Signalling role of skeletal muscle during exercise

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ABSTRACT

Upon acute exercise skeletal muscle is immediately and heavily recruited, while other organs appear to play only a minor role during exercise. These other organs show significant changes and improvements in function, although they are not directly targeted by exercise. These improvements suggest that skeletal muscle can communicate with other organs. In the past 15 years it became clear that skeletal muscle produces and secretes a variety of signalling proteins that are able to interact and communicate with other organs. These signalling proteins are called myokines and are likely the link between exercising muscle and the rest of the body.

The aim of the research presented this thesis is to study the signalling role of skeletal muscle during exercise and to gain further insight in the local molecular changes in skeletal muscle induced by exercise.

In the first part of this thesis the focus was on the local changes induced in skeletal muscle by acute exercise and exercise training. The aim was to gain more insight in the molecular basis of exercise-induced changes in skeletal muscle. For exercise training we performed a microarray analysis on human muscle biopsies taken from endurance, resistance and combined exercise training interventions. We showed that despite a substantial overlap between the three exercise training types, each of the exercise training types had an unique gene expression print. The combined exercise training gene expression print lacked some specific oxidative and PPAR related components compared to endurance exercise training. For acute exercise microarray analysis was performed on muscle biopsies taken before and after an one-legged cycling intervention from resting and exercising skeletal muscle. Results showed that acute exercise induced large gene expression changes in active skeletal muscle. Furthermore, results showed that acute exercise also induced gene expression changes in resting skeletal muscle and that these changes were likely systemically induced via free fatty acids.

In the second part of this thesis the focus was on the signalling role of skeletal muscle during exercise. Secretome analysis was performed on the microarrays of the muscle biopsies taken before and after the one-legged cycling intervention. This secretome analysis resulted in a list of putative myokines of which a selection was measured in the plasma. CCL2 (MCP-1) and CX3CL1 (Fractalkine) were the myokines that were induced both in plasma and at the mRNA level. In that same study we showed that the increase in Angptl4 mRNA levels was higher in the resting leg compared to the exercising leg. Follow-up studies using cell culture and mice models revealed that Angptl4 levels were increased in the resting leg

via free fatty acids that activated PPARs. In the exercising leg the increased Angptl4 levels were inhibited via AMPK activation. This resulted in an influx of triglyceride derived fatty acids in the exercising, but not in the resting skeletal muscle.

In conclusion, we showed that exercise not only elicits molecular changes in active or trained skeletal muscle, but also in non-active organs such as resting skeletal muscle. Furthermore, we were able to identify several myokines produced by skeletal muscle during exercise, of which CCL2, CX3CL1 and Angptl4 were the most promising. CCL2, CX3CL1 and Angptl4 all increased plasma levels during acute exercise. It remains unclear what the systemic role is of these myokines. For Angptl4 we were able to provide more insight in the mechanism and local functioning during exercise. We concluded that Angptl4 is important in the substrate distribution during exercise. From the results presented this thesis we conclude that skeletal muscle has an important signalling role during exercise, but that it remains unclear how important this signalling role is systemically.

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Chapter 1

General introduction

DIABETES TYPE 2

Diabetes type 2 and other metabolic diseases such as obesity and metabolic syndrome are affecting an increasing number of people, placing a large burden on health care and society. The increased prevalence of obesity and diabetes is likely attributable to increased prosperity and over-availability of a wide variety of (un)healthy foods in combination with a more sedentary lifestyle [1,2]. The increase in global prevalence of metabolic disease rates shows no signs of slowing down, largely due to the steep incline in metabolic diseases in former developing countries [3].

Diabetes type 2 is characterized by increased insulin resistance in peripheral tissues such as liver, adipose tissue and skeletal muscle [4]. Insulin resistance is one of the first hallmarks of diabetes type 2 [5]. Another hallmark of type 2 diabetes is insufficient insulin production by beta cells [5]. Obesity is one of the major risk factors for diabetes type 2 and insulin resistance, together with the other characteristics of metabolic syndrome, such as high blood pressure, dyslipidaemia, high fasting blood glucose levels [6]. Insulin resistance in peripheral tissues is characterized by impaired GLUT4 translocation, allowing less glucose to enter the cells [7]. Furthermore, insulin receptor substrate (IRS-1) phosphorylation and phosphatidylinositol (PI) 3-kinase activity are impaired in insulin resistance [8]. What causes these impairments in insulin signalling pathway remains unclear, but a role for toxic lipid metabolites such as diacylglycerol, ceramide and fatty acyl CoA is suggested [9].

Several strategies are used to treat type 2 diabetes. Lifestyle and pharmaceutical interventions are currently the most common strategies. Most often diabetic patients are treated with metformin and receive dietary advice to lose weight. In addition, exercise is prescribed as lifestyle intervention to improve health of diabetic patients. Exercise is also often used in the prevention of diabetes type 2. Research shows that of all treatments available to type 2 diabetic patients, exercise is probably the most efficient and most cost-effective [6,7,10-12]. Several large scale interventions studies in humans show that long-term lifestyle interventions consisting of only exercise training or the combination of exercise training and dietary counselling significantly reduces the incidence of type 2 diabetes [12-15]. Short- and long-term exercise training intervention studies show a dramatic effect of exercise training on insulin resistance [16,17]. Accordingly, extensive research is carried out aimed at understanding the exact effects of exercise on health. Also the mechanism behind the positive health effects are extremely important to reveal, since this will provide more insight in the effects of exercise in the context of type 2 diabetes.

EXERCISE

Exercise training generally leads to enhanced performance. Athletes train to get stronger, faster and more skilled. Many studies have addressed the effect of exercise training on the body and have shown that the effects are large and multifactorial. Maximal oxygen uptake increases by more efficient oxygen transport and uptake, leading to improved endurance performance [18,19]. Lactate and CO_2 are more easily removed, reducing the fatigue during acute exercise [20]. Heart rate at a fixed intensity decreases, allowing to perform at a higher intensity [21]. Neural activation of skeletal muscle improves, allowing more efficient movement [22,23]. Since exercise training improves neural activation and induces hypertrophy, muscles get stronger which increases strength and power [22]. All these effects are examples of exercise training induced changes and how these changes result in performance enhancement.

Exercise training also has a large impact on health, especially in diseased conditions. Besides being beneficial for diabetes type 2 and other metabolic diseases, also cardiovascular disease can be prevented or treated by exercise training. Exercise causes a reduction of insulin resistance of peripheral tissues [16,17]. Furthermore, exercise training has been shown to improve factors such as cholesterol levels and blood pressure [24]. Reductions in blood pressure and cholesterol levels reduce the risk for cardiovascular disease and slow down the progression of cardiovascular disease. Another example of the health effects of exercise training is that recovery after chemotherapy is accelerated when exercise is performed [25]. The large impact of exercise training on health implies that exercise is a very important and interesting target for further research.

It is important to realize that acute exercise and exercise training have very different effects. Acute exercise elicits a more stress-like response compared to a chronic adaptive response that is seen after exercise training. Studies assessing the role of exercise on health have focussed on endurance training and/or resistance training. Some studies combined resistance and endurance exercise training. Combined exercise training is thought to be the most effective type of exercise to reduce insulin resistance in diabetes type 2 [26].

To understand how exercise positively affects health and performance, it is important to gain more insight into the molecular biology of exercise and to elucidate the molecular pathways and processes triggered by exercise. Changes and adaptations induced by acute exercise and exercise training occur throughout the body. Changes in enzymes activity levels are known to play a crucial role, but also changes in mRNA levels are critical

in the regulation of the response to exercise. Literature has shown that whole genome gene expression profiling or transcriptomics is an efficient and valuable way to gain insight into molecular mechanisms [27], as it provides a complete overview of the gene expression changes on a specific time point.

TRANSCRIPTOMICS

In the past, when researchers wanted to assess the effect of treatments on mRNA levels, most common techniques were Northern Blotting and qPCR. Both techniques allow only a limited number of genes to be measured at a time. Currently qPCR is still in use, but as technology evolved, high throughput gene expression profiling techniques became available. These high throughput gene expression profiling techniques include microarray and RNA-sequencing, allowing simultaneous measurement of all ~20,000 genes present in the human genome. The general term for high throughput mRNA profiling is transcriptomics, covering all techniques assessing mRNA levels of large subsets of genes. Microarray analysis or transcriptomics is used in many different research fields, assessing a wide variety of questions. Besides the human genome, also the genome of other species and plants are mapped and available for transcriptomics.

In human studies microarrays are used for many applications, including determination of the effects of pharmaceutical compounds or nutrition on gene expression. Microarray analysis is also used in exercise studies to assess effects on several organs such as adipose tissue [28] and peripheral blood mononuclear cells (PMBCs)[29]. Research in humans is limited by the fact that not all organs are reachable for tissue collection. The organ most heavily influenced by exercise is skeletal muscle, and this tissue can be relatively easily sampled. Several studies therefore focus on the changes in gene expression induced by exercise in skeletal muscle [27,30,31].

SKELETAL MUSCLE

As stated before, of all organs skeletal muscle is probably the most influenced by exercise. Skeletal muscle generates movements during exercise, which necessitates the initiation of many biological pathways. Mechanical work has to be performed, substrates and oxygen have to be taken up and metabolized and waste products have to be carried out of the muscle. What is remarkable, is that all these actions and many more occur in a very limited timespan, showing that skeletal muscle is an extremely rapidly and effectively

responding organ. Skeletal muscle is metabolically very active, since skeletal muscle is one of the main utilizers of glucose and free fatty acids and has a high turnover of ATP [32]. During exercise, glucose, liver and muscle glycogen storages, albumin-bound free fatty acids, intramyocellular triglyceride content and triglyceride-derived free fatty acids are used as fuel [33-35]. The balance between those substrates is largely determined by the intensity of the exercise: the higher the intensity, the higher the rate of glucose utilization [33]. The amount of energy derived from triglyceride-derived free fatty acids was long underestimated, but currently the opinion is that this source of energy is also important during exercise [36].
Besides all acute effects of exercise described in the previous paragraph, exercise training causes a more adaptive response in skeletal muscle. Exercise training for example affects the protein synthesis and degradation balance, causing hypertrophy [37]. Another example is that mitochondrial oxidative capacity is increased during exercise training, allowing more ATP to be generated via oxidative pathways [38]. These two examples illustrate all the adaptations induced by exercise training in skeletal muscle and show how closely exercise and skeletal muscle are linked.

SIGNALLING PROTEINS FROM NON-TRADITIONAL SECRETORY ORGANS

Since the initial discovery of secretion, numerous hormones have been identified. Until approximately 25 years ago, the general belief was that only traditional hormone-producing glands could excrete hormones, including the pituitary gland and islet of Langerhans. The discovery of leptin as first adipocyte-produced hormone profoundly changed our view on circulating signalling proteins and their origin [39]. While previously white adipose tissue was thought to merely serve as a depot for energy storage and for insulation [40], the identification of leptin led to the realization that white adipose tissue is a very active endocrine organ [39]. Extensive research has shown that white adipose tissue plays a crucial role in lipid and glucose metabolism and energy balance [40]. The revelations about white adipose tissue as an endocrine organ also resulted in interest in other organs. Subsequent studies showed that organs such as the brain, heart and bone could also secrete signalling proteins [41-43].
Until recently skeletal muscle was seen as an organ that hardly interacted with other organs, except via substrate fluxes. Whereas skeletal and cardiac muscle are immediately and heavily recruited upon exercise, other organs appear to play only a minor role during

exercise. Despite that these organs are not directly targeted by exercise, they still show significant changes and improvements in function [44], suggesting that skeletal muscle can communicate with other organs. In the last decade it has become clear that skeletal muscle produces and secretes a variety of signalling proteins that are able to interact and communicate with other organs [45].

MYOKINES

Signalling proteins produced and secreted by skeletal muscle are called myokines [44]. The definition of a myokine is that the signalling protein is produced by skeletal muscle during any condition, which implicates that a myokine does not have to be produced during exercise. Myokines, but also other compounds, produced by skeletal muscle during exercise are called exercise factors [44]. Exercise factors are a specific group of myokines that are characterized by production during exercise. After the identification of IL-6 as the first myokine, many studies have been performed to further establish the field of myokines [45]. The number of identified myokines increased and the role of those myokines in health and during exercise was studied. Well-known examples of recently identified myokines are Irisin [46], FGF-21 [47], Angptl4 [36], IL-8 [48] and IL-15 [49]. Exercise is an important and strong modulator of health, suggesting that identification of crucial mediators of exercise effects could potentially be relevant in gaining more insight in the effect of exercise on health. Myokines have a great therapeutic potential, since they may be the link between working muscle and the positive health effects of exercise. Currently, many myokines have been identified using a variety of approaches [50-52]. However, some knowledge on the physiological function is available for only a few myokines. Potential effects on development of (metabolic) diseases have remained an uncharted territory up to now.

OUTLINE OF THESIS

The aim of the research presented in this thesis is to study the signalling role of skeletal muscle during exercise and to gain further insight in the local molecular changes in skeletal muscle induced by exercise.

In chapter 2 an overview of the myokine field is given. In this review, all myokines identified in humans are discussed and their role in exercise is addressed. In chapter 3 microarray analysis is used to study the molecular effects of several types of exercise

training to generate more insight in the differences in training effects. We show that combined, endurance and resistance exercise training result in distinctive gene expression patterns. The most remarkable finding is that combined exercise training (endurance and resistance) lacks some crucial oxidative gene expression changes compared to endurance exercise training, implying that combining endurance and resistance exercise training might interfere with some favourable changes induced by endurance exercise training alone. In chapter 4 a human acute one-legged cycling model is used. With that model we assess the effects of acute exercise on gene expression in exercising and non-exercising skeletal muscle. The aim is to gain more insight into whether exercise can directly influence skeletal muscles that are not active during exercise. We show that acute exercise also has a large impact on gene expression in non-exercising skeletal muscle, besides an even larger effect on exercising muscle. These changes in the non-exercising muscle are induced via circulating factors. The presence of many PPAR target genes implicate a role for free fatty acids in the induction in the non-exercising leg. In chapter 5 a screening is performed to identify new myokines in the acute one-legged cycling study. In this chapter a secretome analysis is performed, resulting in a list of new putative myokines, of which a selection is measured in plasma. CCL2 and CX3CL1 are identified as myokines in chapter 5. Known myokines such as IL-6 and Irisin are not or partly confirmed as myokines. In chapter 6 the mechanism and role of one of the myokines identified in the acute one-legged cycling study is studied. This myokine Angptl4 plays an important role in the fuel substrate distribution between exercising and non-exercising skeletal muscle. Higher levels of Angptl4 in the non-exercising skeletal muscle direct triglyceride derived fatty acids to the working muscle where the substrates are needed. Finally, the general discussion and conclusions are presented in chapter 7.

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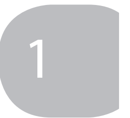

Chapter 2

The search for exercise factors in humans

Milène Catoire
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In preparation

ABSTRACT

Regular exercise has been shown to positively affect the risk for numerous chronic diseases. Exercise not only impacts the contracting skeletal muscle, but also elicits systemic alterations and impacts distant organs. The exact mechanisms driving the more systemic changes have yet to be resolved, but exercise factors are thought to be an important missing link. Exercise factors are proteins that are released from skeletal muscle into the circulation specifically during exercise. They represent a subclass of the myokines, which are classified as proteins secreted from skeletal muscle serving a signalling role. Here we provide an overview of the current literature on myokines. Presently, many studies focus on the identification of new myokines using a variety of approaches, ranging from proteomic analysis of media of cultured myocytes subjected to electrical pulse stimulation, to transcriptomics analysis of skeletal muscle of exercising mice and humans. These studies have generated an extensive list of myokines, yet the functional relevance of many of these novel myokines remain unclear. Few of these myokines represent putative exercise factors. Currently, the myokines IL-6, SPARC, Angptl4, CX3CL1 and CCL2 (MCP-1) have the highest potential to serve as exercise factors, since for all these factors there is clear evidence that plasma levels increase during exercise. While some additional effort into the identification of novel human myokines may be warranted, future research should primarily be dedicated towards determining the functional role of already identified myokines/ exercise factors during exercise.

INTRODUCTION

Exercise is a very powerful and comparatively simple way to treat and prevent metabolic diseases such as type 2 diabetes and obesity. Exercise positively influences many metabolic risk factors including fasting glucose levels [1], lipid profiles [2], blood pressure [3] and insulin sensitivity [4]. Currently, the mechanisms behind these changes are still partly unknown. Exercise has an impact on the entire body, but skeletal muscle is the organ that is activated most strongly by exercise, as skeletal muscle performs the energy demanding mechanical work. To provide the fuel for the contracting muscle, uptake and utilization of oxygen and energy substrates increase dramatically during exercise. All these changes occur within a very limited timespan, showing that skeletal muscle responds rapidly and effectively to physical stress. In addition to the rapid stress-like response during acute exercise, repeated exercise bouts as during chronic exercise training elicit an adaptive response. This adaptive response is associated with increased oxidative capacity and mitochondrial density, as well as by an increase in skeletal muscle insulin sensitivity [4-6]. The adaptations triggered by exercise training are not limited to skeletal muscle but influence the circulation and other organs, as illustrated by the exercise-induced reductions in blood pressure [3], reduced inflammatory status [7] and improved blood lipid profiles [2]. How exercise is able to affect and communicate with distant organs is not fully clear but the most plausible explanation is that these effects are mediated by proteins secreted from muscle called myokines, and more specifically myokines that are induced by exercise, which are often referred to as exercise factors.

It is now well established that skeletal muscle is capable of secreting a variety of proteins. The first protein identified that was found to be released from muscle tissue into the circulation during exercise is IL-6 [8,9]. As such, IL-6 represents the first myokine. From that moment onwards the quest for other myokines started, resulting in an increasing number of papers published on the topic. A variety of different approaches have been used to identify new myokines. Previous reviews on myokines were published several years ago [10-13], or focused on crosstalk with specific organs [14]. Here we give an overview of the current status in the myokine field in humans, with a focus on exercise factors. First a brief explanation of the term "exercise factor" is given, followed by clarification of the distinction between known myokines and exercise factors. Finally, the contemporary literature on myokines and exercise factors in humans is discussed.

WHAT IS AN EXERCISE FACTOR?

An exercise factor has been defined as a humoral factor produced by skeletal muscle in response to exercise [15,16]. Exercise factors have to be secreted into the circulation, implicating that they potentially have systemic effects [15,16]. Myokines on the other hand are proteins produced by skeletal muscle, that are not necessarily induced upon exercise nor do they have to have a systemic function [16]. Accordingly, a subset of myokines may be classified as exercise factors. A potential additional criteria for myokines is that they are exclusively produced by skeletal muscle. However, none of the factors currently labelled as myokine fulfils that criteria, with the possible but controversial exception of irisin [17], and it is questionable whether any protein will. Indeed, all of the known myokines are produced by a wide variety of tissues. For example, IL-6 is a well-known pro-inflammatory cytokine produced by many different organs and cells [18]. It should be emphasized that none of the proteins presently labelled as adipokine are exclusively produced by adipose tissue, with the exception of adiponectin. For this reason we support the following definition of a myokine: a myokine is any protein that is secreted by skeletal muscle that plays a signalling role in an autocrine, paracrine and/or endocrine manner. The classification as a myokine is irrespective of the proteins also being produced by other tissues.

Having defined the term myokine and exercise factor, it is important to make the distinction between exercise factors that are acutely induced by a single exercise bout and exercise factors that are chronically induced upon exercise training. In general, acute exercise and exercise training have very different effects on skeletal muscle, with acute exercise eliciting a more stress-like response compared to a chronic adaptive response following exercise training, likely resulting in production of a different set of myokines.

WHICH MYOKINES ARE EXERCISE FACTORS?

As discussed above, not all myokines are exercise factors and not all exercise factors are myokines, but in general most potential exercise factors are likely myokines. A variety of approaches have been used to find new myokines and explore their function. A possible approach is to perform myokine screenings in different types of models. Cell culture models are often used to perform screenings, since screenings in cell culture models are relatively easy to perform and give a ‘clean’ overview of the secretome of

skeletal muscle cells. The screenings in cell culture models resulted in lists of myokines that are produced by myocytes in vitro in rest [19], during differentiation [20] and with electrical pulse stimulation [21,22]. Another screening approach used human muscle biopsies collected before and after acute exercise as starting material, and was able to generate a list of potential myokines [23]. Other studies first performed a screening, followed by further experimentation to functionally characterize the newly identified myokine. This approach has been pursued for Angptl4 [24], IL-7 [25], Apelin [26], Irisin [17] and SPARC [27]. SPARC, IL-7, Apelin and Irisin were identified using exercise training as a model, whereas Angptl4 was identified using acute exercise as a model. Complementing the screenings to identify new myokines, many studies have followed a targeted approach assessing mRNA and proteins levels of one particular protein, resulting in the identification of several myokines such as IL-6 [28], IL-8 [29], IL-15 [30], BDNF [31] and FGF21 [32]. All these myokines were initially investigated in the context of acute exercise, except for FGF21 which was initially linked to fasting [32].

Adding up all myokines identified in the different studies performed so far results in a list of more than 100 myokines induced at the mRNA and/or protein level by exercise, electrical pulse stimulation, fasting and differentiation. Many of these myokines have only been identified in cell models and have not been studied in humans, making the relevance of those myokines uncertain. This review will focus on myokines identified in humans in the past 15 years. The list of myokines fulfilling these criteria is shown in table 2.1. The table indicates whether a myokine is an (potential) exercise factor, and whether levels are increased upon acute exercise and/or exercise training.

From table 2.1 it is evident that a considerable fraction of the myokines cannot be classified as exercise factors. So far there is no evidence that Apelin, BDNF, FGF21, IL-7 and LIF, despite their regulation by exercise, are actually secreted into the circulation. For other myokines, including IL-8, IL-15 and especially Irisin, it is not clear if plasma levels are increased by exercise. Currently, due to conflicting results, there is a lot of controversy surrounding Irisin and its role during exercise. Promising exercise factors include Angptl4, CX3CL1, IL-6, CCL2 (MCP-1) and SPARC. All exhibit increased muscle gene expression and increased circulating levels upon acute exercise. Expression and circulating levels of SPARC are increased upon exercise training, while levels of IL-6 and CCL2 decrease after exercise training. Few data are available for CX3CL1, SPARC and to a lesser extent CCL2. In the following section all myokines included in table 2.1 will be discussed in more detail separately.

Table 2.1 Myokines identified in humans

Myokines	References	Exercise factor?	Acute	Training
Angptl4	[22-24,33]	Yes	Yes	No
Apelin	[26]	No, not in plasma	? (no data)	Yes
BDNF	[31,34-36]	No, not in plasma	Yes	Yes
BAIBA	[37]	Yes	? (no data)	Yes
FGF21	[23,32,38]	No, not in plasma	Yes	No
CX3CL1	[23]	Yes	Yes	No
IL-6	[8,9,22,23,28,39-60]	Yes	Yes	Yes, reduced
IL-7	[25]	No, not in plasma	? (no data)	Yes
IL-8	[22,49,52,58,59,61-63]	Yes, only in plasma after extreme conditions	Yes	? (no data)
IL-15	[30,64-68]	Yes, but very limited proof	Yes	Yes
Irisin	[17,69-76]	Yes, but contradictory results and protein might not be functional	Yes?, contradictory results	Yes?, contradictory results
LIF	[77,78]	No, not in plasma	Yes	? (no data)
CCL2	[23,50,79-85]	Yes	Yes	Yes, reduced
SPARC	[23,27,86]	Yes	Yes	Yes
Metrnl	[87]	Yes, only in mice	Yes	? (no data)

EXERCISE FACTORS

IL-6

IL-6 is the most studied and discussed of all myokines. The reason for this attention is that IL-6 was the first identified myokine and is also a very well-known cytokine. Indeed, more than 99% of the literature on IL-6 pertains to its non-myokine actions. IL-6 is mainly characterized as a pro-inflammatory cytokine involved in T cell differentiation and activation, and is involved in many (auto)immune diseases such as rheumatoid arthritis, inflammatory bowel disease, and diabetes type 1 [88]. IL-6 has also been connected with development of insulin resistance in adipose tissue and liver [89-91]. In 1998, Ostrowski et al. first showed that mRNA levels and circulating levels of IL-6 are increased during acute exercise [54]. Many other studies have confirmed and have further elaborated on these finding in the years that followed. The increases in IL-6 mRNA and plasma levels were found to be dependent on blood glucose levels and glycogen levels [40,45]. Whereas low glycogen levels were associated with increased IL-6 mRNA and plasma levels [45,57], glucose ingestion led to lower IL-6 plasma levels, although without any corresponding

changes at the gene expression level [40,52]. These results indicate that low glycogen and blood glucose levels trigger IL-6 production and secretion during acute exercise, suggesting that the direct stimulus for increased IL-6 production may not be muscle activity perse but depletion of muscle glycogen.

Despite many different studies showing increased IL-6 levels after acute exercise, some controversy surrounding IL-6 as a myokine remains. To gain more insight in the IL-6 response upon acute exercise and exercise training, we made an inventory of the studies that measured local or plasma IL-6 levels during exercise (see table 2.2). Most studies only assessed IL-6 levels in plasma, and almost all studies found increased plasma levels of IL-6 in response to a variety of acute exercise types. The studies that did not find an increase in plasma IL-6 levels generally involved more moderate exercise that was not weight-baring (such as cycling). When IL-6 mRNA and plasma levels were both assessed, in almost all cases corresponding increases for both IL-6 plasma and mRNA levels were observed. The only discrepant results were described by Catoire et al. and Langsberg et al. Langsberg et al. found an increase in IL-6 plasma level, but not mRNA levels, after a 36 km run [47]. Catoire et al. showed increased mRNA levels but not plasma levels in subjects that performed one-legged cycling exercise [23]. Integrating the results of the different studies, it appears that lack of induction of IL-6 by exercise is confined to acute exercise studies of limited duration and those that employed a relatively moderate type of exercise. Accordingly, the emerging message is that intense weight baring exercise and long exercise session that deplete glycogen storages are needed to change IL-6 levels, which suggest that increased plasma IL-6 levels may be partly driven by muscle damage. In this regard the study of Langsberg et al. is extremely interesting, since they used a long and weight baring type of acute exercise, and found increased IL-6 mRNA levels only in the tendons of the muscle and not in the muscle itself. Remarkably, the increase in IL-6 mRNA in tendons was associated with elevated plasma IL-6 levels. In addition, several studies showed that acute exercise also increased IL-6 mRNA levels in adipose tissue [43,92,93], suggesting that part of the elevation in IL-6 plasma levels during acute exercise might originate from tissues other than skeletal muscle.

