are mediated via the formation of ROS. However, it would be interesting to see why cells show delayed differentiation and not total inhibition. There is abundant evidence that muscle is capable of antioxidative adaptation in response to exercise (reviewed in (211, 212)). For example, SOD has been shown to be increased following exercise in an intensity-dependent manner (213–215). Similar findings were published regarding GPx and Cat (216). Possibly after a few La exposures, cells adapt by increasing their antioxidative capacity, i.e. expression of SOD, GPx, and/or Cat and hence at a later stage La-induced ROS-signalling is inhibited and differentiation can progress.

The third aim of this study was to elucidate whether La levels influence the skeletal muscle adaptation signalling molecule p38 MAPK and its downstream targets H3K4me3 and H3K27me3 to further establish the important role of La as a metabolic signal of gene expression. The results presented here show a clear inhibition of p38 MAPK phosphorylation by 20 mM La DM compared to control treatment, i.e. 0 mM La DM. Serum-deprivation without the addition of La led to increased p38 MAPK activation within one hour of treatment,

whereas La-treated samples failed to increase phosphorylation levels above baseline over the whole time course of the experiment. Further prove for the involvement of p38 MAPK in mediating the La effect is drawn from experiments including the p38 MAPK specific inhibitor SB203580. Clearly, cells treated with 20 mM La DM elicited the same phenotype as the cells treated with 0 mM La DM + SB 203580, i.e. high content of early differentiation markers and low content of late differentiation markers compared to 0 mM La DM-treated samples.

Up to the present time, no report has been published to specifically investigate the activation of p38 MAPK in response to La in myoblasts. Nevertheless, one study found elevated p38 MAPK activation in response to incubation with 20 mM La medium, simulating ischemia in adult rat ventricular myocytes (217). However, ischemic incubation media also contained sodium dithionite, a strong reducing agent that might account for the observed differences in p38 phosphorylation given its redox sensitive activity further explained below. Their finding is nevertheless in line with another study that found increased phosphorylated p38 MAPK, JNK, and ERK levels in stem/progenitor cells grown *in vivo* in matrigel plugs in mice (218). Here, experimental setup and analysed cell type differ immensely and care has to be taken when comparing results from this study with the ones reported here. In contrast, Mendler et al. demonstrated that La inhibits p38 MAPK and JNK activation in cytotoxic t-cells (CTLs) leading to CTL inhibition by tumour lactic acidosis (158). Obviously, La-induced regulation of p38 MAPK depends on many diverse factors, such as cell type or environment that remain largely unknown. p38 MAPK was shown to be activated via the apoptosis-stimulating kinase 1 (ASK1) following elevated ROS formation (146, 219, 220). Another study has shown that especially mitochondrial ROS initiate p38 MAPK activation (221). Although the literature agrees that ROS activates p38 MAPK the underlying mechanisms remain less clear (140). Nevertheless, the here presented findings are in contrast to the literature which would predict increased activation of p38 MAPK mediated via ROS formation. Only little is known about the mechanisms of p38 MAPK activation by ROS, so it can only be speculated about reasons to explain this phenomenon. p38 MAPK activation requires either phosphorylation by an upstream kinase such as ASK1 and/or inhibition of the dephosphorylation by a MAPK phosphatase (MKP). MKPs are negative regulators of MAPKs as their phosphatase activity leads to inactivation of these (222–225). Possibly, one or both of these events were inhibited by the La molecule. For example, exposure to low light associated with lower levels of ROS production elicited lower expression of MKP1 in retinal pigment endothelial cells whereas high light doses decreased its expression and led to increased MAPK activation (226). This implies that the activation of MAPK by ROS depends on their concentration which is in line with findings explained above of ROS effects on skeletal muscle differentiation (140, 145). Another underlying mechanism could be increased Cdkn1a expression. ROS-induced ASK1 activation and subsequent p38 MAPK phosphorylation have been shown to induce apoptosis

