INDIVIDUAL AND COMBINATORY EFFECTS OF VOLUNTARY WHEEL RUNNING AND sActRIIB-Fc ADMINISTRATION ON REDOX-BALANCE IN mdx MICE

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Master’s thesis in Exercise Physiology
Spring 2015
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ABSTRACT

Duchenne’s muscular dystrophy (DMD) is X-chromosome linked muscle wasting disease. It is caused by a mutation in the gene coding protein called dystrophin leading to premature death and significantly impairing the quality of life of DMD patients. Oxidative stress is a contributing factor in the pathology of DMD. Light intensity exercise and interventions that promote sirtuin (SIRT) 1 activity have been shown to be antioxidant for mdx mice and to ameliorate the symptoms of DMD. Also blocking activin receptor IIB (ActRIIB) ligands has been shown in some, but not all studies to improve the pathology of the DMD. The purpose of this study was to find out the individual and combinatorial effects of voluntary wheel running and blocking ActRIIB ligands with soluble fusion protein of ActRIIB (promotes muscle hypertrophy)(sActRIIB-Fc) on redox balance in mdx mice, an animal model for Duchenne’s muscular dystrophy. The mdx mice were randomly divided into 4 groups (n=8 in each): PBS placebo sedentary, PBS placebo running, sActRIIB-Fc administered sedentary, and sActRIIB-Fc administered running group. In addition, wild-type PBS placebo treated mice served as a control group. sActRIIB-Fc was injected intraperitoneally, once a week for 7 weeks, which was also the length of the intervention period. Mice in the running groups had free access to running wheels. Redox balance was assessed by measuring the amount of oxidized (GSSG) and reduced glutathione (GSH) in the gastrocnemius muscle. Outcome of the redox balance i.e oxidative damage was assessed from the carbonylated proteins using western immunoblot analysis. Furthermore, protein expression of SIRT1, the phosphorylation of SIRT1 at ser 46 (p-SIRT1), SIRT3, SIRT6, AMPK, and the phosphorylation of AMPK at thr 172 (p-AMPK) were measured using western immunoblot analysis. There was increasing running effect in the oxidized glutathione (GSSG) (p=0.014), increasing running effect in the ratio of the oxidized glutathione and total glutathione (GSH/TGSH) (p=0.012), increasing running effect in the ratio of oxidized glutathione and reduced glutathione (GSSG/GSH) (p=0.0006) and increasing running effect in protein carbonyls (p=0.026) (2x2 ANOVA). In addition, there was increasing running x sActRIIB-Fc interaction effect in the carbonylated proteins (p=0.018), increasing running x sActRIIB-Fc interaction effect in p-SIRT1 (p=0.025) and increasing running x sActRIIB-Fc interaction effect and in the ratio of p-AMPK and AMPK (p-AMPK/AMPK) (p=0.029) (2x2 ANOVA). However, there were no changes in SIRT1, 3 and 6 protein expressions. The main finding of this study was that combination of exercise and blocking ActRIIB binding ligands with sActRIIB-Fc increased protein carbonyls which was accompanied by increased phosphorylation of SIRT1 at ser 46 and oxidized form of glutathione (GSSG). In addition running independently increased protein carbonyls and oxidized glutathione. These results suggest that voluntary wheel running independently and combined with sActRIIB-Fc administration results in elevated oxidative stress that dystrophic mice can’t fully rescue with endogenous antioxidants.

Key words: Muscular dystrophy, oxidative stress, running, ActRIIB blocking.
ACKNOWLEDGEMENTS

Now that I have finally completed my master’s thesis I would like to thank several people who have helped me in this process.

Firstly I would like to thank my girlfriend Sini and my family: Reijo, Päivi, Henriikka and Annukka for their endless support and understanding in this long process.

Secondly I would like thank my research supervisor Juha Hulmi for giving me this opportunity to do my master’s thesis of such an interesting topic. Especially, I would like to show my greatest gratitude for Juha’s tireless effort in advising, challenging and inspiring me. In addition, I would like to thank Mustafa Atalay, Ayhan Korkmaz and their research team at the University of Eastern Finland for measuring the glutathione metabolism variables and protein carbonyls.

Thirdly I would like to thank my study mate Tuuli Nissinen for opposing my thesis and helping me with the western immunoblot analysis. In addition, I would like to thank my study mate Juho Hyödynmaa for inspiring conversations and for the time we spent together in the laboratory.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ActRIIB</td>
<td>Activin receptor II B</td>
</tr>
<tr>
<td>ALK4/ALK5</td>
<td>Activin-like kinase 4/5</td>
</tr>
<tr>
<td>p-AMPK &amp; AMPK</td>
<td>Phosphorylated adenosine monophosphate-sensitive protein kinase / Adenosine monophosphate-sensitive protein kinase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker’s muscular dystrophy</td>
</tr>
<tr>
<td>BMP1</td>
<td>Bone morphogenetic protein 1</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium-ion</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CaMKK &amp; CaMKK$^\beta$</td>
<td>Calcium-ion/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CDK1 &amp; CDK5</td>
<td>Cyclin dependent kinase-1 &amp; 5</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitolytransferase 1</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxylic acid</td>
</tr>
<tr>
<td>DAP</td>
<td>Dystrophin associated complex</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne’s muscular dystrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual-specificity tyrosine-regulated kinase</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen related receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty-acid</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>Fatty acid translocase CD36</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead transcription factor</td>
</tr>
<tr>
<td>GADD45</td>
<td>Growth arrest and DNA damage-inducible protein 45</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GASP1</td>
<td>Growth differentiation factor-associated serum protein-1</td>
</tr>
<tr>
<td>GDF-8</td>
<td>Growth/differentiation factor 8</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>GTE</td>
<td>Green tea extract</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hemoxygenase-1</td>
</tr>
<tr>
<td>HKII</td>
<td>Hexokinase II</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IDH2</td>
<td>Isocitrate dehydrogenase 2</td>
</tr>
<tr>
<td>IL-6 &amp; IL1β</td>
<td>Interleukin 6 &amp; 1β</td>
</tr>
<tr>
<td>JA16</td>
<td>Neutralizing antibody to myostatin</td>
</tr>
<tr>
<td>JNK1 &amp; JNK 2</td>
<td>Jun N-terminal kinase 1 &amp; 2</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>mdx</td>
<td>Mouse model of Duchenne’s muscular dystrophy</td>
</tr>
<tr>
<td>MKK4/6</td>
<td>Mitogen activated protein kinase kinase 4 and 6</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Myf5</td>
<td>Myogenic factor 5</td>
</tr>
<tr>
<td>MyoD</td>
<td>Myogenic differentiation factor D</td>
</tr>
<tr>
<td>NAD⁺ &amp; NADH</td>
<td>Oxidized nicotinamide adenine dinucleotide &amp; reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP⁺/NADPH</td>
<td>Oxidized nicotinamide adenine dinucleotide phosphate &amp; reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAMPT</td>
<td>Nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrogen mono-oxide</td>
</tr>
<tr>
<td>NRF-1/NRF-2</td>
<td>Nuclear respiratory factor-1 and 2</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrogen mono-oxide</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (placebo)</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase 4</td>
</tr>
<tr>
<td>PGC1-α</td>
<td>Peroxisome-proliferator-activated receptor gamma co-activator 1-alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>Peroxisome-proliferator-activated receptor-alpha</td>
</tr>
<tr>
<td>p38-MAPK</td>
<td>p38-mitogen-activated protein kinase</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>sActRIIB-Fc</td>
<td>Soluble ligand binding domain of type IIb activin receptor fused to the Fc domain of IgG</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>p-SIRT &amp; SIRT</td>
<td>Phosphorylated sirtuin &amp; sirtuin</td>
</tr>
<tr>
<td>SOD &amp; SOD2</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TAK1</td>
<td>Tumor growth factor activated kinase 1</td>
</tr>
<tr>
<td>TGF/TGF-β</td>
<td>Transforming growth factor/Transforming growth factor-β of signalling cytokines</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRPC-1</td>
<td>Transient receptor potential channel-1</td>
</tr>
<tr>
<td>UCP3</td>
<td>Uncoupling protein 3</td>
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INTRODUCTION

Duchenne’s muscle dystrophy (DMD) is X-chromosome linked muscle wasting disease. It is caused by a mutation in the gene coding for a protein called dystrophin leading to premature death and significantly impairing the quality of life of DMD patients. (Thomas 2013). The function of dystrophin protein is to serve as a link between the exterior and the interior of the muscle cell linking the microfilament network and the extracellular matrix. Lack of functional dystrophin makes the muscle fiber sarcolemma susceptible to degeneration during repeated cycles of muscle contraction and relaxation. (Chakallaka et al. 2005.) Muscle tissue is replaced by connective fibrous, tissue and fat as a result of repeated cycles of degeneration, inflammation and regeneration. This will eventually lead to severe loss of muscle function making the DMD patients wheelchair bound and dependent on ventilator during their puberty. (Terrill et al. 2013.) mdx mouse model is the most used animal model to study Duchenne’s muscle dystrophy, but the symptoms of mdx mice are not as severe as in human DMD patients (Willman et al. 2009). Proper cure for DMD remains unresolved. However, based on the studies conducted with mdx mice, voluntary wheel running has either ameliorated (Call et al. 2008; Sveen et al. 2008; Baltgalvis et al. 2010; Selsby et al. 2013) or not (Landisch et al. 2008) the symptoms of DMD. On the other hand, the blocking of TGF-family members such as myostatin have been reported to show beneficial outcomes by ameliorating the symptoms of DMD in some (Bogdanovich et al. 2002; Bogdanovich et al. 2005; Morine et al. 2010; Pistilli et al. 2011), but not in all studies (Relizani et al. 2014).

Oxidative stress (termed as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to cellular damage (Sies 1997)) seems to be contributing factor in DMD (Renjini et al. 2012; Kim et al. 2013; Terrill et al. 2013). Both the administration of antioxidants (Buetler et al. 2002; Call et al. 2008; Whitehead et al. 2008), light intensity exercise (Kaczor et al. 2007), voluntary wheel running (Call et al. 2008) and forced treadmill running (Schill 2014) have been able to decrease the oxidative stress in mdx mice leading to ameliorated symptoms of DMD in some (Buetler et al. 2002; Kaczor et al. 2007; Call et al. 2008; Whitehead et al. 2008) but not all studies (Schill 2014).
Sirtuins are NAD$^+$-dependent protein and histone deacetylases that are involved in energy metabolism, life-span regulation and protection against oxidative stress (Kelly et al. 2010; Merksamer et al. 2013; Radak et al. 2013). According to previous studies, increased catalytic activity of SIRT1 either caused by resveratrol administration or by genetic modifications has been shown to be beneficial for mdx mice. (Hori et al. 2011; Gordon et al. 2013; Chalkiadaki et al. 2014) Already published data from this study showed that blocking ActRIIB ligands with sActRIIB-Fc increased the muscle mass but decreased the aerobic profile of the mdx mice. However, voluntary wheel running shifted the expression of genes involved in aerobic metabolism towards healthy wildtype mice. (Hulmi et al. 2013b; Kainulainen et al. 2015.) In addition, unpublished microarray results suggested that sedentary mdx mice showed decreased expression of genes involved in glutathione metabolism and voluntary wheel running increased their expression suggesting that running changed the redox balance in mdx mice warranting a further analysis of oxidative stress parameters in these mice.

The purpose of this thesis was to find out individual and combinatory effects of sActRIIB-Fc administration and voluntary wheel running on redox balance in mdx mice. Redox-balance was indirectly assessed by measuring protein carbonyls and oxidized and reduced glutathione levels. In addition, protein expression of sirtuins 1, 3, 6 and AMPK were measured.
2 MUSCULAR DYSTROPHIES AND mdx MOUSE MODEL

Duchenne and Becker muscular dystrophies (DMD/BMD respectively) are X chromosome linked muscle wasting diseases accounting for over 80% of all muscle dystrophies. Boys suffering from DMD are diagnosed as little children and most are bound to wheelchair during their puberty. DMD patients often die at the age of 20 from respiratory complications or cardiomyopathy. More patients survive to the age of 30 due to home ventilation and corticosteroids. Even if there are beneficial effects of using corticosteroids, 25% of patients are not treated with them due to severe side-effects or lack of response. (Thomas 2013.) Approximately 1 of 3500 boys suffers from this severe muscle dystrophy disease (Willman et al. 2009).

BMD is milder as a disease than DMD and is more clinically heterogenous than DMD. Muscle weakness often occurs in the adolescence or young adulthood. In BMD, cardiac decline may surpass muscle weakness in the major cause of death. BMD patients often die to cardiac myopathy before the age of 60. (Thomas 2013.)

Monaco et al. (1986) and Koenig et al. (1987) were the first to discover the major genetic mutations causing the DMD and BMD. DMD was discovered to be caused by absence of functional protein called dystrophin. Furthermore, in BMD mutations reduced amount of dystrophin or shortened functional protein was the disease causing factor (Thomas 2013).

Dystrophin is a 427-kDa cytoskeletal protein that is a member of the β-spectrin/α-actinin protein family (Blake et al. 2002). Full length dystrophin is composed of four distinct structural domains, 1) an N-terminal “actin binding” domain, 2) a middle “rod” domain consisting of spectrin-like repeats, 3) a cysteine rich domain and 4) carboxyl-terminal domain. Full-length dystrophin is homogenously located at the cytoplasmic face of the sarcolemma and it is part of a macromolecular group of proteins collectively referred to as the dystrophin associated protein-complex (DAP). It is known that dystrophin links the intracellular microfilament network of actin to the extracellular matrix. The absence of dystrophin leads to the loss of DAPs at the sarcolemma. This leads to the absence of physical link between interior and exterior of the muscle making the
sarcolemma fragile and the muscle fiber susceptible to degeneration during repeated cycles of muscle contraction and relaxation. (Chakallaka et al. 2005.) The damage to sarcolemma leads to myofibre necrosis consequently leading to inflammation, myogenesis and new muscle formation to regenerate the tissue. Furthermore, repeated cycles of damage and inflammation results in replacement of muscle tissue by fat and fibrous connective tissue consequently leading to severe loss of muscle function. (Terrill et al. 2013.)

Dystrophin and its associated components also serve as a signaling scaffold that is responsive to extracellular stressors. Signaling cascades do not work correctly in dystrophin deficient muscle which likely contributes to the disease pathology. DMD patients also show elevated levels of intracellular calcium leading to aberrant hyperactivation of signaling cascades involved in the inflammatory response. (Chakallaka et al. 2005).

mdx mouse model is one of the most used animal model of DMD and was also used in this thesis. mdx mice are genetically mutated so that they do not have full length dystrophin. Such mutation covers one third of DMD patients. The lifespan of mdx mice is shorter than that of wild type animals. (Willman et al. 2009.) However, the pathology of mdx mice is milder compared to human Duchenne’s muscular dystrophy patients and thus is closer to Becker’s muscular dystrophy. The reason for milder pathology for mdx mice has been proposed to be that growth as well as muscle size and mechanical loading increases the severity of dystrophopathy, which is less pronounced for example in mdx mice. (Terrill et al. 2013.) It has to be also mentioned that mdx mice show compensatory expression of utrophin (homologue of dystrophin), which ameliorates the symptoms of muscular dystrophy (Grady et al. 1997). The muscle degeneration in mdx mice comes in waves and is not continuous like in human DMD patients. mdx mice living in cage show symptoms of muscle weakness only when they are old, whereas DMD patients suffer severely from symptoms already during their adolescence and puberty. In addition to deleterious effects on skeletal muscle, mdx mice suffer from abnormal cardiac function and from cardiomyopathy. (Willman et al. 2009.)
3 AEROBIC EXERCISE

Aerobic exercise is defined as exercise where most of the ATP is produced in oxidative phosphorylation in mitochondria (McArdle et al. 2010, 452). Exercise/physical activity is beneficial to health and it prevents the risk of several chronic conditions (Booth et al. 2012), which are summarized in table 1.