A considerable number of papers have focussed on the effect of exercise training on IL-6 levels. Surprisingly, exercise training seems to have the opposite effect on IL-6 as compared with acute exercise. Indeed, studies indicate that exercise training reduces IL-6 gene expression and plasma levels [41,48]. These findings raise questions about the proposed role of IL-6 as mediator of the stimulatory effect of acute exercise on insulin

Table 2.2 Studies assessing IL-6 levels in humans

	Study	Authors	Year	Number of subjects	Characteristics	Exercise type	Exercise intensity	Exercise duration	IL-6 plasma	IL-6 gene expression
1	PMID:21088973	Mendham et al.	2011	12	Sedentary middle aged male	RT and AT	Vigerous (80% RT, 50% AT) AT = cycling	40min	Increase	ND
						RT and AT	Low (60% RT, 30% AT)	40min	No change	ND
2	PMID:20029520	Croft et al.	2009	?	Recreationally active male	AT	High intensity running	50 min	Increase	ND
3	PMID:7961280	Ullum et al.	1994	17	Young moderately exercised healthy male	AT	75% $VO2_{max}$ cycling	1h	Increase	ND
4	PMID:9130176	Bruunsgaard et al.	1997	9	Young moderately exercised healthy male	AT	65% $VO2_{max}$ or eccentric exercise cycling	30 min	Increases after eccentric exercise	ND
5	PMID:9252483	Rohde et al.	1997	6	Young healthy male	AT	Eccentric cycling	1h	Increases with eccentric exercise	ND
6	PMID:9518745	Ostrowski et al.	1998	16	Young healthy trained males	AT	Marathon	Marathon	Increase	Increase
7	PMID:9824725	Ostrowski et al.	1998	10	Young endurance trained males	AT	75% $VO2_{max}$ running	2.5h	Increase	ND
8	PMID:9925898	Ostrowski et al.	1999	10	Young trained healthy males	AT	Marathon	Marathon	Increase	ND
9	PMID:11502577	Steensberg et al.	2001	7	Healthy young males trained	AT	75% $VO2_{max}$ cycling	2.5h	Increase	ND

Table 2.2 *Continued*

10	PMID:11687509	Keller et al.	2001	6	Healthy young males trained	AT	60% W_{max} knee extensions	3h	Increase	Increase
11	PMID:11731593	Steensberg et al.	2001	7	Young healthy males trained	AT	40% W_{max} knee extensions	5h	Increase (locally measured)	Increase
12	PMID:12388119	Steensberg et al.	2002	6	Healthy young males trained	AT	55% W_{max} knee extensions	3h	Increase (locally measured)	Increase
13	PMID:12509497	Helge et al.	2003	7	Healthy young males trained	AT	25% W_{max} knee extensions followed by 65%+85% W_{max} legs	45min + 35min	Increase	ND
14	PMID:12702735	Febbraio et al.	2003	7	Healthy young males trained	AT	65% $VO2_{max}$ cycling	2h	Increase	ND
15	PMID:12937023	MacDonald et al.	2003	8	Healthy young males trained	AT	70% $VO2_{max}$ cycling	1h	Increase	ND
16	PMID:12958150	Penkowa et al.	2003	18	Healthy young males trained	AT	60% $VO2_{max}$ cycling	3h	Increase	Increase
17	PMID:14521945	Keller et al.	2003	7	Healthy young males	AT	60% $VO2_{max}$ cycling	3h	Increase	Increase
18	PMID:15220185	Febbraio et al.	2004	6	Healthy young males recreationally active	AT	70% $VO2_{peak}$ cycling	2h	Increase	ND
						AT	40% $VO2_{peak}$	2h	No change	ND

Table 2.2 continues on next page

	Study	Authors	Year	Number of subjects	Characteristics	Exercise type	Exercise intensity	Exercise duration	IL-6 plasma	IL-6 gene expression
19	PMID:23361845	Christiansen et al.	2013	31	Lean and obese	AT	60% max heart rate cycling	2h	Increase	Increase
20	PMID:23463480	Scott et al.	2013	21	Recreationally and endurance trained males	AT	To exhaustion	variable	Increase	ND
21	PMID:23383690	Pereira et al.	2013	24	Sedentary females with or without MetS	RT	60% 1RM	7*10 reps	Increased in MetS	ND
22	PMID:22810957	Almada et al.	2013	10	Twins	AT	Anaerobic treshold	45min	Increase	ND
23	PMID:22052872	Friedmann-Bette et al.	2012	11	Male young	RT	10RM	5*10 reps	ND	No change
24	PMID:21916608	Nieman et al.	2012	31	Male cyclist	AT	60% $VO2_{max}$ + 10km time trial cycling	2h	Increase	ND
25	PMID:21879351	Waśkiewicz et al.	2012	14	Trained athletes	AT	(ultra) marathon	Marathon	Increase	ND
26	PMID:21552156	Scott et al.	2011	10	Trained young males	AT	55% $VO2_{max}$ running	1h	Increase	ND
						AT	65% $VO2_{max}$ running	1h	Increase	ND
						AT	75% $VO2_{max}$ running	1h	Increase	ND

27	PMID:21153418	Toft et al.	2011	6	Active young males	AT	60% W_{max} knee extensions	2h	Increase	ND
28	PMID:20396982	Leggate et al.	2010	11	Healthy males	AT	High intenstity cycling	1h	Increase	ND
						AT	Moderate intensity cycling	1h	No change	ND
29	PMID:19527938	Gray et al.	2009	9	Healthy males	AT	90% lactate treshold cycling	1.5h	Increase	ND
30	PMID:18341089	Rosa et al.	2007	21	Children	AT	80% $VO2_{max}$ cycling	30min	Increase	ND
31	PMID:18320358	Gray et al.	2008	12	Sedentary middle aged male	AT	To exhaustion	variable	Increase	ND
32	PMID:17823296	Louis et al.	2007	12	Healthy active	RT	70% 1RM knee extensions	3*10 reps	ND	Increase
						AT	75% $VO2_{max}$ running	30min	ND	Increase
33	PMID:15778897	Minetto et al.	2005	17	Trained young	AT	3h spinning	3h	Increase	ND
						RT	160 maximal contractions	15 min	Increase	ND
34	PMID:12736844	Niess et al.	2003	7	Young endurance trained males	AT	90% treshold running	1h	Increase	ND

Table 2.2 *Continued*

Table 2.2 continues on next page

	Study	Authors	Year	Number of subjects	Characteristics	Exercise type	Exercise intensity	Exercise duration	IL-6 plasma	IL-6 gene expression
35	PMID:12582947	Willoughby et al.	2003	8	Young males	RT	Eccentric knee extensions	7*10 reps	Increase	Increase
36	PMID:23226462	Catoire et al.	2012	12	Healthy middle aged males	AT	50% W_{max} one-legged cycling	1h	No change	Increase
37	PMID:11080265	Steensberg et al.	2000	6	Healthy young males	AT	40% W_{max} one-legged cycling	5h	Increase (locally measured)	ND
38	PMID:12870175	Febbraio et al.	2003	17	Diabetic and matched control male subjects	AT	60% $VO2_{peak}$	25 min	Increase (locally measured)	ND
39	PMID:15059966	Hiscock et al.	2004	7	Healthy active males	AT	55% $VO2_{peak}$	2h	ND	Increase
40	PMID:15075299	Holmes et al.	2004	5	Healthy males	AT	62% $VO2_{max}$	3h	Increase	Increase
41	PMID:12154195	Langsberg et al.	2002	6	Healthy trained	AT	36 km run	~3h	Increase	No change
42	PMID:22907232	Mathers et al.	2012	35	Elderly	RT	Maximal unilateral knee extension	3*10 reps	ND	Increase

43	PMID:14672962	Nieman et al.	2004	30	Strength-trained young males	RT	40-60% of 1RM, 10 exercises for all major muscle groups	10*4*10 reps	Increase	Increase
44	PMID:16118573	Nieman et al.	2005	15	Endurance trained young males	AT	60% W_{max} cycling	2.5h	Increase	Increase
45	PMID:12533503	Nieman et al.	2003	16	Endurance trained young males	AT	70% $VO2_{max}$ running	3h	Increase	Increase
46	PMID:21558575	Carol et al.	2011	9	Endurance trained young males	AT	50% W_{max}	1.5h	Incraese	ND
47	PMID:11389214	Starkie et al.	2001	7	Trained young males	AT	Lactate treshold running or cycling	1h	Increase	Increase

MetS = metabolic syndrome, AT = aerobic training, RT = resistance training, $VO2_{max}$ = maximum oxygen uptake, W_{max} = maximum workload, $VO2_{peak}$ = peak oxygen uptake, 1RM = one repetition maximum, ND = not determined.

sensitivity. IL-6 produced during contraction positively affected insulin signalling and improved insulin sensitivity in muscle [94]. These effects were opposite to the effects of increased IL-6 levels in adipose tissue and liver on insulin signalling [89-91]. IL-6 induced insulin resistance in hepatocytes [90]. In vivo, IL-6 induced insulin resistance in liver [91], while in adipose tissue high IL-6 levels are associated to insulin resistance [89]. In literature it was suggested that the effects of IL-6 on insulin sensitivity are very tissue specific and also depend on chronically elevated levels or only short-term increased levels [95].
In conclusion, acute exercise stimulates production and secretion of IL-6 by skeletal muscle, while exercise training reduces IL-6 levels. IL-6 secretion seems to be especially activated by high intensity and long duration exercise, suggesting that some form of muscle damage may be needed to trigger IL-6 release and raising questions about the potential role of IL-6 in mediating the effects of moderate exercise. Finally, it is doubtful that the increase in plasma IL-6 after acute exercise originates solely from skeletal muscle. Other organs such as adipose tissue likely contribute as well .

CCL2

Similar to IL-6, CCL2 is a very well-known inflammatory marker. During inflammation caused by injury or infection, CCL2 recruits monocytes, T cells and dendritic cells. In skeletal muscle CCL2 is involved in macrophage infiltration after tissue damage [96,97]. Recently it was discovered that skeletal muscle CCL2 mRNA levels are elevated during acute exercise, resulting in increased plasma levels [23]. Based on these data, CCL2 can be classified as a myokine and exercise factor. Previous studies already showed that acute moderate intensity cycling, marathon running, and resistance exercise increased CCL2 gene expression levels in skeletal muscle [50,79-81]. CCL2 protein was found to be increased in skeletal muscle after eccentric exercise, but not after concentric exercise [82]. Furthermore there was a clear co-localization of CCL2 with satellite cells and macrophages after the acute eccentric exercise bouts, which could not be observed after concentric acute exercise [82]. Given the role of CCL2 in macrophage infiltration upon muscle damage and a clear co-localization of CCL2 with macrophages, it appears that the local increase in CCL2 might partly originate from immune cells. The induction of CCL2 by trauma or acute exercise and subsequent infiltration of macrophages and other immune cells appears to be crucial for muscle repair and adaptation [96,98]. Infiltration of immune cells was also found to be important for skeletal muscle hypertrophy following exercise [99]. Secretion of CCL2 into the blood is also indicative of a systemic role during

acute exercise, although concrete evidence is lacking. Interestingly, long-term training results in a decrease in plasma CCL2 levels [83,85], which is analogous to the effects of exercise training on IL-6 levels. Furthermore, similar to IL-6, CCL2 is produced by adipose tissue during obesity as part of a chronic low grade inflammation and promotes insulin resistance [100-102]. Evidence suggests that both acute exercise and exercise training cause a shift in adipose tissue macrophages from inflammatory M1 to anti-inflammatory M2 macrophages [103,104]. It can be hypothesized that CCL2 produced in skeletal muscle plays a role in this shift, but more research is necessary to better understand the functional consequences of CCL2 release during exercise. In conclusion, CCL2 is a very interesting exercise factor that may have an important function in mediating (part of) the inflammatory and metabolic responses following an acute exercise bout.

Angptl4

Angptl4 is a secreted protein that regulates the influx of plasma triglyceride-derived fatty acids into tissues by inhibiting the enzyme lipoprotein lipase [105]. Angptl4 is produced by a variety of tissues including adipose tissue, liver, heart, intestine and skeletal muscle. Expression of Angptl4 in skeletal muscle is activated by plasma free fatty acid via the Peroxisome Proliferator–Activated Receptor (PPAR)-δ [106]. Kersten et al. showed that acute exercise increased Angptl4 levels in plasma, but only when the exercise was performed in the fasted condition [33]. Co-ingestion of glucose, which blocks the elevation in plasma free fatty acids during exercise, prevented the exercise-induced increase in plasma Angptl4, suggesting that induction of plasma Angptl4 by exercise is mediated by elevated free fatty acids.

Recently we found that acute exercise also increased Angptl4 gene expression levels in human skeletal muscle, most likely via elevated plasma free fatty acids. Surprisingly, the magnitude of the increase in Angptl4 mRNA was much higher in non-exercising muscle as compared with exercising muscle [23,24]. The selective induction of Angptl4 in non-exercising muscle may serve to prevent local fat overload, while directing fatty acids to the active skeletal muscle as fuel, thus pointing to Angptl4 as a key regulator of lipid homeostasis during exercise. Increased Angptl4 gene expression during exercise was accompanied by increased plasma levels, implying that Angptl4 is a myokine and an exercise factor. However, whether Angptl4 produced by skeletal muscle acts at a distance and impacts other tissues remains unclear [24]. In contrast to acute exercise, exercise training did not change Angptl4 plasma levels [24]. In conclusion, Angptl4 is a

very interesting potential exercise factor. Whether skeletal muscle-derived Angptl4 also has a systemic role requires further study.

CX3CL1

CX3CL1 has been involved in acute skeletal muscle injury and regeneration via its ability to attract macrophages and other immune cells, which is thought to be crucial for hypertrophy [99]. As an inflammatory marker, CX3CL1 is involved in leukocyte adhesion [107], and macrophage-directed rescuing of skeletal muscle cells from apoptosis [108]. Because CX3CL1 was only recently identified as a myokine, there is limited literature on CX3CL1 in the context of exercise and skeletal muscle. Catoire et al. showed that acute exercise induced gene expression and plasma levels of CX3CL1 [23]. Furthermore, it was shown that CX3CL1 is part of the secretome of skeletal muscle [20], supporting the status of CX3CL1 as a myokine. Besides a local role in muscle regeneration, it is unclear whether the increased plasma CX3CL1 may have any systemic effects. Interestingly, plasma levels of CX3CL1 were found to be elevated in subjects with type 2 diabetes [109]. Furthermore, decreased CX3CL1 signalling was suggested to be one of the mechanism underlying β cell dysfunction in type 2 diabetes [110]. Taken together, CX3CL1 is a highly interesting myokine and exercise factor, which may be involved in mediating specific effects of exercise.

SPARC

SPARC was identified as a myokine in a screening study in mice using microarrays. The study revealed that SPARC gene expression levels are increased after acute exercise and exercise training [27]. Acute exercise also significantly increased SPARC plasma levels and this result was reproduced in humans, suggesting a potential systemic role of SPARC during exercise [27]. Plasma levels of SPARC tended to increase in mice following exercise training. The increase in SPARC mRNA levels after acute exercise and exercise training in humans was corroborated in another study. Unfortunately, plasma levels of SPARC were not measured [23]. Locally produced SPARC has been connected to muscle development [86,111] and glucose metabolism [112]. Furthermore, it was shown that SPARC has an inhibitory effect on tumorgenesis in colon during exercise [27]. An intriguing aspect of SPARC is that it is induced during both acute exercise and exercise training, raising the possibility that it may function as general exercise factor.

POSSIBLE EXERCISE FACTORS

Irisin

Irisin was presented as a very promising myokine by Bostrom et al. [17]. Irisin was transcribed and translated from the FCNC5 gene and was induced by exercise training. Data showed that irisin promotes browning of white adipose tissue. Browning of white adipose tissue implicates that white adipose tissue cells develop the properties of brown adipose tissue cells, allowing them to generate heat and increase whole body energy expenditure [17]. Since the identification of irisin, many studies have tried to confirm and further elaborate on the role of irisin in exercise. In general, results of those studies have not been able to consistently reproduce the original findings of Bostrom et al. Indeed, several studies found that irisin levels in plasma and skeletal muscle were not influenced by exercise training [69,70,72,73]. Whereas Bostrom et al. reported no change in plasma irisin following acute exercise [17], other studies found that plasma irisin goes up during acute exercise [72,75,76]. One of the major concerns is that almost all ELISA's used for quantification of plasma irisin have not been properly validated, casting doubt on the reliability of the findings. Another major concern is that irisin levels in human plasma are likely very low, since the rate of translation of the FDNC5 gene is much lower in humans as compared with mice, due to the fact that human FNDC5 misses the usual start codon [74]. Interestingly, a recent study was able to detect irisin protein in human plasma using highly sensitive mass spectrometric analysis [71]. In conclusion, the role of irisin as a myokine remains controversial. The results in mice are encouraging [17,75], but there are serious reservations about the relevance of plasma irisin in humans.

IL-8 & IL-15

IL-8 and IL-15 were both identified as myokines shortly after IL-6 was bestowed with the same label. IL-8 and IL-15 are well-known pro-inflammatory cytokines. IL-8 induces chemotaxis in activated immune cells, and also induces phagocytosis [113]. IL-15 regulates T and natural killer cell activation and proliferation [114]. A number of studies have shown induction of IL-8 and IL-15 by acute exercise [30,49,52,61,68]. IL-15 gene expression levels also went up after exercise training [66]. There are no recent studies on IL-8 and IL-15 as myokines. Plasma levels of IL-8 were found to be increased by strenuous exercise, including 24-h ultra-endurance exercise and 3 hours of running/cycling [52,59,63]. Only one study showed increased plasma levels of IL-15 after exercise,

which consisted of 30 minutes of running at 70% $VO2_{max}$ [67]. This exercise protocol seems relatively modest but nevertheless was associated with considerable muscle damage, as illustrated by increased plasma creatine kinase levels [67]. The above results suggest that only strenuous exercise associated with severe muscle causes plasma levels of IL-8 and IL-15 to go up. IL-8 has been suggested to be involved in angiogenesis in skeletal muscle [115], while IL-15 is may play a role in glucose metabolism and muscle development [116,117]. In conclusion, IL-8 and IL-15 are myokines that likely serve a local role in response to exercise. Their identity as exercise factors is uncertain.

AUTOCRINE AND PARACRINE MYOKINES

FGF21

FGF21 is a member of the fibroblast growth factor family which now comprises 22 members in man. It is produced by a number of tissues, including liver, white adipose tissue and pancreas. Metabolically, FGF21 is probably one of the most promising myokines, since it influences lipid and glucose metabolism, as well as insulin signalling [118-120]. It has been shown that FGF21 is also expressed in skeletal muscle and that expression is increased upon fasting [32]. So far only one study has shown a stimulatory effect of acute exercise on FGF21 mRNA [23]. Other studies on FGF21 in skeletal muscle mainly focussed on the role in insulin signalling. In vitro, in skeletal muscle FGF21 protected against palmatite-induced insulin resistance [121] and was induced by insulin via the Akt/PI-3 kinase pathway [32,122]. Systemically, FGF21 is working as an endocrine factor increasing for example lipolysis in white adipose tissue and gluconeogenesis in liver [123]. In monkeys and rodents, FGF21 administration causes a marked decrease in fasting plasma glucose , insulin, glucagon, and triglycerides [118]. Additionally, FGF21 administration lowered plasma LDL and raised HDL [118]. A recent study indicates that part of the metabolic effects of FGF21 are relayed by the brain [124]. Due to its favourable properties, FGF21 has become a highly promising pharmaceutical target for metabolic diseases, including diabetes type 2. Taken together, FGF21 is a highly interesting putative myokine. Considering the scarce data available, the secretion of FGF21 by muscle into the blood needs experimental confirmation, Furthermore, the possible effect of acute exercise and exercise training on plasma FGF21 needs to be explored further.

BDNF

BDNF was found to be increased in plasma upon acute exercise [125,126]. BDNF is mainly expressed in various parts of the brain where it functions as a regulator of neuron growth and survival [127]. In addition, BDNF is expressed in skeletal muscle, where it is induced by acute exercise [31] and exercise training [34]. Current evidence suggests that skeletal muscle-derived BDNF is not released into the circulation [31]. The increased plasma levels therefore likely originate from the brain, suggesting that muscle-derived BDNF might only serve a local role. Skeletal muscle BDNF has been shown to be involved in satellite differentiation, muscle regeneration and AMPK signalling [31,36,128]. In conclusion, BDNF is a probably myokine but is not released from the muscle into the circulation, indicating it is not an exercise factor.

OTHER MYOKINES/EXERCISE FACTORS

Apelin, BAIBA, IL-7, Metrnl and LIF have all been proposed as myokines in humans, although evidence for all of them is currently limited [25,26,34,37,87]. Since data in humans is so limited for these myokines, we will not extensively address them in this review. Interestingly, BIABA is not a protein but a metabolite released from skeletal muscle during acute exercise and in vitro during electrical pulse stimulation [37]. BAIBA is likely involved in browning of white adipose tissue, making it an interesting potential exercise factor. Very recently a paper on Metrnl was published, adding Mertnl to the myokine family. Mertnl has been linked to increased energy expenditure and improved glucose tolerance [87].

CONCLUSIONS

A considerable number of human myokines have been identified throughout the years, yet limited information is available about the function of most of those myokines. The majority of the myokines identified probably has a local role and is unlikely to carry a systemic function as exercise factor. So far the only well-established myokine and exercise factor is IL-6. Promising exercise factors and myokines include SPARC, CCL2, CX3CL1 and Angptl4. For all the other putative myokines, more evidence is needed that they are actually secreted into the plasma, and that production is increased in response to exercise. In the coming years, emphasis should be placed on better characterizing the functional role of the existing and newly identified myokines in the acute and chronic response to exercise, and explore their potential as target for metabolic diseases.

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Chapter 3

Combined exercise training is less efficient in inducing health beneficial gene expression changes compared to endurance and resistance exercise training alone

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In preparation

ABSTRACT

There is very limited knowledge on molecular changes induced by endurance, resistance and combined (or concurrent) exercise training. This knowledge is important for the use of different types of exercise training in the context of health and disease.

The aim of this study was to assess the effect of combined exercise training on gene expression profiles, and compare this with the gene expression profiles of endurance and resistance exercise training. Microarray dataset from three human intervention studies were included (combined exercise training, resistance exercise training and endurance exercise training). Microarray analysis was performed on muscle biopsies of the vastus lateralis muscle for all three studies. The endurance and resistance exercise training dataset were extracted from GEO.

Our results showed that despite a substantial overlap between the three training types, each of the training types had an own specific gene expression signature. The combined exercise training gene expression print lacked some specific oxidative and PPAR related components compared to endurance exercise training.

In conclusion, the gene expression profile of combined exercise training has a less oxidative signature compared to endurance exercise training. This gene expression profile might have implications for the use of combined exercise training in the context of metabolic disease and sports.

INTRODUCTION

The number of people affected by obesity and diabetes type 2 continues to increase, putting a growing burden on healthcare and society. Research has shown that exercise is one of the most effective ways to prevent and treat metabolic diseases such as type 2 diabetes. Exercise training increases insulin sensitivity[1], restores mitochondrial function [1] and reduces liver fat [2]. The exact mechanisms behind the positive effects of exercise on health remain largely unknown. Better understanding of these mechanisms is crucial to effectively apply exercise for treatment of metabolic disease.

Different types of exercise are beneficial in the treatment of metabolic disease. Endurance and resistance exercise training are the types of exercise that are most often used. Combined (or concurrent) exercise training combines resistance and endurance exercise training and is also frequently used in the treatment of metabolic disease. Combined exercise training has been suggested to be more favourable to treat metabolic disease than endurance or resistance exercise training alone [3]. Currently there is very limited knowledge on the similarities and differences in molecular changes induced by the different types of exercise training. This knowledge is relevant to determine what type exercise should be recommended in disease and as training strategy for a particular sport. Indeed, many athletes use resistance exercise training as support training for their principal endurance sport.

Resistance and endurance exercise training cause profound alterations in gene expression [4,5], which are at the basis of the adaptive changes in exercise capacity. Until now research only assessed the gene expression changes induced by resistance or endurance exercise training separately [6,7]. The molecular effects of combined exercise training were never assessed, nor was a comparison made between the different types of exercise training. Whole genome gene expression profiling by microarray has been shown to be a very effective and valuable way to get information on expression of all genes in the human genome [8]. Therefore the aim of this study was to assess the effect of combined exercise training on gene expression profiles, and compare this profile with the gene expression profiles of endurance and resistance exercise training. For this purpose we utilized whole genome gene expression data from three types of exercise training (resistance, endurance, combined) and compared the gene expression profiles of the three types of exercise training using several bioinformatics tools. The microarray datasets from the resistance and endurance exercise training study were extracted from the Gene Expression Omnibus (GEO). The results show distinct gene

expression profiles for each type of exercise training. The gene expression profile of combined exercise training has a less pronounced oxidative signature compared to endurance exercise training.

METHODS

Human intervention studies

Three human intervention studies were included: a combined exercise training study, a resistance exercise training study, and an endurance exercise training study. Studies were chosen based on the following criteria: supervised and controlled exercise intervention, between 6 weeks and 12 weeks of training and healthy male participants. In addition, whole genome expression data had to be available.

Combined exercise training

Results of this training intervention were previously published in Meex et al. and Catoire et al. [1,9]. In short, 18 healthy middle-aged male subjects performed 12 weeks of combined exercise training. Combined exercise training consisted of 2 endurance exercise sessions and 1 resistance exercise session per week. More details about the training is provided in table 3.1.

Table 3.1 Overview of the exercise training interventions

Training type	Number weeks	Number of sessions per week	Type of training	# of subjects	Type of subjects
Endurance	6	4	45 min cycling on 70% $VO2_{max}$	24	Healthy young
Resistance	12	3	bilateral knee extensions with 3x10 reps at 70% of 1-RM	8	Healthy young
Combined	12	3	2x cycling 30 min at 55% of W_{max}, 1x 1x8 reps at 55% of MVC + 2x 8 reps at 75% MVC for all large muscle groups	18	Healthy middle-aged

Endurance exercise training

The microarray dataset of this study was extracted from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; GSE35659) and previously published in Keller et al. and Timmons et al. [6,10]. The study contained 24 healthy young subjects participating

in 6 weeks of endurance exercise training (4 sessions per week). More details about the exercise training performed is provided in table 3.1.

Resistance exercise training

The microarray dataset of this study was extracted from GEO (GSE28422). The results were previously published in Raue et al. [7]. 8 healthy young subjects performed 3 resistance exercise sessions per week for 12 week. More details about the training regimen are provided in table 3.1.

Microarray analysis

For the three studies the biopsies were taken from the vastus lateralis muscle under resting conditions. For the endurance exercise training intervention at least 24 hours had elapsed between the last exercise session and collection of the muscle biopsy. For the resistance and combined exercise training intervention 48 hours had elapsed between the last exercise session and the muscle biopsy. Processing of the biopsies for microarray analysis for the endurance and resistance exercise training studies is described in the published articles [6,7,10]. The Affymetrix Human Genome U133 Plus 2.0 microarray was used for the endurance and resistance exercise training studies. For the combined exercise training study, RNA was isolated using Trizol (Invitrogen, Breda, NL) and purified using RNeasy columns (Qiagen, Venlo, NL), after which RNA was labelled and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays (Affymetrix, Santa Clara, CA). All three microarray datasets were normalized using median polish and probesets were combined for the three datasets, leaving only the probesets present on both types of Affymetrix microarrays. This reduced the number of genes to 17,309. Statistical differences between baseline and post-training samples were assessed using Intensity-Based Moderated T-statistic (IBMT [11]) for each intervention and considered to be statically significant if $p < 0.01$. For a graphical representation of the workflow, see figure 3.1.

Pathway analysis

Geneset enrichment analysis (GSEA; http://www.broad.mit.edu/gsea/) was performed for the three datasets and genesets with $p < 0.05$ were considered significantly enriched or depleted. The Enrichment plugin in Cytoscape was used to visualize GSEA output, cut offs of $p < 0.01$, $q < 0.02$ and overlap coefficient < 0.5 were used.