in several cancer cell lines. Overexpression of p21 led to its increased binding to ASK1, subsequent ASK1 inactivation and hence, prevention of apoptosis (227). Although this mechanism has not been demonstrated in skeletal muscle cells, possibly increased p21 levels observed following La treatment could prevent the activation of p38 MAPK by ASK1 inhibition. However, expression of Cdkn1a was measured 6 h, p38 MAPK activation was decreased as soon as 15 min after start of the La treatment. So, to prove this concept gene expression and protein levels of p21 have to be closer investigated. However, as overall knowledge about detailed mechanisms on p38 MAPK activation is scarce more research is necessary to address the question which mechanisms are influenced by La to inhibit its phosphorylation.

Collectively the data provide evidence that the delay in late differentiation induced by La treatment is at least partly due to decreased activation of the pro-myogenic p38 MAPK. However, the data is not conclusive on how p38 MAPK is inactivated, as the La-induced increase in ROS should predictably have led to increased activation. Moreover, p38 MAPK was shown to induce cell cycle arrest (102), but here, p38 MAPK is not activated by La but proliferation ceases. Further investigations need to shed light on which p38 MAPK-independent pathways are activated to increase Cdkn1a gene expression.

p38 MAPK has several downstream targets exerting its differentiation driving effect on. Two key targets are the histone modifications H3K4me3 and H3K27me3 (72, 73, 80) which both lead to differentiation progression during myogenesis. Notably, the H3K4me3 pattern strongly resembles that of p38 MAPK activation, and H3K27me3 also increases within the same time frame indicating a direct connection between the two events. Both results confirm the known mechanisms and furthermore support the notion that differentiation delay caused by La is caused by diminished p38 MAPK activation. This observation might also explain the finding that acutely Myf5 gene expression is reduced, as this was also shown to be a p38 MAPK-dependent process (77). However, it does not account for the increased abundance after 5 days of incubation for which the reasons remain unknown with the given data.

When C2C12 cells were intermittently incubated with 20 mM La DM during an experiment lasting 5 days (a simulated microcycle of resistance or high intensity endurance training), H3K4me3 was significantly down-regulated in La-treated myoblasts compared to control samples which is in line with the data from the acute experiments. Interestingly, after data analysis distinguishing myoblasts from myotubes, it was observed that in myotubes no signal could be detected for H3K4me3 in 20 mM La DM-treated samples. This finding indicated that H3K4me3 is massively downregulated, but not necessarily totally abolished. The absence of signal might be explicable by the methodology used. In fluorescent quantification a threshold for a signal has to be set and if this is above a weak signal the measurement will give 0. Nonetheless, a profound reduction of H3K4me3 has to be reported. Further analysis is

however necessary to put this finding in context. To conclusively prove the concept of p38 MAPK inactivation and associated diminished inhibition of differentiation events, rescue experiments by transfection of cells with a p38 MAPK activator, and chromatin immunoprecipitation analysis to investigate genes affected by reduced H3K4me3 and H3K27me3 would be essential. Additionally, SB203580 experiments should be conducted testing the p38 MAPK-dependency of H3K4me3 and H3K27me3.

The fourth aim of the study was to confirm that the aforementioned mechanisms observed in differentiating skeletal muscle stem cells are conserved during differentiation and remain active in differentiated muscle tissue exposed to similar La concentrations evoked by exercise *in vivo*.