TABLE 1. List of the chronic conditions that are prevented by regular exercise (adapted form Booth et al. 2012)

<table>
<thead>
<tr>
<th>Metabolic disorders</th>
<th>metabolic syndrome, obesity, insulin resistance, prediabetes, type 2 diabetes, non-alcoholic fatty liver disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular diseases</td>
<td>coronary heart disease, peripheral artery disease, hypertension, stroke, congestive heart failure, endothelial dysfunction, arterial dyslipidemia, hemostasis, deep vein thrombosis</td>
</tr>
<tr>
<td>Mental illness</td>
<td>cognitive dysfunction, depression, anxiety</td>
</tr>
<tr>
<td>Bones and joints</td>
<td>osteoporosis, osteoarthritis, balance, bone fracture/falls, rheumatoid arthritis</td>
</tr>
<tr>
<td>Cancers</td>
<td>colon cancer, breast cancer, endometrial cancer</td>
</tr>
<tr>
<td>Pregnancy and fecundity</td>
<td>gestational diabetes, pre-eclampsia, polycystic ovary syndrome, erectile dysfunction</td>
</tr>
<tr>
<td>Muscle and ventilatory system</td>
<td>low cardiorespiratory fitness (VO2max), sarcopenia</td>
</tr>
<tr>
<td>Others</td>
<td>constipation, gallbladder diseases, accelerated biological aging/premature death, diverticulitis</td>
</tr>
</tbody>
</table>

3.1 Physiological adaptations to aerobic exercise in skeletal muscle

Regular aerobic exercise changes the metabolic properties and the phenotype of the skeletal muscle. In addition to skeletal muscle, regular aerobic exercise causes beneficial adaptations also in other tissues. (Booth et al. 2012.) Basic physiological and mo-
lecular adaptations to regular aerobic exercise are briefly summarized in the sections 3.1.1 and 3.1.2. The main focus is on skeletal muscle.

### 3.1.1 Basic physiological adaptations of to aerobic exercise

Regular aerobic exercise training leads to increased mitochondrial biogenesis, which is a process that increases the amount of total mitochondria and mitochondrial proteins in skeletal muscle. In addition, the aerobic metabolism enzyme activity is increased in trained muscles. This enables larger capacity to produce ATP aerobically without accumulation of metabolic byproducts from anaerobic glycolytic metabolism delaying the onset of muscle fatigue. (Hood 2001.) However, it has to be mentioned that byproducts from anaerobic metabolism are just one factor causing muscle fatigue and inhibiting muscle contraction (Allen et al. 2008). Aerobically trained muscles possess increased ability to oxidize fatty acids during submaximal workloads and increased ability to utilize carbohydrates during maximal workloads. This is a consequence of increased fatty-acid and carbohydrate enzyme activity and larger amount of mitochondria. (McArdle et al. 2010. 459–460.) In addition, transport of glucose and free fatty acids to working skeletal muscles is enhanced in response to regular exercise. This is due to greater expression of glucose and free fatty acid transport proteins on sarcolemma. (Richter & Hargreaves 2013; Talanian et al. 2010.)

Regular exercise has been shown to increase the transformation of glycolytic type IIb to more oxidative muscle fiber type IIa. In addition, professional endurance athletes that have practiced for years have increased amount of type I fibers. However, exercise-induced transformation to type I fibers remains unsolved. (Yan et al. 2010.) Furthermore, aerobic exercise training leads to increased skeletal muscle capillary density. This allows greater blood flow to the working muscle enabling greater supply of oxygen and nutrients to the muscle and removal of metabolic by-products from the muscle. (Barry et al. 2004.)
3.1.2 Global gene expression level adaptations of skeletal muscle to aerobic exercise

Gene expression level adaptations of skeletal muscle to aerobic exercise were first studied using RT-PCR techniques (mRNA expression) and immunoblotting (protein expression). In these studies, it was found that genes regulating energy metabolism like PDK4, UCP3, HO-1, LPL, HKII and GLUT4 were the most up-regulated during recovery from exercise (Pilegaard et al. 2000). After that, micro-array techniques have enabled the global gene expression studies in response to aerobic exercise. Mahoney et al. (2005) examined global mRNA expression in human skeletal muscle during recovery after aerobic endurance exercise (high-intensity cycling ~ 75 min.). Statistically significantly up-regulated genes were involved in 1) fatty acid and carbohydrate metabolism and mitochondrial biogenesis, 2) oxidant stress response, 3) electrolyte transport across membranes and 4) genes involved in cell stress, proteolysis, apoptosis, growth, differentiation and transcriptional activation. In addition to global mRNA expression after one single bout of exercise, Timmons et al. (2005) examined the global mRNA expression after 6 weeks of exercise training using microarray analysis. The most upregulated gene groups were related to calcium ion binding and extracellular matrix related proteins. Gene expression adaptations related to mitochondrial biogenesis and energy metabolism are briefly reviewed in the next paragraph. Gene expression adaptations to oxidants are reviewed in chapter 5.6.

Increased mitochondrial biogenesis, carbohydrate and fatty acid oxidative capacity and determination of muscle fiber type is at least to some extent being mediated by a well-known regulator of aerobic metabolism, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α) and its receptor peroxisome-proliferator-activated receptor-alpha (PPAR-α). To support this claim, regular aerobic exercise training leads to increased expression of them both. PGC-1α is a transcription factor that activates PPAR-α and its isoforms which regulate transcription of many genes involved in glucose metabolism and fatty acid metabolism. (Baar et al. 2002; Lin et al. 2002; Russell et al. 2003.) Furthermore, PGC-1α activates proteins such as NRF-1, -2 and ERRs, which will increase the transcription of mitochondrial proteins related to oxidative capacity, such as cytochrome c and cytochrome oxidase IV (Geng et al. 2010; Jornavayz &
Shulman, 2010). Aerobic exercise leads to PGC-1α activation by post-translational modifications. These modifications are mediated at least by AMPK (AMP-sensitive protein kinase that phosphorylates PGC-1α), SIRT1 (deacetylates and activates PGC-1α), and p38MAPK (mitogen-activated protein kinase that also phosphorylates PGC-1α). (Jornayvaz & Shulman, 2010). During exercise, intracellular concentration of calcium ions increases in skeletal muscle, which leads to upregulated expression of PGC-1α by calcineurin and CaMKK (Kusuhara et al. 2007). PGC-1α mediated signalling pathways regulating mitochondrial biogenesis and fatty-acid metabolism are summarized in figure 1. In addition, PGC-1α also up-regulates the transcription of GLUT4, thus promoting skeletal muscle glucose uptake (Baar et al. 2002). While PGC-1α has been suggested to be a “master regulator” of aerobic exercise (Chinsomboon et al. 2009), some studies suggest that PGC-1α is not needed for some exercise adaptations (Rowe et al. 2012). It has been shown that in response to aerobic exercise, the most upregulated genes involved in fat oxidation are those genes that are involved in regulating FA uptake across the plasma membrane e.g fatty acid translocase (FAT/CD36) and across the mitochondrial membrane e.g carnitine palmitoyltransferase (CPT1) (Bonen et al. 1999; Tunstall et al. 2002).

FIGURE 1. Signalling pathways thought to be mediated by PGC-1α regulating mitochondrial biogenesis and fatty acid metabolism. (Ventura-Claperier et al. 2007).
3.3 Muscle dystrophies and aerobic exercise

The health benefits of regular exercise are widely known (for a review see Booth et al. 2012), but the benefits of exercise for DMD patients are not totally clear. The problem is that, it is not known what kind of exercise could be suitable for DMD patients without increased muscle damage from exercise. In order to study this, mdx mouse model has been the most used animal model. Both voluntary wheel running and forced treadmill running are used as exercise mode and it seems that voluntary wheel running is more suitable mode of exercise for mdx mice. (Grange & Call 2007.) It is known that mdx mice run voluntarily significantly less than wild-type mice (Dupont-Versteegden et al. 1994; Carter et al. 1995). In addition, young mdx mice run more than old mdx mice (Carter et al. 1995). Many studies that have found that aerobic exercise is beneficial for human dystrophy patients and mdx mice follow. Sveen et al. (2008) showed that sub-maximal aerobic cycle ergometer training improves aerobic physical fitness and force production of muscles involved in cycling without damage to muscles in BMD human patients. Baltgalvis et al. (2010) showed that 12 week period of voluntary low-intensity running leads to improved resistance to fatigue and to improved muscle force production of plantar-flexor muscles in mdx mice. Selsby et al. (2013) showed that long-term voluntary wheel running improves functions of cardiac and plantar flexor muscles, but may have some side-effects on diaphragm muscle. Hourde et al. (2013) found that voluntary wheel running was beneficial for skeletal muscle of the mdx mice, but detrimental to the heart muscle. Call et al. (2008) showed that 3 weeks of low resistance voluntary wheel running increased extensor digitorum longus tetanic stress, total contractile protein content, heart citrate synthase and quadriceps beta-hydroxyacyl-CoA dehydrogenase (enzyme in β-oxidation) activity. Most recently from the datasets used in the present thesis, Kainulainen et al. (2015) showed that 7 weeks of voluntary running increases the gene expression of aerobic metabolism and oxidative capacity on mdx mice. All in all, it seems that at least voluntary low-resistance dynamic exercise has many positive effects on the skeletal muscle of mdx mice, but may have some side-effects for example on diaphragm and heart muscle. However, in one study, 9 weeks of voluntary wheel running did not improve muscle’s oxidative capacity like it did for wild-type mice (Landisch et al. 2008). In contrast, Kainulainen et al. (2015) showed that 7 weeks of voluntary running increases the gene expression of aerobic metabolism and
oxidative capacity on mdx mice. It needs to be mentioned that forced high intensity treadmill running has been shown to worsen the pathology of DMD in mdx mice (De Luca et al. 2003; Cameroni et al. 2014).
4 MYOSTATIN AND ACTIVINS AND THEIR RECEPTORS

Myostatin, also known as growth/differentiation factor 8 (GDF-8) is a member of transforming growth factor (TGF-β) of signalling cytokines (Matsakas & Diel 2005; Elkina et al. 2011). It is known to be a negative regulator of skeletal muscle mass: myostatin null or myostatin gene mutated mammals show 2–3 times bigger skeletal muscle mass compared to wild type mammals (McPherron et al. 1997; Clop et al. 2006; Mosher et al. 2007). Myostatin controls the differentiation and proliferation of skeletal muscle throughout the embryonic development. In addition, it controls muscle homeostasis in the adult (Walsh & Celeste 2005). Activins are also members of TGF-β superfamily (Wrana 2013). Activins A and B promote muscle wasting pathways, as overexpression of these proteins leads to severe loss of muscle mass (Chen et al. 2014). Myostatin and activins mediate their signalling through activin receptors of which type IIB is especially important in skeletal muscle (Lee & McPherron 2001). ActRIIB receptor binding ligands including myostatin and activins were blocked in this thesis using soluble fusion-protein of ActRIIB (sActRIIB-Fc) in order to promote muscle hypertrophy. Summary of the basics of myostatin and activins and their receptor ActRIIB are summarized below. In addition, a short summary of myostatin/activin blocking strategies focusing on administration of sActRIIB-Fc and its effect on the phenotype of the skeletal muscle are summarized below.

4.1 Myostatin Structure

Myostatin is a homodimer protein with a molecular weight of 25 kDa. During embryogenesis, myostatin is exclusively expressed at skeletal muscle, but during adulthood it is also expressed to some extent in other tissues (e.g. heart, adipose-tissue and mammary gland) (Elkina et al. 2011). Myostatin is first translated in skeletal muscle as a precursor 375 amino-acid propeptide, which is proteolytically cleaved after its formation (figure 2). After that, myostatin is secreted as a latent complex (Thomas et al. 2000). The secreted configuration of myostatin is biologically inactive. It contains propeptide region (38 kDa) and disulfide linked dimer mature C-terminal peptide domains (12 kDa). These compartments are non-covalently associated with each other. Myostatin propeptide region helps to guide the proper folding and dimerization of the mature C-
terminal peptide and regulates the biological activity of the C-terminal dimer through the formation of a latent complex. Myostatin is found in latent inactive form in circulation. (Walsh & Celeste 2005; Huang et al. 2011.)

**FIGURE 2.** Proteolytic processing of myostatin protein. Precursor myostatin that is composed of a signal peptide (SP), an N-terminal propeptide domain and a C-terminal domain are first cleaved by furin family enzymes to remove the signal peptide. The resulting peptides are dimerized, through disulfide bonds at the indicated position form latent myostatin and are then cleaved by BMP1/Tolloid matrix metalloproteinase enzymes producing mature myostatin. (Huang et al. 2011.)

### 4.2 ActRIIB receptor and its signalling pathways

As mentioned, myostatin and activins mediate the signal through activin receptors (mostly binds to ActRIIB) like many other members of TGF-β family. Activin receptors are transmembrane threonine/serine kinases are divided into two types. Type I receptor (ALK4 and ALK5 for myostatin) has a unique GS domain, which is rich in glycine- and serine-residues located closely to the intracellular space and adjacent to the kinase domain, which is absent in type II receptors. Binding of ligand to activin receptor II causes its assembly with type I receptor and phosphorylation of its GS domain. Thus, the signal of ActRIIB ligands is mediated through activated complex of two receptors. (Elkina et al. 2011; Huang et al. 2011.)
The molecular mechanisms of ActRIIB receptor signalling actions are not very well understood. It is suggested that binding of activins and myostatin to ActRIIB and thus, formation of type I and II receptor complex results in the phosphorylation of two serine residues of Smad 2 or Smad 3 at carboxyl (COOH) domains. This leads to the assembly of Smad2/3 with Smad 4 to the heterodimer that is able to translocate to nucleus where it is able to activate transcription of target genes, eventually suppressing e.g. myogenesis. MyoD (a transcriptional factor that is involved in skeletal muscle development and repair of damaged skeletal muscle) is one of the known downstream signalling targets of Smad signalling. Furthermore, Smad signaling targets other genes such as myogenic factor 5 (Myf5) and myogenin, which are known to be important for myogenesis. (Elkina et al. 2011; Huang et al. 2011.)

There are also other non-Smad pathways that participate in myostatin signal transduction. One of these pathways is MAPK signalling pathway. It is shown that myostatin activates the p38 MAPK through the TAK1-MKK6 cascade. In addition, it is shown that p38 MAPK plays an important role in myostatin-regulated inhibition of myoblast proliferation. The other known non-Smad pathways are Ras/Erk 1/2 and JNK signalling which is activated by TAK1-MKK4 cascade. In both of these signalling pathways ActRIIB is involved in signal transduction. Ras/Erk 1/2 signalling pathway is shown to suppress myoblast proliferation in C2C12 myoblasts. Furthermore, JNK signalling pathway is shown to participate in myostatin-regulated inhibition of myoblast proliferation and differentiation. (Huang et al. 2011.) ActRIIB-mediated signalling pathways are summarized in figure 3.
As it was mentioned earlier, myostatin and activins signal through ActRIIB receptors. Myostatin and activin binding to the ActRIIB can be inhibited using certain exogenous molecules for example follistatin (targets e.g. myostatin and activins) and its modified forms (targets mostly only myostatin), myostatin propeptide, JA16 (neutralizing antibody to myostatin), sActRIIB-Fc (soluble ligand binding domain of type Iib activin receptor (ActRIIB) fused to the Fc domain of IgG) and GASP1. (Whittemore, et al. 2003; Bogdanovich et al. 2005; Chiu et al. 2013; Smith & Lin 2013.) sActRIIB-Fc was used
in this thesis in order to block myostatin and activin binding to their receptor. Thus, the focus will be on sActRIIB-Fc in this literature review.