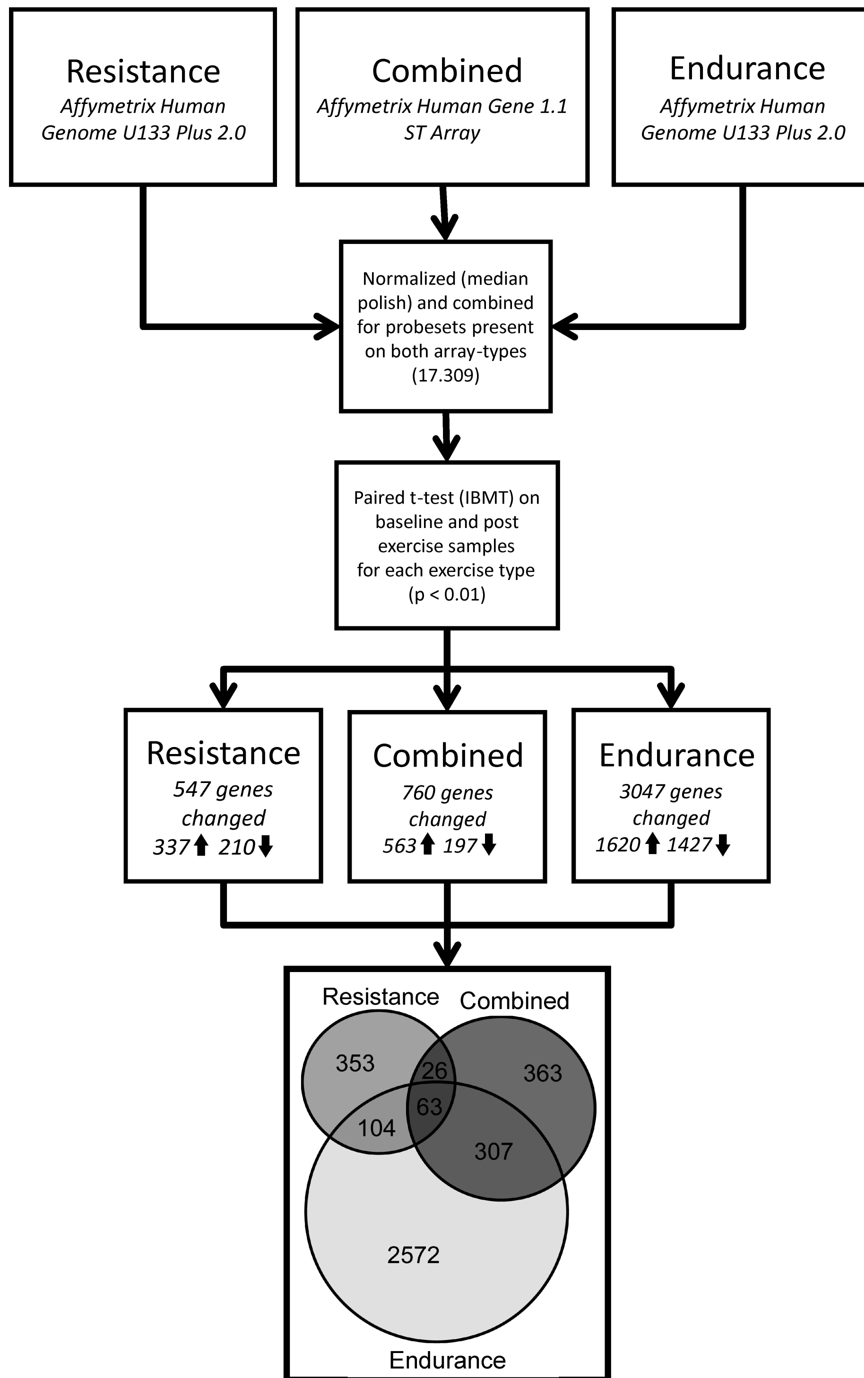

Figure 3.1 *Flow chart of the work flow of the microarray analysis. The Venn diagram represents the overlapping genes for the three training types. Combined N = 18, Endurance N = 24, Resistance N = 8.*

RESULTS

After combining the three microarray datasets, 17,309 common genes remained. Of these 17,309 genes, 547 genes were significantly changed by resistance exercise training, 3047 genes were significantly changed by endurance exercise training and 760 genes were significantly changed in combined exercise training (figure 3.1; $p < 0.01$). For all three types of exercise training, the majority of the genes was upregulated, which was most pronounced for the combined exercise training.

Comparative analysis showed an overlap of 63 genes significantly induced by all three training types (figure 3.1), representing 8.3%, 2.1% and 11.5% of the total number of significant genes for combined, endurance, resistance exercise training, respectively (figure 3.2). Interestingly, a large portion of the genes altered by combined exercise training overlapped with the genes altered by endurance exercise training (307 genes, 40.4% of the total number of genes altered by combined training). The overlap between the genes altered by combined exercise training and resistance exercise training was limited to 3.4%.

Next, the significantly induced genes for each type of training were ranked based on fold change. The top 30 for each type of training is depicted in figure 3.3. The top 20 of the

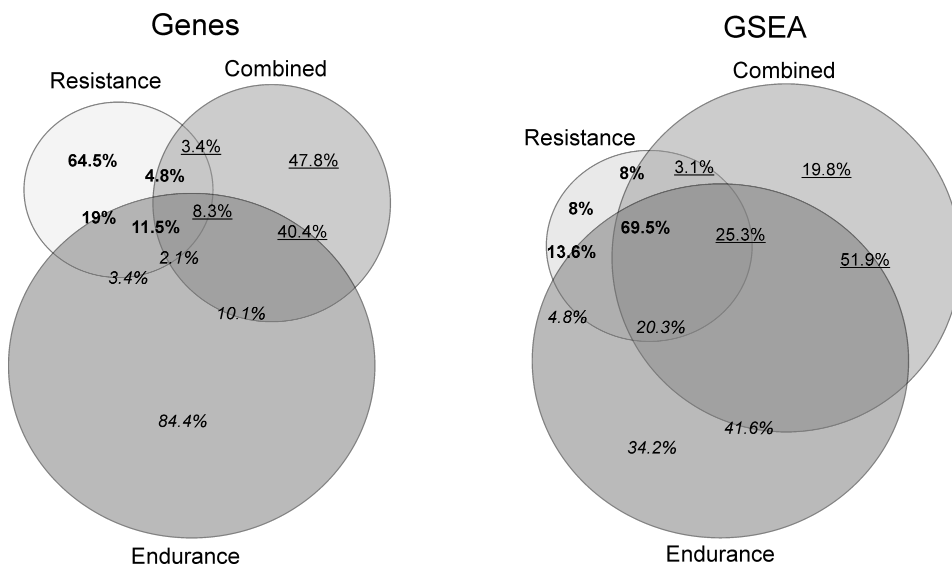

Figure 3.2 *Relative overlap of the three exercise training types for genes (left Venn diagram) and GSEA genesets (right Venn diagram). Underlined represents combined exercise training, bold resistance exercise training and italic endurance exercise training.*

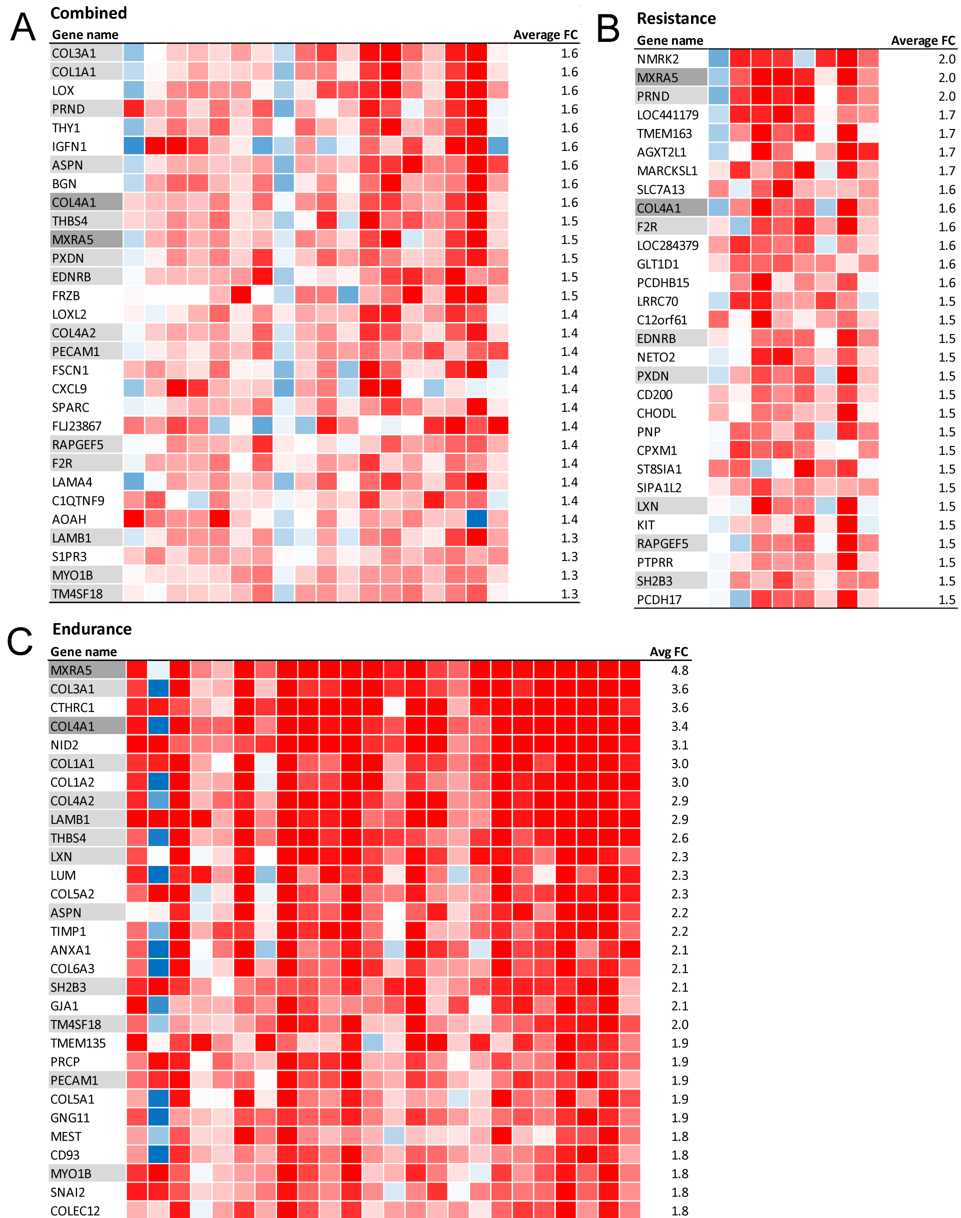

Figure 3.3 *Top 30 genes of combined **(A)**, resistance **(B)** and endurance **(C)** exercise training ($p < 0.01$). Individual expression levels of subjects are showed in the colums. Red is a signal log ratio of 2, blue of -2. Overlap between the top 30s are indicated with dark grey (present in 3 training types) or light grey (present in 2 training types).*

combined exercise training study shown in figure 3.3A deviates from a top 20 that was previously published based on the same dataset [9]. The explanation for this is that two types of microarrays were combined. Our analysis only took into account probes that were present on both types of microarrays. This selection of genes resulted in a difference in number of probes for the combined microarrays compared to the number of probes per gene for the two types of microarrays separately. A difference in number of probes can results in subtle changes in expression levels. Close inspection of the top 30 of the three different training types revealed overall higher fold changes following endurance exercise training and more limited variation between the subjects, as compared with combined and resistance exercise training (figure 3.3). Comparison of the top 30 genes for the three training types yielded 2 common genes: COL4A1 and MXRA5. Of the top 30 genes induced by combined exercise training, 9 genes overlapped with endurance exercise training and 5 genes with resistance exercise training.

To gain more insight into the processes changed by the different types of exercise training, geneset enrichment analysis (GSEA) was performed. GSEA examines if certain processes or pathways (genesets) are over- or underrepresented in the dataset. In the GSEA expression levels of all genes are taken into account, not only the genes that are significantly induced. The analysis resulted in 162 enriched genesets for combined exercise training, 202 enriched genesets for endurance exercise training and only 51 enriched genesets for resistance exercise training ($p < 0.05$). Taking into account the relatively limited overlap in significantly altered genes between the different training types, the overlap in enriched genesets between the three types of training was surprisingly large: 41 genesets (69.5% of resistance exercise training, 25.3% of combined exercise training and 20.3% of endurance exercise training genesets; figure 3.2). Eighty-four genesets were overlapping between combined and endurance exercise training (40.4% of combined exercise training and 41.6% of endurance exercise training).

To obtain a better overview of the results, the GSEA output was loaded into the Enrichment tool of Cytoscape and a very strict cut-off was used to visualize the most enriched genesets ($p < 0.01$, $q < 0.02$). A very strict cut-off was used to be able to generate a clear overview of the most enriched (or depleted) genesets. Results of the GSEA of the combined exercise training were consecutively compared with GSEA results of endurance and resistance exercise training (supplemental figure S3.1). The comparison between combined and resistance exercise training showed that the overlap is relatively limited (supplemental figure S3.1B). Overlapping geneset were related to immune response

and homeostasis. Only one overlapping geneset was related to metabolism: Reactome geneset "Respiratory electron transport, ATP synthesis by chemi-osmotic coupling and heat production by uncoupling proteins". The comparison between combined and endurance exercise training revealed a more substantial overlap (supplemental figure S3.1A). A clear difference in enrichment of genesets involved in metabolism was observed between combined and endurance exercise training. Genesets involved in fatty

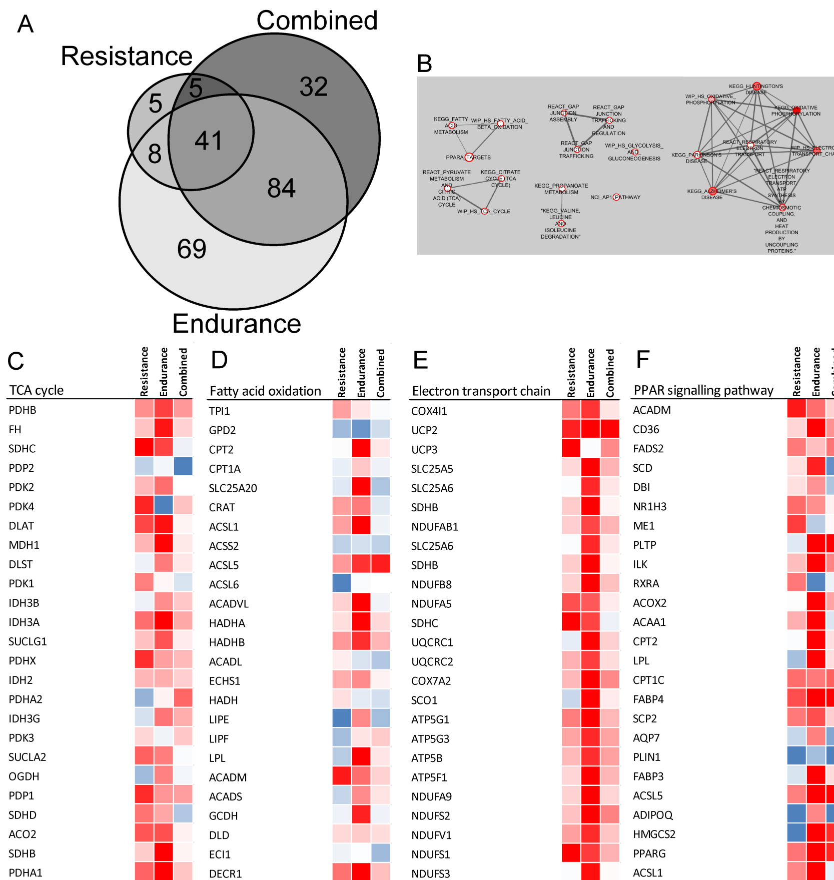

Figure 3.4 *Results of the Geneset Enrichment Analysis (GSEA) of the three types of exercise training.* ***(A)*** *Venn diagram overlapping the enriched geneset of the three exercise types (p < 0.05),* ***(B)*** *selection of the enrichment map showing the genesets different between combined and endurance exercise training. Red is enriched, blue is depleted. The inner parts of the circles represent combined exercise training, the outer parts endurance exercise training.,* ***(C)-(F)*** *heatmaps showing 25 most representative individual genes for 4 selected genesets: TCA cycle (WIP, sign. enriched in resistance + endurance), fatty acid oxidation (WIP, sign. enriched in endurance), electron transport chain (WIP, sign. enriched in resistance, endurance & combined), PPAR signalling (KEGG, sign. enriched in endurance). Red is a signal log ratio of 0.25, blue -0.25.*

acid metabolism, citrate cycle and glycolysis were not enriched in combined exercise training, but strongly enriched in endurance exercise training (figure 3.4B). Other genesets related to oxidative phosphorylation and electron transport chain were enriched in both combined and endurance exercise training, but the enrichment in endurance exercise training was again more pronounced (figure 3.4B).

Four representative metabolic genesets are displayed in figure 3.4C-F. The electron transport chain geneset was enriched in all three training types (figure 3.4E). Interestingly, genes within this geneset showed larger variation and more modest induction by combined exercise training as compared with resistance and endurance exercise training. The PPAR signalling pathway was only significantly enriched in endurance exercise training. This enrichment is caused by a small set of highly induced PPAR targets (figure 3.4F). Some of those PPAR targets were also induced by combined exercise training alone or by both resistance and combined exercise training (PPARG, FABP4, CPT1C, ACSL5, PLTP, ILK). The induction of those genes was in almost all cases lower in combined exercise training as compared with endurance exercise training. Genes within genesets of the TCA cycle (figure 3.4C) and fatty acid oxidation (figure 3.4D) showed a clear induction by endurance exercise training. The TCA cycle geneset was also significantly enriched in resistance exercise training. Genesets of the TCA cycle and fatty acid oxidation were not significantly enriched in combined exercise training, and genes within these genesets showed minor and variable changes in expression.

DISCUSSION

We are the first to compare gene expression profiles of combined exercise training with expression profiles of resistance and endurance exercise training. Our results showed a substantial overlap between gene expression profiles of combined and endurance exercise training, whereas the overlap between combined and resistance exercise training was less pronounced. Despite the substantial overlap between the three training types, each of the training types had a specific gene expression signature. The expression signature of combined exercise training was characterized by fewer and smaller changes in oxidative metabolism as compared with endurance exercise training.

In this study we showed that the gene expression profile of combined exercise training overlapped more with the profile of endurance exercise training then with the profile of resistance exercise training. A similar pattern is seen in literature for other physiological

adaptations induced by exercise training [12]. For example, combined exercise training increased $VO2_{max}$ and time to exhaustion and these increases were comparable to adaptations induced by endurance exercise training [13]. Combined exercise training increased strength, however to a lesser extent then in resistance exercise training [14]. The predominance of endurance exercise training effects in combined exercise training effects was probably caused by a relatively larger fraction of endurance sessions over resistance sessions [13]. In this study combined exercise training also consisted of two endurance exercise sessions and one resistance exercise session per week, explaining why the gene expression profile of combined exercise training overlapped more with the profile of endurance exercise training.

Despite an substantial overlap between the three training types, each of the training types had a specific gene expression profile. The distinct gene expression profiles of the three different training types indicate that each type of exercise training elicits unique molecular adaptions. Interestingly, the genes MXRA5 and COL4A1 were the only genes present in the top 30 of all three training types. This presence can indicate that these genes can be markers of exercise training. Currently there is very limited data on the function of MRXA5 besides it being linked to and serving as potential biomarker for colorectal cancer [15,16]. Inspection of BioGPS (www.biogps.org) showed that MXRA5 is weakly expressed in skeletal muscle under baseline conditions, when compared with small intestine and colon. MRXA5 was linked to Duchenne muscular dystrophy in a large scale genetic screening study [17], suggesting an potential role in muscle development. COL4A1 is a certain type of collagen that is in cooperated into the membrane of cells and is linked to angiopathy and haemorrhagic stroke [18]. Mutations in the COL4A1 gene are thought to play a role in the HANAC syndrome (hereditary angiopathy with nephropathy, aneurysm, and muscle cramps[19]). In skeletal muscle COL4A1 is linked to myopathy [20]. Similar to MRXA4, baseline expression of COL4A1 in skeletal muscle is low (www.biogps.org).

To gain more insight in the processes changed by the different types of exercise training, we performed a GSEA. This GSEA showed that in resistance exercise training many oxidative genesets were enriched. This finding is not completely in line with expectations based on literature. In general it is suggested that resistance exercise training induces changes in genes involved in protein degradation, angiogenesis and growth [4,21]. Gordon et al. [4] showed that genes involved in glucose metabolism and oxidative phosphorylation were downregulated after resistance exercise training [4],

while we showed a clear enrichment in genes involved in oxidative phosphorylation. The differences in outcome can be explained by the timing of the muscle biopsies. Gordon et al. performed the biopsies 4h after an acute exercise bout, while in the study we performed the biopsies were taken at least 48h after the last acute exercise bout. Both studies thus assessed a different type of response. Gordon et al. assessed the effect of training on the acute exercise gene expression response. This response was reduced for gene expression of genes involved in oxidative phosphorylation. This finding does not give any information about the effect of resistance exercise training on baseline levels, it can even be hypothesized that the reduced response size is caused by increased baseline levels. We assessed the effect of training on baseline gene expression levels and found that baseline levels of gene involved in oxidative phosphorylation were increased. Another interesting finding was that genesets involved in neuronal adaptations were enriched in resistance exercise training. This would explain many of the neuronal adaptations after resistance exercise training.

The analysis performed in this study were limited to gene expression changes. Health or physiological parameters were not measured. We concluded from the results that PPAR signalling and fatty acid oxidation were only weakly activated by combined exercise training when compared with endurance exercise training. It is known that PPAR signalling plays a crucial role in the improvements in dyslipidaemia and insulin sensitivity after exercise in skeletal muscle [22-24]. The limited induction of PPAR targets might therefore have important implications for the positive health effects of combined exercise training. Also the reduced enrichment of genesets involved in oxidation hold an important message, namely that oxidative capacity in skeletal muscle might show reduced adaption in combined exercise training compared to endurance exercise training. This is important in a context of health, since Sparks et al. [25] showed that combined exercise training increased mitochondrial capacity in diabetes type 2. For future research it is therefore important to assess the adaptive consequences for exercise-induced improvements in metabolic disease of a gene expression signature that is less oxidative in combined exercise training compared to the signature of endurance exercise training. This remark is supported by the literature assessing the muscle fibre type – fiber size paradox showing that there is an inverse relation between the size of a muscle and its oxidative capacity [26]. Muscle fibres stimulated to induce hypertrophy and increase oxidative capacity increased these properties to a lesser extent compared to fibres stimulated to induce hypertrophy or increased oxidative capacity alone.

Combined exercise training is advised and performed based on research showing a more beneficial effect on metabolic diseases compared to endurance and resistance exercise training alone [27]. In addition, many endurance athletes perform complement their usual training schedule with additional resistance exercise training to gain better performance. Several studies assessing the addition of resistance exercise training to the training program of (elite) endurance athletes showed increased endurance performance [28-30]. This increased performance was characterized by increased cycling economy, but not increased $VO2_{max}$ [28-31]. Furthermore training power and muscle size were increased by the added resistance exercise [28,29]. The increased cycling economy points to neuronal optimization as one of the most important factors that result increased performance. As stated previously, in this study we showed that combined exercise training had a clearly less oxidative signature compared to endurance exercise training. These findings might hold an important message for sports practice, namely that combining resistance and endurance exercise training might interfere with the oxidative adaptations induced by endurance exercise training alone. Important here to note is that the subject populations of studies on combined exercise training in sport sciences are very different from the subject populations used in studies related to health.

There are some factors that might have influenced the results of the study. Our analysis included three studies that differed in other parameters besides the type of exercise. First, the number of subjects differed between the studies. Second, subjects participating in the combined exercise training intervention were middle-aged, while the subjects in the other exercise training interventions were relatively young. Third, the subjects of the combined exercise training study were healthy but overweight while subjects in the other studies had normal weights. Finally, the timing of the muscle biopsies after the last exercise session differed across the studies. All these factors can have influenced the outcomes of this study. This study is performed as a first pilot and clearly shows the importance of a comparison between the three types of exercise training. The limitations of this study implicate that no strong conclusions can be made from the results of this study, but give direction for future research.

We conclude that despite the large overlap in gene expression between combined, endurance and resistance exercise training, each of the training types had an unique gene expression signature, indicating that each training type induced specific molecular changes. The minimal activation of oxidative and PPAR related pathways by combined exercise training might have implications for the use of combined exercise training in

the context of metabolic disease and sports. It can be hypothesized that addition of resistance exercise training potentially interferes with the positive effects of endurance exercise training on the gene expression profile of skeletal muscle. This hypothesis is an interesting topic for future research.

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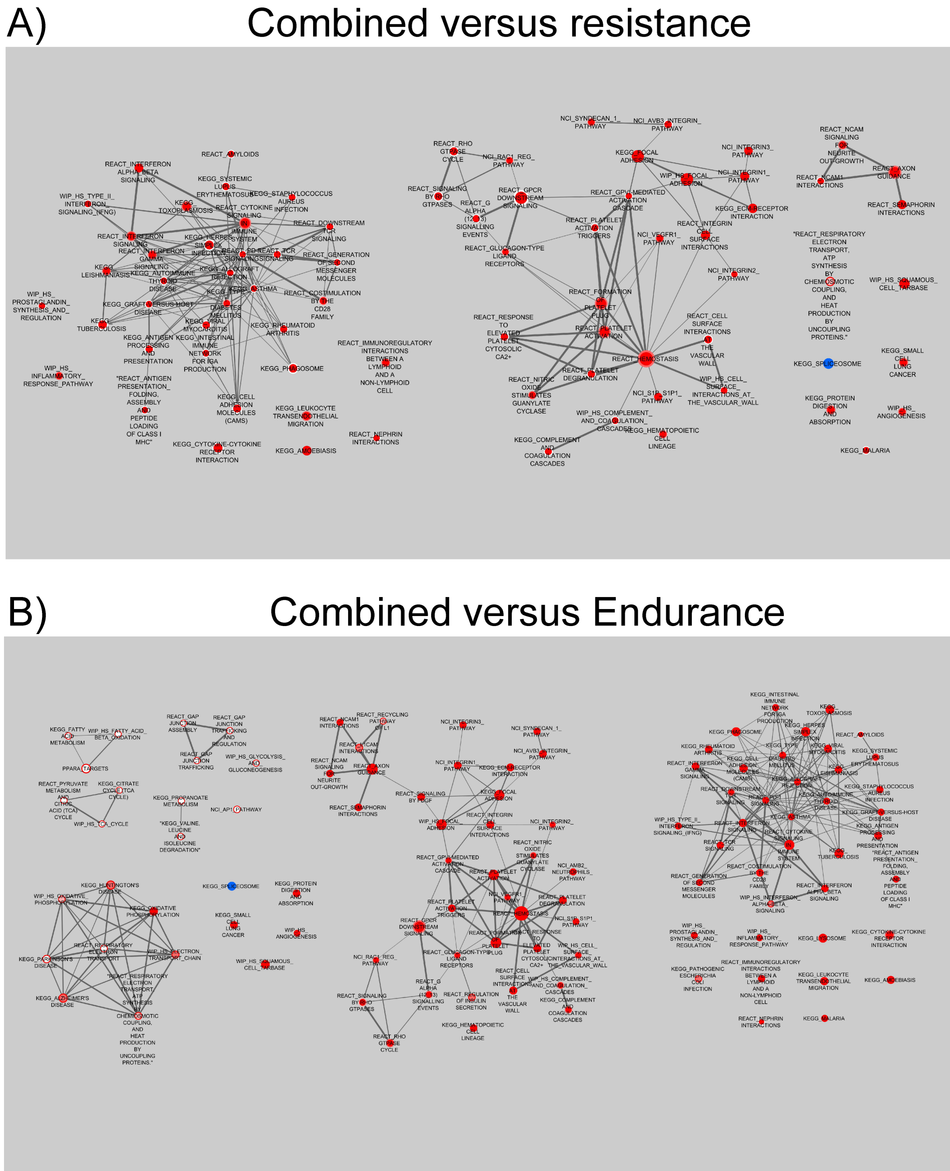

Supplemental figure S3.1 *GSEA enrichment maps of combined exercise training compared with endurance **(A)** and resistance **(B)** exercise training. Red is enriched, blue is depleted. The inner parts of the circles represent combined exercise training, the outer parts endurance **(A)** or resistance **(B)** exercise training.*

Chapter 4

Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle

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ABSTRACT

Regular physical activity positively influences whole body energy metabolism and substrate handling in exercising muscle. While it is recognized that the effects of exercise extend beyond exercising muscle, it is unclear to what extent exercise impacts non-exercising muscles. Here we investigated the effects of an acute endurance exercise bout on gene expression in exercising and non-exercising human muscle. To that end, 12 male subjects aged 44-56 performed one hour of one-legged cycling at 50% W_{max}. Muscle biopsies were taken from the exercising and non-exercising leg before and immediately after exercise and analyzed by microarray. One-legged cycling raised plasma lactate, free fatty acids, cortisol, noradrenalin, and adrenalin levels. Surprisingly, acute endurance exercise not only caused pronounced gene expression changes in exercising muscle but also in non-exercising muscle. In the exercising leg the three most highly induced genes were all part of the NR4A family. Remarkably, many genes induced in non-exercising muscle were PPAR targets or related to PPAR signalling, including PDK4, Angptl4 and SLC22A5. Pathway analysis confirmed this finding.