High intensity resistance training was chosen as a model of increased metabolic stress accompanied by high intramuscular La levels (228–231). Several considerations led to the fact that La levels were not determined in this study: [1] A method to determine intramuscular La levels during and/or after exercise was not available. [2] The exercise stimulus was only applied in one leg and exercise time was short. Measuring systemic La levels would have possibly led to distorted results. Systemic La levels depend on several factors, such as La production, muscular La export into the bloodstream, and uptake by different organs that use La as an energy substrate, e.g. heart, liver, brain, passive musculature. All these factors vary widely between subjects and are therefore unlikely to reflect local La production. This notion is supported by Buitrago et al. who could not detect any increases in systemic La levels following a HIT protocol although the intensity of work was found to be higher by measurement of oxygen used. The group explained their finding by the shortened exercise time of HIT protocols not allowing enough time for La to accumulate in the blood (232). In this study presented her exercise time varied immensely (HIT: 1 set of 20 repetitions; STD: 3 sets with 10 repetitions with a 3 min break in between) further justifying this approach. [3] The tight time frame of muscle biopsies placed discomfort on the subjects and taking blood for La measurement would have further distressed them. As results obtained for the above mentioned reasons might not be reliable, it was decided against additionally measuring systemic La levels. The HIT and STD protocols nevertheless differed massively in their design and information from the literature listed above suggests considerably higher metabolic stress, amongst other by higher La levels, during the HIT protocol.

Up to the present time, no study has investigated the effects of a certain training stimulus on trimethylation of H3K4 or H3K27. In general, knowledge regarding histone modifications following exercise is humble. The present study was designed to address this topic and additionally added a tight time frame to be furthermore able to assess temporal relationships between key regulators, such as p38 MAPK, and histone modifications, such as H3K4me3

and H3K27me3. Comparison of a HIT to a STD stimulus revealed that HIT leads to a pronounced decrease in p38 MAPK phosphorylation in human muscle immediately after exercise that only starts recovering 4 h post-exercise. The results further showed a pronounced loss of H3K4me3 as soon as 15 min following HIT, with a significant reduction in H3K4me3 still being present after 4 h post-exercise. H3K27me3 was positively affected for by HIT, both modifications indicating an expression-repressive epigenetic landscape following HIT. Only a few studies have been published that describe the changes on histone modifications associated with promoter regions of specific genes in order to gain a better understanding of how exercise may differentially affect transcription in muscle in response to different modes of exercise (reviewed in (233)). A single bout of endurance exercise (cycling for 60 min at 76% VO2peak) led to a global increase in H3K36 acetylation, a modification that is associated with transcriptional elongation. Furthermore, the same study showed that AMPK and CaMKII activation led to nuclear export of histone deacetylases 4 and 5 (HDAC4 and 5) rendering these enzymes to suppress histone acetylation (115). Additionally, epigenetic modifications have been shown to depend on metabolic signals, such as AMPK and CaMK II (10). This delivers a possible explanation for the finding of a study where a reduction of DNA methylation of the promoters for e.g. PGC1α, and subsequent increased gene expression was found to be exercise intensity dose-dependent (11). Taking all this evidence into account, it appears that a metabolic stimulus such as La is able to induce epigenetic modifications that alter gene expression and hence, skeletal muscle adaptation. Although other stimuli arising with exercise and physical activity, e.g. mechanical stress, might also affect epigenetic modifications occurring with resistance exercise, together with the in vitro data, it can be assumed that the HIT effect exerted on histone modifications is primarily of metabolic nature. The HIT stimulus applied in this study was very high and evoked histone modifications related to inhibition of gene expression. With regards to studies finding a positive correlation of exercise intensity and gene expression induction, findings from the study presented here imply that very high exercise intensities might regulate gene expression negatively. Further research is nevertheless necessary, e.g. gene expression analysis, chromatin immunoprecipitation to determine genes labelled with the H3K4me3 and H3K27me3 mark, and further training intervention studies *in vivo* to elucidate the functional relevance of the here described modification and additionally involved mechanisms.

Conclusively, these data are first evidence to show that in differentiating myoblasts La reduces p38 MAPK activation and subsequent H3K4me3 and H3K27me3. Furthermore, it was possible to demonstrate for the first time that this mechanism is conserved in differentiated muscle tissue and also applies *in vivo*. Taken together, it is concluded that epigenetic modifications are inducible by La as a metabolic signal and that epigenetic

responses to exercise and physical activity are exercise-intensity and hence, metabolic environment dependent.