Administration of soluble activin type IIB receptor is shown to increase muscle growth independent of muscle fiber type in wild-type mice. According to Cadena et al. (2010), the increase in wet muscle mass is approximately 30–40% in wild-type mice. Administration of sActRIIB has become promising treatment to muscle dystrophies and other muscle wasting conditions. It is shown in some studies that blocking ActRIIB ligands with sActRIIB-Fc improves skeletal muscle mass (Pistilli et al. 2011; Hoogars et al. 2012) and functional strength of mdx mice, thus ameliorating the symptoms of DMD (Pistilli et al. 2011). In addition, blocking ActRIIB ligands with sActRIIB-Fc attenuated the hypoxia induced loss of muscle mass (Pistilli et al. 2010). Furthermore, other myostatin blocking strategies have also shown beneficial effects for treatment of DMD similarly to sActRIIB-Fc (Bogdanovich et al. 2002; Bogdanovich et al. 2005). It is shown that myostatin directly stimulates the proliferation of muscle fibroblasts and the production of extracellular matrix proteins e.g. collagen, thus increasing muscle fibrosis (Li et al. 2008). To support this, mdx mice that are myostatin blocked either by exogenous agents or by genetic modifications show decreased level of muscle fibrosis (Bogdanovich et al. 2002; Wagner et al. 2002; Qiao et al. 2008; Nakatani 2008). In addition, adeno associated virus mediated gene transfer of a soluble form of the extracellular domain of the activin IIB receptor to liver and thus, into circulation has been shown to increase the muscle mass and force production of EDL muscle in mdx mice (Morine et al. 2010).

sActRIIB-Fc binds also to multiple ligands and thus, inhibits their binding to ActRIIB receptor. Souza et al. (2008) found using mass spectrometry-based proteomics and *in vitro* assays that ActRIIB-Fc binds to myostatin, activins A, B and AB, and bone morphogenetic proteins -9, -10, -11. They also found *in vitro* that activins -A, -B, -AB and BMP-11 may negatively regulate muscle growth similarly as myostatin, because they could be blocked from inhibiting the myoblast to myotube differentiation with soluble ActRIIs. Thus, it can not be said that alterations to muscle phenotype would be only a result of blocking myostatin signalling, when soluble ActRIIs are used.
Blocking ligands of ActRIIB has also unfavourable effects to skeletal muscle of mdx mice. It has been shown that hypertrophied muscles of mdx mice treated with sActRIIB-Fc show decreased oxidative capacity and lower levels of key oxidative enzymes and transcription factors. (Kainulainen et al. 2015; Relizani et al. 2014; Hulmi et al. 2013a; Rahimov et al. 2011.) However, voluntary wheel running seems to reverse these side effects shifting the phenotype of skeletal muscle towards healthy wild-type mice (Kainulainen et al. 2015).
5 OXIDATIVE STRESS AND REDOX IN SKELETAL MUSCLE

Oxidation is defined as the removal of electrons and reduction as the gain of electrons. Oxidation is always accompanied by reduction of an electron acceptor (Martin et al. 1983, 124). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal metabolism (Sies 1997) and they are both beneficial (e.g. Gomez-Caprera et al. 2008b; Ristow et al. 2009) and harmful (for a review see Valkon et al. 2007) to living species. An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed oxidative stress (Sies 1997).

Oxygen is poisonous and aerobic organisms only survive its presence, because they contain antioxidants. Antioxidants can be synthesized in vivo or taken in from the diet. Halliwell & Gutteridge (2007) define the antioxidant as any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. Oxidizable substrates include every molecule found in vivo. However, the definition is somewhat imperfect, because it does not for example take into account the chaperones or inhibitors of reactive species generation. Thus, the definition is simplified as “any substance that delays, prevents or removes oxidative damage to a target molecule”. (Halliwell & Gutteridge 2007.)

5.1 Free radicals and ROS

Free radicals also known as ROS and RNS, can be defined as molecules or molecular fragments containing one or more unpaired electrons on their atomic or molecular orbitals. Radicals containing oxygen represent the most important group of radical species generated in living systems. Molecular oxygen is in itself a free radical due to its unique electronic configuration. (Valkon et al. 2007.) Below is brief overview of ROS.

Superoxide. Superoxide (O$_2^-$) is mostly formed as an intermediate in biochemical reactions. These biochemical reactions include e.g. the electron transport chain as a part of oxidative phosphorylation. In addition, inflammatory cells produce relatively large amounts of superoxide in the process as they defend cells from invading organisms. Superoxide has relatively long half-life compared to other radicals, which enables its dif-
fusion within the cytoplasm and increasing the number of its potential targets. Superoxide has both reducing and oxidizing capabilities, as it can reduce some biological materials (e.g. cytochrome c) and oxidize others (ascorbate). Spontaneous and catalyzed dismutation reaction of superoxide creates hydrogen-peroxide molecules. In fact, superoxide dismutation is major source of hydrogen peroxide in cells. (Powers & Jackson, 2008.)

*Hydrogen peroxide.* Hydrogen peroxide ($H_2O_2$) is a reactive compound that has stable structure, is membrane permeable and has relatively long half-life within the cells. Hydrogen peroxide is able to generate free radicals, such as the hydroxyl radical in specific circumstances. However, hydrogen peroxide does not have the capability of oxidizing DNA or lipids directly, but can inactivate some enzymes. $H_2O_2$ is considered as a relatively weak oxidizing agent, but it is cytotoxic to cells primarily due to its ability to generate hydroxyl radicals through metal catalyzed reactions. (Powers & Jackson, 2008.)

*Hydroxyl radicals.* Hydroxyl radicals (OH$^-$) are potentially the most damaging ROS in biological organisms. They are not permeable to membranes and damage molecules close to their site of generation. (Powers & Jackson, 2008.)

There are also other major primary radicals in cells. Singlet oxygen, nitric oxide ($NO^-$), peroxynitrite (ONOO$^-$) and hyperchlorite (HOCl$^-$) are few to be mentioned. Singlet oxygen is an electronically excited form of oxygen that has very short half-life but is capable of diffusion and is membrane permeable. Nitric oxide is synthesized from amino acid L-arginine in reaction that occurs in many cell types. In this reaction, the nitric oxide synthases convert L-arginine into NO and L-citrulline utilizing NADPH. NO is a weak reducing agent that reacts with oxygen to form nitric dioxide. In addition, it reacts very rapidly with superoxide anion to produce peroxynitrite. Peroxynitrite is a strong oxidizing agent that can damage DNA, lead to depletion of thiol groups and nitration of proteins. Hyperchlorite is mostly formed by neutrophils by the action of myeloperoxidase utilizing hydrogen peroxide. Hyperchlorite has the capability of oxidizing thiols, lipids, ascorbate and NADPH with the generation of various secondary products. (Powers & Jackson, 2008)
5.2 Antioxidants

Because of the continuous free radical production, living organisms have developed defence mechanisms against oxidative stress (Valkon et al. 2007). In healthy aerobic organisms, the reactive oxygen species are approximately balanced with the antioxidant defence. However, this balance is not constantly perfect and ROS mediated damage occurs continuously. Thus, antioxidant defences rather limit the ROS damage than eliminate it. This is because of two reasons. First, maintaining excess antioxidant defence would be energetically too expensive compared to repairing or replacing damaged biomolecules. Secondly, antioxidants can not simply handle some very reactive species such as free hydroxyl radicals (OH\(^-\)) that react with anything. (Halliwell & Gutteridge 2007.) In addition, ROS are needed to some extent e.g. in optimal force production (Reid et al. 1993) and exercise derived adaptations (Gomez-Caprera et al. 2008b; Ristow et al. 2009).

Aerobic organism protects itself from oxidative stress at many levels (Valkon et al. 2007). The first level of protection is prevention. For example, enzymes which are prone to generate free radicals are designed in such a way that they do not release free radicals to their surrounding space. A good example of such enzyme is cythocrome oxidase, which is mostly responsible for cellular oxygen reduction. Its three-dimensional “cage-like” structure inhibits the release of free radicals. (Sies 1997.)

The interception of oxidants is the second level of anti-oxidative defence and it can be divided into non-enzymatic antioxidant defence and enzymatic antioxidant defence (Sies 1997). Enzymatic and non-enzymatic antioxidants exist both in both extracellular and vascular spaces. The most important anti-oxiadative enzymes are glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutases (SOD). (Powers & Jackson 2008). Below is a brief overview of these enzymes

**Superoxide dismutase (SOD).** SOD forms the first line of defence against superoxide radicals as it catalyses the dismutation reaction, in which superoxide radicals form hydrogen peroxide and oxygen. There are three isoforms of SOD found in mammals. Two
of these isoforms are found within the cells and one of them is found in the extracellular space. (Powers & Jackson 2008.)

*Glutathione peroxidase (GPx).* There are five different glutathione peroxidase isoforms. All of these GPxs catalyze the reduction of hydrogen peroxide or organic hydroperoxide to water and alcohol. This reaction uses reduced glutathione (GSH), or in some cases thioredoxin or glutaredoxin as an electron donor. GSH donates a pair of hydrogen ions, when it is the electron donor. This reaction leads to oxidation of GSH and formation of glutathione disulfide (GSSG). GSSG is then reduced back to GSH by glutathione reductase. NADPH provides the reducing power of this reaction. (Powers & Jackson 2008.)

*Catalase (CAT).* Catalase catalyzes the break-down of $\text{H}_2\text{O}_2$ into water and oxygen among other cellular functions that are not listed here. Although it has the same substrate as GPx, CAT has lower affinity to $\text{H}_2\text{O}_2$ compared to GPx at low concentrations of $\text{H}_2\text{O}_2$ (Powers & Jackson 2008.)

Non-enzymatic antioxidants include e.g. glutathione (GSH), uric acid, bilirubin and α-lipoic acid (Powers & Jackson 2008). In addition, there are many other non-enzymatic antioxidants, which are not listed here. Brief summary of aforementioned non-enzymatic antioxidants follows.

*GSH.* Glutathione is a tri-peptide and is one of the most important non-enzymatic antioxidants in muscle fibers. GSH is primarily synthesized in liver and transported to other tissues via the circulation. GSH concentration is much higher in type I fibers compared to type IIb fibers. GSH can react with radicals itself by donating electrons to them thus, oxidizing itself to GSSG. As previously was mentioned, it also serves as a substrate to GPx to eliminate hydrogen- and hydroperoxide levels. GSH can also reduce other antioxidants for example vitamin E and C. (Powers & Jackson, 2008.) Oxidative stress can be measured e.g. from the ratio of oxidized glutathione and reduced glutathione (GSSG/GSH). When the ratio is bigger the more there is oxidative stress. (Valkon et al. 2007.)
α-Lipoic acid. α-Lipoic acid is a natural compound that can be obtained from different kinds of foods. α-Lipoic acid is usually bound to enzyme complexes, in which it serves as a co-factor for α-dehydrogenase to participate in S-O transfer reactions. Reduced and unbound form of α-lipoic-acid and several of its metabolites are effective antioxidants, which can also participate in vitamin C recycling. (Powers & Jackson, 2008.)

Uric acid. Uric acid is a by-product of purine metabolism and almost all of uric acid is converted to urate at physiological pH. Urate is able to scavenge levels of peroxyl radicals, hydroxyl radicals and singlet oxygen by donating electrons to them. It is considered that urate is an important low-molecular-mass antioxidant in human biological fluids. It is also able to chelate metal ions (copper and iron) in order to prevent them from catalyzing hydroxyls radicals. (Powers & Jackson, 2008.)

Bilirubin. Bilirubin is the final product of hemoprotein catabolism and it is a strong antioxidant against peroxyl radicals although bilirubin is reducing species. Bilirubin is oxidized back to biliverdin and then recycled back to bilirubin via biliverdin reductase. (Powers & Jackson, 2008.)

Dietary antioxidants. There are a vast number of antioxidants that are obtained from diet, from which vitamin E, vitamin C, and carotenoids are probably the most important ones. Both vitamin E and carotenoids are located in the membrane of tissues and they protect cells from lipid peroxidation scavenging several different ROS species including superoxide and peroxyl radicals. Whereas carotenoids and vitamin E are lipid-soluble, vitamin C is hydrophilic and thus, functions better as an antioxidant in aqueous environments. Ascorbate anion is the predominant form of vitamin C and its role as an antioxidant is twofold compared to vitamin C. Vitamin C can directly scavenge superoxide, hydroxyl, and lipid hydroperoxide radicals. Secondly, vitamin C plays an important role in the recycling of vitamin E oxidizing itself to vitamin C radical (semiascorbyl). Semiascorbyl radical is then reduced back to vitamin C by NADPH semiascorbyl reductase, glutathione or dihydrolipoic acid. (Powers & Jackson, 2008.)

Because prevention and interception against ROS do not work perfectly, cells have the capacity to repair the cellular compartments that are damaged by ROS. Oxidative damage includes DNA damage (base damage, single- or double-strand bond breakage),
membrane lipid damage, damage to proteins and other cellular compartments. There are several enzyme systems in cells, which are responsible for the repairing damaged DNA, lipids, proteins and other compartments. (Sies 1997.)

5.3 Oxidative damage and its major consequences

ROS can damage cells compartments, if they are present in high concentrations. It is known that hydroxyl radicals react with all the compartments of DNA damaging its structures. This can cause permanent mutations in DNA, leading to first steps of mutagenesis, carcinogenesis and ageing. Furthermore, ROS generated by metal ions can damage polyunsaturated fatty acids, leading to a peroxidation process. The major final product of this process is malondialdehyde (MDA), which is mutagenic in mammals and carcinogenic in rats. The other major end-product of this process is 4-hydroxy-2-nonenal (HNE), which is weakly mutagenic, but the major toxic product of lipid peroxidation. ROS and also RNS (reactive nitrogen species) cause damage to all amino acid residues especially to cysteine and methionine. Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups and low molecular weight thiols particularly in GSH. (Valkon et al. 2007.) Oxidative damage of proteins can be measured e.g. from the concentration of carbonyl groups. The protein carbonyls are formed by a direct metal catalyzed oxidative attack on the side chains of certain amino acids. The level of protein carbonyls are increased due to increased levels of ROS, or due to decreased levels of anti-oxidative system. Carbonylated protein aggregates can become cytotoxic and are associated with a large number of age-related diseases. (for a review see Nyström 2005.)

ROS is linked to several pathological processes such as cachexia, atherosclerosis, cancer, ischemia/perfusion, inflammation, rheumatic arthritis and neurodegenerative diseases such as Alzheimer and Parkinson diseases. In addition, ROS is believed to affect the process of aging. The major consequence of chronic elevated ROS regarding skeletal muscle, is muscle atrophy. (Radak et al. 2008.)
5.4 Sirtuins and oxidative stress

Sirtuins are NAD$^+$-dependent protein and histone deacetylases or mono-ADP ribosyltransferases. In addition, they have demalonylation and desuccinylation activity. (Kelly et al. 2010.) Sirtuins catalyze the removal of acetyl groups from the side chain amino acid group of lysine residues. This reaction consumes NAD$^+$ and generates nicotinamide and 2’-O-acetyl-ADP-ribose. (Merksamer et al. 2013.) There are seven different sirtuins found in mammals. These seven sirtuins are located in three different parts of the cell. SIRT6 and 7 are localized in nucleus, SIRT3, 4 and 5 are found in mitochondria and SIRT1 and 2 are found in nucleus and cytoplasm. Sirtuins regulate many cellular functions such as aerobic metabolism, oxidative stress management and longevity. They regulate cellular functions either by increasing or decreasing gene expression, or by regulating several enzymes either by activating or silencing them. (Kelly et al. 2010.) The activity of most of the sirtuins is regulated by post-translational modifications, as well as by the availability of NAD$^+$, in other words redox-balance of the cell. One post-translational modification of sirtuins is phosphorylation at N- and C-terminals, which plays a role in substrate binding. (Radak et al. 2013.) Sirtuin 1 is phosphorylated at 13 different serine and threonine residues in vivo in human cells (Sasaki et al. 2008). Sirtuin 1 is at least phosphorylated by Cyclin B/Cdk1 (threonine 530 & serine 540), DYRK1A (Threonine 522), DYRK3 (threonine 522), JNK1 (serine 27, serine 47 & threonine 530), CAMKKβ (serine 27 & serine 47) in different types of cells (Sasaki et al. 2008; Nasrin et al. 2009; Guo et al. 2010; Wen et al. 2013). SIRT1, p-SIRT1 at Ser 46 (was measured using an antibody specific to a sequence against human sirtuin at Ser47), as well as SIRT3 and 6 were studied in this thesis. Brief summary of their role regarding oxidative stress and exercise follows.