In conclusion, our data indicate that acute endurance exercise elicits pronounced changes in gene expression in non-exercising muscle, which are likely mediated by changes in circulating factors such as free fatty acids. The study points to a major influence of exercise beyond the contracting muscle.

INTRODUCTION

Regular exercise training is generally recognized as a powerful preventive and therapeutic strategy for diseases such as type 2 diabetes, obesity and cardiovascular disease. At a systemic level, regular exercise training improves cardiac and lung function [1,2,3], reduces the amount of adipose tissue [4], increases muscle mass [5,6,7], and decreases liver fat [8,9], representing chronic adaptations to repeated exercise bouts. Interestingly, the observation that unilateral training also improves strength in the immobilized or untrained limb indicates that the beneficial effects of exercise are not limited to the tissues directly engaged in exercise [10,11,12].

Immediately upon initiation of exercise, local demand for ATP, oxygen, glucose and fatty acids increases dramatically. These demands are accommodated by rapid changes in skeletal muscle activity of key enzymes and transporters involved in glucose and fatty acid oxidation via allosteric regulation and phosphorylation of rate-limiting enzymes. In addition, regulation at the mRNA level importantly contributes to the acute response and chronic adaptations to exercise. A large number of studies have shown that acute exercise induces genes involved in a variety of processes, including energy metabolism, hypertrophy and signalling [13,14,15,16,17,18,19,20,21]. Whole genome mRNA profiling has confirmed these findings, revealing major changes in skeletal muscle gene expression from 1 hour to even 48 hours after cessation of exercise [22,23,24,25]. All efforts to characterize exercise-induced changes in mRNA have so far focused on the exercising muscle. To what extent exercise influences gene expression in non-exercising muscles remains completely unclear. Conceivably, exercise may elicit changes in gene expression in non-exercising muscle via circulating mediators and metabolites. Such a mechanism may provide a conceptual framework for the impact of exercise on non-contractile tissues such as liver. In the present study, we have employed the one-legged exercise model and pre- and post-exercise muscle biopsies to study the acute effects of exercise on whole genome gene expression in exercising and resting human skeletal muscle. The results reveal that acute endurance exercise elicits pronounced changes in gene expression in non-exercising muscle, which are likely mediated by changes in circulating factors such as free fatty acids (FFA).

SUBJECTS AND METHODS

Subjects

Twelve healthy middle-aged men (age 51.5 ± 5.1 years, body weight 88 ± 17 kg, body mass index 26 ± 4) participated in the study. All subjects exercised less than 4 hours per week. Anthropometric parameters, $VO2_{max}$ and W_{max} (1 and 2 legged) values can be found in table 4.1. The study was approved by the medical ethical committee of Wageningen University and all subjects received oral and written information about the experimental procedures and provided written informed consent.

Table 4.1 Subject characteristics (n = 12)

Age (years)	52 ± 5
Length (cm)	184 ± 5
Weight (kg)	88 ± 17
BMI (kg/m^2)	26 ± 4
Rest HR (bpm)	60 ± 11
Maximum HR (bpm)	170 ± 17
$VO2_{max}$ (ml/min/kg)	35 ± 10
$VO2_{peak}$ 1 leg (ml/min/kg)	28 ± 10
W_{max} 1 leg (watts)	164 ± 37
W_{max} 2 legs (watts)	260 ± 64

HR = heart rate, BMI = body mass index, $VO2_{max}$ = maximum oxygen uptake, $VO2_{peak}$ = peak oxygen uptake, W_{max} = maximum work load.

Experimental design

All subjects performed a single 60 minutes experimental endurance exercise bout, which was preceded by two preliminary exercise tests and two familiarization trials (figure 4.1A). During the endurance exercise bout subjects had to perform one-legged cycling on a cycle ergometer (Excalibur Sport, Lode, Groningen NL) adapted with a custom-made leg support. Skeletal muscle biopsies were taken from both legs immediately before and shortly after exercise.

Preliminary exercise tests

Two graded cycling exercise tests to exhaustion were performed, the first with both legs, the second with one leg (Excalibur Sport, Lode, Groningen NL). The first two-legged exercise test was used to determine the subjects' maximum aerobic capacity ($VO2_{max}$;

table 4.1), while the second test was used to determine maximum workload of the dominant experimental leg ($W_{max-1-leg}$; table 4.1). Both tests started at a set workload (100 watt 2 legs, 20 watt 1 leg), which increased gradually until exhaustion (15 watts per minute 2 legs, 10 watts per minute 1 leg), determined as the participant not being able to continue cycling at 60 rounds per minute for longer than 15 seconds. Respiratory quotient (RQ) was above 1 at the end of the test in all subjects. During the tests oxygen uptake (VO_2) and heart rate (HR) were measured (Oxycon Pro, Jaeger, Hoechberg, Germany). The tests were performed at the same ergometer as the endurance exercise bout and familiarization trails and were completed at least 14 days before the endurance exercise bout and 3 days apart to avoid a training effect. The day prior to both tests subjects were asked to refrain from alcohol and heavy exercise.

Familiarization trials

All participants were unfamiliar with one-legged exercise. Therefore the two familiarization trials were performed before the endurance exercise bout to make sure that subjects became familiar with one-legged cycling (figure 4.1A). The familiarization trials consisted of 20 minutes of one-legged cycling at a self-chosen workload. All subjects performed the one-legged exercise with the dominant leg, which was determined via a number of daily life related questions.

Experimental endurance exercise bout

Subject refrained from heavy exercise the last 4 days prior to the experimental day. The last day before the experimental exercise bout subjects received a standardized evening meal and refrained from alcohol. On the morning (8.15h) of the experimental test subjects reported to the research facility, after an overnight fast (>10 hours). The experimental exercise bout consisted of 60 min one-legged cycling at 50% of the one-legged W_{max}. Before (T0) and shortly after (T1) the exercise a venous blood sample was drawn and muscle biopsies were taken from both legs (see figure 4.1B). Two hours after cessation of the exercise a third blood sample was taken (T3). During the experimental exercise HR was recorded continuously. Subjects remained fasted until after the last blood sampling, but were allowed to drink water ad libitum.

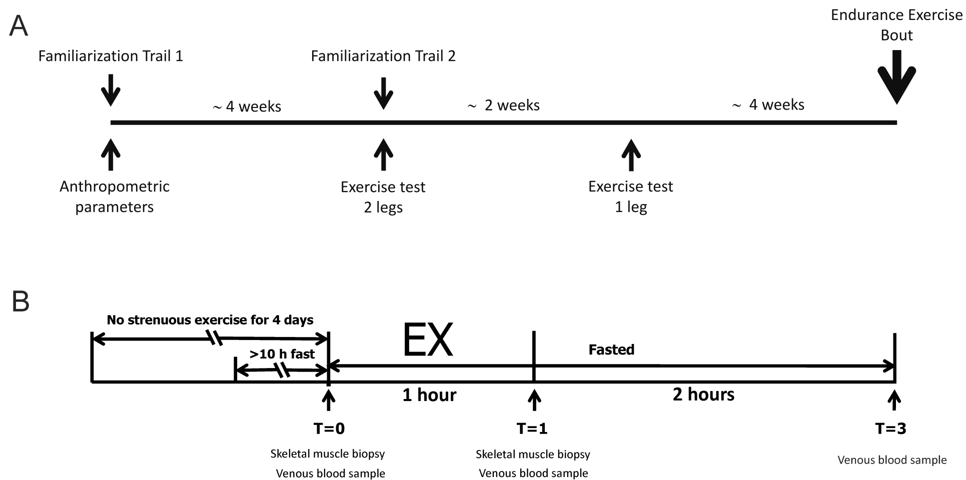


Figure 4.1 *Experimental design. The timeline of the study* ***(A)*** *and set-up of the endurance exercise bout* ***(B)****. After 2 familiarization trails and 2 exercise tests, subjects performed 1 hour of submaximal one-legged endurance exercise. Before and after the exercise bout muscle biopsies and venous blood samples were taken, and another blood sample was taken 2 hours after the end of exercise.*

Blood samples

Blood was collected in EDTA containing tubes. The samples were immediately centrifuged at 1000*g* at 4°C for 10 minutes, after which plasma was stored in -80°C until further analysis. Blood samples were analysed for free fatty acids (Centre for Medical Diagnostics (SHO), Velp, NL), glucose, triglycerides, cortisol, lactate (Gelderse Vallei hospital, Ede, NL), catecholamines (laboratory of clinical chemistry, Radboud Medical Center, Nijmegen, NL), and insulin (enzyme-linked immunosorbent assay, Mercodia, Uppsala, Sweden).

Muscle biopsies

Percutaneous needle biopsies were taken before (T0) and shortly after (T1) exercise from the vastus lateralis muscle from both legs (4 biopsies in total), using the Bergström technique with suction [26]. Skin was anesthetized with Xylocaine 2% with Adrenaline. All biopsies were taken from a separate incisions. There was at least a 2 cm gap between the biopsies of T0 and T1 to prevent influence of the earlier biopsy. The second biopsy from the same leg was taken from a more proximal position. Pre-exercise biopsies were taken just before the exercise; post-exercise biopsies within 30 minutes after termination of the exercise bout, on average it took ~15 minutes before the first post-exercise biopsy was taken. Biopsies of the exercising leg were taken first, followed shortly afterwards

by the biopsy of the non-exercising leg. After each biopsy, the collected tissue sample was carefully cleared from visible adipose tissue and blood and divided into four pieces. Three pieces were directly frozen into liquid nitrogen and one piece was embedded into Tissue-Tek O.C.T. compound (Sakura Tissue Tek, Alphen a/d Rijn, NL) and frozen in liquid-nitrogen cooled isopentane, and stored at -80°C for further analysis. We were not able to collect muscle biopsies of one of the participants due to hypersensitivity of the participant to the biopsy procedure.

RNA extraction

Total RNA was isolated from the skeletal muscle tissue by using Trizol reagent (Invitrogen, Breda, NL). Thereafter RNA was purified using the Qiagen RNeasy Micro kit (Qiagen, Venlo, NL) and RNA quality was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, NL).

Microarray processing

Total RNA (100 ng) was labelled using an Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands) and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays coding 19,732 genes, (Affymetrix ,Santa Clara, CA). Sample labelling, hybridization to chips and image scanning was performed according manufacturer's instructions.

Microarray data analysis

Microarray analysis was performed using MADMAX pipeline for statistical analysis of microarray data [27]. Quality control was performed and all arrays met our criteria, except arrays from 2 participants that showed a clearly distinct clustering and pattern after normalization. Those microarrays were excluded from further analysis. For further analysis a custom annotation was used based on reorganized oligonucleotide probes, which combines all individual probes for a gene [28]. Expression values were calculated using robust multichip average (RMA) method, which includes quantile normalisation [29]. Microarray data were filtered, and probe sets with expression values higher than 20 on more than 5 arrays were considered to be expressed and selected for further statistical analysis. In addition, an Inter Quartile Range (IQR) cut-off of 0.2 was used to filter out genes that showed no variation between the conditions. Significant differences in expression were assessed using Intensity-Based Moderated T-statistic (IBMT [30]).

Genes were defined as significantly changed when the p value was < 0.01. Differences in gene expression between the legs were determined using a paired IBMT test on the difference between T0 and T1 for both legs ($p < 0.05$).

Two subjects (4 and 8) were classified as outliers based on their aberrant response to exercise in the non-exercising leg. In subject 4 and 8, expression of 6 out of the 10 most highly upregulated genes was higher than the average plus two times the standard deviation. Accordingly, the analysis was repeated without these two subjects.

All microarray data are MIAME compliant and have been submitted to the Gene Expression Omnibus (accession number GSE41769).

Pathway analysis

Geneset enrichment analysis (GSEA; http://www.broad.mit.edu/gsea/) was performed for both legs using MADMAX and genesets with a false discovery rate (FDR) < 0.2 were considered significantly enriched. Possible transcription factors playing a role in the activation and inhibition of genes were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA). The ClueGO plugin in Cytoscape was used for gaining insight into processes activated during exercise in both legs [31,32].

cDNA synthesis and quantitative real time PCR

Total RNA was reverse transcribed with a cDNA synthesis kit (Promega, Leiden, NL). Standard qPCR was performed using SensiMix real time PCR reagents (Bioline, London, UK) and a Bio-Rad CFX384 machine (Bio-Rad laboratories, Veenendaal, NL). Primer sequences were based on availability in the PRIMERBANK (http//pga.mgh.harvard.edu/primerbank/index.html) and can be found in table 4.2. qPCR data were normalized using GAPDH as housekeeping gene for the human samples, since it was shown to be stable within skeletal muscle during exercise [33], and was stable between the time points according to our microarray analysis.

Statistical analysis

Statistical analysis for the plasma parameters and qPCR results were performed using SPSS (version 18, SPSS, Chicago, IL). Differences between the different time points for the plasma parameters (TG, glucose, FFA, lactate, insulin, cortisol, adrenaline and noradrenaline) were determined using a repeated measure one-way ANOVA. Differences between T0 and T1 in qPCR were evaluated using a paired t-test, differences between the

Table 4.2 Primer sequences used for qPCR

Gene name	Primer sequence
NR4A1-F	ATGCCCTGTATCCAAGCCC
NR4A1-R	GTGTAGCCGTCCATGAAGGT
NR4A2-F	GTTCAGGCGCAGTATGGGTC
NR4A2-R	AGAGTGGTAACTGTAGCTCTGAG
NR4A3-F	CAGCACTGAGATCACGGCTAC
NR4A3-R	CCCTCCACGAAGGTACTGATG
FOS-F	CACTCCAAGCGGAGACAGAC
FOS-R	AGGTCATCAGGGATCTTGCAG
JUNB-F	CCTACCGGAGTCTCAAAGCG
JUNB-R	CGAGCCCTGACCAGAAAAGTA
GAPDH-F	GAAGGTGAAGGTCGGAGTC
GAPDH-R	GAAGATGGTGATGGGATTTC

legs in qPCR were evaluated using a paired t-test for the changes between T0 and T1 in each leg. Data are mean ± standard deviation and $p < 0.05$ was considered statistically significant.

RESULTS

Systemic effects of one-legged exercise

Subjects performed one-legged exercise for one hour at 50% of their maximal workload with an average heart rate during the last five minutes of 132 ± 18 beats per minute. The percentage heart rate reserve (%HRR) during the last 5 minutes was 65 ± 12.5% (figure 4.2). Plasma glucose and triglyceride (TG) levels were not altered by one-legged cycling, while plasma FFA and lactate increased at T3, but mostly returned to baseline at T3 (figure 4.2). Insulin, cortisol and noradrenalin increased during exercise, with insulin and cortisol returning to baseline at T3 (figure 4.2). Adrenalin tended to increase during exercise, although the increase was not statistically significant ($p = 0.08$; figure 4.2).

One-legged cycling exercise altered gene expression in skeletal muscle of the exercising and non-exercising leg

We first ruled out that there was a significant difference in baseline expression between the two legs. Only eight out of 19,732 genes were found to be significantly different between the two legs at baseline ($p < 0.01$; supplemental table S4.1).

Statistical comparison of the baseline and post-exercise samples revealed that in the exercising leg (E) one-legged exercise significantly changed expression of 938 genes (p

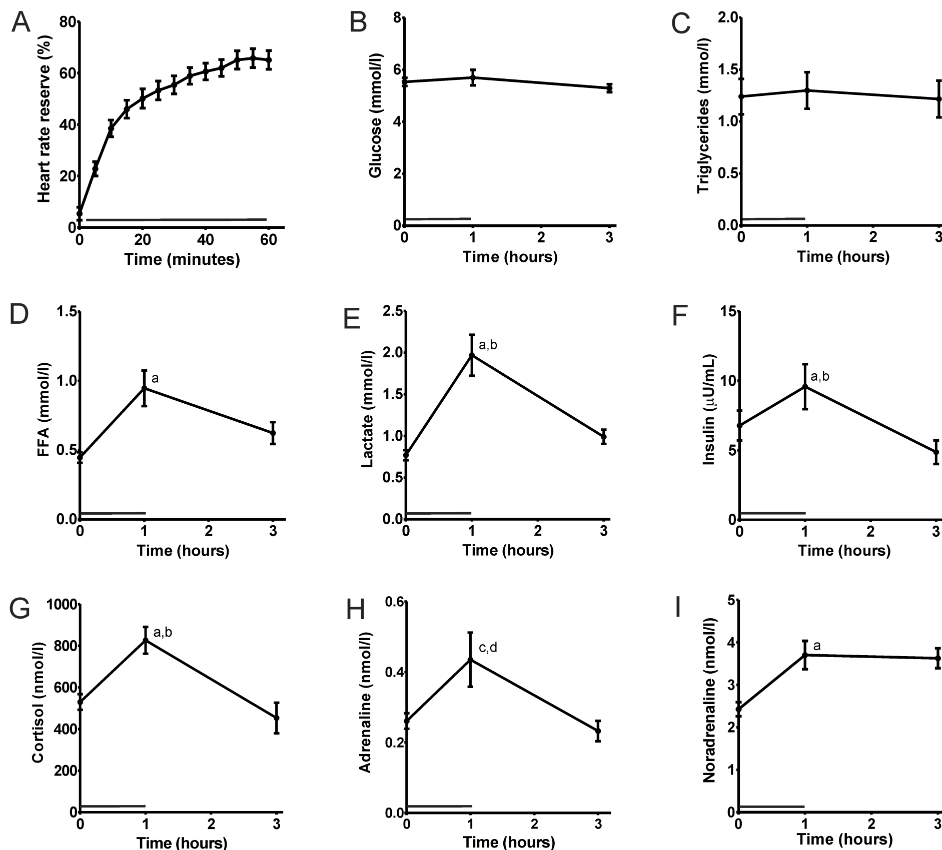


Figure 4.2 *Exercise increases heart rate and plasma levels of FFA, insulin, cortisol and noradrenaline. Heart rate reserve (%) was calculated based on the heart rate measured during the exercise (n = 12). Plasma glucose, triglyceride, free fatty acids, lactate, insulin, cortisol, adrenaline and noradrenaline were measured before and after exercise (T0 and T1; n = 12) and after 2 hours of recovery (T3; n = 12). a = p <0.05 compared to T0, b = p < 0.5 compared to T3, c = p < 0.1 compared to T0, p < 0.1 compared to T3, repeated measures ANOVA. Depicted is mean ± SEM.*

< 0.01), with the majority of genes being upregulated (figure 4.3A and D). The number of genes significantly changed by exercise in the non-exercising leg (NE) was lower but still remarkably high ($p < 0.01$; 516 genes), and also here the majority of genes was upregulated (figure 4.3A and C). Intriguingly, the majority of genes altered in the non-exercising leg were also altered in the exercising leg (figure 4.3A). Overall, the data indicate that a single exercise bout of exercise caused marked changes in gene expression not only in exercising muscle, but also in non-exercising muscle and that exercise mainly promotes upregulation of gene expression.

To gain more insight into the changes induced by exercise, genes were subsequently ranked according to mean fold-change in expression in the exercising leg and the changes in expression compared between the individual subjects (figure 4.4A, left panel). Expression changes of the same set of genes in the non-exercising leg are presented in parallel (figure 4.4A, right panel). Remarkably, the most highly induced genes in the exercising leg are the three members of the nuclear receptor subfamily NR4A. Of the 20 most highly induced genes in the exercising leg, 17 were also significantly upregulated in the non-exercising leg although to a lesser extent. Figure 4.4A clearly illustrates the marked inter-individual variation in response to exercise in the non-exercising leg. qPCR of selected genes largely confirmed the results of the microarray (supplemental figure S4.1).

To examine the impact of exercise on gene expression in parts of the body not directly influenced by exercise, we next focused our attention on genes upregulated in the non-exercising leg. Similarly to the exercising leg, genes were ranked according to mean fold-change in the non-exercising leg (supplemental figure S4.2, left panel) and changes in the exercising leg are shown in parallel (supplemental figure S4.2, right panel). Many of the most highly upregulated genes in the non-exercising leg were also highly induced in the exercising leg (supplemental figure S4.2). The two most highly upregulated genes were again NR4A2 (FC = 5.3) and NR4A3 (FC = 4.5). Importantly, subject 4 and 8 both showed a distinct profile from the other subjects illustrated by marked induction of numerous genes in the non-exercising leg that are not shared with other subjects. Based on the notion that gene expression was clearly distinctive from other subjects and using specific criteria outlined in the methods, we classified them as outliers and repeated statistical analysis without subject 4 and 8. We suspect that these subjects engaged in involuntary (isometric) contractions or other type of stimulation of the non-exercising leg. Removal of both subjects markedly reduced the number of significantly changed genes (209 vs.

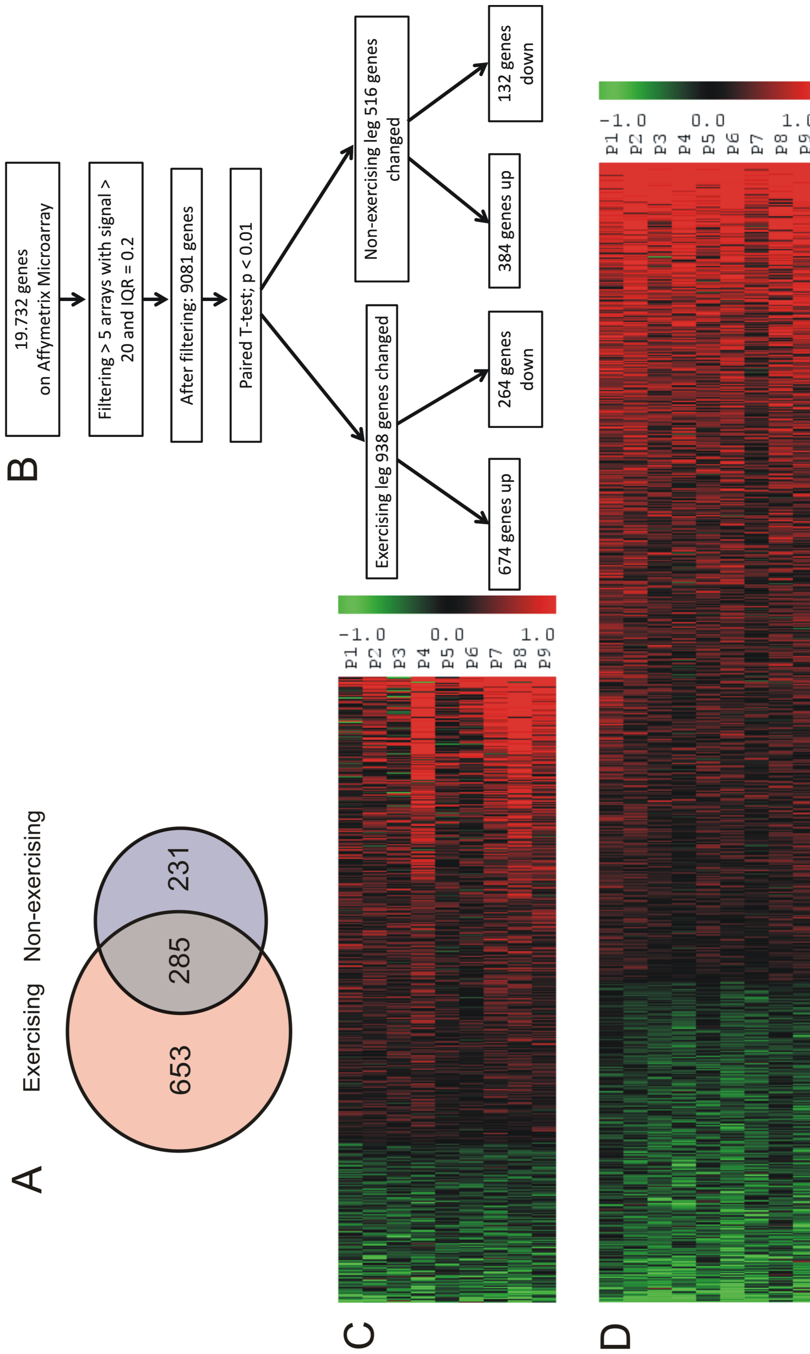


Figure 4.3 *Exercise mainly causes upregulation of gene expression in both the exercising and non-exercising leg.* ***(A)*** *Venn diagram of significantly regulated genes and their overlap.* ***(B)*** *Flowchart of microarray analysis. Heatmaps of all significant genes in the non-exercising* ***(C)*** *and exercising leg* ***(D)*** *and number of genesets significantly regulated by exercise in both legs. n = 9, IQR = interquartile range.*

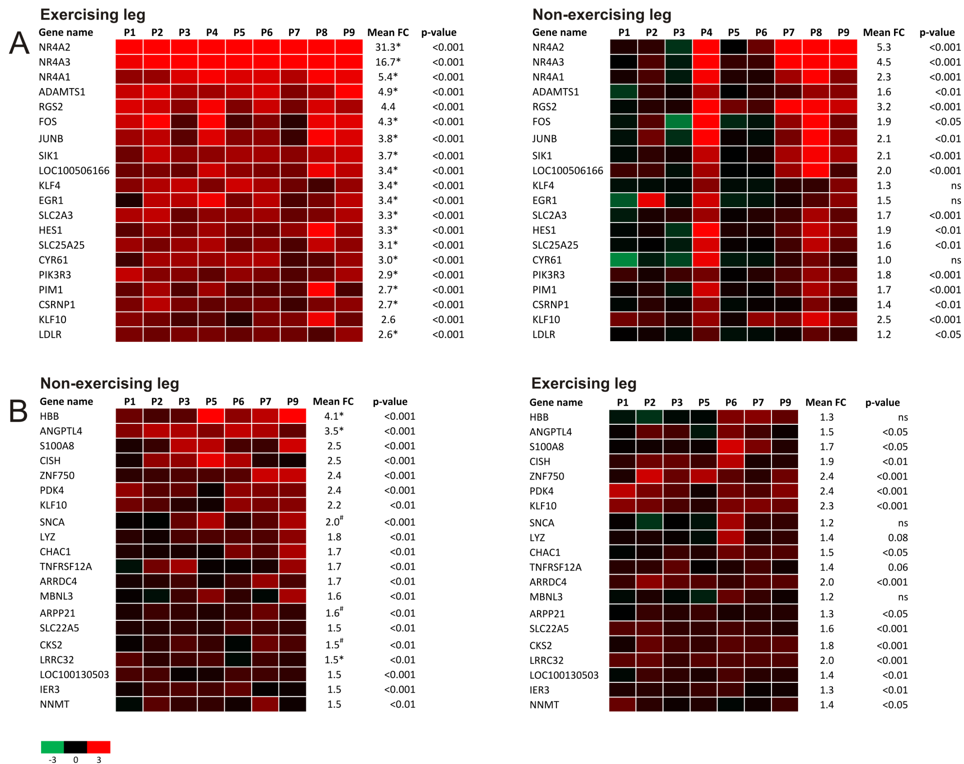


Figure 4.4 *Top 20 of most highly induced genes in exercising and non-exercising leg.* ***(A)*** *Left panel shows the top 20 of upregulated genes in the exercising leg (n = 9), right panel the corresponding genes in the non-exercising leg.* ***(B)*** *Left panel shows the top 20 of upregulated genes in the non-exercising leg (n = 7), right panel the corresponding genes in the exercising leg. Green is a signal log ratio of -3, red a signal log ratio of 3. Values are displayed per subject to visualize inter-individual differences. FC = fold change, * = $p < 0.05$, # = $p < 0.1$ between exercising and non-exercising leg.*

516 genes). Furthermore, the overlap in gene regulation between the exercising and non-exercising leg decreased from 285 to 85 genes. After removing subject 4 and 8, many of the most highly upregulated genes in the non-exercising leg were characterized by lower induction in the exercising leg. Other genes showed similar fold-induction in the exercising and non-exercising leg, including ZNF750, PDK4, KLF10, and SLC22A5 (figure 4.4B), which are established or suspected target genes of PPARs [34].