Exercise and physical activity adaptations with the desired outcome being improved function and/or increased muscle mass requires the preservation of nuclear domains, and therefore the activation, proliferation, differentiation and fusion of SCs with skeletal muscle fibres. The data obtained from in vitro experiments imply that common views on design and periodisation of training (as well as the prescription of antioxidant nutritional supplements) should be reassessed. La modifies skeletal muscle adaptation by delaying late differentiation of activated SCs via a ROS-sensitive signalling pathway. Suggestively, if this mechanism also exists *in vivo*, it might lead to diminished or delayed fusion of SCs with existing skeletal muscle fibres and hence, impaired skeletal muscle adaptation. So in order to design an optimal training regime with the best possible adaptational response, the following proposed training regimen considers these results.

Suggestively, one should start with a type of training to increase the SC pool most efficiently. Several human studies reported an increase in SC number of over 80% 5-8 days after a single bout of maximal eccentric contraction (234–236). In contrast, other studies applying an endurance type training (38, 39) or lower intensity resistance training (39, 237–239) in humans could only demonstrate markedly smaller changes in SC pool expansion of 30-62.5%. The next cycle within the training regimen should lead to the withdrawal from the cell cycle and promotion of early differentiation. Considering the in vitro data reported here, a high intensity endurance training cycle accompanied by high La levels should be an efficient way to drive proliferating SCs to withdraw from the cell cycle and into the early differentiation phase. Although several molecular promoters (240) and involved signalling pathways (reviewed in (241)) of myoblast fusion into myotunbes or with existing myofibres were identified, no data is available on how different types of exercise modify this process. However, it should not be accompanied by high La levels as our data implies that these prevent further differentiation and fusion of myoblasts with already existing myofibres, possibly eventually suppressing hypertrophy. Nevertheless, further research is necessary to elucidate which sequential pattern and timing of training stimuli elicits the most effective training programme.

The *in vivo* study was predominantly conducted to establish whether the mechanism of decreased p38 MAPK activation and subsequent histone modifications caused by high La levels is conserved during differentiation. Unfortunately, no measurements were included to elucidate the meaning of the intracellular signalling modifications and its functional consequences regarding skeletal muscle adaptation. Hence, from these data no practical application can be derived. However, the conservation of the signalling pathways during

differentiation implies that differentiated myoblasts might be a suitable model for the study of histone modifications (at least for H3K4 and H3K27) exerted by different training stimuli, such as metabolic stress or mechanical strain.

The finding that the La effects are at least partly mediated by ROS furthermore suggests that the use of antioxidants as nutritional supplements could influence the process of skeletal muscle adaptation. The use of antioxidants is common among athletes with the intention to avoid deleterious effects of oxidative stress arising with exercise (242). Recently, more and more studies imply that antioxidative substances attenuate beneficial effects of exercise and physical activity due to the inhibition of essential ROS-signalling and subsequent cellular adaptation (144, 243–246). For La-induced adaptations as in high intensity endurance training the use of antioxidants might be deleterious at first as it might prevent cell cycle withdrawal and induction of early differentiation. However, at some point it could be beneficial as it possibly accelerates the delayed differentiation process induced by the metabolic signal. Obviously, the form of exercise also elicits implications on nutritional supplementation recommendations and even the timing of their application has to be considered. However, advising on nutritional supplements needs precaution due to its complicated nature and is not further discussed here.