5.4.2. Sirtuin 1

SIRT1 is known to mediate the oxidative stress directly by deacetylating several transcription factors, which promote the expression of antioxidative genes (Merksamer et al. 2013). Brunet et al. (2004) and Motta et al. (2004) showed that in response to oxidative stress SIRT1 deacetylates several FOXO family members of transcriptors, which promote the expression of anti-oxidative enzymes including SOD and catalase. Further-
more, it is shown by Kobayashi et al. (2005) that SIRT1 plays an important role in FOXO4-mediated GADD45 expression in response to oxidative stress that may contribute to cellular stress resistance and longevity by DNA repair, because GADD45 is required for efficient DNA base excision repair and nucleotide excision repair. SIRT1 deacetylation of FOXO-family members primarily occurs at nucleus, when FOXOs are migrated there as a consequence of elevated ROS levels (Radak et al. 2013). SIRT1 also deacetylates PGC-1α, which consequently increases mitochondrial mass and up-regulates the expression of oxidative stress genes including glutathione peroxidase (GPx), catalase (CAT) and manganese SOD (MnSOD) (Merksamer et al. 2013; Radak et al. 2013). SIRT1 inactivates the p65 subunit of nuclear factor kappa B (NF-kB) through direct deacetylation, which suppresses the transcriptional activity of NF-κB. This decreases the NF-κB mediated inflammatory processes of the cells and prevents the TNF-α mediated activation of matrix metalloproteinase 9, interleukin 1β, IL6, nitric oxide synthase and inducible nitrous oxide production, consequently suppressing the inflammation processes and ROS production. (Merksamer et al. 2013; Radak et al. 2013.) Furthermore, SIRT1 deacetylates p53 thus, inactivating its enhancement of ROS production. p53 enhances ROS production through mitochondrial dysfunction and/or increased expression of genes that are involved in redox modulation such as p53-upregulated modulator of apoptosis (PUMA), NADPH activator A (NOXA) and p53 induced gene 3 (PIG-3). (Radak et al. 2013.)

SIRT1 protein expression seems to increase acutely after one exercise training session in parallel with other metabolic enzymes such as GLUT4, HKII and PGC-1α, at least in healthy rat soleus muscle, which contains mainly oxidative slow-twitch muscle fibers (Suwa et al. 2008). Furthermore, it has been shown that exercise increases the activity of SIRT1 due to increased activity of NAMPT (nicotinamide phosphoribosyltransferase, which is involved in NAD⁺ synthesis) in old exercising rats. Increased activity of NAMPT consequently led to increased levels of NAD⁺, which is the catalytic fuel of SIRT1. The increased activity of SIRT1 due to exercise decreased the hallmarks of oxidative stress measured by the amount of carbonylated proteins. (Koltai et al. 2010.) In contrast to these results, there are studies that have not seen any changes in the expression or activity of SIRT1 in response to longer period of voluntary wheel running in mice. (Chabi et al. 2009). In addition, Gurd et al. (2009) showed that chronic electrical stimulation increased mitochondrial biogenesis, but decreased the expression of SIRT1
in skeletal muscle. However, the intrinsic activity of SIRT1 was increased. Thus, the role of SIRT1 in exercise is not fully clear, but it seems that as an adaptation to longer term endurance training the protein expression of sirtuin 1 does not change. However, exercise seems to regulate sirtuin 1 activity at substrate level by increasing the NAD$^+$ levels (Koltai et al. 2010).

JNK1 is known to phosphorylate SIRT1 at Ser 27, Ser 47 and Thr 530 as an outcome of oxidative stress, which increases the nuclear localization, protein stability and enzymatic activity of SIRT1 in human kidney cells. However, it was found that mouse SIRT1 does not have the phosphorylation site at Ser 27 and Thr 530. In addition, it was concluded that JNK1 increases the phosphorylation at Ser 46 instead of at Ser 47 in mice. (Nasrin et al. 2009.) In human cells, JNK 2 also phosphorylates SIRT1 at serine 27 especially increasing its stability and half-life, while no correlation between serine 47 and SIRT1 half-life was observed (Ford et al. 2008). In contrast to these results, SIRT1 phosphorylation at Ser 47 by mammalian target of rapamycin (mTOR) alone results in decreased catalytic activity of SIRT1 resulting in reduced survival of DNA damage-induced prematurely senescent human cancer-cells (Back et al. 2011). In addition, hyperphosphorylation of SIRT1 at serine 47 is shown to be enhanced in senescent endothelial cells of female pigs promoting senescent phenotype. Cyclin dependent kinase (CDK5) seems to be the kinase modulating this phosphorylation. (Bai et al. 2012.) On the other hand, CaMKKβ is shown to phosphorylate SIRT1 at Ser 27 and Ser 47 increasing the stability, the anti-inflammatory and anti-oxidative properties of SIRT1 in human umbilical vein endothelial cells (Wen et al. 2013). In summary, it seems that SIRT1 is phosphorylated at many sites by several kinases and all of their functions are not even known yet. Furthermore, it seems that regulation of SIRT1 by phosphorylation has combinatorial effects and independent phosphorylation of Ser 47 does not promote its catalytic activity in humans. However, it needs to be noted that most of these studies did not use skeletal muscle cells and the phenotypes of the studied cells differ significantly from those that are used in this thesis.

Activation of SIRT1 by resveratrol has been shown to decrease the level of oxidative stress and to ameliorate the symptoms of mdx mice. (Hori et al. 2011; Gordon et al. 2013). In addition, it has been shown that transgenic mdx mice overexpressing SIRT1 gene specifically in skeletal muscle have ameliorated pathophysiology of DMD
(Chalkiadaki et al. 2014). According to Hourde et al. (2013), mdx mice have reduced mRNA expression of SIRT1 compared to wild-type counterparts and 4 months of voluntary wheel running increases the mRNA expression of SIRT1 in mdx mice. In addition, according to Chalkiadaki et al. (2014), SIRT1 protein expression levels of mdx mice do not differ from the levels of wild-type mice. However, NAD$^+$-levels were significantly lower in mdx mice compared to wild-type mice, suggesting that SIRT1 activity is reduced in mdx mice. Another study conducted by Camerino et al. (2014), showed that mRNA expression of SIRT1 is increased in mdx mice compared to wild-type mice. It was also concluded that 4 weeks of treadmill running (12 m/min) did not change the expression of SIRT1, but 12 weeks of treadmill running led to significant downregulation of SIRT1 mRNA expression. All in all, the exercise derived changes in SIRT1 mRNA and protein expression are inconsistent and seem to be dependent on the exercise volume and intensity.

5.4.3 Sirtuin 3 and 6

SIRT3 is localized in mitochondria, which is the place where the major part of the reactive oxygen species is formed. SIRT3 deacetylates and activates several enzymes that maintain cellular ROS levels. SIRT3 deacetylates SOD2, which increases its catalytic activity (dismutation of superoxide anions). In addition, SIRT3 stimulates the activity of mitochondrial isocitrate dehydrogenase (IDH2) by direct deacetylation. IDH2 promotes the conversion of NADP$^+$ to NAPDH, which increases the turnover of glutathione from oxidized to reduced form. (for a review see, Merksamer et al. 2013.) In addition to the antioxidative role of SIRT3, it also deacetylates and activates mitochondrial enzymes involved in fatty acid $\beta$-oxidation, amino acid metabolism and the electron transport chain (Kincaid & Bossy-Wetsel 2013). According to animal model studies, SIRT3 expression may increase with aerobic exercise and its catalytic activity is dependent on NAD$^+$-levels, in other words redox-state of the cell (Palacios et al. 2009; Hokari et al. 2010). Caloric restriction and fasting also increases SIRT3 protein expression and its deacetylase activity (Palacios et al. 2009; Qiu et al. 2010).

SIRT 6 is localized in nucleus and and its role in cellular functions is not as clear as the role of SIRT1 and SIRT3 (Kelly et al. 2010). However, it is linked to DNA damage prevention, genomic instability promotion, lifespan regulation (Mostoslavksy et al.
to the inhibition of glycolysis related gene expression and promotion of fatty acid oxidation related gene expression (Houtkooper et al. 2012). SIRT6 knock-out mice are small and have severe development abnormalities and eventually die at the age of 4 weeks (Mostoslavsky et al. 2006). Little is known about the molecular mechanisms of SIRT6, but according to Michishita et al. (2008) SIRT6 deacetylates histone H3 lysine 9 (H3K9) residues, which modulates telomeric chromatin. H3K9 also inhibits NF-κB transcription and signalling, which is involved in inflammatory, oxidative stress and apoptotic processes (Kawahara et al. 2009). Moreover, SIRT6 is needed in proper telomere function as SIRT6 knockout mice show telomere dysfunction that is related to premature ageing disorder called Werner syndrome (Michishita et al. 2008). Male rats overexpressing SIRT6 have significantly longer lifespan than their wild-type counterparts (Kanfi et al. 2012).
5.5 mdx mice and oxidative stress and the effects of myostatin/activin blocking

mdx mice and DMD human patients show elevated levels of oxidative stress (oxidized DNA, proteins and lipid-peroxidation) and it seems to be a contributing factor to the pathology of DMD (Kazcor et al. 2007; Renjini et al. 2012). It seems that depletion of glutathione (GSH) and lowered antioxidant activities (GSH peroxidase, GSH-S transferase) contribute to DMD pathology significantly (Falst et al. 1998; Dudley et al. 2006; Renjini et al. 2012). Furthermore, it has been concluded that lower levels of GSH was due to lowered activity of gamma-glutamyl cysteine ligase, which is the rate limiting factor in GSH synthesis. (Renjini et al. 2012.)

5.5.1 mdx mice and oxidative stress

The mechanism that promotes oxidative stress in mdx remains still quite unclear. However, excessive intracellular calcium levels and inflammation are proposed mechanisms. It has been theorized that excessive cytosolic calcium leads to increased mitochondrial calcium concentration, which results in increased ATP synthesis consequently leading to increased ROS production of mitochondria due to increased oxygen consumption and enhanced electron flow through electron transport chain. (Terrill et al. 2013.)

According to Whitehead et al. (2010), NADPH oxidase is an important source of ROS in mdx mice and pharmacological inhibition of it decreases the intracellular Ca^{2+} rise following stretch contractions, which is thought to be a key mechanism for muscle damage and functional impairment in mdx mice. To support the role of NF-κB in pathophysiology on DMD, blocking NF-κB with pyrroline dithiocarbamate reduces skeletal muscle degeneration and enhances muscle function in mdx mice (Messina et al. 2006).

Finally, sarcolemma of mdx mice is prone to damage due to the lack of functional dystrophin linking the cell’s cytoskeleton to the extracellular matrix. The damage to the muscle fiber’s membrane increases the inflammation process within the cell, which can be another process that leads to increased levels of ROS formed e.g. in phagosytosis of
cellular organisms by neutrophils and macrophages. (Terrill et al. 2013.) It seems that
the activation of NF-κB and its downstream targets: oxidative stress, and pro-
inflammatory cytokines (e.g., TNF-α, IL-1β, TGF-β) play an important role in inflam-
mation-mediated processes linked to the progression of muscular dystrophy. In addition,
it seems that oxidative stress amplifies the activation NF-κB signalling pathway and
thus pathology of DMD via NAD(P)H oxidase, which triggers signalling cascade in-
cluding caveolin-3, transient receptor potential channel 1 (TRPC-1), matrix metallopro-
teinase 9 (MMP-9), and NF-κB. (Kim et al. 2013.)

5.5.2. Myostatin/activin blocking and oxidative stress

Sriram et al. (2011) showed that myostatin causes elevation of ROS production and ab-
sence of myostatin enhances the antioxidant response in C2C12 myoblasts in vitro. In
addition, they showed that increased ROS levels induce myostatin activation. These
findings identify mechanism for a sustained ROS production in myostatin-elevated situ-
ations such as aging and other muscle wasting conditions (Figure 4.) Thus, blocking
myostatin could be a possible treatment to scavenge sustained ROS levels and inhibit
muscle wasting processes. In addition, in a previous study, it was shown that Smad3
null mice have elevated levels of ROS in skeletal muscle and inactivation of myostatin
partially decreases the amount of oxidative stress. It was shown that increased ROS was
due to increased p38, ERK, MAPK signalling and not via NF-κB signalling. Further-
more, TNF-α, NADPH oxidase and xanthine oxidase levels were upregulated leading to
increased formation of ROS. (Sriram et al. 2014.)
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**FIGURE 4.** Proposed mechanism of myostatin induced ROS in skeletal muscle. Increased levels of myostatin result in TNF-α production via NF-κB signalling, which leads to increased production of ROS by NAPDH oxidase. Increased levels of ROS cause a feed forward loop increasing myostatin levels via NF-κB signalling. Elevated ROS levels may in part cause a decrease in muscle protein synthesis and increase in protein degradation consequently leading to skeletal muscle wasting. Antioxidants (ascorbic acid and 5-ASA) reduce levels of ROS, myostatin and TNF-α by blocking NF-κB signalling. (Shriram et al. 2011.)

### 5.6 Aerobic exercise – pro- or antioxidative?

It is well documented that aerobic exercise leads to increased production of ROS and skeletal muscle is considered to be the major source of it. Other major sources of ROS during exercise are heart, lungs and white blood cells. (Powers & Jackson, 2008.) Focus on this literature review is on skeletal muscle. Skeletal muscle produces superoxide at multiple subcellular sites and the production rate of superoxide is increased during exercise at several of these sites. The potential sites for superoxide production in skeletal muscle are mitochondria, sarcoplasmic reticulum, transverse tubules, plasma membrane, phospholipase and xanthine oxidase dependent processes. (Powers & Jackson, 2008.)
The question is: is the increased production of ROS caused by exercise harmful to skeletal muscle and other tissues? One perspective to answer to this question is that chronic exposure to ROS is linked to several pathological processes such as cachexia, atherosclerosis, cancer, ischemia/perfusion, inflammation, rheumatic arthritis and neurodegenerative diseases such as Alzheimer and Parkinson diseases. In addition, ROS is believed to affect in the process of aging. Even though it is well known that exercise training leads to increased production of ROS, exercise is also known to decrease the incidence of previously mentioned oxidative-stress associated diseases. It has to be also mentioned that chronic disuse of muscle leads to elevated levels of ROS, which plays a role in muscle atrophy. (Radak et al. 2008.) According to Shriram et al. (2011), elevated levels of myostatin play an important role in promoting ROS mediated process of atrophying muscle. All in all, it seems that regular exercise causes adaptations that promote anti-oxidative system. A brief summary of exercise-derived adaptations follows.

As it was mentioned in chapter 3.1.2, aerobic exercise training increases the expression of genes that are involved in the anti-oxidative system (Mahoney et al. 2005). These genes involved all seven metallothionein genes, superoxide–responsive transcription factor (interferon regulatory factor 1) and an enzyme involved in DNA repair from free radical damage (tyrosyl-DNA phosphodiesterase 1). This data supports the fact that even one exercise training session activates the oxidant stress management and signalling processes. Furthermore, regular exercise training increases the amount and activity of enzymatic antioxidants such as superoxide dismutases and glutathione peroxidases in healthy (Criswell et al. 1993; Siu et al. 2004). Furthermore, it is known that high intensity exercise leads to increased levels of glutathione (GSH), which is a non-enzymatic anti-oxidant. This is speculated to be a consequence of increased expression of enzymes that are involved in GSH synthesis. (Powers & Jackson 2008.)

It is well documented that ROS play an important role in skeletal muscle adaptation to aerobic exercise training by being an activator to several signalling pathways. It is also thought that a short-period exposure to ROS may activate signalling pathways that promote anti-oxidative capacity and defence against ROS, whereas chronic long-time exposure to ROS may activate signalling processes involved in proteolysis and programmed cell death. (Powers et al. 2010.) Moderate intensity exercise has been shown
to increase the expression of enzymes that protect against oxidants (e.g. superoxide dismutase) (Gomez-Caprera et al. 2008a). In addition, ROS seem to play an important role in PGC-1α mediated skeletal muscle adaptations in response to exercise. Gene expression responses of PGC-1α and mitochondrial biogenesis are decreased in response to exercise, if high doses of supplementary antioxidants are taken before exercise. In addition, high doses of vitamin C independently and in combination with vitamin E decreases the exercise derived expression of endogenous antioxidants and anti-oxidative enzymes. (Gomez-Caprera et al. 2008b; Ristow et al. 2009.) This data indicates that ROS play an important role in exercise mediated skeletal muscle adaptation.