Processes during one legged cycling in the exercising leg and non-exercising leg

We suspected that exercise-induced changes in gene expression were mediated by specific transcription factors. To identify these transcription factors and to identify pathways regulated by exercise, we performed Ingenuity pathway analysis (IPA) (figure 4.5 and supplemental table S4.2). IPA uses information from literature combined with gene expression changes to predict a role of transcription factors in the dataset. The most significant set of target genes in the exercising leg was the set controlled by CREB1. CREB1 and also ATF4 (lower in the list) are both induced by cAMP and mediate cAMP-dependent gene regulation. SREBF1, SREBF2 and NR1H3 (LXR) are all involved in lipid and especially cholesterol homeostasis and have extensive cross-talk. Other sets of target genes shown in figure 4.5 are involved in growth (sets under control of STAT3, FOXO1, NOTCH1, MYC and NR3C1) and inflammation (sets under control of NR3C1 and STAT3). HIF1A is a major regulator in the adaptive responses to hypoxia.

The only significant set of target genes in the non-exercising leg was the set controlled by PPARα, which is consistent with the marked upregulation of several existing and putative PPARα target genes (supplemental table S4.2). Since PPARα is activated by fatty acids [35], these data point to a role of elevated plasma FFA levels in exercise-induced changes in the non-exercising leg, and to a lesser extent in the exercising leg, as other processes seem to play a more pronounced role there.

To further determine the biological processes activated during exercise, we used ClueGO and GSEA. ClueGO integrates GO categories and creates a functionally organized GO category networks based on the overlap between the different GO categories and the significance [31]. According to ClueGO analysis, a substantial number of processes was induced during exercise in both legs (figure 4.6). In the exercising leg the important processes were growth and development of skeletal muscle, neurons and vessels, metabolism (mostly basal and protein metabolism) and transcriptional regulation (figure 4.6A). Also kinase cascade and signalling were induced. GSEA showed an comparable picture, with upregulation of genesets involved in growth (hypertrophy model), MAPK signalling and stress response (AP1 pathway; supplemental figure S4.3). ClueGO revealed that most processes induced by exercise in the non-exercising leg are involved in basal metabolism and signalling/transport (figure 4.6B). GSEA showed a clear upregulation of PPAR target genes, (cytokine) signalling and growth and stress response (hypertrophy & AP1 pathway; supplemental figure S4.3).

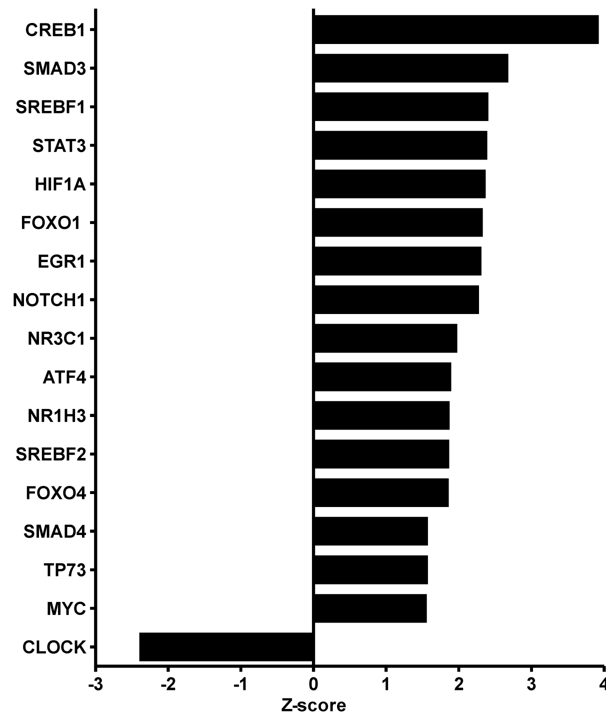
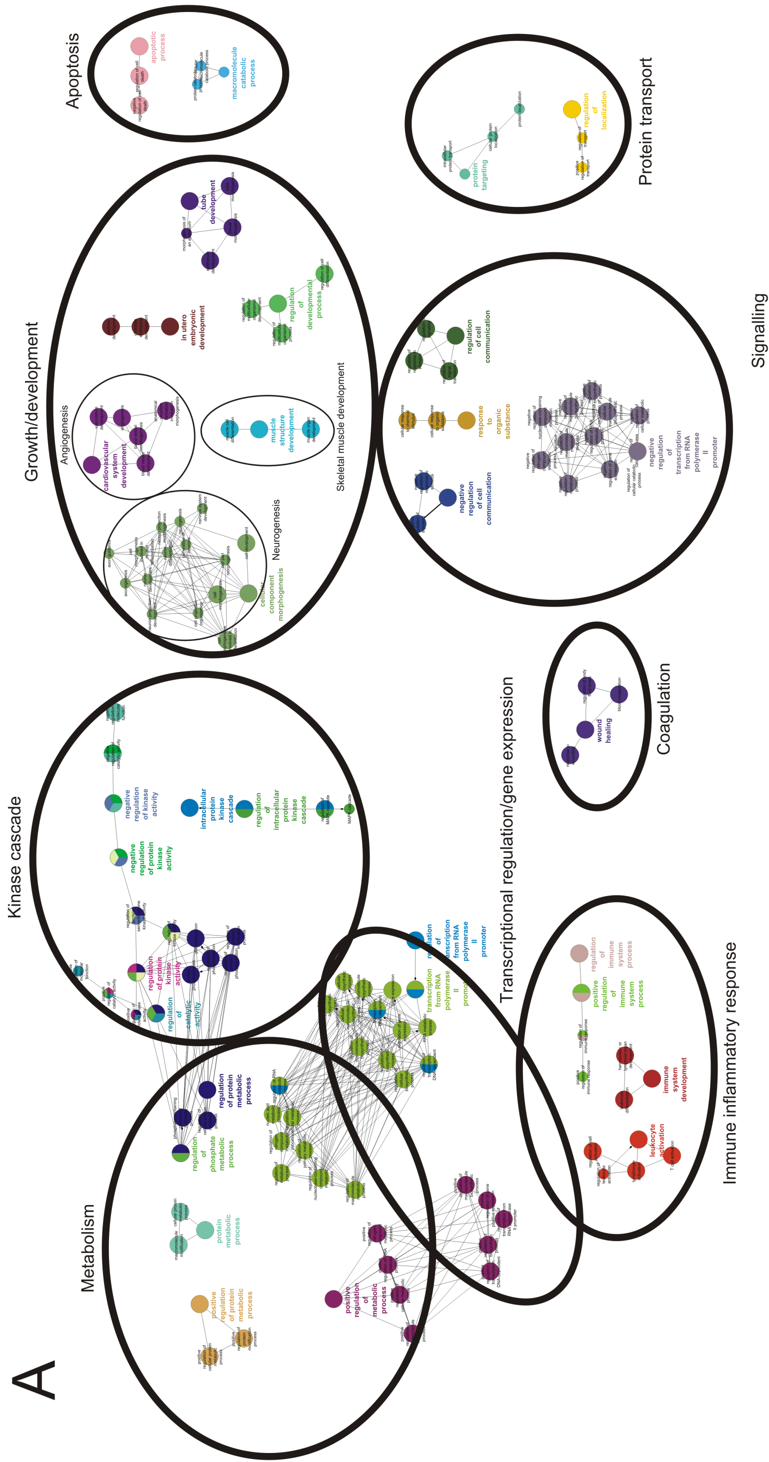


Figure 4.5 *Induction of transcription factor pathways by exercise. Transcription factor pathways related to growth, stress response, cAMP signalling and hypoxia were induced by exercise. Transcription factor pathways were identified for the exercising leg using IPA and are displayed in a bar diagram. Genes induced by exercise for the different transcription factors can be found in supplemental table S4.1. Transcription factors with a z-score above 1.5 (or under -1.5) are considered as biologically relevant.*

DISCUSSION

Here we used the one-legged exercise model to study the effect of acute endurance exercise on whole genome muscle gene expression and determine the relative importance of systemic versus local contraction-related stimuli. We found that acute exercise induced immediate and dramatic gene expression changes in the exercising leg, with the most dramatic inductions observed for the NR4A family. Strikingly, acute exercise also caused substantial gene expression changes in non-exercising leg. Overall, our data indicate that the molecular responses to acute exercise are not confined to the exercising muscle but also extend to resting muscle. The notion that exercise alters gene expression in non-exercising muscles is new. Our data provide a conceptual and molecular framework for the observation that immobilized muscle can experience


A
Metabolism
Kinase cascade
Growth/development
Angiogenesis
Neurogenesis
Skeletal muscle development
Apoptosis
Protein transport
Signalling
Coagulation
Transcriptional regulation/gene expression
Immune inflammatory response
positive regulation of protein metabolic process
protein metabolic process
regulation of phosphate metabolic process
regulation of protein metabolic process
positive regulation of metabolic process
regulation of catalytic activity
regulation of protein kinase activity
negative regulation of protein kinase activity
negative regulation of kinase activity
intracellular protein kinase cascade
regulation of intracellular protein kinase cascade
cardiovascular system development
muscle structure development
in utero embryonic development
tube development
regulation of developmental process
apoptotic process
macromolecule catabolic process
protein targeting
regulation of localization
regulation of cell communication
response to organic substance
negative regulation of cell communication
negative regulation of transcription from RNA polymerase II promoter
wound healing
regulation of immune system process
positive regulation of immune system process
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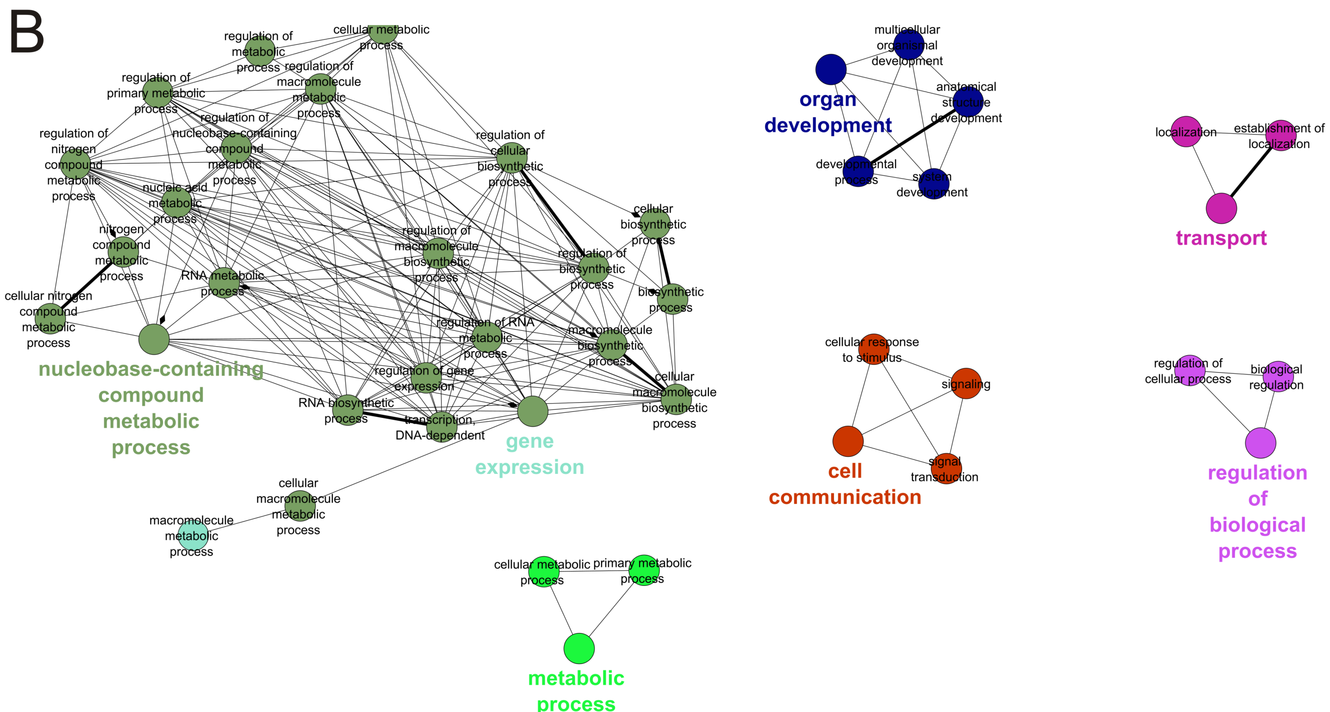

Figure 4.6 *ClueGO network analysis. Analysis shows significant regulation of several GO categories involved in skeletal muscle development, angiogenesis, inflammation and MAPK cascade in the exercising leg (**A**; n = 9) and basal metabolism and signalling in the non-exercising leg (**B**; n = 7). The nodes represent significantly changed GO categories. Lines represent the overlap between different categories. All nodes with a large overlap have a similar colour.*

favourable metabolic adaptations in response to repeated contractile activity in non-immobilized muscle [10,11,12].

In general, changes in gene expression in non-exercising muscle were less pronounced compared to the exercising muscle, both in magnitude of fold-changes and number of genes changed. A relatively small number of genes, many of which represent known target genes of the PPAR transcription factors including Angptl4, KLF10, SLC22A5, ZNF750, PDK4, was induced equally in exercising and non-exercising muscle. PPARs play a key role in regulation of lipid metabolism in a variety of tissue, including skeletal muscle [36]. Ingenuity pathway analysis and GSEA further indicated significant enrichment of PPAR target genes in set of genes upregulated in the non-exercising leg. Induction of PPARα targets is expected to lead to enhanced fatty acid oxidation as an acute but perhaps also adaptive response to endurance exercise. Inasmuch as plasma FFA levels go up during exercise and fatty acids are known ligands of PPARs and Angptl4 and PDK4 [37,38], our data may point to an important role of elevated FFAs as systemic factor driving gene expression changes in exercising and non-exercising muscle during exercise. One gene (Angptl4) was more highly induced in the non-exercising leg compared to the exercising leg. Detailed exploration of the regulation and role of Angptl4 during exercise will be reserved for a future publication. Apart from FFAs, it could be hypothesized that other factors including other metabolites and circulating hormones may also impact gene expression in non-exercising muscle. NR4A transcription factors are known to be regulated via β-adrenergic signalling [39]. Elevated catecholamines (via sympathetic innervation or via the circulation) may at least partially account for the induction of NR4A genes in the exercising leg, which was supported by Ingenuity Pathway Analysis showing the CREB1 pathway as most significant transcription factor pathway. However, even though circulating levels of catecholamines were increased, expression of NR4A genes was not increased in the non-exercising leg, indicating only a minor role of catecholamines in the non-exercising leg. It can be hypothesized that gene expression changes in the non-exercising leg may also be elicited by myokines secreted from the exercising leg. In a future publication we will address the impact of exercise on secretion of myokines using a so called secretome approach followed by measurement of numerous potentially novel and existing myokines in plasma. However, this type of analysis is beyond the scope of the present manuscript.

We are the first to use microarray to assess the immediate effect of acute endurance exercise on human skeletal muscle gene expression. Prior studies focused on the recovery

after exercise [22,24,25], or used animal models [22,23]. Exercise causes immediate perturbations of homeostasis that are gradually restored during recovery. Based on this notion, it can be suspected that changes in gene expression elicited by exercise gradually fade out during post-exercise recovery. In support, in our study a total of 938 genes was altered post-exercise in the exercising leg, whereas previously 173 genes were found to be increased 3 hours after termination of exercise and 37 genes 48 hours after exercise [24]. However, direct comparison is complicated by the use of different array platforms and statistical cut-offs. Mahoney et al. [24] used an custom made array, and only signal log ratios were available, which did not allow us to use the same statistical cut-off.

Many of the observed exercise-induced changes in gene expression are likely part of an acute stress response related to disturbances in homeostasis elicited by exercise. The most highly induced genes in the exercising leg were all members of the NR4A family, a subgroup of orphan receptors within the nuclear receptor superfamily. NR4A1 and NR4A3 have been reported to be upregulated shortly after acute exercise and during recovery in rat [40,41], pig [23], and human [24], and this upregulation likely occurs locally by contractile stimuli [40]. This finding was confirmed by our study in which we observed an upregulation of NR4As in the exercising, but not in the non-exercising leg. NR4A transcription factors are also known to be induced by adrenaline and noradrenaline [39]. Circulating adrenalin and noradrenalin levels were increased in our study but must exert only a minor effect as NR4As were exclusively induced in the exercising leg,. Upregulation of NR4A2 has been observed once in human but its role during exercise is unknown [24]. NR4A1 and 3 are thought to play a key role in regulating energy metabolism and early adaptation [42,43,44]. Combined analysis of our study and the study of Mahoney et al. suggests that NR4A2 is induced immediately during exercise and decreases relatively quickly after exercise during recovery, whereas NR4A3 shows a different pattern characterized by a relative small induction during exercise followed by a large induction in the recovery phase (supplemental figure S4.4 [24]). The results may imply that NR4A family might play an important role in the regulation of metabolic responses after exercise.

Unlike in other subjects, in two subjects the overall magnitude of gene expression changes in the exercising and non-exercising leg were very similar. It is unclear how such an extreme response can occur in a resting leg that was not engaged in any concentric contractions. We cannot completely exclude these two subjects performed significant isometric contractions in the non-exercising leg, although subjects were instructed not

to. Alternatively, a potential role for (involuntary) neural stimulation muscles may be envisioned. It is known that neural stimulation can induce gene expression changes via increased calcium concentrations in the skeletal muscle [45,46,47], as well as via other mechanisms [48]. Since energy utilization and metabolic flux must have been much lower in the non-exercising leg, which showed gene expression changes comparable to the exercising leg, it can be inferred that the majority of gene expression changes are unrelated to metabolic flux. The exception are genes induced similarly in the exercising and non-exercising leg in all subjects.

One-legged cycling is frequently used as a model to enable direct comparison between exercising and resting muscle. Earlier studies encountered problems with repeated biopsies, showing inflammatory gene expression changes induced by repeated biopsies [49] . Other studies showed no effect of repeated biopsies on gene expression [50]. The main difference between these studies is that the study that did show an effect of repeated biopsies on gene expression used one incision for all biopsies [49], whereas the other study showing no effect performed new incisions for each biopsies [50], analogous to our protocol. This indicates that separate incisions might be crucial for reducing the effect of repeated biopsies, which was also verified by the lack of inflammatory genes changed in the resting muscle in our study.

In conclusion, exercise has profound effects on gene expression in human skeletal muscle, not only in exercising muscle but also in resting muscle. The latter effects are likely mediated by changes in circulating factors such as FFA and may explain why muscles not involved in the exercise movement may undergo favourable adaptations in response to exercise.

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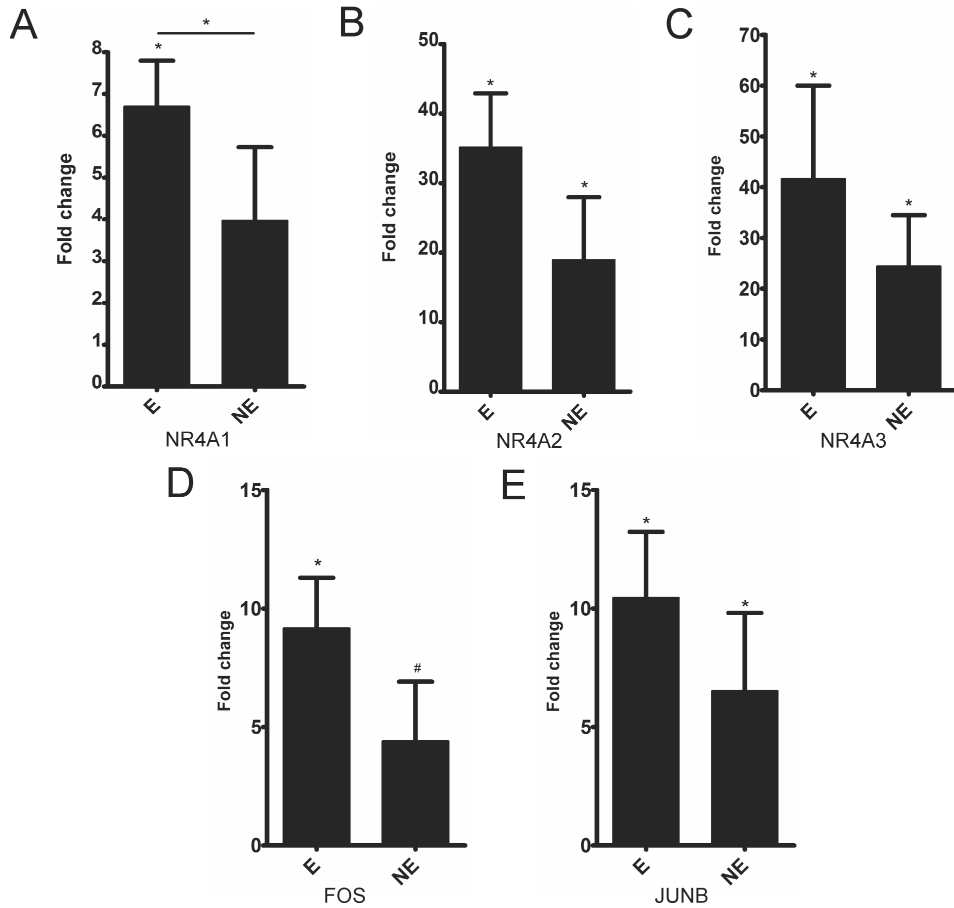
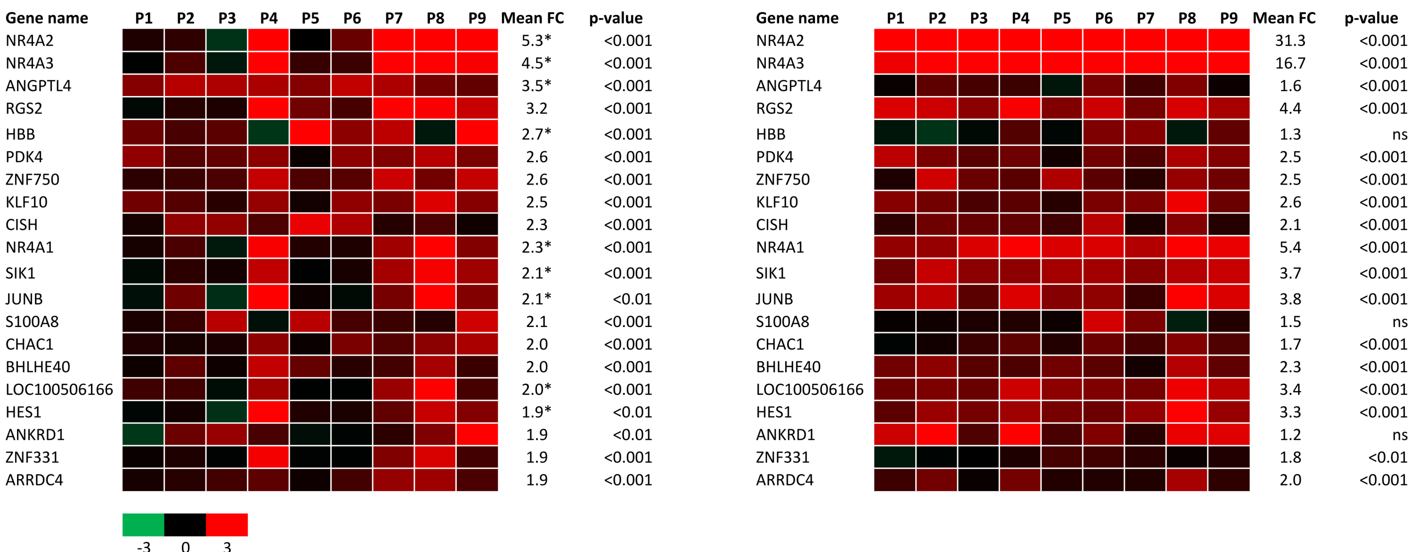
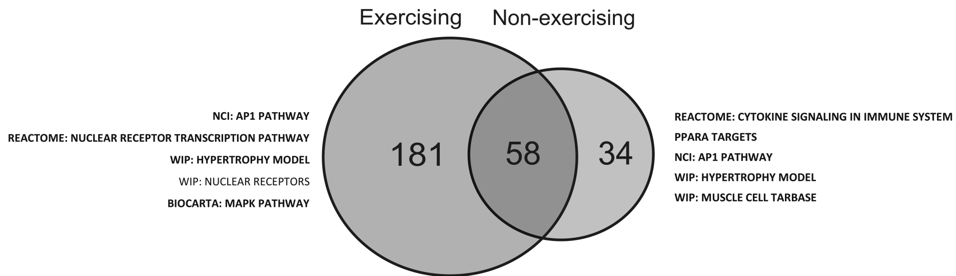


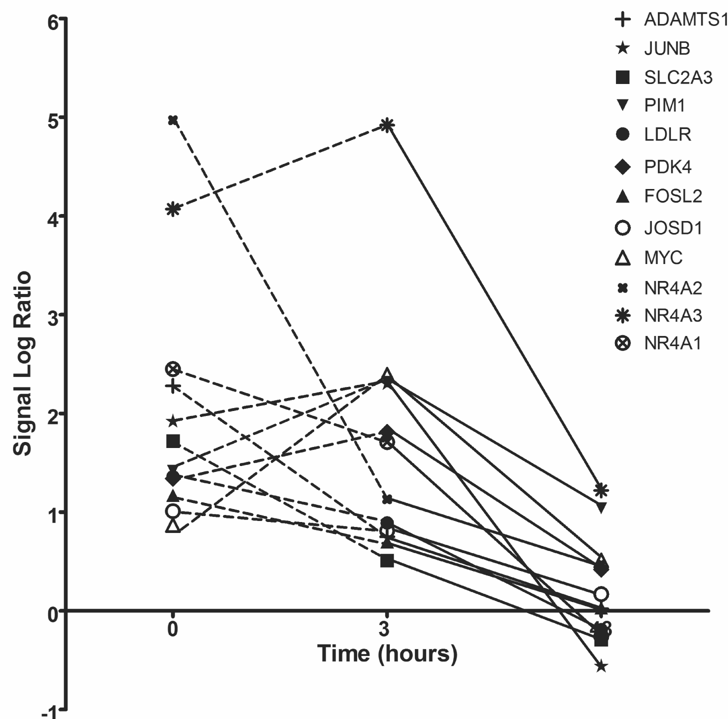
Supplemental figure S4.1 *qPCR confirming microarray results of NR4A family, JUNB and FOS (n = 11). Fold changes of both legs are displayed, which are calculated by dividing the post-exercise by the baseline sample for both legs. Before expression values were normalized by the housekeeping gene GAPDH. * = p < 0.01. Depicted is mean ± SEM.*



Supplemental figure S4.2 *The 20 most highly induced genes in the non-exercising leg, including subjects 4 and 8. Heatmap of the top 20 upregulated genes in the non-exercising (n = 9), left panel shows top 20 of the non-exercising leg, right the corresponding genes in the exercising leg. Green is a signal log ratio of -3, red a signal log ratio of 3. Values are displayed per subject to visualize inter-individual differences. FC = fold change,* $* = p < 0.05$, $^{\#} = p < 0.1$ *between exercising and non-exercising leg.*



Supplemental figure S4.3 *Acute endurance exercise induces several genesets in both legs related to immune response, skeletal muscle hypertrophy and stress. Venn diagram shows the overlap between the upregulated genesets in both legs. Next to the circles are the top 5 enriched genesets. Genesets depicted in bold are overlapping between exercising and non-exercising leg, whereas geneset with a normal font are unique for that leg. FDR = 0.2, exercising leg n = 9, non-exercising leg n = 7.*



Supplemental figure S4.4 *Combined time course of gene expression of selected genes of Mahoney et al. and this study: signal log ratios of selected genes (based on presence in datasets and expression) are displayed directly after exercise (this study) and 3 and 48 hours after exercise (Mahoney et al. 2005).*

Supplemental table S4.1 List of differentially expressed genes at baseline (T0) in exercising and non-exercising leg

Gene name	Mean FC	P value	FDR value
XPOT	-1.28545	0.0014	0.99962
PROCR	1.17887	0.0038	0.99962
C3orf71	1.191506	0.0049	0.99962
BANP	1.22251	0.0052	0.99962
MALL	1.283441	0.0088	0.99962
SPRR4	1.250174	0.0094	0.99962
LOC100505587	1.150362	0.0096	0.99962
THAP5	1.230518	0.0098	0.99962