5 Conclusion

The results presented here provide strong evidence that La is indeed a metabolic signal for skeletal muscle adaptation in vitro (Figure 5.1). In differentiating myoblasts it induces the formation of ROS which downstream lead to several signalling events. Firstly, it leads to the withdrawal from the cell cycle by up-regulation of Cdkn1a, the gene encoding the cell cycle control protein p21 which induces cell cycle arrest. Markers of the early differentiation phase (Pax7 and Myf5) are also induced. However, late differentiation is timely delayed in a dose-dependent manner. This effect is possibly mediated via the inhibition of p38 MAPK and subsequent diminished H3K4me3 and H3K27me3. Conduction of a human intervention study revealed that p38 MAPK is also regulated by metabolic signals *in vivo* and that the mechanism of inactivation of p38 MAPK and down-regulation of H3K4me3 and H3K27me3 is conserved during differentiation and still present in differentiated skeletal muscle tissue. Furthermore, the findings propose that traditional views on training design should be reassessed and possibly nutritional supplementation be reconsidered. Additionally, differentiated C2C12 myoblasts might propose a usable model to study epigenetic modifications as at least histone modifications are conserved in differentiated tissue *in vivo*.

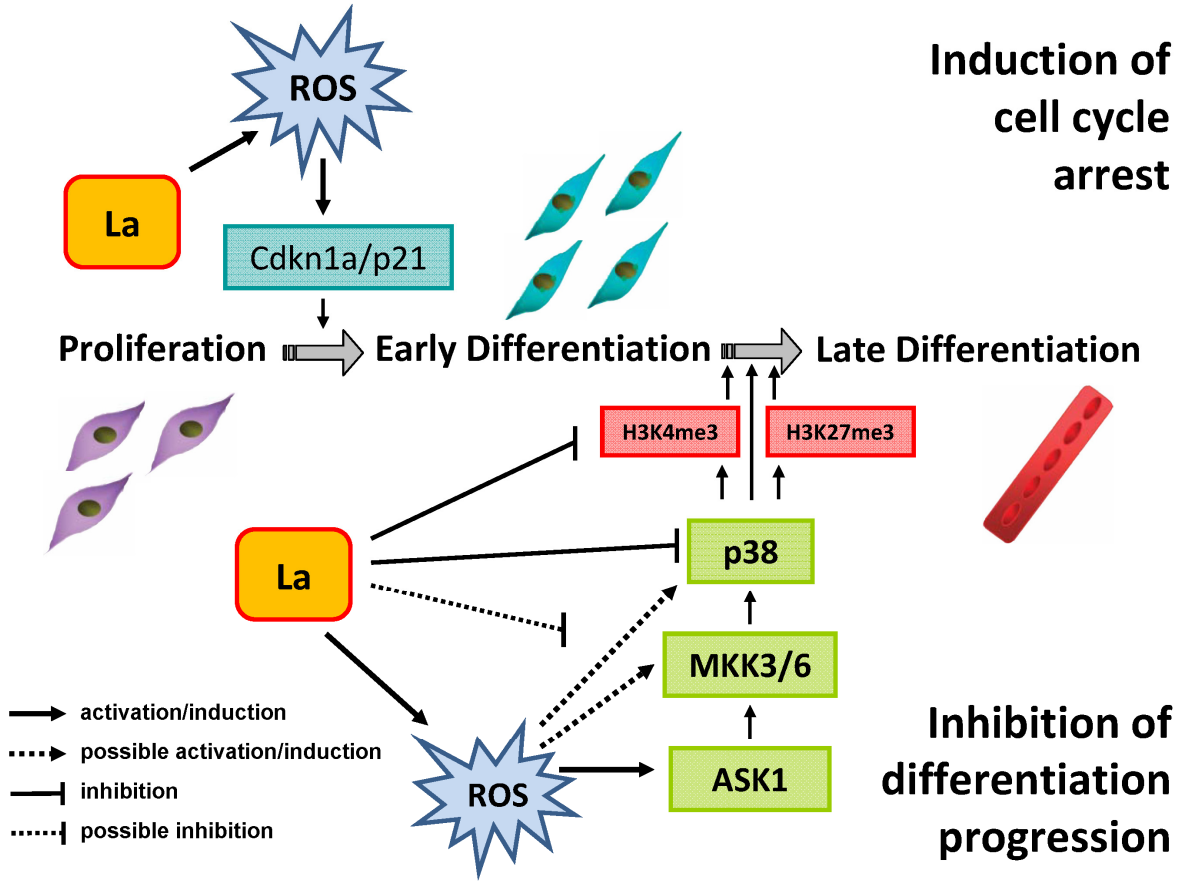

Figure 5.1: La induces cell cycle arrest and inhibits differentiation progression. Incubation with La enhances the serum deprivation-induced withdrawal from the cell cycle. This is possibly mediated by the ROS-induced