5.6.1 Exercise and exogenous antioxidants in muscular dystrophy and oxidative stress

As it was discussed in chapter 5.5., mdx mice show increased markers of oxidative stress. The question is whether aerobic exercise could be also anti-oxidative to mdx mice similarly to healthy and thus, could ameliorate the symptoms of DMD. There is limited data to answer this question properly, but all the studies that include exercising mdx mice and/or antioxidant supplementation and oxidative stress are summarized below. Kaczor et al. (2007) showed that low intensity, low-volume exercise decreases the markers (malondialdehyde and protein carbonyls) of oxidative stress and increases anti-oxidative enzyme activity in white mdx muscle. According to one unpublished MSc thesis, forced treadmill running has been to shown to decrease the levels of oxidized glutathione in mdx mice. However, it was also shown that running led to increased muscle fibrosis. (Schill 2014.) Call et al. (2008) studied the combinatorial and independent effects of green tea extract (GTE) (anti-oxidant) supplementation and voluntary wheel running on oxidative stress. They found that running independently increased anti-oxidative capacity of blood serum by 22 %. In addition, GTE alone and in combination with running increased the anti-oxidative capacity of blood serum. Buetler et al. (2002) also showed that GTE decreases the muscle necrosis and scavenges ROS in mdx mice. Another study conducted by Whitehead et al. (2008), showed that administration of N-acetylcysteine (antioxidant) decreased the ROS levels and ameliorated pathophysiology of DMD. This was accompanied by decreased expression of NF-κB, a transcription factor that is involved in pro-inflammatory cytokine expression. It also increased
the expression of sarcolemmal β-dystroglycan and utrophin (dystrophin homologue). All in all, it seems that there are several strategies that increase antioxidative capacity of mdx mice and they have beneficial outcome to the pathophysiology on DMD. To the author’s knowledge, this is the first study to elucidate the combined and independent effects of ActRIIB-blocking and voluntary wheel running on oxidative stress in mdx mice.
6 RESEARCH QUESTIONS AND HYPOTHESES

6.1 Research questions

1. How does 7 weeks of voluntary wheel running and blocking ActRIIB ligands with sActRIIB-Fc independently and in combination influence on the protein carbonyls and glutathione metabolism of the gastrocnemius muscle.

2. How does voluntary wheel running and blocking ActRIIB ligands with sActRIIB-Fc independently and in combination influence on the protein expression of sirtuins 1, 3, and 6, phosphorylation of SIRT1 at serine 46 and AMPK and phosphorylation of AMPK.

6.2 Hypotheses

1. Voluntary wheel running and sActRIIB-Fc administration independently and in combination decreases the levels of protein carbonyls and decreases the level of oxidized glutathione and increases the level of reduced glutathione.

Arguments: According to previous studies, both human DMD patients and mdx mice show increased level of oxidative stress. (Ragusa et al. 1997; Kazcor et al. 2007; Renjini et al. 2012). However, light intensity low volume treadmill running (Kazcor et al. 2007) and voluntary wheel running (Call et al. 2008) have been shown to scavenge protein carbonyls and lipid peroxidation in mdx mice. In addition, forced treadmill running has been shown to decrease the levels of oxidized glutathione in mdx mice in one unpublished MSc thesis (Schill 2014). Very little is known about the oxidative stress in sActRIIB-Fc administered mdx mice. However according to Shriram et al (2011), myostatin plays an important role in increasing the levels of oxidative stress in aging muscle. In addition, they showed that myostatin null myoblasts show increased activity of anti-oxidative enzymes. Thus, it could be hypothesized that ActRIIB ligand blocking with sActRIIB-Fc could improve the anti-oxidative capacity of mdx mice which could translate into decreased levels of protein carbonyls and oxidized glutathione. In addi-
tion, high intensity high volume endurance training has been shown to increase total glutathione levels of healthy skeletal muscle as an adaptive mechanism to defend against free radical production (Sen et al. 1992; Leeuwenburgh et al. 1997).

2. Voluntary wheel running independently or in combination with sActRIIB-Fc increases or does not change the protein expression of sirtuins 1, 3, 6, phosphorylation of sirtuin 1 at ser 46, total AMPK and p-AMPK at thr 172.

Arguments: According to previous studies, sirtuins 1, 3 and 6 have important anti-oxidative properties by deacetylating several proteins or histones that are involved in anti-oxidative defense (Kobayashi et al. 2005; Kawahara et al. 2009; Merksamer et al. 2013; Radak et al. 2013). Phosphorylation of SIRT1 at ser 47 (homologue of serine 46 in mice) has been shown to increase and decrease the anti-oxidative activity of SIRT1 in different types of human cells. It seems that at least in humans, phosphorylation at different residues than ser 47 is also needed to promote the anti-oxidative properties of SIRT1 (Ford et al. 2008; Nasrin et al. 2009; Back et al. 2011; Bai et al. 2012 Wen et al. 2013). The interaction between p-SIRT1 at ser 46 and exercise is unknown, but it could be speculated that as exercise increases the activity of SIRT1, this could be seen as increased phosphorylation of SIRT1 at ser 46. According to one previous study, it has been shown that long term voluntary wheel running does not increase the protein expression of SIRT1 in healthy rats (Chabi et al. 2009), but one previous study showed that acutely after a bout of exercise protein expression of SIRT1 increases in healthy rats (Suwa et al. 2008). In addition, exercise has been shown to increase the activity of the SIRT1 by increasing the NAD\(^+\)/NADH ratio (Koltai et al. 2010). According to Hourde et al. (2013) mdx mice have reduced mRNA expression of SIRT1 compared to wild-type counterparts, but 4 months of voluntary wheel running increases the mRNA expression of SIRT1 in mdx mice. In contrast, Camerino et al. (2014) found that mRNA expression of SIRT1 is increased in mdx mice compared to wild-type mice. It was also concluded that 4 weeks of treadmill running (12 m/min for 30 mins 2 x/week) did not change the expression of SIRT1 but 12 weeks of treadmill running led to significant downregulation of SIRT1 mRNA expression. Thus, it seems that the exercise derived changes in SIRT1 protein and mRNA expression are dependent on exercise intensity and volume. Since the exercise mode in the current thesis was voluntary wheel running and it is considered beneficial for the mdx mice, it is hypothesized that running may in-
crease the protein expression of SIRT1. According to animal studies that used healthy animals as subjects, SIRT3 expression increases with exercise and its catalytic activity is regulated by the NAD⁺/NADH ratio. (Palacios et al. 2009; Hokari et al. 2010) Thus, it is hypothesized that voluntary wheel running may increase the protein expression of SIRT3. The interaction between exercise and SIRT6 is unknown, but it could be hypothesized that as exercise increases the NAD⁺-levels, which is catalytic fuel for SIRT6, voluntary wheel running could increase protein expression of SIRT6. Previous studies do not provide any proper arguments for the sActRIIB-Fc administration regarding sirtuin protein expression in mdx mice. However, myostatin blocking may decrease the level of oxidative stress (Shriram et al. 2011) as it was speculated earlier and this could also be mediated to some extent via sirtuins, which could translate into increased protein expression of sirtuins 1, 3 and 6 or phosphorylation of SIRT1 at ser 46.

AMPK is an AMP-sensitive protein kinase that regulates glucose and fatty acid metabolism and it also seems to play an important role in skeletal muscle adaptations to exercise (Lira et al. 2010). Its signaling activity is significantly increased during exercise due to break down of ATP to AMP. In addition, phosphorylation at thr 172 by its upstream kinases is needed to activate AMPK signalling. (Hardie 2008.) Chronic hyperactivation of AMPK by pharmaceutical agent (amino-4-imidazolecarboxamide riboside (AICAR) has been shown to shift the muscle fiber phenotype of mdx mice into slow oxidative phenotype. This has been accompanied by ameliorated symptoms of DMD. (Ljubicic et al. 2011; Al-Rewashdy et al. 2015.) One acute bout of treadmill running has been shown to increase the levels of pAMPK at thr 172, but no changes in total AMPK protein content was observed in mdx mice (Ljubicic et al. 2012). AMPK has also been shown to be linked to exercise derived actions of SIRT1 and PGC-1α, both of which are master regulators of aerobic metabolism (Jäger et al. 2007; Canto et al. 2010) and possess anti-oxidative properties (Merksamer et al. 2013 Radak et al. 2013). Based on these studies it seems that increased activity of AMPK signalling is beneficial for mdx mice and we hypothesize that voluntary wheel running could chronically increase the protein content of AMPK and pAMPK at thr 172.
7 METHODS

7.1 Animals

The mice used in the study were male, 6–7 weeks old, from a C57B1/10nJ background. All mice were from Jackson Laboratory (Bar Harbor, Maine, USA). They were housed in individual cages with standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM.) The mice had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden).

7.2 Ethics statement

The treatment of the animals was in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocol was approved by the national animal experiment board (permit number: ESLH-2009-08528/Ym-23). All efforts were made to minimize suffering.

7.3 Experimental design

The mdx mice were randomly divided into 4 groups (n=8 in each): PBS sedentary (mdx PBS), PBS running (mdx RUN), sActRIIB-Fc administered sedentary (mdx AcR) and sActRIIB-Fc administered running (mdx AcR RUN). sActRIIB-Fc or PBS (5 mg/kg) was injected intraperitoneally once a week for 7 weeks. In addition, PBS was administered to wild type (n=5), which were used as a healthy control group (wt PBS). A schematic representation of the experimental design is shown in figure 5.
Voluntary wheel running was chosen as exercise modality, during the 7 weeks of the study intervention. The running wheels were locked during the first injection day and the next day to prevent mice from exercising and to follow the treatment take effect. The mice didn’t have access to the running wheels during the last two days, so that the outcome effects would not reflect the acute effects of exercise. During the experiment all conditions were standardized. The mice were sacrificed after the experiment by cervical dislocation and blood and tissue samples were collected.

7.4 sActRIIB-Fc production

The recombinant fusion protein was produced and purified \textit{in house} at the University of Helsinki, as it is described in the publications of Hoogars et al. (2012) and Hulmi et al. (2013). It has been reported previously to normalize (LPS- or activin a overexpression-induced) lung pathology in a mouse model and to increase muscle size in mdx mice. The ectodomain (ecd) of human sActRIIB was amplified via PCR with the following primers: 5’-GGACTAGTAACATGACGCAGATCTGCGGTGGGGGCTGTCGG-3’ and 5’-CCAGATCTCGGTTGGGGCTGTCGG-3’ from a plasmid containing the human ActRIIB sequence (in pCR-Blunt II-TOPO AM2 G17 ActRIIB, IMAGE clone no. 40005760; The IMAGE Consortium). A human IgG1 Fc domain with a COOH-terminal His6 tag was amplified by PCR (5’-GCAGATCTAATCGAAGGTCGTGGATCCCAAATCTTGTGAC-3’ and 5’-TCCCTGTCTCGGTTAAAACACCATCACCATCACCATTGAGCGCGCCGCTT-3’) from the pIgPlus expression plasmid. The subcloning of these products was done into
the pEGM-T easy (Promega) vectors, sequenced, and fused before cloning into the expression vector pEFIRES-p. For the final protein production, Chinese hamster ovary (CHO) cells were transfected with the above mentioned ActRIIBecd-FcHis6 expression vector via lipofection (Fugene 6; Roche) and selected with puromycin (Sigma-Aldrich, Lyon, France). During selection cells were grown in Dulbecco’s modified Eagle media (DMEM) supplemented with 2 mmol/l L-glutamine, 100 g/ml streptomycin, 100 IU/ml penicillin, and 10 % fetal calf serum (FCS). For large-scale expression, cells were adapted to CD OptiCHO medium (Gibco) supplemented with 2 mmol/l L-glutamine and grown in suspension in an orbital shaker. Cell culture supernatants were clarified by filtration through a 0.22-m membrane (Steritop; Millipore). Next, NaCl and imidazole were added, and the solution was pumped through a Ni2-loaded HiTrap Chelating column (GE Healthcare Life Sciences, Uppsala, Sweden) at 4°C. Protein was eluted by raising imidazole concentrations, dialyzed against PBS, and finally concentrated with Amicon Ultra concentrator (30 00 MWCO; Millipore). The purity of our sActRIIB-Fc preparation after IMAC purification was estimated to be 90 % based on on silver-stained SDS-PAGE.

7.5 Voluntary wheel running, activity index and feed intake

The running mice were housed individually in cages where they had free access to custom-made running wheels (diameter 24 cm, width 8 cm) 24/day. The sedentary mice were housed in similar cages without the running wheel. Total running distance was recorded daily, while body mass and feed consumption were measured weekly. Vertical groundreaction forces were measured using a custom-made force plate (linearity 99%, cross-talk 2%). Forces were measured by four uniaxial strain gauges, which were rectangularly arranged under the corners of a glass plate on which the animal housing cage was located. A 14-bit A/D converter (DI-710; DATAQ Instruments, Akron, OH) was used for digitizing the force data at a sampling rate of 80 Hz. The measurement range of the strain gauge was 250 g, while the digitization precision was 0.02 g. The outcome variable distance was calculated similarly as previously (Rantalainen et al. 2011). The force plate recording has been validated against video recording and found to be perfectly valid (Rantalainen et al. 2011). After the animal housing, the cage without the animal was positioned on the glass plate and the force levels of all gauges were
adjusted to zero. Twenty-four-hour measurements were conducted for the mice without
running wheels. The three time points selected were 1–5 days before the first treatment
injections, during the third week, and the end of the sixth week. The measurement was
conducted in the animal’s own cage and in the same room with the other mice in this
experiment. Force-plate data and voluntary wheel running data were combined to assess
the activity index of all the groups even if they did not run.

7.6 Muscle and fat sampling

The mice were sacrificed by cervical dislocation. Immediately after, lower leg muscles:
soleus, gastrocnemius, musculus quadriceps femoris (MQF), extensor digitorum longus
(EDL) and tibialis anterior were removed, weighed and frozen in liquid nitrogen.
Epididymal fat pad was collected for the estimation of fat mass. Muscle weights report-
ed are always average weights of left and right legs.

7.7 Grip strength test, hang wire test and muscle fiber CSA measure-
ment

Before euthanization, grip strength was measured with a custom made device (Universi-
ty of Jyväskylä) as previously described (Hulmi et al 2013a). Strength endurance and
body coordination were tested by a hanging wire test as previously described (van
Putten et al. 2010). Mice were suspended above a metal wire secured 30 cm above a
padded cell and released. Depending on the functional ability of the mouse, use of all
limbs and the tail were accepted during a 10-min hanging session. Three trials were giv-
en and the longest hanging time (up to the maximum of 10 min) was used for the further
analysis.

Gastrocnemius muscle cross-sections cut with cryomicrotome were stained for sarco-
lemma using antibody against caveolin 3 (ab2912; Abcam) and capillaries with
isolectin-GS-IB4 containing fluorescent Alexa Fluor 488 label (Molecular Probes, Eu-
egene, OR), as described earlier (Hulmi et al. 2013a). Image analysis was performed with
Image J software (National Institutes of Health, Bethesda, MD).
7.8 Western immunoblot protein analysis

Western immunoblot protein analysis was carried out in order to measure the protein quantity of SIRT1, SIRT3, SIRT6, p-SIRT1 at ser 46, p-AMPK at thr 172, AMPK and total amount of carbonylated proteins of gastrocnemius muscle. Sirtuin and AMPK measurement protocol was carried out slightly differently than protein carbonyl measurement protocol. Before the western immunoblot procedures were carried out gastrocnemius muscle samples were pulverized and homogenized as previously described (Hulmi et al. 2013a).