Supplemental table S4.2 Induced transcription factor pathway and the genes contributing to this induction

Transcription factor	Target genes in dataset
Exercising leg	
CREB1	APOLD1, ATF3, BHLHE40, BTG2, CEBPB, CIITA, CSRNP1, CYR61, DUSP14, EGR1, EGR2, ERRFI1, FLT1, FOS, FOSB, GADD45B, IDI1, IRS2, JUNB, LDLR, LSS, MCL1, MIDN, MKNK2, MYC, NAB2, NFIL3, NFKBID, NOS2, NR4A1, NR4A2, NR4A3, PDXK, PER1, PPARGC1A, PPP1R15A, RGS2, SERTAD1, SIAH2, SIK1, SLC2A3, VEGFA
SMAD3	BCL2L11, CCL2, CDKN1A, CTGF, ESR1, FOS, HEY1, JAM2, JUNB, JUND, MXD1, POR, RHOB, SKIL, SMAD1, SMAD7, TGFB1, TGFB3
SREBF1	ACLY, ADH1C, CDKN1A, CFD, FASN, HK2, HMOX1, HSPA1A/HSPA1B, HSPA5, IDI1, INSIG1, IRS2, LDLR, LGALS3, LSS, PIK3R3, SREBF1, TGFB1, VEGFA
STAT3	A2M, BCL2L11, BCL3, CCL2, CD9, CDKN1A, CIITA, CXCL2, DLL1, EGR1, FASN, FOS, GADD45A, ICAM1, IFI30, IFITM3, IL6R, JUNB, MCL1, MYC, NOS2, PCNT, PIM1, PPARGC1A, RORC, SGK1, SOCS3, SREBF1, STAT1, TAP1, TLR3, TNFRSF1B, VEGFA, WARS
HIF1A	ADAM17, ADM, AKAP12, AURKA, BIRC2, CDKN1A, CHKA, CXCL12, CYR61, ERO1L, FAM13A, FLT1, FOS, HK2, HMOX1, INHBB, IRS2, ITGA5, ITGB3, KDM3A, LIFR, MCL1, MYC, NDRG1, NOS2, NOS3, NOTCH1, NRARP, PDGFA, PDGFB, PFKFB3, SLC40A1, SMAD7, SOX9, TGFB1, TGFB3, VASP, VEGFA
FOXO1	ACLY, BCL2L11, CDC42EP3, CDKN1A, CTGF, ELOVL5, FASN, FOXO1, GADD45A, GADD45B, HSPA5 ,IER3, IRS1, IRS2, KLF2, LPL, ME1, PPARGC1A, SGK1, SLC25A1, SREBF1
EGR1	ATF3, CASP3, CASP9, CCL2, CDKN1A, CXCL2, FOSL1, GADD45A, GDF15, HMOX1, ICAM1, JUNB, JUND, ME1, MXD1, MYC, NAB2, NDRG1, PDGFA, PDGFC, TGFB1, THBS1, VEGFA
NOTCH1	CDKN1A, CEBPD, CFD, DLL1, FLT1, FOSB, HES1, HEY1, HEY2, ICAM1, MYC, NOS2, NR2F2, REL, RORC, TGFB1, TGFBR2, TP63
CLOCK	ANGPTL4, ARNT2, BHLHE40, BPHL, CBR1, CCRN4L, CHST3, DBP, DDIT3, DNAJB9, DUSP8, FASN, FKBP5, GADD45A, GADD45B, GDNF, GIMAP4, HSPA1A/HSPA1B, HSPH1, ICAM1, ID1, JMJD6, JUNB, KLF13, MAP2K3, MKNK2, NFIL3, PER1, PER2, POR, SLC20A1, ST3GAL5, TNFAIP1, TOR1B, USP2, WEE1
NR3C1	A2M, ABHD2, ABL1, ACTB, ACTN1, ADH1C, ADM, ANGPTL4, APOL3, AQP1, ARID4B, BAG2, BAG3, BCL2L11, BCOR, BHLHE40, BIRC2, BRAF, CASP9, CCL2, CDC42EP3, CDKN1A, CLK2, CSRNP1, CSRNP2, DAPK2, DAPK3, DDIT4, DDX5, DEDD2, DUSP1, DUSP16, DYNLL1, EGR1, EMP1, ERRFI1, FASTKD1, FKBP5, FOS, FOXO1, GADD45A, GADD45B, GLUL, HNRNPU, ICAM1, IER2, IER3, IGF1, ING2, IP6K3, JMJD6, JUND, LRRC8A, MCL1, MKNK2, MYC, NFKBIA, PARP4, PDE4B, PDGFA, PDPN, PIK3CB, PIK3R3, PLEKHF1, PPP1R13L, PPP1R15A, PPP2CA, PPP2R1B, RELT, RGS2, RHOB, RRAGC, SEMA3C, SERINC3, SERPINB9, SERTAD2, SGK1, SIAH1, SIAH2, SLC19A2, SLC38A1, SMAD1, SNAI2, STOM, THBD, TLE3, TNFAIP1, TNFAIP3, TNFRSF10B, TNFRSF12A, TNFRSF1B, TP53BP2, TSC22D3, UGCG, VGLL2, YWHAH
ATF4	ATF3, CDKN1A, CEBPB, CTH, DDIT3, DDIT4, ERO1L, GDF15, KLF4, LGALS3, MAP1LC3B, NDRG1, NID2, OSMR, PMP22, PYCR1, SLC7A5, SNAI2, TNFRSF12A, VEGFA, WARS
NR1H3	ACSL3, ARG2, FASN, LDLR, LPL, MYLIP, NFKBIA, NOS2, PPARGC1A, SREBF1
SREBF2	ACLY, CDKN1A, FASN, IDI1, INSIG1, IRS2, LDLR, LSS, SREBF1
FOXO4	BCL6, CDC42EP3, CDKN1A, CTGF, FASN, GADD45A, GADD45B, IDI1, IER3, SGK1

Supplemental table S4.2 continues on next page

Supplemental table S4.2 *Continued*

Transcription factor	Target genes in dataset
ELK1	CDKN1A, EGR1, EGR2, FOS, FOSL1, JAM2, JUNB, MCL1
ETS1	CDKN1A, CTGF, DIAPH1, FLT1, HSPA1A/HSPA1B, INSIG1, ITGB3, MCL1, MYC, NPR1, PLAU, PVR, SP100, ZNF302
FOXL2	ATF3, CXCL2, FOS, ICAM1, IER3, MAFF, NR4A3, PPARGC1A, PPP1R15A, RGS2, SMAD6, TNFAIP3
SMAD4	ANGPT2, CDC42EP3, CDK17, CDKN1A, CTGF, GADD45A, GADD45B, ID2, IER3, IRAK3, ITGA6, JAM2, MYC, POR, RASSF1, SERTAD1, SGK1, SLC6A1, SMAD3, SMAD6, SMAD7, TGFB1, THBS1, VEGFA
TP73	ABCC1, ADAM17, BHLHE40, CDKN1A, CTH, DBP, DDIT3, FASN, HIVEP1, IL4R, KLHL21, MIR22HG, PDGFB, PMP22, PTPN3, SAT1, TCEAL1, TGFB1, THBS1, TNFRSF1B, VEGFA
MYC	ADM, AKAP12, AMD1, ANGPT2, BIRC2, CDKN1A, CHKA, CLEC3B, COL15A1, COL4A1, COL6A3, COX7A2L, DDIT3, DUSP1, EIF4A1, FABP4, FASN, FBLN2, FBN1, GADD45A, GADD45G, GLS, HIST1H4A, HK2, HMOX1, HSP90AA1, HSPH1, ID2, ITGA6, KLF4, KRAS, MAT2A, MNT, MYC, MYO1C, ODC1, PFKP, PLAU, PLAUR, PMP22, SDCBP, SERINC3, SERPINH1, SGK1, THBS1, THBS2, TIMP2, TXNIP, VEGFA
Non-exercising leg	
PPARA	ANGPTL4, CES3, CPT1A, MVK, NR1D1, PDK4, PPM1D, RETSAT, SLC25A20, UCP2

Chapter 5

Identification of human exercise-induced myokines using secretome analysis

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ABSTRACT

Endurance exercise is associated with significant improvements in cardio-metabolic risk parameters. A role for myokines has been hypothesized, yet limited information is available about myokines induced by acute endurance exercise in humans. Therefore the aim of the study was to identify novel exercise-induced myokines in humans. To this end, we carried out a one hour one-legged acute endurance exercise intervention in 12 male subjects and a 12 week exercise training intervention in 18 male subjects. Muscle biopsies were taken before and after acute exercise or exercise training, and were subjected to microarray-based analysis of secreted proteins (secretome). For acute exercise, secretome analysis resulted in a list of 86 putative myokines, which was reduced to 29 by applying a fold-change cut-off of 1.5. Based on that shortlist, a selection of putative myokines was measured in the plasma using ELISA or multiplex assay. From that selection, CX3CL1 (Fractalkine) and CCL2 (MCP-1) increased at both mRNA and plasma level. From the known myokines, only IL-6 and FGF21 changed at the mRNA level, whereas none of the known myokines changed at the plasma level. Secretome analysis of exercise training intervention resulted in a list of 69 putative myokines. Comparing putative myokines altered by acute exercise and exercise training revealed a very limited overlap of only 13 genes.

In conclusion, this study identified CX3CL1 and CCL2 as myokines that were induced by acute exercise at the gene expression and plasma level and that may be involved in communication between skeletal muscle and other organs.

INTRODUCTION

Physical exercise can serve as a very powerful tool in the prevention and treatment of metabolic diseases, including obesity and type 2 diabetes. In type 2 diabetic patients, exercise increases insulin sensitivity in liver and muscle [1], improves cardiorespiratory fitness and cardiovascular health [2], and reduces dyslipidaemia [3,4]. While skeletal muscle is the predominant organ impacted by acute exercise, specific metabolic alterations elicited by exercise may be accounted for by changes at the level of other organs such as the liver and adipose tissue. Very little is known about mechanism of crosstalk between muscle and other organs that could underlie effects of exercise on systemic health.

One possible mechanism is via secretion of proteins that signal between muscle and the rest of the body. A recent publication showed that acute exercise not only changes gene expression in the exercising muscle, but also in the non-exercising muscle [5], supporting a crosstalk between active skeletal muscle and inactive organs. In recent years, it has become clear that skeletal muscle releases a variety of proteins into the circulation. These proteins are referred to as myokines and represent putative "exercise factors" [6-8]. To serve as exercise factor, production of the myokine should be altered upon muscle contraction and/or by chronic exercise training. A number of myokines have been shown to be released from muscle during and shortly after exercise, with interleukin-6 (IL-6) being the most prominent example [9]. Evidence suggests that IL-6 released from muscle during exercise may contribute to the exercise-induced increase in hepatic glucose production [10]. Other presumed myokines include IL-8, IL-15 [11], leukemia inhibitory factor (LIF; [12]), and fibroblast growth factor 21 (FGF-21;[13]). None of these factors are solely produced by muscle and all have major functions elsewhere in the body. Due to their potential as pharmacological targets, there is an on-going quest to find additional myokines and studies are undertaken to determine their role during acute exercise and in the adaptive response to chronic exercise training.

Different approaches towards identification of novel myokines have been pursued, mainly focusing on animal models or on cultured myocytes subjected to electrical pulse stimulation [14-18]. The studies undertaken in humans focus on specific genes and proteins [19-21] or are limited to exercise training [14,22]. So far no untargeted screenings have been undertaken to identify novel myokines during acute exercise in humans. Furthermore, there are data on myokines commonly regulated by acute exercise and exercise training. Myokines increasing in both exercise modalities would be highly

interesting targets for development of exercise mimetics and are also prime candidates to serve as general exercise (bio)marker.
Accordingly, in this study we have utilized the one-legged exercise model and performed whole genome gene expression analysis in pre- and post-exercise muscle biopsies to screen for new putative myokines in humans. Results of the myokine screening of the acute one-legged exercise were compared with a myokine screening of a 12-week exercise training intervention, to assess the overlap between acutely and chronically induced myokines. The overall results of the whole genome gene expression analysis of the one-legged cycling have been published elsewhere [5]. The present paper is focused specifically on the study of myokines. Our results point to a number of novel myokines that may be involved in cross-talk between skeletal muscle and other organs.

METHODS

Acute exercise intervention

Twelve healthy middle-aged men (age 51.5 ± 5.1 years, body weight 88 ± 17 kg, body mass index (BMI) 26 ± 4 kg/m^2; average±standard deviation) participated in the study. Inclusion criteria were: between 40 and 60 years of age, BMI under 30 kg/m^2 and male gender. Subjects had an average maximal oxygen uptake of 35 ± 10 ml/min/kg and were sedentary to moderately active (not more than 3 hours of exercise per week). Subjects did not use any medicine, nor did they smoke. More details about the study population can be found elsewhere [5]. This age group was chosen because they are part of the population mostly affected by diseases such as obesity and diabetes type 2, and at the same time are able to perform more intense exercise without causing potential health risks. The study was approved by the medical ethical committee of Wageningen University and all subjects received oral and written information about the experimental procedures and provided written informed consent.
Detailed microarray analysis of this study was published elsewhere [5]. This study is a detailed follow up on this article to provide more insight on the myokines induced by exercise. All subjects performed a single 60 minute experimental endurance exercise bout, which was preceded by two preliminary exercise tests and two familiarization trials. During the endurance exercise bout subjects had to perform one-legged cycling at 50% of maximal wattage reached during a one-legged maximal exercise test (W_{max}) on a cycle ergometer (Excalibur Sport, Lode, Groningen NL), adapted with a custom-made

leg support. Exercise was reported to be fairly difficult and subjects reached a maximum of ~60% of the heart rate reserve after one hour. Skeletal muscle biopsies were taken from both legs before and within 15 minutes after exercise. Subjects were fasted during the whole experimental exercise bout, in total for approximately 15 hours, and had ad libitum access to water. Temperature in the exercise room was ~20° Celcius.

Exercise training intervention

18 healthy middle-aged male subjects (at baseline: age 59 ± 3.7 years, weight 95.1 ± 12 kg, BMI 29.8 ± 6.6 kg/m^2, $VO2_{max}$ 28.9 ± 4.4 ml/min/kg; average ± standard deviation) performed a 12 week mixed exercise training intervention. The 18 healthy subjects participated in a larger study and were matched for age, BMI and maximal oxygen uptake with 18 diabetic subjects. The mixed exercise training consisted of 2 endurance exercise session and 1 resistance exercise session per week (45 minutes per session). More details about the study and participants can be found in Meex et al. [1].

Tissue collection

Blood was collected in EDTA containing tubes. The samples were immediately centrifuged at 1000*g* at 4°C for 10 minutes, after which plasma was stored in -80°C until further analysis.

For the acute exercise study, percutaneous needle biopsies were taken before (T0) and shortly after exercise (T1) from both legs (4 biopsies in total). For the exercise training study, needle biopsies were taken before and 3 days after termination of the training period after an overnight fast. Biopsies were collected from the vastus lateralis muscle using the Bergström technique with suction [23]. All biopsies were taken from a separate incision. After collections biopsies were immediately frozen into liquid nitrogen and stored at -80°C for further analysis. We were not able to collect muscle biopsies of one of the participants due to hypersensitivity of the participant to the biopsy procedure.

RNA extraction, microarray processing and microarray data analysis

RNA extraction, microarray processing and microarray analysis for the acute exercise study are described in detail elsewhere [5]. In short, RNA was isolated using Trizol and purified using RNeasy columns, after which RNA was labelled and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays coding 19,732 genes (Affymetrix ,Santa Clara, CA). Microarray analysis was performed using MADMAX pipeline for statistical

analysis of microarray data [24]. Quality control was performed and all arrays met our criteria, except arrays from 2 participants that showed a clearly distinct clustering and pattern after normalization. Those microarrays were excluded from further analysis. Microarray data were filtered (expression values > 20 on more than 5 arrays, interquartile range > 0.2). Significant differences in expression were assessed using Intensity-Based Moderated T-statistic (IBMT [25]). Genes were defined as significantly changed when the p value was < 0.01. Differences in gene expression between the legs were determined using a paired IBMT test on the difference between T0 and T1 for both legs ($p < 0.05$). The microarray dataset is MIAME compliant and have been submitted to the Gene Expression Omnibus (accession number GSE41769).

Several participants were excluded through the pipeline of the acute exercise study. To provide an overview the excluded subjects and reason for exclusion are summed up in chronological order. The study started with 12 participants, one participant was excluded due to hypersensitivity to the biopsy procedure, two participants were excluded based on a distinct clustering in the quality control of the microarray, and two participants were excluded solely in the non-exercising leg based on their aberrant response to exercise in that leg (rationale in Catoire et al. [5]) , leaving the acute exercise study with N = 9 for the exercising leg and N = 7 for the non-exercising leg.

The processing and analysis of the muscle biopsies derived from the exercise training study was as described above. All samples passed quality control. The microarray data were filtered (expression values > 20 on more than 5 arrays, interquartile range > 0.2) and subjected to statistical analysis ($p < 0.01$). The primary microarray dataset was submitted to Gene Expression Omnibus (GSE53598).

cDNA synthesis and quantitative real time PCR

Total RNA was reverse transcribed with a cDNA synthesis kit (Promega, Leiden, NL). Standard qPCR was performed using SensiMix real time PCR reagents (Bioline, London, UK) and a Bio-Rad CFX384 machine (Bio-Rad laboratories, Veenendaal, NL). Primer sequences were based on availability in the PRIMERBANK (http//pga.mgh.harvard.edu/primerbank/index.html) and can be found in table 5.1. GAPDH was used as housekeeping gene [26], as its expression did not change upon acute exercise or exercise training (measured by qPCR and/or microarray)

Table 5.1 Primer sequences used for qPCR

Gene	Primer sequence
hIL6-F	AAACAACCTGAACCTTCCAAAGA
hIL6-R	GCAAGTCTCCTCATTGAATCCA
hIL15-F	TTTCAGTGCAGGGCTTCCTAA
hIL15-R	GGGTGAACATCACTTTCCGTAT
hMCP1-F	CAGCCAGATGCAATCAATGCC
hMCP1-R	TGGAATCCTGAACCCACTTCT
hVEGFA-F	CGCAGCTACTGCCATCCAAT
hVEGFA-R	GTGAGGTTTGATCCGCATAATCT
hCX3CL1-F	ACCACGGTGTGACGAAATG
hCX3CL1-R	TGGATGAGCAAAGCTACAGGTAT
hFNDC5-F	TGGAGGAGGATACGGAGTACA
hFNDC5-R	CCACATGAACAGGACCACGA
hADAMTS1-F	GAGGCGTATTTCATCCAGCC
hADAMTS1-R	GTCCTCGTCTTCGGTCTCC
hCYR61-F	CTCGCCTTAGTCGTCACCC
hCYR61-R	CGCCGAAGTTGCATTCCAG
GAPDH-F	GAAGGTGAAGGTCGGAGTC
GAPDH-R	GAAGATGGTGATGGGATTTC

Secretome analysis

Microarray-based secretome analysis was performed on all significantly changed genes in the acute exercise and training studies by screening for genes likely encoding secreted proteins using Gene Ontology Classification and SignalP predicting tools. Specifically, significantly changed genes annotated in Gene Ontology Cellular Component (GO CC) as extracellular region or space (GO:0005576/GO:0005615) were selected. Remaining genes that were not assigned to a GO category were screened for presence of a signal peptide using SignalP [27].

Plasma measurement of known and new myokines

IL-6, IL-18, IL-15, BDNF, CCL2 (MCP-1) and VEGF were measured in plasma of the acute exercise study using a multiplex immunoassay as described by Schipper et al. [28]. CYR61, CXCL2 (USCN Life Science, Houston, TX), and CX3CL1 (fractalkine, R&D Systems, Minneapolis, MN) were measured using enzyme-linked immunosorbent assay (ELISA).

Bioinformatics tools used for microarray analysis

Using an interquartile range > 0.5, 821 genes were selected from the acute exercise dataset and used for further analysis. Those genes showed the largest variability through

the experiment. For the plasma levels, values measured at T0 and T1 were used as input. MixOmics, a R package [29], was used to perform sparse partial least square regression (sPLS) to assess the relationship between plasma parameters and gene expression. A canonical sPLS was used. Furthermore a hierarchical clustering was performed on the sPLS results. Clusters were extracted in R and analysed using Ingenuity (www.ingenuity.com) and ClueGO [30]. Correlation of the top 200 most variable genes were calculated using GItools.

Statistical analysis

Statistical analysis for the plasma myokines and qPCR data was performed using SPSS (version 18, SPSS, Chicago, IL). Differences between the different time points for the plasma parameters were determined using a repeated measure one-way ANOVA. Gene expression differences between T0 and T1 were evaluated using a paired t-test. Gene expression differences between the legs for the changes from T0 to T1 were evaluated using a paired t-test. Data are mean ± SD and $p < 0.05$ was considered statistically significant.

RESULTS

Acute exercise-induced myokines determined by secretome analysis

One hour of one-legged cycling significantly changed the expression of 938 genes in the exercising leg ($p < 0.01$, $N = 9$), compared to 209 genes in the non-exercising leg ($p < 0.01$, $N = 7$). The overall analysis of the whole genome expression data has been published elsewhere [5]. The significantly changed genes were further analysed to identify potential candidate myokines by so called secretome analysis, which involved screening of the microarray dataset for genes likely encoding secreted proteins using Gene Ontology Classification and SignalP predicting tools. We found that 80 genes encoding secreted proteins were changed in the exercising leg, 52 of which were upregulated and 28 were downregulated (Figure 5.1; $p < 0.01$). In the non-exercising leg, 17 genes encoding secreted proteins were significantly changed (12 up- versus 5 down-regulated; figure 5.1). Upregulated genes showed an overlap between the exercising and non-exercising leg, which was not observed for the down-regulated genes. The complete list of putative myokines can be found in supplemental table S5.1. We further reduced the number of exercise-induced myokines by applying a fold-change (FC) cut-off of 1.5, resulting in 29

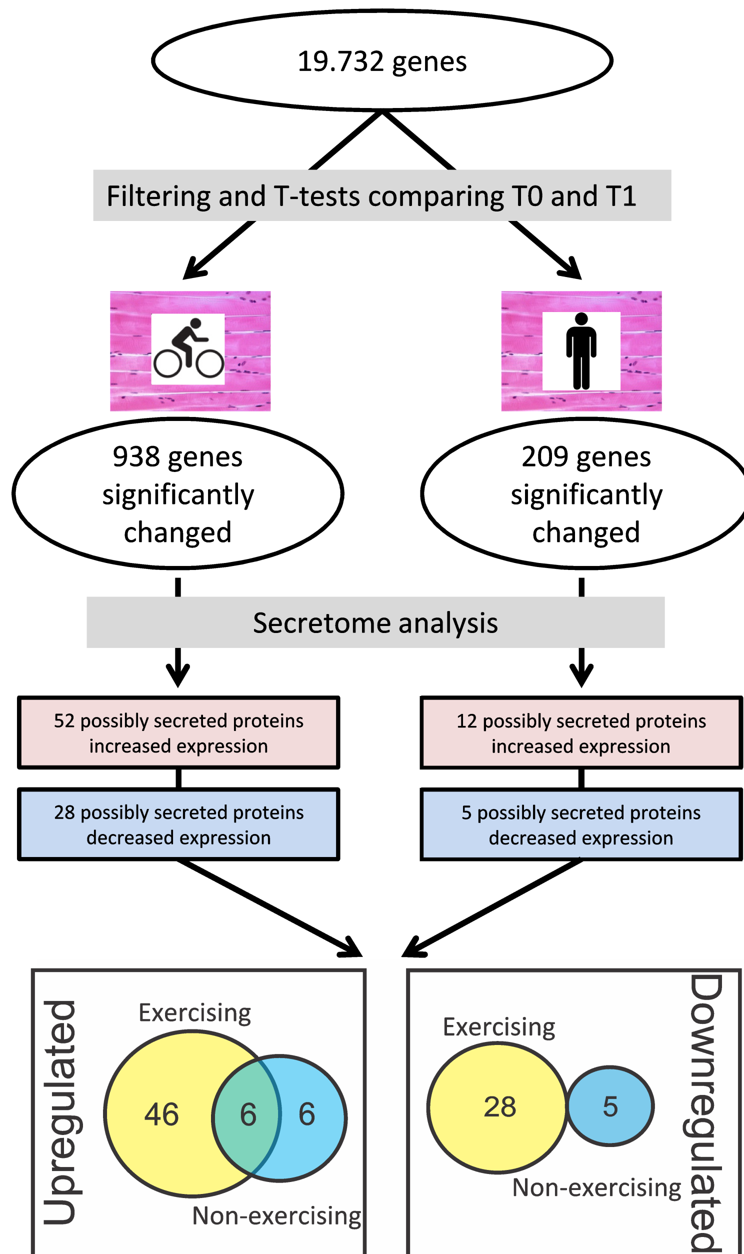

Figure 5.1 *Secretome analysis reveals a large number of new putative myokines in the acute exercise study. Flow chart showing the various steps for identifying new myokines. Genes were considered significantly changed when $p < 0.01$. T0 is before exercise, T1 directly after exercise. Geneset was filtered using signal cut-offs > 20 on more than 5 arrays, and an interquartile range of 0.2 was applied. Exercising leg N = 9, non-exercising leg N = 7.*

putative myokines. The 1.5 FC cut-off was applied to narrow down our search for new systemic myokines, based on the notion that minor changes in gene expression are less likely to have physiological consequences. Only 2 putative myokines were significantly regulated in both legs, while a further 23 putative myokines were specifically regulated in the exercising leg. 4 myokines were specifically regulated in the non-exercising leg (figure 5.2). Putative myokines with the highest fold-change were ADAMTS1 (exercising leg; FC 4.9), Angptl4 (exercising leg: FC 1.6, non-exercising leg 3.5) and S100A8 (non-exercising leg: FC 2.5). The set of myokines that was significantly induced in the exercising leg included the chemokines CXCL2, CX3CL1 (fractalkine) and CCL2. CCL2 has already been reported as a myokine whose mRNA and protein level are increased locally in the muscle during exercise [31,32]. Interestingly, only one putative myokine (Angptl4) was

	Gene name	Description	E	NE	FC E	FC NE	Muscle specific
Both legs	ANGPTL4	angiopoietin-like 4			1.5*	3.5	NO
	SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, 3F			1.5*	1.2	NO
Only exercising leg	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1			5.2*	1.3	NO
	CYR61	cysteine-rich, angiogenic inducer, 61			3.2*	-1.5	NO
	APOLD1	apolipoprotein L domain containing 1			2.6*	1.3	NO
	THBD	thrombomodulin			1.9*	1.3	NO
	CX3CL1	chemokine (C-X3-C motif) ligand 1			1.9*	-1.2	NO
	CXCL2	chemokine (C-X-C motif) ligand 2			1.8*	1	NO
	LOC100505657	hypothetical protein LOC100505657			1.8*	1.2	?
	FGF6	fibroblast growth factor 6			1.7	1.4	NO
	DLL1	delta-like 1 (Drosophila)			1.7*	1.4	NO
	SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A			1.6*	1.1	NO
	HSPH1	heat shock 105kDa/110kDa protein 1			1.7*	1.0	NO
	THBS1	thrombospondin 1			1.6	1.3	**
	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9			1.6*	1.1	NO
	VEGFA	vascular endothelial growth factor A			1.5*	1.3	NO
	SDC4	syndecan 4			1.5*	1.2	NO
	GDNF	glial cell derived neurotrophic factor			1.5*	1.2	NO
	CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2			1.5#	1.1	NO
	PDGFB	platelet-derived growth factor beta polypeptide			1.5*	-1.2	NO
	DHRS4L2	dehydrogenase/reductase (SDR family) member 4 like 2			-1.6*	-1.1	NO
	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10			-1.8*	1.0	NO
Only NE leg	S100A8	S100 calcium binding protein A8			1.7	2.5	NO
	LYZ	lysozyme			1.4	1.8	NO
	SNCA	synuclein, alpha			1.2*	2	NO
	MSTN	myostatin			-1.2*	-1.5	NO
			-2	2			

Figure 5.2 *Heatmap of putative myokines induced in the acute exercise study with a fold change > 1.5. Heatmap showing the 29 most highly induced genes encoding secreted proteins. Genes were divided into three categories: significantly induced in both legs, significantly induced in exercising leg and significantly induced in non-exercising leg. Muscle-specific gene expression was verified using BioGPS and is indicated for all putative myokines. * = $p < 0.05$, # = $p < 0.1$ between the legs, Paired t-test was performed for the difference between the legs for N = 7. ** = genes selectively expressed in cardiomyocytes and smooth muscle cells (BioGPS), ? = not present in BioGPS. Exercising leg N = 9, non-exercising leg N = 7.*

more highly induced in the non-exercising compared to the exercising leg. The changes in gene expression of selected putative myokines as determined by microarray were confirmed by qPCR (figure 5.3A), except for VEGFA.