expression of Cdkn1a, the gene encoding the cell cycle regulator p21 which leads to cell cycle arrest. ROS were shown to activate ASK1, MKK3/6, and p38. Although La induces the production of ROS, p38 MAPK and subsequent H3K4me3 and H3K27me3 are inactivated resulting in late differentiation delay. Suggestively, La regulates a signalling event downstream of ROS that prevents p38 MAPK activation and subsequently H3K4me3 and H3K27me3 and associated differentiation progression. La – lactate; ROS – reactive oxygen species; Cdkn1a – cyclin-dependent kinase inhibitor 1a; p38 – p38 mitogen-activated protein kinase; MKK3/6 – MAPK kinase 3/6; ASK1 – apoptosis-stimulating kinase 1; H3Kxme3 – trimethylated histone 3 at lysine x.

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7 Appendix

Table A.1: La delayed myogenin synthesis in C2C12 cells

A

Myogenin [%]	D1	D2	D3	D4	D5
0 mM La DM	0.06 ± 0.04	2.78 ± 0.39	5.79 ± 1.14	10.01 ± 1.05	7.87 ± 1.45
10 mM La DM	0.0	1.66 ± 0.30	6.47 ± 0.67	6.92 ± 0.72	9.85 ± 1.69
20 mM La DM	0.0	1.09 ± 0.21	2.65 ± 0.31	4.14 ± 0.66	5.07 ± 0.63

B

p-values	D1	D2	D3	D4	D5
0 vs. 10 mM	-	-	-	-	-
0 vs. 20 mM	-	0.011	< 0.001	0.018	-
10 vs. 20 mM	-	-	0.001	-	-

Table A.2: La delayed MHC synthesis in C2C12 cells in a dose-dependent manner

A

MHC [%]	D1	D2	D3	D4	D5
0 mM La DM	0.0	0.47 ± 0.22	2.89 ± 0.47	10.03 ± 1.26	31.51 ± 1.75
10 mM La DM	0.0	0.13 ± 0.08	1.49 ± 0.22	12.56 ± 1.14	29.48 ± 1.49
20 mM La DM	0.0	0.0	0.02 ± 0.02	0.93 ± 0.26	6.08 ± 0.70

B

p-values	D1	D2	D3	D4	D5
0 vs. 10 mM	-	0.011	<0.001	-	-
0 vs. 20 mM	-	< 0.001	< 0.001	< 0.001	0.027
10 vs. 20 mM	-	< 0.001	0.001	-	-

Table A.3: La altered gene expression of cell cycle control genes Cdkn1a and Trp53

A

x-fold increase	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Trp53	1	0.61 ± 0.03	0.55 ± 0.05	0.79 ± 0.06	0.56 ± 0.02	1.98 ± 0.03
Cdkn1a	1	1.24 ± 0.32	2.67 ± 0.30	1.56 ± 0.04	2.03 ± 0.15	1.98 ± 0.18

B

Trp53 p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		-	0.017	-	-	-
0 mM	-		0.038	-	-	-
20 mM	0.017	0.038		-	-	-
20mM + AA	-	-	-		-	-
20 mM + NAc	-	-	-	-		-
20 mM + LA	**-**	**-**	**-**	**-**	**-**	

C

Cdkn1a p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		0.001	p < 0.001	0.022	p < 0.001	0.002
0 mM	0.001		-	0.008	-	-
20 mM	p < 0.001	-		0.008	-	-
20mM + AA	0.022	0.008	0.008		0.011	-
20 mM + NAc	p < 0.001	-	-	0.011		-
20 mM + LA	0.002	**-**	-	**-**	**-**	