7.8.1 Protocol of sirtuins and AMPK

*SDS-Page.* The homogenized gastrocnemius muscle samples were diluted with ddH$_2$O so that 15 µl of each sample contained 35 µg of total protein. 15 µl of diluted sample was then combined with 15 µl of 2 x Laemmli sample buffer (Bio-Rad #161-0737) which contained 5 % β-mercaptoethanol. After that, sample mixtures were briefly centrifuged and heated for 10 min at 95°C. Samples were again briefly centrifuged after the heating and were placed on ice for approximately 5 minutes. Then samples were pipetted to the electrophoresis gel (4–20% Criterion™ TGX™ Precast Gels, Bio-Rad #567-1094) so that each well contained approximately 30 µg of total protein. Molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad #161-0374) was pipetted to the first well of each gel. Then the electrophoresis chamber was filled with electrophoresis running buffer (2.5 mM Tris Base, 19.2 mM glycine, 0.01% SDS, ddH$_2$O). Electrophoresis was run at 250 V for approximately 30 minutes in order to separate the proteins, according to their molecular weight.

*Blotting.* After the electrophoresis phase proteins were transferred from the electrophoresis gel to an absorbent PVDF membrane (Hybond-P, GE Healthcare Life Sciences, RPN303F). Gel was put into transfer buffer (2.5 mM Tris Base, 19.0 mM glycine, (pH adjusted to 8.3 with HCl), 10% methanol, ddH$_2$O) so that its electric charge would be balanced. PVDF membrane was activated by exposing it to methanol for approximately 10 seconds. After that, PVDF membrane was also put into transfer buffer for approximately 15 minutes in order to balance its electric charge. Then the blotting sandwich
was built. A scotch-brite pad and sheet of blotting paper were submerged in transfer buffer. Then electrophoresis gel was put onto the sheet of blotting paper and PVDF membrane onto the gel. After that another sheet of blotting paper and scotch-brite pad were put onto PVDF membrane and blotting cassette was closed. Blotting cassettes were submerged in transfer buffer in the blotting chamber. Blotting was conducted by using electric current of 300 mA, for approximately 2 h 30 minutes, at 4°C on ice. Transfer buffer was stirred by magnetic mixer during the blotting.

**Ponceau S staining blocking and primary antibodies.** Membranes were stained with Ponceau S after the blotting in order to ensure that proteins were successfully transferred from the gel to the PVDF membrane. In addition, the staining was later used for the quantitation of relative protein content. Membranes were imaged with Molecular imager ChemiDoc XRS system (Bio-Rad) and Quantity One 4.6.3.-software (Bio-Rad). After that, membranes were cut into strips which contained one protein of interest. Then strips were put into blocking solution (TBS + 0,1% Tween-20 + 5% non-fat milk) for 2 hours and were gently rocked in the room temperature. Blocking was carried out to avoid unspecific protein binding. After that strips were incubated overnight at 4°C with primary antibodies in gentle rocking. More details of primary antibodies are in appendix 2.

**Secondary antibodies and detection.** After the overnight incubation, strips were rinsed and washed 4 x 5 minutes with TBS-tween in hard rocking. Then strips were incubated with secondary antibodies for 1 hour at room temperature in gentle rocking. See appendix 2 for more detailed information about the secondary antibodies. After the incubation, strips were rinsed and washed 5 x 5 minutes in hard rocking with TBS-Tween. After the washing and rinsing, strips were incubated with detection kit (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Protein Biology Products, Thermo Scientific #34096). After the incubation, strips were imaged with Molecular Imager ChemiDoc XRS System (Bio-Rad) and Quantity One 4.6.3.-software (Bio-Rad).

**Stripping.** In some cases same blotting strip was used to detect two different proteins (p-SIRT1 was stripped to detect total SIRT1 and p-AMPK was stripped to detect total AMPK). In these cases protocol called stripping was carried out to remove the bound antibodies enabling the binding of new antibodies. Strips were incubated with Restore™ Western Blot Stripping Buffer (Thermo Scientific, #21059) for approximate-
ly 20 minutes with hard rocking at room temperature. After the incubation, strips were rinsed five times with ddH$_2$O and washed 3 x 5 minutes in TBS-Tween. After the stripping protocol, strips were again put into blocking solution, which was followed by already explained steps with new antibodies.

Quantitation and data analysis. The relative quantities of proteins were determined with Quantity One 4.6.3.- software. GAPDH and Actin band of Ponceau S staining were quantified in order to normalize the raw data and to exclude the effect of different protein loading. All the values were normalized as a mean to Ponceau S staining and GAPDH. Then this GAPDH and Ponceau S staining normalized mean value was further divided by the mean value of the whole gel in order to avoid the gel to gel variation. In addition, the Ponceau S staining and GAPDH normalized value was further normalized to mdx PBS value which was set to be 1.00.

7.8.2 Protein carbonyls

Protein carbonyls were measured at the University of Eastern Finland by Drs. Mustafa Atalay and Ayhan Korkmaz. The Western immunoblot protocol was carried out in similar way as in this already published paper (Atalay et. al. 2004).

7.9 Glutathione metabolism measurements

Glutathione metabolism variables were also measured at the University of Eastern Finland by Drs. Mustafa Atalay and Ayhan Korkmaz as previously described (Lappalainen et. al. 2009).

7.10 Data processing and statistical analyses

All the statistical tests were conducted using IBM SPSS statistics version 20. Comparison between mdx PBS and wt PBS groups was done by the LSD- t-test, whereas the comparisons between the mdx groups were done by Tukey’s post hoc test. In addition, running, administration of sActRIIB-Fc and their interaction effect was conducted by using the 2 x 2 ANOVA-test. Pearson’s correlation was used to find correlations be-
etween the studied variables. Results were statistically significant if \( p < 0.05 \). All the figures and were done by Power point and Excel softwares (Microsoft).
8 RESULTS

8.1 Background information

sActRIIB-Fc administration decreased the daily voluntary wheel running distance during the first few weeks of the intervention period as it was already earlier published (Hulmi et al. 2013b). There was a significant difference in the daily mean running distances between the PBS treated and sActRIIB-Fc administered groups during the weeks 2 and 3 (P<0.05) (Figure 6A). Decreased grip strength and worse performance in hang wire test was observed in mdx mice (P<0.05) (Figure 7A; 7B). No changes in mean fiber cross-sectional area (CSA) was observed in mdx mice, but percentage of very small (<600 µm$^2$) or small (<1000µm$^2$) fibers was higher than in healthy wildtype controls (P<0.05) (Figure 8B). Blocking ActRIIB ligands by sActRIIB-Fc increased muscle mass in all the muscles examined as published earlier (Hulmi et al. 2013b), but this did not translate into increased muscle fiber size, grip strength or better performance in the hangwire test (figure 6B; 7A; 7B). No effect of exercise was noticed on grip strength, but exercise shifted the CSA distribution further towards smaller fibers. However, no statistically significant difference was noted in the percentage mean count of small fibers (< 600 µm$^2$) by running vs. no running (2x2 ANOVA P=0.295) and thus the decreasing effect of exercise was throughout the fiber size range. The body mass increased in every group during the 7 weeks intervention period (Figure 6B). sActRIIB-Fc administration increased the mass of gastrocnemius muscle both in the sedentary and in the running group compared to mdx PBS (P<0.05) (figure 8A). In addition, the mass of gastrocnemius was higher in mdx PBS compared to wt PBS (P<0.05), suggesting that there was “pseudo-hypertrophy” in the mdx muscle. In addition, the mass of gastrocnemius was significantly lower in the sActRIIB-Fc running group compared to sedentary sActRIIB-Fc group (P<0.05).
FIGURE 6. A) Weekly voluntary wheel running distance per day during 7 weeks intervention period. The symbol * denotes for the significant difference between the groups (P<0.05). B) Percentual change in body mass during the 7 weeks intervention period. Abbreviations: mdx = the mouse model of Duchenne’s muscular dystrophy. wt = wild-type healthy mice. PBS = placebo treated. AcR = sActRIIB-Fc administered. Run = voluntary wheel running.
FIGURE 7. A) Longest hanging time in hang wire test measured in seconds. The symbol * denotes for the significant difference between the mdx PBS group and wt PBS groups (P<0.05). B) Grip strength measured in grams. The symbol * denotes for the significant difference between the mdx PBS group and wt PBS groups (P<0.05). Abbreviations: mdx = the mouse model of Duchenne’s muscular dystrophy. wt = wild-type healthy mice. PBS = placebo treated. AcR = sActRIIB-Fc administered. Run = voluntary wheel running.
FIGURE 8. A) Mass of gastrocnemius muscle after 7 weeks intervention period. The symbol * denotes for the significant difference to the mdx PBS group (P<0.05). The symbol # denotes for the significant difference between mdx AcR and mdx AcR Run. (P<0.05)    B) The gastrocnemius muscle fiber cross sectional area distribution measured in percents of total fibers. The symbol * denotes for the significant difference between wt PBS and mdx PBS (P<0.05). Abbreviations: mdx = the mouse model of Duchenne’s muscular dystrophy. wt = wild-type healthy mice. PBS = placebo treated. AcR = sActRIIB-Fc administered. Run = voluntary wheel running.

8.2 Glutathione metabolism and protein carbonyls

Glutathione metabolism. There was increasing running (P=0.0006) and sActRIIB-Fc administration effect (P=0.082) in the ratio of oxidized glutathione and reduced glutathione (GSSG/GSH) (2x2 ANOVA) (Figure 9A). However, significant difference between the groups was not observed. There was increasing running effect (P=0.012) in the ratio of oxidized glutathione and total glutathione (GSSG/TGSH) (2x2 ANOVA) but there was not significant difference between the groups (figure 9B). Wild-type group had significantly less reduced glutathione than all of the mdx mouse groups (P=0.0013) (LSD post hoc test). However, there was not significant difference in the
mdx mice (figure 9C). There was increasing running effect (P=0.014) in the amount of oxidized glutathione (GSSG) (2x2 ANOVA). In addition, wild-type mice had significantly less oxidized glutathione compared to mdx PBS group (P=0.033) (LSD post hoc test). Nevertheless, there was not significant difference between the mdx mouse groups (figure 9D).

FIGURE 9. A) Ratio of oxidized glutathione (GSSG) and reduced glutathione (GSH) after 7 week intervention period. B) Ratio of oxidized glutathione (GSSG) and total glutathione (TGSH) after the 7 week intervention period C) Reduced glutathione levels after 7 weeks intervention period. ** denotes for significant difference between mdx PBS group and wild-type mice group (P<0.01) D) Oxidized glutathione levels after 7 weeks intervention period. * denotes for significant difference between wild-type and mdx mice (P<0.05). Abbreviations: mdx = the mouse model of Duchenne’s muscular dystrophy. wt = wild-type healthy mice. PBS = placebo treated. AcR = sActRIIB-Fc administered. Run = voluntary wheel running.

Protein carbonyls. There was increasing running (P=0.026) and running x sActRIIB-Fc administration interaction effect in the carbonylated proteins (P=0.018) (2x2 ANOVA). Running increased protein carbonyls in sActRIIB-Fc administered groups (Tukey’s post hoc test) (P=0.013) (figure 10A). Representative western immunoblot image of protein carbonyls is presented in the figure 10B.
8.3 Sirtuins and AMPK

In total SIRT1 there was not significant difference between the groups in mdx mice. However, protein expression of SIRT1 was significantly higher in wild-type than in mdx PBS group (LSD post hoc test P=0.0046). There was significant difference in the p-SIRT1 at ser 46 between sActRIIB-Fc administered running group and mdx PBS treated running group (Tukey’s post hoc test P=0.011). In addition, there was an increasing sActRIIB-Fc administration effect (P=0.011) and an increasing running x sActRIIB-Fc interaction effect in the phosphorylation of SIRT1 at ser 46 (P=0.025) (2x2 ANOVA). Phosphorylation of SIRT1 at ser 46 was significantly lower in wild-type mice compared to mdx PBS group. (LSD post hoc test P=0.027). Phosphorylation level of SIRT1 at ser 46 and total SIRT1 levels are presented in the Figure 11A. Running in PBS and sActRIIB-Fc administered groups had tendency to increase SIRT3 protein expression, but no significant difference was observed between any of the groups (Figure...
Sirtuin 6 protein expression tended to increase in running PBS and in both of the sActRIIB administered groups, but no significant difference was noted between the groups (Figure 11C). Running and sActRIIB-Fc administration had decreasing interaction effect (P=0.029) in the ratio of phosphorylated AMPK and total AMPK (2x2 ANOVA). In addition, Tukey’s post-hoc test revealed significant difference in the ratio of pAMPK and total AMPK between mdx PBS group and mdx AcR group (P=0.033). pAMPK and total AMPK levels are presented in the figure 11D. Western blot images are presented in Figure 12.

FIGURE 11. A) Protein expression levels of total Sirtuin 1 and phosphorylation of sirtuin 1 at ser 46 after 7 weeks intervention period. The symbol * denotes for significant difference between wt PBS and mdx PBS (P<0.05). The symbol ** denotes for significant difference between wt PBS and mdx PBS groups (P<0.01). The symbol # denotes for the significant difference between mdx Run and mdx AcR run groups in p-SIRT1 at ser 46. B) Protein expression levels of Sirtuin 3 after 7 weeks intervention period. C) Protein expression levels of Sirtuin 6 after 7 weeks intervention period. D) Protein expression levels of phosphorylated AMPK and total AMPK. The symbol * denotes for the significant difference in the pAMPK/AMPK between mdx PBS and mdx AcR groups. Abbreviations: mdx = the mouse model of Duchenne’s muscular dystrophy. wt = wild-type healthy mice. PBS = placebo treated. AcR = sActRIIB-Fc administered. Run = voluntary wheel running.
8.4 Correlations

Strong correlation between the activity index and protein carbonyls was found in sActRIIB-Fc administered mdx mice (Figure 13A) \((R=0.866; p=0.000063)\). In contrast, no significant correlation was observed when placebo treated groups were combined (Figure 13B).
FIGURE 13. A) Pearson’s correlation coefficient and scatter dot plot between protein carbonyls and activity index from the whole intervention period in sActRIIB-Fc administered mdx mice. B) Pearson’s correlation coefficient and scatter dot plot between protein carbonyls and activity index from the whole intervention period in PBS treated mdx mice.

There was significant correlation between p-SIRT1 and p-AMPK in sActRIIB-Fc administered mdx mice (R=0.610; p=0.027) and running mdx mice (R=0.614; p=0.034) when outlier was excluded (circled with red in Figures 14A & 14B). Strong correlation in sActRIIB-Fc administered running group was found between p-AMPK and protein carbonyls (R=0.878, p=0.021) (Figure 14C). In addition, almost statistically significant correlation was found in running mdx mice between p-AMPK and protein carbonyls (R=0.551; P=0.051) (Figure 14D).
FIGURE 14. A) Pearson’s correlation coefficient and scatter dot plot between pAMPK and pSIRT1 after the 7 week intervention period in sActRIIB-Fc administered mdx mice. When outlier is excluded (circled with) $R^2=0.5774$. B) Pearson’s correlation coefficient and scatter dot plot between pAMPK and pSIRT1 after the 7 week intervention period in running mdx mice. When outlier is excluded (circled with red) $R^2=0.5866$. C) Pearson’s correlation coefficient and scatter dot plot between pAMPK and protein carbonyls after the 7 week intervention period in sActRIIB-Fc running group. D) Pearson’s correlation coefficient and scatter dot plot between pAMPK and protein carbonyls after the 7 week intervention period in running mdx mice.


9 DISCUSSION

The main finding of this study was that combination of exercise and blocking ActRIIB binding ligands with sActRIIB-Fc increased protein carbonyls, which was accompanied by oxidized form of glutathione (GSSG) and increased phosphorylation of SIRT1 at ser 46. In addition, running independently increased protein carbonyls and oxidized glutathione. These results suggest that voluntary wheel running independently and combined with sActRIIB-Fc administration results in elevated oxidative stress that dystrophic mice can not fully rescue with endogenous antioxidants.