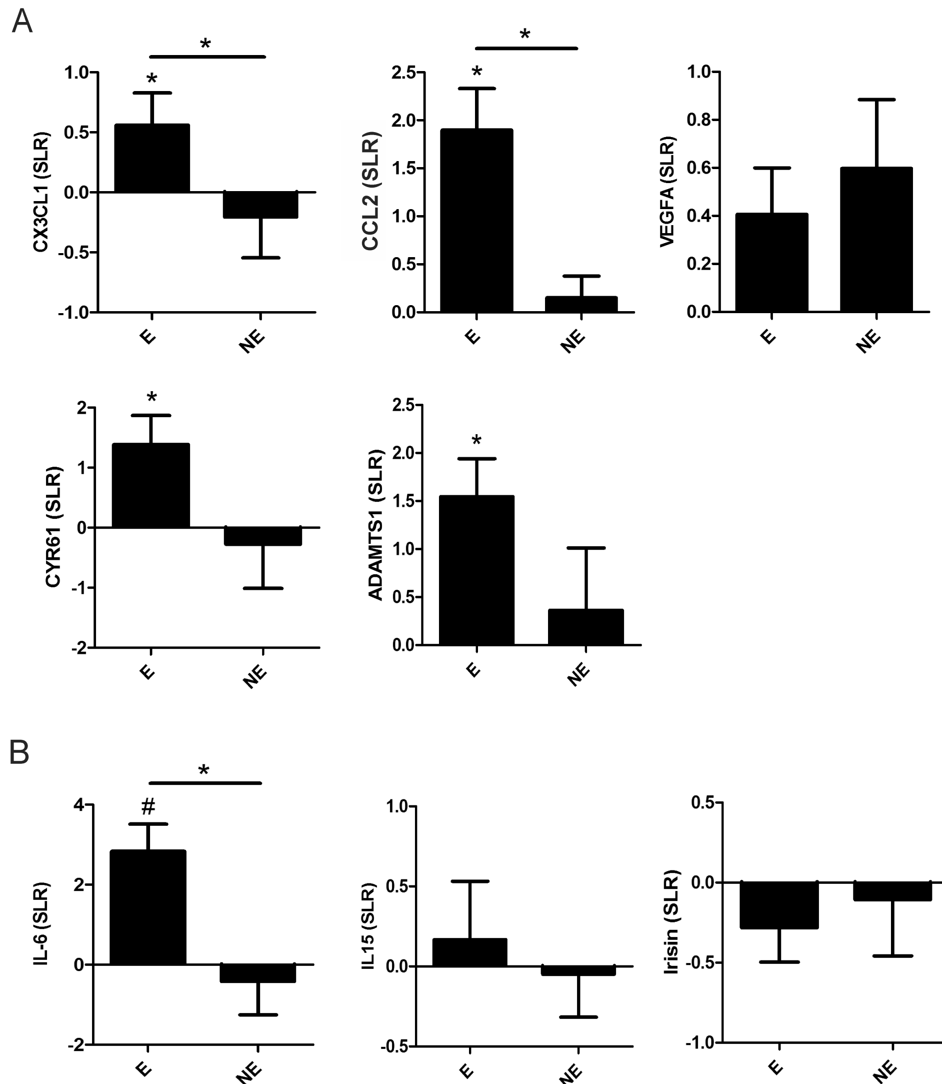

Figure 5.3 *Verification of microarray data by qPCR. Gene expression of selected novel* ***(A)*** *and known* ***(B)*** *myokines as determined by qPCR in the acute exercise study. Signal log ratios (SLR) for both legs are displayed. Expression values were normalized by the housekeeping gene GAPDH. * = $p < 0.05$, # = $p < 0.1$. Depicted is mean ± SEM, N = 11.*

We examined whether any of the 29 exercise-induced genes encoding secreted proteins were selectively expressed in muscle, as assessed using BioGPS (http://biogps.org). Only 2 of the putative myokines were expressed selectively in muscle, THBS1 and CTGF, which showed the highest expression in smooth muscle and cardiomyocytes (figure 5.2).
Several known myokines, including IL-6, IL-8, IL-15, and FNDC5 (Irisin) did not appear in the total list of 96 significantly changed genes that encoded secreted proteins. Detailed analysis revealed that most known myokines were indeed not significantly changed in either the exercising or non-exercising leg (table 5.2). IL-6 and FGF21 were both significantly induced in the exercising leg, but due to their low expression signal did not pass the filtering process. To confirm the microarray results we performed qPCR on a selection of known myokines (figure 5.3B), which largely confirmed the microarray results. Indeed, IL-15 and FNDC5 were not significantly changed, and a trend was observed for increased expression of IL-6 in the exercising leg.

Table 5.2 Microarray results of majority currently known myokines for the exercising (N = 9) and non-exercising leg (N = 7)

Gene name	FC exercising leg	p value	FC non-exercising leg	p value
IL6	1.6	0.0002	1.2	0.1
IL8	-1.0	0.7	-1.1	0.3
IL15	-1.1	0.2	-1.1	0.2
BDNF	1.1	0.2	1.1	0.2
IL7	-1.1	0.2	1.1	0.3
LIF	1.1	0.5	-1.0	0.9
FGF21	1.1	0.01	-1.0	0.9
Irisin	-1.0	0.7	-1.1	0.2

Acute exercise-induced in plasma levels of myokines

Based on the list of 29 putative myokines altered > 1.5 fold, we created a shortlist of promising myokines that we measured in plasma using either ELISA or multiplex immunoassay.
In line with the increased mRNA levels, plasma levels of CCL2 and CX3CL1 increased during one legged exercise and during recovery, which was consistently observed in all subjects (figure 5.4A). Despite increased mRNA expression, CYR61 and CXCL2 did not show any change in plasma levels in response to exercise. Plasma VEGFA levels seemed to be slightly increased by exercise but the effect did not reach statistical significance

(figure 5.4A). Also, plasma levels of the known myokines IL-6, IL-8, IL-15 and BDNF did not change during or after exercise (figure 5.4B).

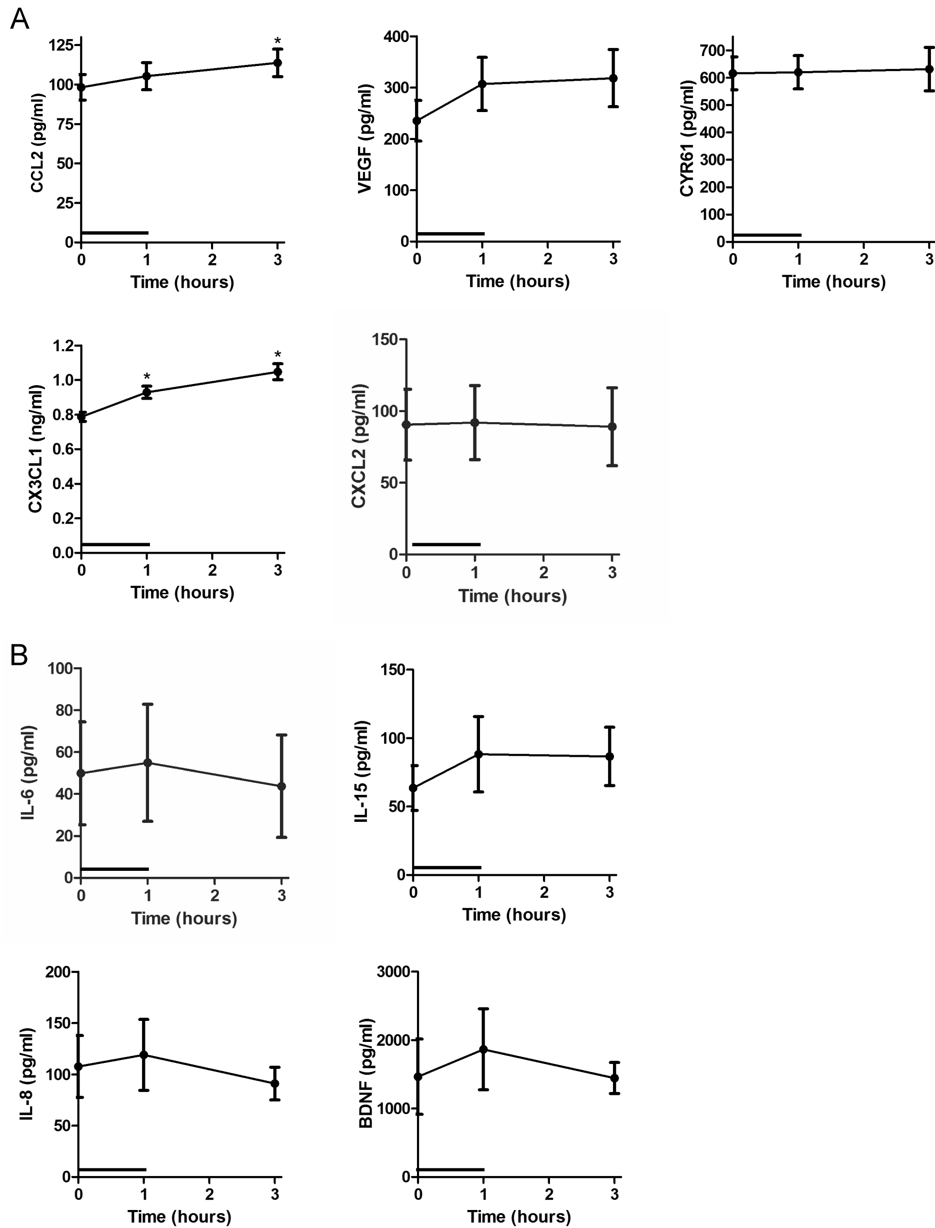

Figure 5.4 *Plasma levels of myokines. Selected known myokines were measured in plasma of the acute exercise study by multiplex immunoassay or ELISA. Black bar between time points 0 and 1 indicates exercise period. Depicted is mean ± SEM, * = p < 0.05 compared to baseline, N = 12.*

Relationship between plasma myokine levels and muscle gene expression in acute exercise

To better understand the underlying molecular basis for the changes in plasma levels of the putative myokines, we determined the correlation between plasma levels of myokines and genes regulated in the exercising leg using sparse partial least square (sPLS) followed by clustering (figure 5.5). This analysis showed that CX3CL1 and CCL2 are part of one cluster, which also includes BDNF, VEGF, and to a lesser extent IL-15. Those 5 myokines are highly correlated with a subset of 242 genes shown in the upper left corner of the heatmap in figure 5.5. To gain insight into the function and regulation

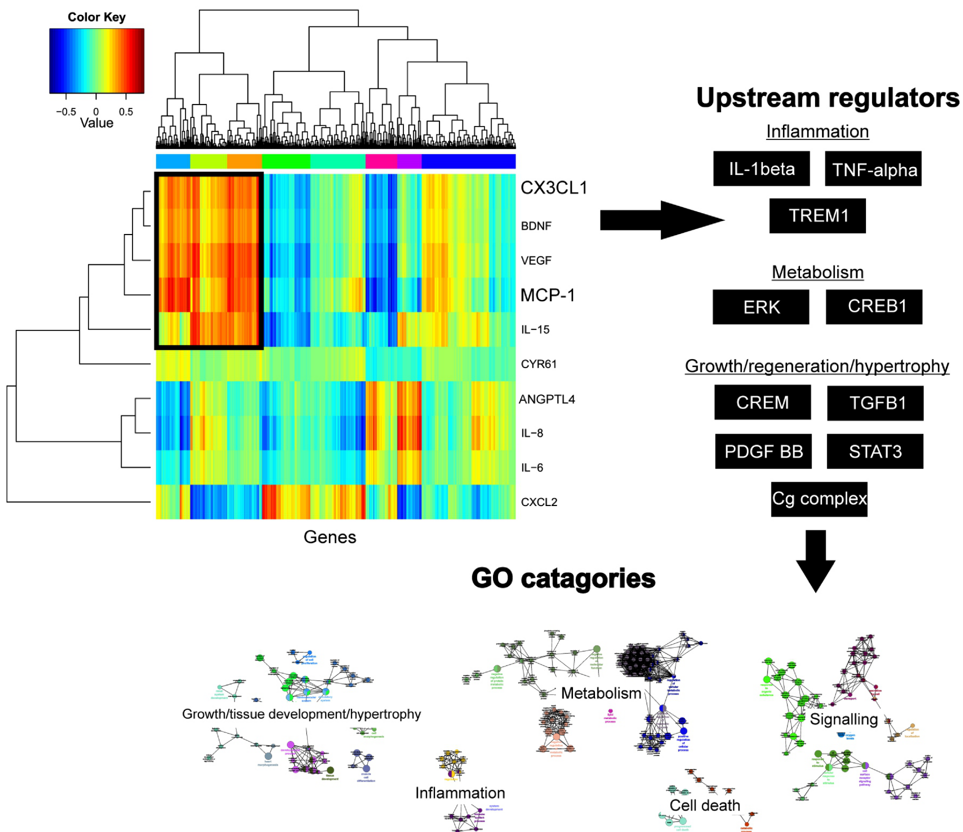

Figure 5.5 *Hypertrophy, metabolism and inflammation characterize the extracted set of genes resulting from a sPLS regression analysis of exercising leg gene expression and myokine plasma levels. sPLS was performed on the most variable genes (IQR > 0.5) and myokine plasma levels of the acute exercise study and hierarchically clustered (upper left window). A cluster highly correlating with CCL2 and CX3CL1 was extracted and analysed with Ingenuity (right upper window) and ClueGO (lower window).*

of these genes, upstream regulator analysis was performed using Ingenuity, which indicates which regulators (transcription factor, cytokine, enzyme complex) might be responsible for the observed gene expression changes. The analysis yielded upstream regulators mainly involved in metabolism (e.g. CREB1, ERK), growth/hypertrophy (e.g. TGFB1, STAT3) and inflammation (IL-1beta, TNFalpha). In addition, a Gene Ontology (GO) category analysis was performed using ClueGO, which yielded the specific processes that were represented by the genes highly correlated with the five myokines: metabolism, inflammation, signalling, growth/tissue development/hypertrophy, cell death.

We further extended this analysis by correlating gene expression of myokines with gene expression of 200 other highly regulated genes in the exercising leg. This resulted in a subset of 44 genes having a correlation coefficient > 0.5 with the CX3CL1 or CCL2 gene, or both (figure 5.6). 68% of those genes were also present in the subset generated by correlating plasma levels and gene expression. Similarly, the upstream regulators for the 44 genes largely overlap with the upstream regulators for the genes clustering with plasma CX3CL1 and CCL2. To assess the similarities between exercising and non-exercising leg, sPLS using MixOmics was performed on the non-exercising (figure 5.7). Again, a clear cluster was obtained that exhibited a high correlation with CX3CL1, CCL2,

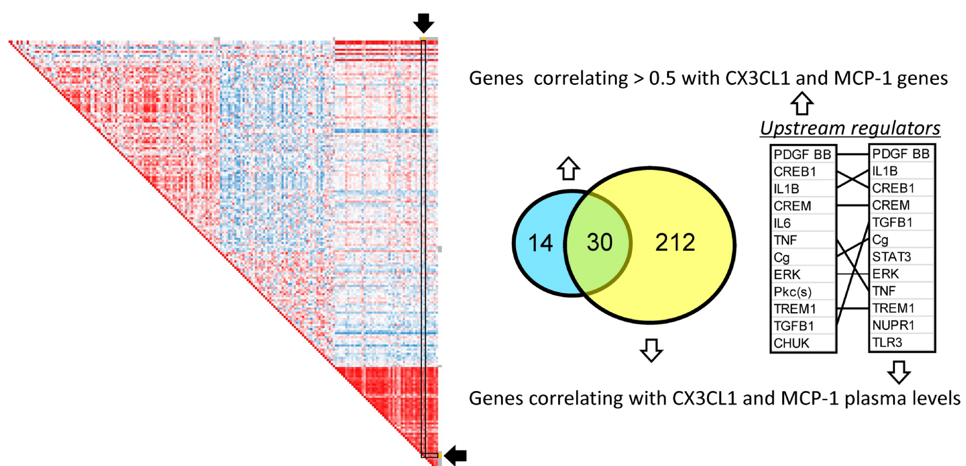

Figure 5.6 *Similar processes also characterize genes with a high correlation with the CX3CL1 and CCL2 genes. A correlation analysis was performed using GItools on gene expression of the 200 most variable genes and genes coding for myokines of the acute exercise study. 44 genes had a correlation coefficient > 0.5 with the CX3CL1 and/or CCL2 gene. Overlap was determined between those 44 genes and the subset of genes clustering with CCL2 and CX3CL1, highlighted in the heatmap in figure 5.5 (middle part) and analysed for upstream regulators with Ingenuity (right part).*

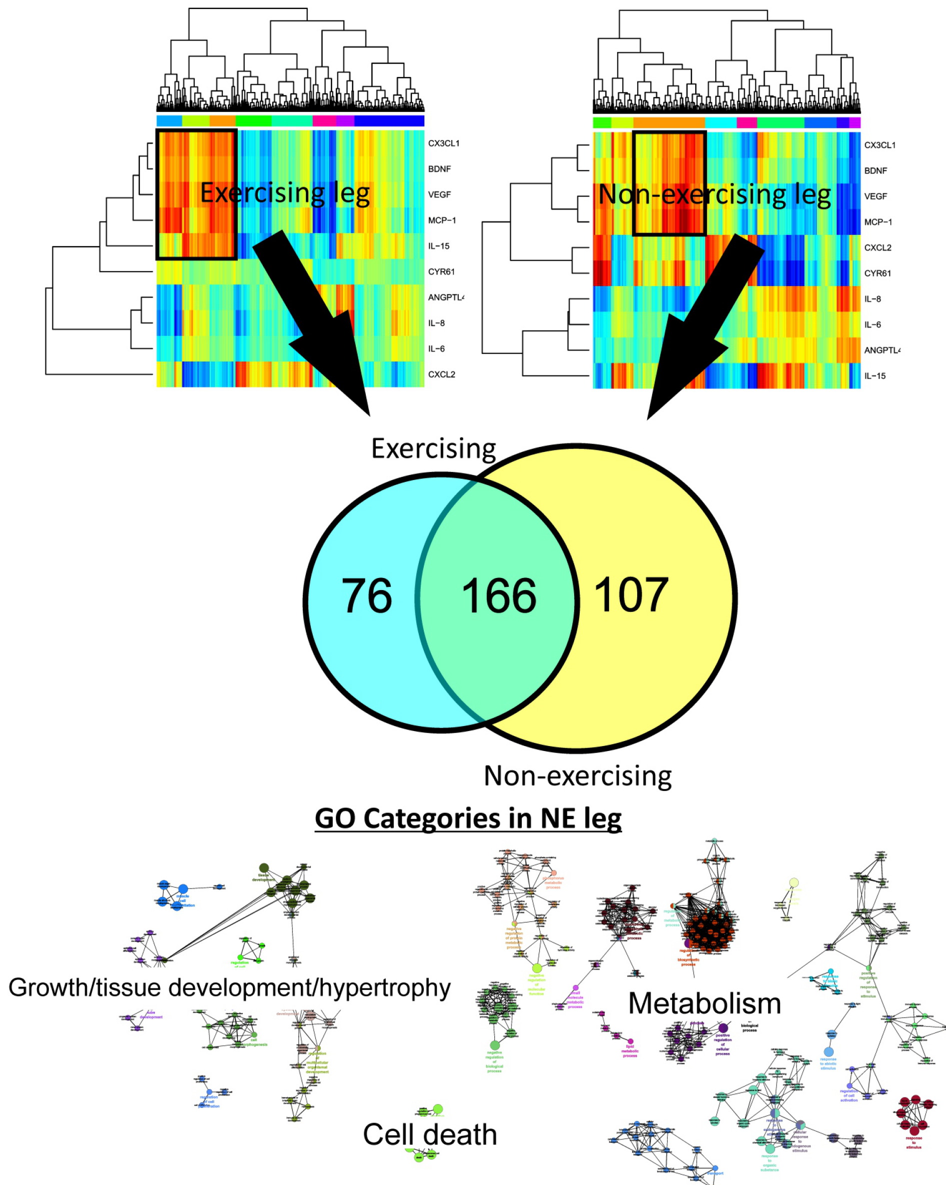

Figure 5.7 *The genes in the non-exercising leg correlating with CX3CL1 and CCL2 plasma levels correspond largely to the set of genes of exercising leg. sPLS of both legs is shown, for both legs expression values of the most variable genes (IQR > 0.5) and myokine plasma levels were used (upper part). Both clusters were extracted and overlapped (middle part). The non-exercising cluster was analysed with Ingenuity (lower part).*

BDNF, and VEGF. Genes in this cluster showed marked overlap (68%) with the gene cluster from the exercising leg. Also, GO categories determined with ClueGO were highly similar between the two gene clusters.

Comparison between secretome analysis of acute exercise versus exercise training

Microarray analisys was performed and the 20 highest induced genes are depicted in figure 5.8A. Secretome analysis of the exercise training intervention resulted in a list of 69 genes potentially coding for myokines, which can all be found in supplemental table S5.1. A large subset of those 69 genes encoded proteins that play a role in the extracellular matrix of the cell and therefore are unlikely to be involved in signalling, In general the fold-induction of the putative myokines was much lower in exercise training compared with acute exercise (figure 5.8A), and the majority of putative myokines was upregulated (figure 5.8C). Although the total number of putative secreted proteins was comparable between exercise training and acute exercise, the overlap was relatively small, with only 12 genes shared between the two exercise modalities (figure 5.8B). All 13 genes exhibited a very modest change in expression, which for most genes went in opposite directions by acute exercise and exercise training (figure 5.8D). Only SHE, ENG, EFNA1 and ACTN4 were upregulated by both acute exercise and exercise training.

DISCUSSION

Here we applied secretome analysis to identify novel myokines induced by acute exercise or exercise training. We are unaware of any other unbiased screenings for novel myokines that used muscle biopsies from exercising humans as starting material. Our results reveal a number of exercise-induced myokines that are secreted into plasma, including CX3CL1 and CCL2. In contrast, one-legged exercise had no effect on plasma levels of several known myokines, including IL-6, IL-8 and IL-15. Intriguingly, we observed only very minor overlap between the secretome analysis of acute exercise and exercise training, reflecting the very different effects of acute exercise and exercise training on gene expression in muscle.

CCL2 and the newly discovered myokine CX3CL1 are well-known chemokines. CCL2 is a key mediator of macrophage recruitment during the inflammatory response and was reported to be increased at the mRNA level by resistance exercise [19,31] and moderately-intensive cycling [32]. Immunohistochemical studies indicate that CCL2 protein is present

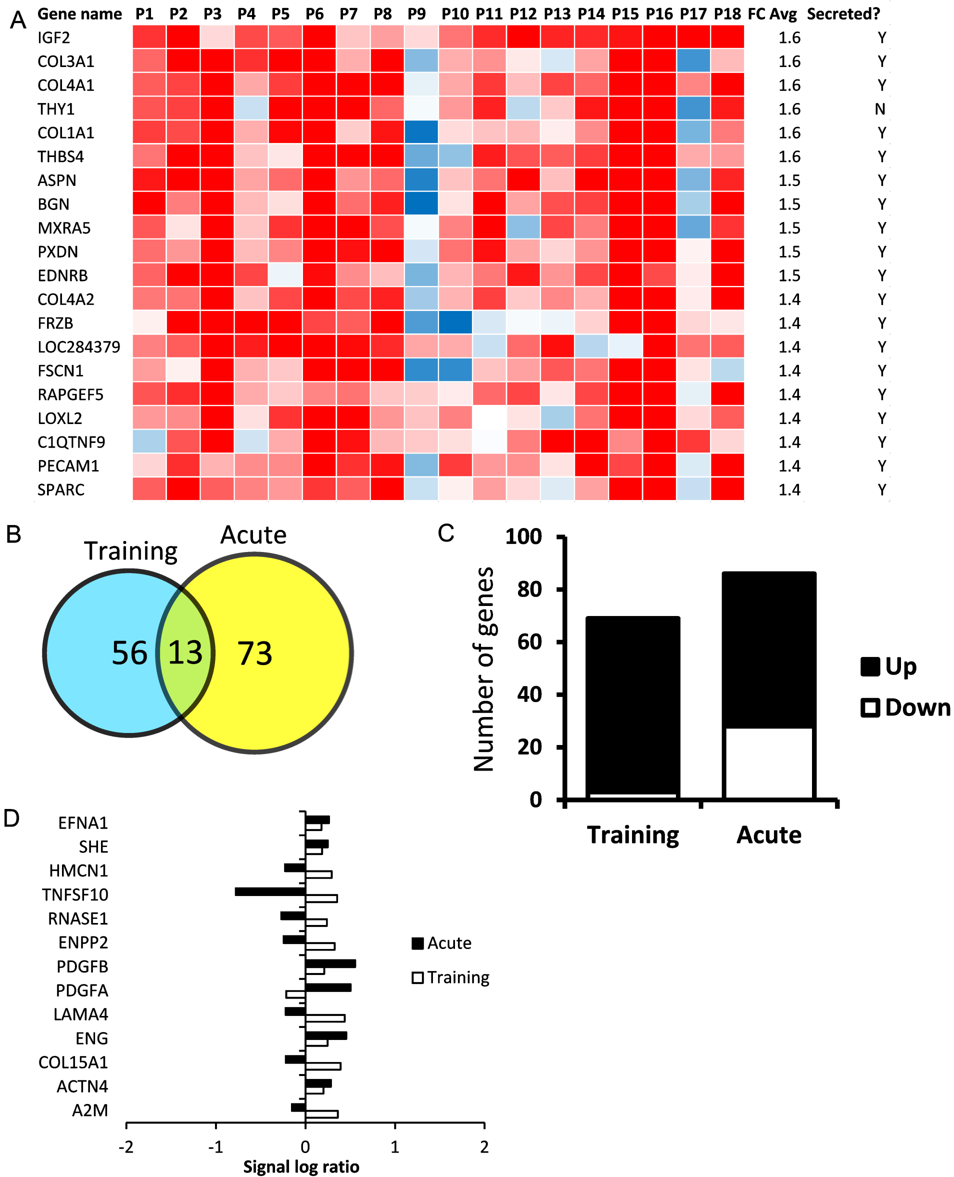

Figure 5.8 *Overlap between acutely induced and training induced myokines.* ***(A)*** *Top 20 upregulated genes during exercise training.* ***(B)*** *Venn diagram of overlap of putative myokines induced in acute exercise and exercise training.* ***(C)*** *Number of putative myokines up- or downregulated in acute exercise and exercise training.* ***(D)*** *Bar chart of the expression of the overlapping putative myokines. Acute N = 9, training N = 18.*

in skeletal muscle, being co-localized with satellite cells and macrophages [33]. CX3CL1 (also referred to as Fractalkine) plays a role in leukocyte adhesion [34] and is involved in macrophage-directed rescuing of skeletal muscle cells from apoptosis [35]. Both CCL2 and CX3CL1 are involved in acute skeletal muscle injury and regeneration by attracting macrophages and other immune cells recruited for repair and growth of skeletal muscle, and may play a role in the adaptive response to exercise [36]. Infiltration of macrophages is believed to be crucial for exercise-induced hypertrophy and it is possible that CX3CL1 and CCL2 play a role in this process [37]. To what extent exercise-induced local changes in CX3CL1 and CCL2 influence skeletal muscle insulin sensitivity will be the subject of our future investigations. Existing data suggest that CCL2 is negatively related to insulin sensitivity [38,39] and increased CCL2 plasma levels are associated with diabetes [40]. The influence of CX3CL1 on insulin signalling is not known, but plasma levels of CX3CL1 were found to be elevated in subjects with type 2 diabetes [41].