Table A.4: La influenced gene expression of Pax7, Myf5, myogenin, MHC 1, and MHC2

A

x-fold increase	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pax7	1	0.88 ± 0.02	0.42 ± 0.03	1.45 ± 0.18	1.22 ± 0.08	1.06 ± 0.08
Myf5	1	0.97 ± 0.02	0.72 ± 0.01	1.15 ± 0.03	1.11 ± 0.02	1.17 ± 0.19
Myogenin	1	2.64 ± 0.18	1.40 ± 0.12	3.31 ± 0.45	2.65 ± 0.45	3.15 ± 0.11
MHC1	1	2.33 ± 0.01	1.40 ± 0.16	1.87 ± 0.49	2.45 ± 0.30	2.59 ± 0.15
MHC2	1	2.40 ± 0.65	1.22 ± 0.48	3.6 ± 0.27	1.75 ± 0.13	1.86 ± 0.49

B

Pax7 p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		-	0.01	0.03	-	-
0 mM	-		0.033	0.009	-	-
20 mM	0.01	0.033		p < 0.001	0.002	0.006
20mM + AA	0.03	0.009	p < 0.001		-	*0.067*
20 mM + NAc	-	-	0.002	-		-
20 mM + LA	**-**	**-**	0.006	*0.067*	**-**	

C

Myf5 p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		-	-	-	-	-
0 mM	-		-	-	-	-
20 mM	-	-		0.027	0.046	0.022
20mM + AA	-	-	0.027		-	-
20 mM + NAc	-	-	0.046	-		-
20 mM + LA	**-**	**-**	0.022	**-**	**-**	

D

Myogenin p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		0.016	-	0.003	0.016	0.004
0 mM	0.016		-	-	-	-
20 mM	-	*0.066*		0.007	*0.064*	0.012
20mM + AA	0.003	-	0.007		-	-
20 mM + NAc	0.016	-	*0.064*	-		-
20 mM + LA	0.004	-	0.012	-	-	

E

MHC1 p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		0.027	-	-	0.018	0.011
0 mM	0.027		-	-	-	-
20 mM	-	-		-	*0.089*	0.049
20mM + AA	-	-	-		-	-
20 mM + NAc	0.018	-	*0.089*	-		-
20 mM + LA	0.011	-	0.049	-	-	

F

MHC2 p-values	Pre	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pre		-	-	0.01	-	-
0 mM	-		-	-	-	-
20 mM	-	-		0.015	-	-
20mM + AA	0.01	-	0.015		*0.053*	*0.071*
20 mM + NAc	-	-	-	*0.053*		-
20 mM + LA	-	-	-	*0.071*	-	

Table A.5: La-induced delayed differentiation in C2C12 cells was reversible by the use of antioxidants

A

[%] positive cells	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
Pax7	3.6 ± 0.8	10.3 ± 1.2	4.7 ± 0.9	6.1 ± 1.2	8.6 ± 1.9
Myf5	1.7 ± 0.5	3.6 ± 0.9	0.9 ± 0.2	2.1 ± 0.4	1.6 ± 1.5
Myogenin	30.3 ± 2.4	17.2 ± 1.2	34.1 ± 1.3	32.1 ± 1.5	34.9 ± 1.4
MHC	28.7 ± 2.0	15.4 ± 1.8	30.5 ± 1.7	30.3 ± 1.4	29.4 ± 1.6

B

Pax7 - p-values	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
0 mM		0.007	-	-	-
20 mM	0.007		0.049	-	-
20mM + AA	-	0.049		-	-
20 mM + NAc	-	-	-		-
20 mM + LA	-	-	-	-	