9.1 Oxidative damage

In this study it was hypothesized that voluntary wheel running and sActRIIB-Fc administration alone and combined would decrease the levels of protein carbonyls (a marker of oxidative damage of the gastrocnemius muscle). However, running alone and combined with sActRIIB-Fc administration increased protein carbonyls. In previous studies, it has been shown that 17–19 weeks old mdx mice have more thiobarbituric acid reactive substances (marker of oxidative stress) in extraocular muscle, diaphragm, gastrocnemius and soleus compared to wild-type (Ragusa et al. 1997) and 13 weeks old mdx mice have more protein carbonyls in red and white part of the gastrocnemius muscle compared to wild-type mice (Kaczor et al. 2007). Studied muscle in the current study was gastrocnemius and mice were approximately 14 weeks old, when their muscle samples were collected. This makes the comparison of this study and the above mentioned studies reasonable. In contrast to findings of Ragusa et al. (1997) and Kaczor et al. (2007), no significant difference was found between wild-type control and mdx mice in protein carbonylation in the current study. However, both running independently and in combination with sActRIIB-Fc increased protein carbonyls. This is consistent with the observation that running increased the oxidized form of glutathione and combination of exercise and sActRIIB tended to increase oxidized glutathione. It seems that running alone and especially in combination with sActRIIB-Fc shifts the redox-balance of the skeletal muscle to more oxidizing direction. Oxidative stress wasn’t fully rescued by anti-oxidant system since increased protein carbonyls, in other words oxidative damage was observed. Habitual physical activity level of mice, measured by force
plates and protein carbonyls showed strong correlation in sActRIIB-Fc-treated group but not in the PBS-treated group, which may indicate that combination of exercise and sActRIIB-Fc administration provokes too much cellular stress for mdx mice that resulted in oxidative damage. However, according to an MSc thesis from the same data sets as in the current study (Papaionnu 2013), some signs of adaptation can be seen in the gene set analysis, since running independently and in combination with sActRIIB-Fc administration increased the mRNA expression of glutathione metabolism gene set (for a list of genes involved in GSH metabolism, see appendix 1).

In contrast to our results, Kaczor et al. (2007) reported that 8 weeks of very low-intensity treadmill running (2 x/week, 9 m/min for 30 min) decreased the level of protein carbonyls and lipid peroxidation in exercising mdx mice compared to sedentary mdx mice. The difference in protein carbonyls of sedentary and exercising mice was greater in the white gastrocnemius than in the red gastrocnemius. Exercise intensity and volume in the study of Kaczor et al. (2007) was so low that it did not increase antioxidative enzyme activities or anti-oxidative protein expression levels of wild-type mice. In addition, expression and activity levels of anti-oxidative enzymes tended to be lower in exercising mice compared to sedentary mice. Based on these findings, authors concluded that protein carbonylation was reduced as a result of low-intensity training promoted adaptations in reduced ROS production rather than due to increased antioxidative capacity. Based on the study of Kaczor et al. (2007), it is tempting to say that in this thesis mdx mice voluntarily ran too much and their anti-oxidative capacity was not sufficient to scavenge all the free radicals, which led to cellular damage (protein carbonyls) especially when sActRIIB-Fc was administered. To support this claim, the study designs of this thesis and study conducted by Kaczor et al. (2007) only differed in exercise mode, since the mice were approximately at same age when euthanized and studied muscle was gastrocnemius in both of the studies. In the study of the Kaczor et al. (2007), mice ran 270 meters only twice per week, which is much less compared to running distances of this thesis, which were approximately 2000 m–6000 m per day. On the other hand, previously published data of this study have shown that voluntary wheel running has many positive effects on the phenotype of the activin/myostatin blocked muscle and PBS treated muscle (increased expression of gene sets of aerobic metabolism and increased protein expression of certain transcription factors regulating aerobic metabolism). (Hulmi et al. 2013b; Kainulainen et al. 2015). There are also several other
studies that have indicated that voluntary wheel running improves skeletal muscle function of the mdx mice (Call et al. 2008; Baltgalvis et al. 2012; Selsby et al. 2013; Hourde et al. 2013). It has to be mentioned that in the study conducted by Selsby et al. (2013), the length of the intervention period was one year and the peak mean weekly distance that mdx mice ran was approximately 100 km. However, the weekly running distances significantly decreased during the one year intervention and the distance peaked very early in the study intervention. Oxidative stress was not measured, but running improved heart and muscle function making the interpretation of the results of this thesis even more puzzling as it was speculated that there was excess running in this study that provoked oxidative stress. Compared to study conducted by Selsby et al. (2013), the running volume in this study was not exceptionally high for mdx mice. It could be speculated that perhaps increased oxidative stress is transient and after a while mice start to tolerate exercise.

Even though there were running and running x sActRIIB-Fc effect in protein carbonyls, the only significant difference in the mean values of protein carbonyls between the groups was observed between sActRIIB-Fc administered sedentary group and sActRIIB-Fc administered running group. No significant difference was found between both of the running groups and wild-type group. This may indicate that running independently and in combination with sActRIIB-Fc did not provoke oxidative stress in such a magnitude that it would be seriously harmful to the mdx mice or would exacerbate the symptoms of DMD. However, already published data from this same study showed that running had positive effect on the markers of aerobic metabolism. In addition, blocking ActRIIB ligands induced muscle growth. These favourable effects did not translate into clear improvement in strength endurance performance, test or muscle pathology (Hulmi et al. 2013b). Based on this study, it is suggested that excess voluntary wheel running caused oxidative stress, which might have turned over the positive effects of the running and sActRIIB-Fc administration.

It could be speculated that mdx mice need exogenous anti-oxidants in order to promote their anti-oxidative capacity and to improve the pathophysiology of DMD, if the amount and the intensity of running is such that it was in this study. To support this claim, Call et al. (2008) showed that 3 weeks of voluntary wheel running in combination with green tea extract (antioxidant) improved muscle pathology, serum anti-oxidative capacity and
reduced lipid peroxidation in gastrocnemius and heart muscle. However, it has to be mentioned that running alone also improved all of the above mentioned variables in that study. The difference between this thesis and study made by Call et al. (2008) was that training time was 4 weeks shorter and mice were approximately 7 weeks younger in the study of Call et al. (2008). The total amount of exercise and the fact that young (4 weeks old) mdx mice seem to tolerate better exercise induced stress than adult mdx mice (6 months old) (Carter et al. 1995) may explain the different results in this thesis and the study of Call et al. (2008). Although the age affects the running tolerance, the difference between the age of the mice in this thesis and in the study made by Call et al. (2008) was not significant, which emphasizes the difference in the total amount of voluntary wheel running. Furthermore, to support the role of exogenous anti-oxidants in ameliorating the symptoms of DMD previous studies have shown that administration of green tea extract decreases muscle necrosis and scavenges ROS in sedentary mdx mice (Buetler et al. 2002) and administration of N-acetylcysteine (Whitehead et al. 2010) (antioxidant) decreased the ROS levels of mdx mice and was accompanied with increased expression of utrophin (homologue of dystrophin) in sedentary mdx mice. It has to be mentioned that in the future clearly more studies are needed to examine dose and mode of exercise in muscular dystrophy.

9.2 Glutathione metabolism

It was hypothesized that running and sActRIIB administration independently and combined would decrease the levels of oxidized glutathione and increase the levels of reduced glutathione. However, the results were not consistent with hypotheses. Unpublished data from the current study showed that mdx mice tended to have decreased gene sets of glutathione metabolism and oxidoreductase pathway using micro-array method. However, both running and combination of running and administration of sActRIIB-Fc tended to increase the both gene sets (GSEA) (for a list of genes involved in GSH metabolism, see appendix 1). (Kainulainen et al. 2015.) The present thesis examined this further analyzing glutathione levels in muscle. No changes in total glutathione were observed between the treatments. In fact, mdx phenotype had higher levels of glutathione compared to wild-type control. It could be speculated that as an adaptation to chronic ROS exposure, mdx mice had elevated levels of glutathione. To support this
claim, mdx mice had also higher oxidized glutathione level compared to wild-type mice. However, this claim is only partially consistent with the results of Dudley et al. (2006) who reported that 6–8 weeks old mdx mice have reduced total glutathione levels and increased ratio of oxidized and reduced glutathione compared to healthy mice.

Contrary to no observed changes in glutathione, combination of running and sActRIIB-Fc administration tended to increase the oxidized glutathione levels. Thus, it seems that running independently and in combination with sActRIIB-Fc evoked free radical production, which did not translate into increased levels of total glutathione as an adaptation to increased levels of free radicals. It has been shown by Schill (2014) in unpublished MSc thesis that four weeks of forced treadmill running (12 m/min for 30 min 2 x per week) leads to decreased oxidation of glutathione in quadriceps and abdominal muscle in mdx mice, showing that moderate-intensity low-volume exercise might have an anti-oxidative effect in mdx mice. In addition, the study also showed that, even if exercise had positive effect on oxidative profile of the skeletal muscle, it also increased muscle fibrosis, which is not favourable, because DMD also itself increases muscle fibrosis. In addition, high intensity high volume endurance training has been shown to increase total glutathione levels of healthy skeletal muscle as an adaptive mechanism to defend against free radical production (Sen et al. 1992; Leeuwenburgh et al. 1997), but this effect was not seen in the current thesis in mdx mice. Thus, although positive effects of exercise were reported earlier (Hulmi et al. 2013a; Kainulainen et al. 2015) it can be speculated that partially due to oxidative stress no positive effects were seen in muscle endurance or muscle strength.

9.3 Sirtuins and AMPK

In this study, it was hypothesized that voluntary wheel running would increase or would not change the expression of sirtuins and AMPK. The activity of the sirtuins is regulated at least at transcriptional level, by post-translational modifications and by formation of functional complexes with other proteins and at substrate level (NAD$^+$) (Houtkooper et al. 2012). In this thesis the catalytic activity of the sirtuins was indirectly assessed by measuring protein expression of SIRT1, SIRT3 and SIRT6 and by measuring the phosphorylation of SIRT1 at ser 46. There was no difference in the expression of any of the
sirtuins between the treatments but mdx phenotype showed decreased expression of SIRT1 compared to wild-type mice. This is not in line with the previous study, in which there was no difference in SIRT1 protein and mRNA expression between wild-type and mdx mice (age 10–12 weeks) (Chalkiadaka et al. 2014). Another study showed that mdx mice have lower mRNA expression levels of SIRT1 and 4 months of voluntary wheel running seems to increase it (Hourde et al. 2013). According to Camerino et al. (2014) mRNA expression of SIRT1 is increased in mdx mice compared to wild-type mice. It was also concluded that 4 weeks (starting at the age of 4 weeks) of treadmill running (12 m/min for 30 mins 2 x/week) did not change the mRNA expression of SIRT1 but 12 weeks of treadmill running led to a significant downregulation of SIRT1 mRNA expression. The exercise protocol that was used in the study of Camerino et al. (2014) was planned to exacerbate the symptoms of DMD. In that study, it was concluded that mdx mice can not tolerate 12 weeks of exercise of such intensity and volume, which led to increased expression of inflammatory cytokines (TNF-α) and downregulation of PPAR-α, PGC-1α and SIRT1 mRNA expression. It must be mentioned that exercise protocol in the current thesis was not planned to exacerbate the symptoms of DMD, but it shifted the redox-balance for oxidants. However, voluntary wheel running in the current thesis was not as deleterious for mdx mice (to see more information besides this thesis see Hulmi et al. 2013a, Hulmi et al. 2013b; Kainulainen et al. 2015) as forced treadmill running was in the study of Camerino et al. (2014).

In this thesis the phosphorylation of SIRT1 was measured using an antibody specific to a sequence against human sirtuin at Ser 47. According to Nasrin et al. (2009) and Gao et al. (2011), equivalent phosphorylation site of human SIRT1 at ser 47 is at serine 46 amino acid of the protein in mice. In addition, phosphorylation site at Ser 47 is conserved between human and mice (Sasaki et al. 2008). In mice, the phosphorylation of SIRT1 at Ser 46 has been measured using the same antibody as was used in this thesis with success (antibody for human Ser47, Cell Signalling product ID: 2314) (Gao et al. 2011; Lu et al. 2011). In addition, phosphorylation of SIRT1 at Ser 47 was measured in female pigs using the same antibody (Bai et al. 2012). Therefore, it was valid to measure the phosphorylation of SIRT1 at Ser 46 with antibody for human ser 47.

The combination of voluntary wheel running and sActRIIB-Fc and voluntary wheel running alone increased the phosphorylation of SIRT1 at serine 46. This coincided with
increased protein carbonyls and the ratio of oxidized glutathione and reduced glutathione. Thus, it can be speculated that cellular/oxidative stress created by the combination of exercise and sActRIIB-Fc increased the phosphorylation of SIRT1 at serine 46 in order to promote the anti-oxidative properties of the SIRT1. To support this claim, Wen et al. (2013) showed that the phosphorylation of SIRT1 at Ser 46 has been shown to increase the anti-oxidative properties of SIRT1 in thoracic aorta in mice. In addition, phosphorylation of SIRT1 at serine 47 (same as ser 46 in mice) in combination with phosphorylation at serine 27 and threonine 530 has been shown to increase the nuclear localization of SIRT1 and enzymatic activity in response to H$_2$O$_2$ exposure in human kidney cells \textit{in vitro}. However, the outcome, in other words, cellular oxidative damage was not measured. (Nasrin et al. 2009.) In contrast, the phosphorylation of SIRT1 at ser 47 has been shown to decrease the enzymatic activity of SIRT1 in humans (Back et al. 2012) and pigs (Bai et al. 2012). As the catalytic properties of SIRT1 were not measured, it can be only speculated whether the outcome of the increased phosphorylation at ser 46 is protective against oxidants or not. At least in mice according to Wen et al. (2013), phosphorylation of SIRT1 at ser 46 alone is enough to promote the anti-oxidative properties of SIRT1. In contrast, in humans phosphorylation of p-SIRT1 at Ser 47 does not seem to independently increase its catalytic activity and at least phosphorylation at Ser 27 is also needed (Nasrin et al. 2009; Back et al. 2011; Bai et al. 2012; Wen et al. 2013). It has to be mentioned that phosphorylation site at ser 27 does not exist in mice, suggesting that more research is needed in the future to fully understand the mechanisms and the outcomes of the phosphorylation of the SIRT1 in response to oxidative stress in mice. Even if the increased phosphorylation of SIRT1 at ser 46 caused by exercise alone and in combination with sActRIIB-Fc would be anti-oxidative, the outcome of the running combined with the sActRIIB-Fc was increased amount of protein carbonyls and increased ratio of oxidized glutathione and reduced glutathione that are indicators of elevated levels of oxidative stress and damage.

Interventions that increase the enzymatic activity of SIRT1 seem to be beneficial for muscular dystrophies. Administration of resveratrol is pharmaceutical way to activate SIRT1 and increase its enzymatic activity by increasing the cellular NAD$^+$-levels (Park et al. 2012). Activation of SIRT1 by resveratrol has been shown to reduce myofiber loss and muscle fibrosis, which are accompanied with reduced ROS production in mdx mice. However, in that study resveratrol administration was not able to inhibit the cyto-
kine and inflammatory cell infiltration, but was able to inhibit their inflammatory processes in SIRT1 dependent-manner. (Hori et al. 2011.) In addition, 10 days of resveratrol administration has been shown to increase dystrophin homologue protein utrophin and PGC-1α mRNA expression and to reduce inflammation in skeletal muscle of the mdx mice, which is beneficial for the mdx mice (Gordon et al. 2013). Furthermore, transgenic mice over-expressing SIRT1 have significantly ameliorated symptoms of DMD (Chalkiadaki et al. 2014). The proposed mechanism is the SIRT1-mediated shift from glycolytic muscle phenotype to more oxidative muscle phenotype which is less susceptible to muscle damage that occurs in DMD (for a review see Ljubicic et al. 2013). In addition, SIRT1 over-expression has been shown to increase the neuromuscular junction proteins and dystrophin homologue protein utrophin, which improved functional properties of the dystrophin deficient muscle (Chalkiadaki et al. 2014). These studies indicate that interventions, in order to promote the catalytic activity of SIRT1, are beneficial for the mdx mice. Results shown in this thesis are consistent with results of Chabi et al. (2009), who showed in their study that SIRT1 expression does not change in response to eight week voluntary wheel running in wild-type rat skeletal muscle. Furthermore, decrease in SIRT1 expression in response to long-term electric stimulation (Gurd et al. 2009) and increase in SIRT1 protein expression after one acute bout of endurance exercise has been reported in rats (Suwa et al. 2008). However, as it was mentioned earlier, the catalytic activity of the sirtuins is regulated in addition to post-translational modifications, which was already discussed earlier, by formation of protein complexes and at substrate level (cellular NAD⁺-levels) (Houtkooper et al. 2012). In addition, the acetylation levels of SIRT1 targets such as PGC-1α and several histones could be measured in order to assess the catalytic properties of the SIRT1. These should be measured in the future.

Wen et al. (2013) showed that CaMKK-β phosphorylates not only SIRT1 at ser 46, but also AMPK at thr 172 in response to oxidative stress in thoracic aorta in healthy mice. Significant correlation was found in the current thesis between p-SIRT1 at Ser 46 and p-AMPK at Thr 172 in running and sActRIIB-Fc administered mdx mice. In addition, pAMPK and protein carbonyls correlated almost significantly in sActRIIB-Fc administered running group. Thus, it is suggested that mice exposed to increased oxidative stress due to either running alone or combined with sActRIIB-Fc administration also increased the phosphorylation of AMPK at thr172, perhaps in order to increase the anti-
oxidative system in parallel with the phosphorylation of SIRT1 at ser 46. However, it must be mentioned that the analyzed cells in the current thesis were from muscle and Wen et. al (2013) used endothelial cells from thoracic aorta.

Previous studies have shown that SIRT3 and SIRT6 have anti-oxidative properties (Kong et al. 2010; Kawahara et al. 2009; Mao et al. 2011) and at least two previous studies have shown that exercise increases the protein expression of SIRT3 in healthy mice and rats (Palacios et al. 2009; Hokari et al. 2010), whereas Koltai et al. (2009) showed that treadmill running did not change the SIRT6 expression in young healthy mice, but attenuated the age related increase in SIRT6 expression. Since exercise training increases the cellular NAD$^+$-levels (Koltai et al. 2010), which is the catalytic fuel of the sirtuins, we hypothesized that voluntary wheel running would increase catalytic activity of the SIRT3 and SIRT6 and this could be mediated at least to some extent via protein expression. However, there were not any significant differences in the expression of SIRT3 and SIRT6 between any of the treatments. Finally, even if exercise has been shown to increase the activity of sirtuins in some studies and at least the increased activity of SIRT1 is beneficial for the the pathology of the DMD, it is shown in the current study that voluntary wheel running does not change the protein expression levels of sirtuins 1, 3 and 6 chronically as an adaptation to voluntary wheel running. In addition, mdx mice overexpressing SIRT3 and SIRT6 needs to be studied to find out whether treatments in order to increase their activity would be beneficial for the pathology of the DMD.

It was hypothesized that voluntary wheel running could increase the protein expression of AMPK and phosphorylation of AMPK at thr 172. However, no changes in total protein content of AMPK or p-AMPK was observed between the treatments. However, there was decreasing running and sActRIIB-Fc administration interaction effect in the ratio of pAMPK and AMPK (pAMPK/AMPK) and significant difference in pAMPK/AMPK between sedentary mdx placebo group (mdx PBS) and sedentary mdx sActRIIB-Fc administered group. To speculate this, it seems that sedentary mdx mice that are chronically administered with sActRIIB-Fc show increased ratio of pAMPK/AMPK perhaps due to some kind of energy stress. However, as sActRIIB-Fc administration is combined with 7 weeks of voluntary wheel running, pAMPK/AMPK shows decreasing effect. This effect however is due to increased total AMPK levels and
not due to decreased levels of pAMPK. Thus, it is suggested that voluntary wheel running tended to increase the total AMPK levels, but the change was not statistically significant in both of the running groups. To speculate the timing effect on the results of AMPK, it was shown in the study of (Ljubicic et al. 2012) that acutely after an exercise bout, phosphorylation of AMPK increases in mdx mice but there is no change in total AMPK levels. Mice in the current thesis did not have access to running wheels for 48 hours before they were euthanized to prevent the acute effects of exercise and, thus it is speculated that at least the levels of pAMPK could have been higher, if the muscle samples had been collected closer to the last training session. Even though chronic activation of AMPK signalling pathways have shown beneficial effects on mdx mice (Ljubicic et al. 2011; Al-Rewashdy et al. 2015), it is shown in the current study that 7 weeks of voluntary wheel running alone and combined with sActRIIB-Fc administration do not change the protein expression levels of AMPK and pAMPK chronically.

The timing of mouse euthanization and collecting muscle samples may also explain some of the sirtuin protein expression results that we obtained. Mice in the current thesis did not have access to running wheels for 48 before they were euthanized to prevent the acute affects of exercise. This claim is supported at least by Suwa et al. (2008), who showed that SIRT1 protein expression increases acutely after one exercise session in healthy rat skeletal muscle, but does not change in response to long term voluntary wheel running in healthy rats. Unfortunately, the timing of euthanization was not reported. (Chabi et al. 2009.) SIRT3 expression was shown to increase in response to 6 weeks of voluntary wheel running in healthy mice. It was mentioned that muscle samples were collected after the intervention period. (Palacios et al. 2009.) Thus, it is assumed that muscle samples were collected closer to the last training session than in the current study. To support the role of timing in collecting muscle samples regarding protein expression of SIRT3, Hokari et al. (2010) showed that 3 weeks 7 times per week of treadmill running increases the protein expression of SIRT3 in healthy rat skeletal muscle. The muscle samples were collected approximately 21 h after the last exercise session, which is significantly less than than in the current study. Thus, it could be speculated that the protein expression of SIRT1, 3 and 6 could have been higher, if the timing of the muscle sample collection had been closer to the last running session.
9.4 Sources of ROS/oxidative stress

Impaired calcium homeostasis has been suggested to play an important role in promoting the pathology of the DMD. Previous studies have shown that mdx mice show elevated resting cytosolic Ca\textsuperscript{2+}-ion levels (Turner et al. 1991; Imbert et al. 1995; Hopf et al. 1996), while some studies have not seen differences in resting cellular Ca\textsuperscript{2+}-levels between wild-type and mdx mice e.g. (Leijendekker et al. 1996). According to Whitehead et al.’s (2005) review, the differences in the resting sarcoplasmic Ca\textsuperscript{2+}-levels between different studies are firstly dependent on the methods that were used to measure Ca\textsuperscript{2+}-levels, secondly on the age of the cells and thirdly on the previous contractile activity. The proposed mechanism leading to increased cytoplasmic Ca\textsuperscript{2+}-levels in mdx mice is not fully clear. However, at least increased influx of Ca\textsuperscript{2+}-ions through sarcolemma, more specifically through mechanosensitive TRPC channels and increased leakage of Ca\textsuperscript{2+}-ions through sarcoplasmic reticulum via calcium-ion channels (RyRs and IP3Rs) are associated with increased cytosolic Ca\textsuperscript{2+}-ion levels in mdx mice. In addition, reduced activity and protein expression of the SERCA (protein that transports Ca\textsuperscript{2+}-ions from cytosol back to sarcoplasmic reticulum) has been linked to increased cytosolic Ca\textsuperscript{2+}-ion levels in mdx mice. (reviewed in Vallejo-Illarramendi et al. 2014.)

According to Shkryl et al. (2009), nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H) oxidase is a major source of ROS in mdx mice following a mechanical stress. In that study, mechanical stress was applied by osmotic shock, which increases the cytosolic Ca\textsuperscript{2+}-levels transiently. The duration of the “Ca\textsuperscript{2+}-spark” however, is longer in mdx mice than in wild-type mice. Authors speculated that mechanical stress leads to increased NAD(P)H oxidase dependent production of ROS, which may create a vicious circle, in which ROS increases the calcium influx to the cytosol either reacting with sarcoplasmic reticulum (SR) calcium channel protein (RyR) increasing the Ca\textsuperscript{2+}-release from the SR into cytosol, or by oxidizing the sarcoplasma making the sarcolemma more permeable to Ca\textsuperscript{2+}-ions, enabling the influx of Ca\textsuperscript{2+}-ions into the cytosol. Increased cytosolic Ca\textsuperscript{2+}-levels increase the mitochondrial uptake of Ca\textsuperscript{2+}-ions, which in turn increase the mitochondrial ROS production. Increased cytosolic Ca\textsuperscript{2+}-ion level may also increase NAD(P)H oxidase dependent ROS production. To support the role of NAD(P)H oxidase and ROS in generating the increased cytosolic Ca\textsuperscript{2+}-levels, blocking
NAD(P)H oxidase with pharmaceutical agents and administration of antioxidants has been shown to prevent the influx of Ca\textsuperscript{2+}-ions into cytosol in mdx mice (Gervasio et al. 2008; Shkryl et al. 2009; Whitehead et al. 2010).

Fraysse et al. (2004) showed that forced treadmill running (12 m/min on a horizontal treadmill, twice a week, for 4–8 weeks) significantly increased already elevated Ca\textsuperscript{2+}-levels in mdx mice. To speculate the mechanisms of increased ROS production in the current study, it is suggested that mechanical stress induced by voluntary wheel running may have increased the NAD(P)H-oxidase dependent ROS production, consequently leading to increased sarcoplasmic Ca\textsuperscript{2+} levels, further increasing the NAD(P)H oxidase and mitochondrial dependent ROS production. However, this is only speculation since, neither sarcoplasmic Ca\textsuperscript{2+}-levels, nor NAD(P)H-oxidase dependent ROS production were measured due to methodological challenges in \textit{in vivo} studies. It has to be also kept in mind that running clearly acutely increases the calcium levels in muscles in mdx mice. However, in the current study mice did not run for two days before they were euthanized. Thus, it could be speculated that redox-balance would have been even more in the oxidant direction if mice had been euthanized closer to the last running session. However Liu et al. (2000), showed that 8 weeks of high intensity high volume treadmill running leads to greater oxidative damage than one acute high intensity running session in healthy rat skeletal muscle. In the chronic situation mice rested 48 h before they were euthanized and in acute protocol mice were euthanized immediately after the exercise session. Thus, it seems that oxidized cellular compartments remain in muscle cells at least 48 after chronic exercise protocol. Furthermore, the study conducted by Liu et al. (2000) did not resolve the question, whether the cellular oxidation would be even higher, if mice are immediately euthanized after the last exercise session without any recovery.
10 CONCLUSION

As a conclusion, this study suggests that in mdx mice voluntary wheel running especially combined with sActRIIB-Fc increases oxidation of glutathione and production of protein carbonyls, which are indicators of increased oxidative stress and damage. Furthermore, it is concluded that voluntary wheel running and blocking ActRIIB-Fc either alone or combined do not change the protein expression of sirtuins 1, 3, 6, AMPK and pAMPK in mdx mice. However, the combination of voluntary wheel running and administration of sActRIIB-Fc, increased the phosphorylation of SIRT at serine 46 perhaps in order to promote the anti-oxidative properties of the SIRT1, due to increased oxidative stress.

Previously published data from this same study suggested that voluntary wheel running and blocking ActRIIB ligands using sActRIIB-Fc either independently or combined do not ameliorate the pathophysiology and functional properties of the mdx mice, even though exercise had positive effect on biomarkers of the aerobic metabolism pathways and blocking sActRIIB-Fc ligands increased skeletal muscle mass. Based on the data from this study, it is suggested that increased oxidative stress may play a role in turning over the positive effects of exercise and ActRIIB ligand blocking. Suggested combinatorial effects of sActRIIB-Fc administration and voluntary wheel running are summarized in figure 15 and suggested independent effects of voluntary wheel running are summarized in figure 16.
FIGURE 15. Combination of voluntary wheel running and sActRIIB-Fc administration increased: the markers of oxidative capacity (Ox. capac.: citrate synthase, succinate dehydrogenase (SDH) and (MA) micro-array gene sets), reactive oxygen species (ROS: ratio of oxidized glutathione and reduced glutathione), phosphorylation of SIRT1 at ser46 (perhaps to promote the anti-oxidative properties of SIRT1) and increased the glutathione metabolism related micro-array genesets but didn’t affect reduced glutathione levels or antioxidative enzyme activity levels. Endogenous anti-oxidative capacity (Endog. antioxid. capac.) wasn’t able to rescue the cellular compartments from increased ROS and the result was increased oxidative damage (Ox. damage: protein carbonyls). Increased oxidative capacity was suggested to improve the performance in hangwire test but increased oxidative damage perhaps blocked the positive effects of running.
FIGURE 16. Combination of voluntary wheel running and sActRIIB-Fc administration increased: the markers of oxidative capacity (Ox. capac.: citrate synthase, succinate dehydrogenase (SDH) and (MA) micro-array gene sets), reactive oxygen species (ROS: ratio of oxidized glutathione and reduced glutathione and increased the glutathione metabolism related micro-array gene sets but didn’t affect reduced glutathione levels or antioxidative enzyme activity levels. Endogenous anti-oxidative capacity (Endog. antioxid. capac.) wasn’t able to rescue the cellular compartments from increased ROS and the result was increased oxidative damage (Ox. damage: protein carbonyls). Increased oxidative capacity was suggested to improve the performance in hangwire test but increased oxidative damage perhaps blocked the positive effects of running.
11 FUTURE STUDY PROPOSALS

Duchenne’s muscular dystrophy is a severe disease leading to premature death without proper cure. In addition, the quality of life of DMD patients is significantly worsened due to difficulties in locomotion and breathing, making everyday life very difficult. Exercise has been shown to be beneficial at least for mdx mice in some of the previous studies. Also other strategies such as blocking activins/myostatin and administration of exogenous anti-oxidants have been shown to ameliorate the symptoms of the DMD in mdx mice. Based on this study, it is suggested that the role of exogenous antioxidants combined with activin/myostatin blocking and running needs to be further studied in the future. The volume, mode and the intensity of the exercise needs to be further studied since there is no clear consensus in what kind of exercise is the most appropriate for the mdx mice or DMD human patients. Furthermore, the accumulation of data obtained from the animal model studies needs to be applied to human DMD patients, in the future.

This thesis clearly demonstrated that voluntary wheel running alone and combined with sActRIIB-Fc administration shifted the redox-balance to oxidants. However, mechanisms causing the shift in redox balance were not studied. Thus, it is suggested that mechanisms causing the running induced increase in ROS production in mdx mice needs to be further studied, in the future.

Even though the above mentioned strategies show promising results that ameliorate the pathophysiology of DMD, any of them is not perfect cure that could completely cure the disease. DMD originates from the absence of the functional protein called dystrophin. Thus, gene therapy strategies that would replace the mutated dystrophin gene with functional dystrophin gene should be further developed in the future, together with lifestyle strategies.


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myostatin in adult mice increases skeletal muscle mass and strength. Biochemical and Biophysical research communications. 300: 965–971.


APPENDIX 1: Leading-edge genes in sActRIIB-Fc running vs. PBS sedentary genes

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Leading-edge genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione metabolism</td>
<td>GSTA3, MGST1, GSTT2, GPX7, IDH1, GSTT1, RRM2, IDH2, GSTM5, GPX3, GSTZ1, GSTK1, GSTA1, MGST2, GSTP1, GGT5, GSTO2, MGST3, GSTM3, GPX5, GSTM4, RRM2B, GSTA4, TXNDC12, GSS</td>
</tr>
</tbody>
</table>

APPENDIX 2: List of primary and secondary antibodies used in this study

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Ab to SIRT1</td>
<td>abCam ab28170</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Goat Ab to SIRT3</td>
<td>abCam ab118334</td>
<td>horseradish peroxidase-conjugated secondary anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Rabbit Ab to SIRT6</td>
<td>abCam ab62739</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Rabbit Ab to p-SIRT1</td>
<td>Cell Signalling 2314</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Rabbit Ab to AMPK</td>
<td>Cell Signalling 2532</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Rabbit Ab to pAMPK</td>
<td>Cell Signalling 2531</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
</tr>
<tr>
<td>Rabbit Ab to GAPDH</td>
<td>abCam ab9485</td>
<td>horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)</td>
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<td>-------------------</td>
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<td>------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Rat Ab to DNP</td>
<td>Cat# 04-8300, Zymed Laboratories, San Francisco, CA.</td>
<td>anti-rat IgG AlexaFluor 680 conjugated secondary antibody (Life Technologies, Carlsbad, CA, USA)</td>
</tr>
</tbody>
</table>