C

Myf5 - p-values	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
0 mM		-	-	-	-
20 mM	-		0.009	-	-
20mM + AA	-	0.009		-	-
20 mM + NAc	-	-	-		-
20 mM + LA	-	-	-	-	

D

Myogenin - p-values	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
0 mM		<0.001	-	-	-
20 mM	< 0.001		<0.001	<0.001	<0.001
20mM + AA	-	<0.001		-	-
20 mM + NAc	-	<0.001	-		-
20 mM + LA	-	<0.001	-	-	

E

MHC - p-values	0 mM	20 mM	20mM + AA	20 mM + NAc	20 mM + LA
0 mM		<0.001	-	-	-
20 mM	<0.001		<0.001	<0.001	<0.001
20mM + AA	-	<0.001		-	-
20 mM + NAc	-	<0.001	-		-
20 mM + LA	-	<0.001	-	-	

Table 5: La-induced delayed differentiation in C2C12 cells is comparable to p38 MAPK-inhibition

A

[%] positive cells	0 mM	0 mM + DMSO	0 mM + SB203580	20 mM
Pax7	3.84 ± 0.81	4.69 ± 0.86	11.35 ± 1.20	9.57 ± 0.89
Myf5	0.77 ± 0.23	0.79 ± 0.21	3.74 ± 0.43	5.89 ± 2.21
Myogenin	29.79 ± 0.82	32.48 ± 0.60	17.96 ± 1.48	18.62 ± 1.91
MHC	35.43 ± 2.55	32.20 ± 1.73	22.58 ± 0.95	19.22 ± 2.00

B

Pax7 - p-values	0 mM	0 mM + DMSO	0 mM + SB203580	20 mM
0 mM		-	p < 0.001	0.001
0 mM + DMSO	-		p < 0.001	0.005
0 mM + SB203580	p < 0.001	p < 0.001		-
20 mM	0.001	0.005	-	

C

Myf5 - p-values	0 mM	0 mM + DMSO	0 mM + SB203580	20 mM
0 mM		-	p < 0.001	0.046
0 mM + DMSO	-		p < 0.001	0.047
0 mM + SB203580	p < 0.001	p < 0.001		-
20 mM	0.046	0.047	-	

D

Myogenin - p-values	0 mM	0 mM + DMSO	0 mM + SB203580	20 mM
0 mM		-	0.019	0.035
0 mM + DMSO	-		p < 0.001	p < 0.001
0 mM + SB203580	0.019	p < 0.001		-
20 mM	0.035	p < 0.001	-	

E

MHC - p-values	0 mM	0 mM + DMSO	0 mM + SB203580	20 mM
0 mM		-	p < 0.001	p < 0.001
0 mM + DMSO	-		0.006	p < 0.001
0 mM + SB203580	p < 0.001	0.006		-
20 mM	p < 0.001	p < 0.001	-	

8 Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von den unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. med. Wilhelm Bloch betreut worden.

Teilpublikationen

Lena Willkomm, Sarah Schubert, Raphael Jung, Manuela Elsen, Julika Borde, Sebastian Gehlert, Frank Suhr, Wilhelm Bloch, „Lactate regulates C2C12 myogenesis *in vitro*“, in revision.

Lena Willkomm, Sebastian Gehlert, Daniel Jacko, Thorsten Schiffer, Wilhelm Bloch, “p38 MAPK activation and H3K4 trimethylation is decreased by La *in vitro* and by high intensity resistance exercise *in vivo*“, submitted.

Teile dieser Arbeit wurden auf folgenden Kongressen vorgestellt:

Poster:
58th Annual Meeting of the American College of Sports Medicine, 2011
90th Annual Meeting of the German Physiological Society, 2011
59th Annual Meeting of the American College of Sports Medicine, 2012
7th International Stem Cell Meeting of the Stem Cell Network North Rhine Westphalia, 2013
44. Deutscher Sportärztekongress, 2013

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44. Deutscher Sportärztekongress, 2013

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Köln, den 14. Januar 2014

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