Since both chemokines are expressed and secreted from a wide variety of tissues, as is true for all myokines identified so far, it is unclear whether the changes in plasma levels are exclusively driven by increased production in skeletal muscle. Expression profiling of human primary myotubes reveals that expression levels of CCL2 in skeletal muscle are extremely high (data not shown, GSE18589; ranked 145 out of 19,741 genes), which is supported by BioGPS (http://biogps.org). CX3CL1 expression levels in primary myotubes are markedly lower . Staining of CCL2 in skeletal muscle biopsies showed CCL2 to be present in skeletal muscle cells [33]. At the same time, we cannot rule out that macrophages or other cell types present in the biopsies play a role in the induction of CCL2 and CX3CL1 gene expression upon exercise. It is important to note that the number of macrophages in resting skeletal muscle is limited, and that infiltration of macrophages reaches its peak 24 hours post-exercise [42]. Accordingly, it can be argued that myocytes most likely account for the elevated CCL2 and CX3CL1 mRNA levels after acuter exercise.

Several studies have shown that IL-6 is increased upon endurance exercise, both at the mRNA and plasma level [9,20,43]. We found a significant increase in IL-6 mRNA in the exercising leg, which was not associated with any change in plasma IL-6 levels, suggesting a more local role of IL-6. It should be emphasized that IL-6 mRNA levels were very low and failed to pass the initial filtering of the microarray data. The reason for the discrepancy with several published studies is unclear [9,20,43]. One potential explanation is that the size of muscle mass used in one-legged cycling was insufficient to elicit any changes in IL-6 plasma levels. Another explanation is that the exercise intensity was relatively modest

(50% W_{max} versus 75% $VO2_{max}$) compared to other studies with similar exercise duration [44,45], although we did observe an increase in IL-6 mRNA. Recent data showed that carbohydrate ingestion attenuates the increase in plasma IL-6 but not skeletal muscle IL-6 mRNA during exercise in humans [46]. Overall, the degree of correlation between muscle IL-6 mRNA expression and plasma IL-6 levels is clearly dependent on the specific exercise conditions.

Of all the other proteins that have been suggested to be myokines, only FGF-21 showed a significant increase in mRNA levels in response to exercise, although the magnitude of induction was very modest. Limited research has been performed on FGF-21 as a myokine [13], and there are no reports indicating that exercise increases FGF-21 mRNA levels. Limited information is also available on other putative myokines, including IL-8, IL-15, BDNF, and LIF, and their induction in skeletal muscle during exercise has not been consistently demonstrated [21,47,48]. The latter observation may be related to differences in type, intensity or duration of exercise. Recently, irisin was discovered as a novel muscle specific protein that is induced by exercise-training via the transcriptional co-activator PGC1α. Irisin was proposed to be released into the circulation and was found to stimulate browning and associated thermogenesis in subcutaneous fat in mice [18]. It was demonstrated that plasma levels of irisin are induced specifically by exercise training and not upon acute exercise, which is consistent with our observation that acute exercise did not alter expression of the gene coding for irisin (FNDC5). However, in the meantime serious concerns have arisen about the sequence of human FNDC5 and the secretion of irisin into human blood [49]. For this reason we did not attempt to measure plasma irisin levels in our study.

The main outcome of the mixomics analysis was the emergence of a clear cluster of genes correlating with plasma levels of CCL2 and CX3CL1. Interestingly, only CCL2 and CX3CL1 correlated with a clear cluster, whereas much weaker correlations were seen for other myokines. Combined with the observed induction of CCL2 and CX3CL1 mRNA, these data suggest that changes in plasma levels of CCL2 and CX3CL1 are driven by changes in muscle gene expression. Additionally, the processes characterizing the genes present in the CCL2 and CX3CL1 cluster nicely corresponded with the known role of CX3CL1 and CCL2 in growth and hypertrophy, inflammation, and apoptosis [35-37], pointing to a potential role of CCL2 and CX3CL1 in exercise-induced gene expression changes.

There was only very limited overlap between the putative myokines altered by acute exercise compared with myokines altered by exercise training. In general, acute exercise

and exercise training have very different effects on gene expression, with acute exercise eliciting a more stress-like response compared to a chronic adaptive response to exercise training. Whereas the adaptive response causes the improvements in exercise capacity and cardiometabolic fitness, it is still unclear which type of response is most important for conferring the positive effect of exercise on other parameters, such as insulin sensitivity. Based on this notion, it is difficult to define which types of myokines represent the best candidates as pharmacological target for metabolic disturbances such as insulin resistance. Interestingly a large part of the genes resulting from the secretome analysis of the exercise training study encoded extracellular matrix (ECM) proteins. Although ECM proteins are secreted out of the cells, they are unlikely to have an effect on distant cells or tissues. However, they could play a more indirect role in endocrine signalling by influencing the release and activation of other proteins.

One of the major limitations of our approach is that it precludes identification of novel myokines that are regulated exclusively at the post-transcriptional level. Another limitation is that the microarray datasets used in this article are derived from two separate relatively small sets of subjects. Subjects participating in both studies would have made the analysis more powerful. Despite the relatively small sample sizes, in both studies the changes identified were very consistent between the subjects.

In conclusion, we identified several myokines that may be involved in cross-talk between skeletal muscle and other organs. The most promising candidates were CX3CL1 and CCL2 (MCP-1), which were increased at the gene expression level and in blood plasma. Furthermore, there was no substantial overlap of genes encoding potentially secreted proteins induced by acute exercise and exercise training, emphasizing the difference in response to acute exercise or exercise training.

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Supplemental table S5.1 Results secretome analysis acute exercise and exercise training

	Acute exericse study			
	Gene name	Description	FC E	FC NE
Both legs	ANGPTL4	angiopoietin-like 4	1.6	3.5
	SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, 3F	1.5	1.2
	ENG	endoglin	1.4	1.3
	SRGN	serglycin	1.3	1.4
	NOTCH1	notch 1	1.3	1.2
	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	1.2	1.3
Only exercising leg	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	4.9	1.3
	CYR61	cysteine-rich, angiogenic inducer, 61	3.0	-1.5
	APOLD1	apolipoprotein L domain containing 1	2.5	1.3
	THBD	thrombomodulin	2.0	1.3
	FGF6	fibroblast growth factor 6	1.9	1.4
	CXCL2	chemokine (C-X-C motif) ligand 2	1.9	1.0
	CX3CL1	chemokine (C-X3-C motif) ligand 1	1.8	-1.2
	DLL1	delta-like 1 (Drosophila)	1.8	1.2
	THBS1	thrombospondin 1	1.8	1.3
	LOC100505657	hypothetical protein LOC100505657	1.8	1.2
	SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	1.7	1.1
	CCL2	chemokine (C-C motif) ligand 2	1.7	1.0
	HSPH1	heat shock 105kDa/110kDa protein 1	1.6	1.0
	VEGFA	vascular endothelial growth factor A	1.6	1.3
	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	1.6	1.1
	GDNF	glial cell derived neurotrophic factor	1.6	1.2
	CTGF	connective tissue growth factor	1.5	-1.2
	SDC4	syndecan 4	1.5	1.2
	CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	1.5	1.1
	PDGFB	platelet-derived growth factor beta polypeptide	1.5	-1.2
	PLAU	plasminogen activator, urokinase	1.5	1.3
	PDGFA	platelet-derived growth factor alpha polypeptide	1.4	1.3
	LPL	lipoprotein lipase	1.4	1.2
	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	1.4	1.2
	FLT1	fms-related tyrosine kinase 1	1.4	1.1
	IFFO2	intermediate filament family orphan 2	1.4	1.3
	ANGPTL2	angiopoietin-like 2	1.3	1.2
	INHBB	inhibin, beta B	1.3	1.1
	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	1.3	1.1
	C8G	complement component 8, gamma polypeptide	1.3	-1.1

Supplemental table S5.1 continues on next page

Supplemental table S5.1 *Continued*

	Gene name	Description	FC E	FC NE
Only exercising leg	ICAM1	intercellular adhesion molecule 1	1.3	1.1
	GLA	galactosidase, alpha	1.3	1.0
	CHSY1	chondroitin sulfate synthase 1	1.2	1.1
	TINAGL1	tubulointerstitial nephritis antigen-like 1	1.2	1.0
	CD55	CD55 molecule, decay accelerating factor for complement	1.2	1.0
	ACTN4	actinin, alpha 4	1.2	1.1
	MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	1.2	1.1
	TGFB3	transforming growth factor, beta 3	1.2	1.1
	MMRN2	multimerin 2	1.2	1.1
	C2orf69	chromosome 2 open reading frame 69	1.2	-1.0
	SHE	Src homology 2 domain containing E	1.2	1.1
	C3orf64	chromosome 3 open reading frame 64	1.2	1.2
	HSPA5	heat shock 70kDa protein 5	1.2	-1.0
	C1orf170	chromosome 1 open reading frame 170	1.2	1.0
	VCL	vinculin	1.2	1.0
	TXLNA	taxilin alpha	1.1	1.0
	CXCL12	chemokine (C-X-C motif) ligand 12	-1.1	-1.1
	A2M	alpha-2-macroglobulin	-1.1	-1.0
	ASB14	ankyrin repeat and SOCS box containing 14	-1.1	-1.3
	APOL6	apolipoprotein L, 6	-1.2	-1.0
	COL15A1	collagen, type XV, alpha 1	-1.2	1.1
	LAMA4	laminin, alpha 4	-1.2	-1.1
	PCOLCE	procollagen C-endopeptidase enhancer	-1.2	-1.0
	LY6G5B	lymphocyte antigen 6 complex, locus G5B	-1.2	1.1
	HMCN1	hemicentin 1	-1.2	-1.1
	GPX7	glutathione peroxidase 7	-1.2	-1.1
	THBS2	thrombospondin 2	-1.2	-1.1
	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-1.2	-1.0
	OLFML3	olfactomedin-like 3	-1.2	-1.1
	C3	complement component 3	-1.2	-1.0
	COL6A3	collagen, type VI, alpha 3	-1.2	-1.0
	HSD17B11	hydroxysteroid (17-beta) dehydrogenase 11	-1.2	-1.0
	APOL3	apolipoprotein L, 3	-1.2	1.1
	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-1.2	-1.1
	MAMDC2	MAM domain containing 2	-1.2	-1.2
	C1RL	complement component 1, r subcomponent-like	-1.2	1.0
	CTSW	cathepsin W	-1.2	-1.0
	LCN12	lipocalin 12	-1.2	-1.2
	IGF1	insulin-like growth factor 1 (somatomedin C)	-1.3	-1.1
	ADM	adrenomedullin	-1.3	-1.1
	MMP28	matrix metallopeptidase 28	-1.3	1.1
	DHRS4L2	dehydrogenase/reductase (SDR family) member 4 like 2	-1.5	-1.2
	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	-1.7	1.0

Supplemental table S5.1 *Continued*

	Gene name	Description	FC E	FC NE
Only non-exercising leg	S100A8	S100 calcium binding protein A8	1.5	2.5
	SNCA	synuclein, alpha (non A4 component of amyloid precursor)	1.2	2.0
	LYZ	lysozyme	1.2	1.8
	EFNA1	ephrin-A1	1.0	1.2
	PPT1	palmitoyl-protein thioesterase 1	1.0	1.2
	EDN1	endothelin 1	1.0	-1.3
	MSTN	myostatin	-1.2	-1.5

Exercise training study		
Gene name	Description	FC Average
IGF2	insulin-like growth factor 2 (somatomedin A)	1.6
COL3A1	collagen, type III, alpha 1	1.6
COL4A1	collagen, type IV, alpha 1	1.6
COL1A1	collagen, type I, alpha 1	1.6
THBS4	thrombospondin 4	1.6
ASPN	asporin	1.5
BGN	biglycan	1.5
MXRA5	matrix-remodelling associated 5	1.5
PXDN	peroxidasin homolog (Drosophila)	1.5
COL4A2	collagen, type IV, alpha 2	1.4
FRZB	frizzled-related protein	1.4
LOXL2	lysyl oxidase-like 2	1.4
PECAM1	platelet/endothelial cell adhesion molecule 1	1.4
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	1.4
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	1.4
LAMA4	laminin, alpha 4	1.4
F2R	coagulation factor II (thrombin) receptor	1.3
NOV	nephroblastoma overexpressed	1.3
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	1.3
LAMB1	laminin, beta 1	1.3
NID2	nidogen 2 (osteonidogen)	1.3
COL15A1	collagen, type XV, alpha 1	1.3
SULF2	sulfatase 2	1.3
A2M	alpha-2-macroglobulin	1.3
PAMR1	peptidase domain containing associated with muscle regeneration 1	1.3
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1.3
COL5A2	collagen, type V, alpha 2	1.3
HSPG2	heparan sulfate proteoglycan 2	1.3
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	1.3
CECR1	cat eye syndrome chromosome region, candidate 1	1.2
IGFBP7	insulin-like growth factor binding protein 7	1.2

Supplemental table S5.1 continues on next page

Supplemental table S5.1 *Continued*

Gene name	Description	FC Average
NID1	nidogen 1	1.2
SERPINE2	serpin peptidase inhibitor, clade E , member 2	1.2
CTHRC1	collagen triple helix repeat containing 1	1.2
HMCN1	hemicentin 1	1.2
GLIPR1	GLI pathogenesis-related 1	1.2
FAP	fibroblast activation protein, alpha	1.2
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	1.2
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	1.2
IGFBP2	insulin-like growth factor binding protein 2, 36kDa	1.2
IGFBP4	insulin-like growth factor binding protein 4	1.2
TCN2	transcobalamin II	1.2
IL34	interleukin 34	1.2
CPE	carboxypeptidase E	1.2
IGFBP3	insulin-like growth factor binding protein 3	1.2
TGFBI	transforming growth factor, beta-induced, 68kDa	1.2
CXorf36	chromosome X open reading frame 36	1.2
ENG	endoglin	1.2
VWF	von Willebrand factor	1.2
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	1.2
LAMA3	laminin, alpha 3	1.2
TGFB1	transforming growth factor, beta 1	1.2
CD109	CD109 molecule	1.2
ELMO1	engulfment and cell motility 1	1.2
THSD1	thrombospondin, type I, domain containing 1	1.2
PDGFB	platelet-derived growth factor beta polypeptide	1.2
ACTN4	actinin, alpha 4	1.2
F8	coagulation factor VIII, procoagulant component	1.1
BMP1	bone morphogenetic protein 1	1.1
LAMC1	laminin, gamma 1 (formerly LAMB2)	1.1
SHE	Src homology 2 domain containing E	1.1
EFNA1	ephrin-A1	1.1
HLA-C	major histocompatibility complex, class I, C	1.1
NRP1	neuropilin 1	1.1
LGALS8	lectin, galactoside-binding, soluble, 8	1.1
GRN	granulin	1.1
PDGFA	platelet-derived growth factor alpha polypeptide	-1.2
IGFBP6	insulin-like growth factor binding protein 6	-1.2
IGHG3	immunoglobulin heavy constant gamma 3 (G3m marker)	-1.3

Chapter 6

Fatty acid-inducible Angptl4 governs lipid metabolic response to exercise

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ABSTRACT

Physical activity increases energy metabolism in exercising muscle. Whether acute exercise elicits metabolic changes in non-exercising muscles remains unclear. We show that one of the very few genes that during acute exercise is more highly induced in non-exercising muscle compared to exercising human muscle encodes Angiopoietin-like 4 (Angptl4), an inhibitor of lipoprotein lipase-mediated plasma triglyceride clearance. Using a combination of human, animal and in-vitro data, we show that induction of Angptl4 in non-exercising muscle is mediated by elevated plasma free fatty acids via PPARδ, presumably leading to reduced local uptake of plasma triglyceride-derived fatty acids and their sparing for use by exercising muscle. In contrast, in exercising muscle induction of Angptl4 is counteracted likely via AMPK-mediated downregulation, promoting use of plasma triglycerides as fuel for active muscles. Our data suggest a key role of non-exercising muscle and local regulation of Angptl4 via AMPK and FFA in governing lipid homeostasis during exercise.

INTRODUCTION

Acute exercise greatly increases cellular demand for ATP, oxygen, glucose and fatty acids. To meet these demands, acute exercise is associated with marked changes in skeletal muscle activity of key transporters and enzymes involved in glucose and fatty acid transport and oxidation [1]. Much of the regulation occurs via allosteric regulation and covalent modification of rate-limiting enzymes. In addition, alterations at the level of mRNA are increasingly believed to represent an important regulatory mechanism in the acute response to exercise [2]. Indeed, acute exercise induces mRNA expression of many genes involved in a variety of processes, including energy metabolism, hypertrophy and signaling [3-6]. Not surprisingly, most studies have focused on the events occurring in exercising muscle. In contrast, much less is known about the exercise-induced changes in non-exercising muscle. Studies have shown that resting skeletal muscle is crucial in the removal of lactate from the circulation during high-intensity exercise [7], and also plays a role in adrenaline and noradrenaline production during exercise [8]. In addition, similar to exercising muscle, resting muscle exhibits enhanced phosphorylation of mTOR following resistance exercise [9]. Overall, however, the impact of exercise on metabolic processes and gene expression in non-exercising muscles remains ill-defined. It can be envisioned that exercise may elicit changes in gene expression in non-exercising muscle via circulating mediators including muscle-derived myokines and metabolites [10]. The present study was undertaken to try to elucidate the role of inactive muscle in the metabolic response to acute exercise.

EXPERIMENTAL PROCEDURES

Human intervention studies

Twelve healthy men (age 51.5 ± 5.1 years, body weight 88 ± 17 kg, body mass index 26 ± 4) participated in the study, referred to as study A [4]. Subjects were asked to follow healthy eating guidelines the day before the experiment and to refrain from alcohol consumption. Subjects fasted from 10 p.m. the prior evening until the end of the intervention. During the exercise session subjects were allowed to drink water ad libitum. All subjects performed a single endurance exercise bout, consisting of 60 minutes one-legged cycling at 50% of one-legged W_{max} (determined by graded one-legged cycling test). One-legged cycling was performed on a cycle ergometer (Excalibur Sport, Lode, Groningen NL) adapted with a custom-made leg support. Skeletal muscle biopsies were

taken from 11 subjects from both legs, before and after exercise, with the average time of collection at approximately 15 minutes pre- or post-exercise. One subject became apprehensive about the needle biopsy immediately prior to the first biopsy collection. Two subjects were later excluded from the microarray analysis because the microarrays failed to meet quality control criteria. In addition, a venous blood sample was taken before and shortly after exercise. Other human intervention studies included in this manuscript have been previously published and are briefly outlined below.

Study B: 8 young untrained and healthy male subjects (age: 23.3 ± 3.2 years) performed 3h of cycling on an electromagnetically braked ergometer at an intensity of 40% of the predetermined W_{max} [11]. To facilitate completion of the exercise test, subjects received two 125 mL servings of a maltodextrine drink during the second half of the test. Subjects were allowed to drink water ad libitum during the entire test. Plasma was collected before and after 3h cycling exercise and used for measurement of Angptl4.

Study C: 8 young untrained and healthy male subjects (age: 23.3 ± 3.2 years) followed a two week exercise training program on a cycling ergometer [11]. Training consisted of alternating days of interval and endurance training and always started with 7.5 min of warming up at 40% W_{max} and ended with 7.5 min of cooling down at 40% W_{max}. Fasting plasma was collected before and after the 2 week training program and used for measurement of Angptl4. Plasma was collected 3 days after the last training session.

Study D: 6 healthy non-obese male subjects (age: 42.7 ± 2.0 years) followed a 12 week exercise training program on a cycling ergometer [12]. Subjects trained three times per week for 12 weeks for 47.5 ± 2.5 min at 40% of predetermined $VO2_{max}$. Fasting plasma was collected before and after the 12 week training program and used for measurement of Angptl4. Plasma was collected 3 days after the last training session.

Study E: seven healthy, untrained male volunteers participated in the study (age: 22.7 ± 0.6 years). After an overnight fast, a Teflon cannula was inserted in an antecubital vein for sampling of blood. Subjects rested on a bed, and a baseline blood sample was taken. Immediately thereafter, subjects ingested 1.4 g/kg bodyweight glucose or water. Subject exercised at 50% $VO2_{max}$ for 2h and then rested for 4h. At regular intervals subjects ingested 0.35 g/kg bodyweight glucose or water. Muscle biopsies were collected before exercise and after the 4h rest period. All subjects underwent the experimental protocol two times, once with glucose ingestion and once while fasting [13].

Study F: twelve healthy lean male volunteers participated in the study (age: 23.6 ± 1.0 years). Subjects stayed in the respiration chamber for 60h in the normal fed condition

or while being completely fasted, according to a randomized crossover design with a 2-week washout period. Blood samples were collected at the end of the study after an overnight fast (fed condition), or after a cumulative 60h fast. Around the same time, a muscle biopsy was taken [14].

Study G: nine healthy lean male volunteers participated in the study (age: 24.4 ± 1.3 years). After an overnight fast, two Teflon cannulas were inserted into an antecubital vein of each arm. One cannula was used for the infusion of Salbutamol and one cannula for sampling of blood. A first blood sample and muscle biopsy was taken, followed by a continuous infusion of salbutamol for 3h. In addition, two doses of 250 mg acipimox or placebo were given orally at -120 min and time 0. Blood samples were taken at regular intervals throughout the study [15]. A second muscle biopsy was taken after the 3h infusion.

The studies were approved by the medical ethical committee of the institute involved (Wageningen University or Maastricht University).

Blood samples

Blood was collected in EDTA containing tubes and immediately centrifuged for 10 minutes (1000*g*, 4°C). Blood samples were analyzed for FFA levels (Centre for Medical Diagnostics, Velp, NL) and Angptl4 levels (see below).

Skeletal muscle biopsies

Percutaneous needle biopsies were taken before and shortly after exercise from the vastus lateralis muscle from both legs, using the Bergström technique with suction. All biopsies were taken from separate incisions, with the second biopsy in the leg located 2 cm more proximal to the first. Biopsies were taken on average 10-15 minutes before and after the exercise bout. All biopsies were collected, processed, and frozen within 30 minutes post-exercise. After each biopsy, the collected tissue sample was carefully cleared from adipose tissue and blood and directly frozen into liquid nitrogen or embedded into Tissue-Tek O.C.T. compound (Sakura Tissue Tek, Alphen a/d Rijn, NL) and frozen in liquid-nitrogen cooled isopentane.

Muscle biopsy lysates were prepared using a lysis buffer consisting of 50 mM Tris HCl (pH 7.5), 150 mM NaCL, 1 mM EGTA, 1 mM EDTA, 0.27 M sucrose and 2% Triton X-100. Protease and phosphatase inhibitor were added to the buffer (Complete and PhosSTOP, Roch diagnostics GmbH, Mannheim, Germany). Buffer was added to a 1:15 ratio (tissue:buffer) and the tissue was lysed using TissueLyser II (Qiagen, Venlo, NL).

Immunofluorescence

5-μm thick frozen muscle sections were treated with 0.1% Triton X-100 in PBS and incubated for 45 min at room temperature (RT) with the primary antibody mix diluted in 0.05% Tween20 in PBS: a polyclonal rabbit hAngptl4, a mouse monoclonal IgM antibody directed to MHC1 (type 1 muscle fibers; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) and a mouse monoclonal IgG_1 antibody to caveolin-3 (BD Biosciences, Breda, NL). After three washing steps with PBS sections were incubated for 45 min at RT with the appropriate fluorescent-labeled secondary antibodies. The specificity of the antibody for Angptl4 was previously demonstrated via immunoblot of human plasma and use of appropriate peptide controls and corroborated by immunochemical and immunofluorescence staining of Angptl4 in human heart, intestine, and skin wounds, using appropriate negative controls [16-18].

Animal experiments

Exercise studies were carried out using the TSE PhenoMaster treadmill module (TSE Systems, Bad Homburg, Germany), allowing four mice to run simultaneously. Four to five month old male Wildtype (WT) and *Angptl4*-transgenic (*Angptl4*-Tg) mice on C57Bl/6 background were used for the exercise experiments. The *Angptl4*-Tg mice used for the experiments had been bred on C57Bl/6 background for 15 generations. Mice were littermates. Number of mice per group varied between 6 and 9. All mice were acclimatized to the treadmill (10 m/min for 10 minutes) on three consecutive days. On the day of exercise, mice were fasted (4 hours) and were subjected to either a moderate exercise protocol (12 m/min at 0% incline for 90 minutes) or ran until exhaustion (30 minutes at 12m/min, then every 5 minutes an increase of 2.5m/min and 2 degrees until exhaustion). A mouse was considered exhausted if it failed to run after 3 times prompting within 1 minute with a slider with plastic bristles. The moment of exhaustion was determined by an investigator unaware of the genotype of the mice. A control group of non-exercising mice from each genotype remained sedentary inside the treadmill. Upon cessation of exercise mice were immediately anaesthetized using isoflurane and blood was collected by orbital puncture. Mice were then sacrificed by cervical dislocation and tissues were quickly excised and immediately frozen in liquid nitrogen.

The horizontal wire test was performed by placing a steel wire between a gray PVC stand at a height of 30 cm. Mice were hung on the wire with their forepaws and time measurements started at the moment that the researcher judged there was a good grip.

Time measurement was ended as the animal grasped the wire with at least one hind paw. If the animal fell, it was placed back in its cage for a 3 minutes rest, and measurements were repeated.

Animals experiment were approved by the animals ethics committee of Wageningen University.

Plasma triglyceride and free fatty acid clearance

After a 6 hour fast, 6-month old male WT and *Angptl4*-Tg mice were put on a treadmill at a speed of 14m/min. After 30 minutes of running, mice were injected with a single bolus of [^{14}C]-oleate and glycerol tri[3H]oleate-labeled VLDL-like particles and continued to run for another 15 minutes. After 15 minutes, blood was collected, followed by immediate sacrifice of the mice and collection of tissues. Tissues and serum were processed for determination of ^{3}H-activity and ^{14}C-activity. Preparation of VLDL-like particles, complexing of [^{14}C]-oleate with BSA, infusion protocol, and calculations were carried out as described previously [19-21].

Cell culture and treatment

C2C12 myoblasts were maintained in DMEM (Lonza, Breda, NL) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin/ amphotericin (PSA) under 5% CO_2 at 37°C. At ~70-90% confluency, myoblasts were either treated or full growth medium was replaced with alphaMEM supplemented with 2% FCS and 1% PSA to promote differentiation into myotubes. Differentiation medium was changed every 2-3 days. C2C12 myotubes were used for treatment after 1 week of differentiation. All experiments with C2C12 myotubes were performed in differentiation medium.

C2C12 myotubes were treated with human serum by replacing FCS with human serum and incubated for 3h to 6h. For siRNA-mediated knock-down, C2C12 cells were transfected with siRNA sequences using Lipofectamin RNAiMax the day before starting the differentiation. ON-TARGETplus SMARTpool for PPARδ, AMPKα1, AMPKα2 and non-targeting was used (Dharmacon/Thermo-Fisher Scientific, Etten-Leur, NL) at 100 pmol/mL. The medium was replaced by differentiation medium (DMEM supplemented with 2% horse serum, 100 µg/ml penicillin and 100 µg/ml streptomycin) after 24h. After 4 days of differentiation, cells were treated with oleic acid and AICAR as indicated below. Differentiated human primary myotubes were prepared as described previously [22].

Oleic acid (200 µM) was added to cells complexed with BSA (2.5:1). AICAR and metformin

were used at 1 mM. The AMPK inhibitor compound C was used at concentrations indicated in the legend. Cells were treated for 12h unless indicated otherwise.

RNA isolation and qPCR

Total RNA was isolated using Trizol reagent (Invitrogen, Breda, NL) and purified for microarray analysis using the Qiagen RNeasy Micro kit (Qiagen, Venlo, NL).
RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was carried out using SensiMiX (Bioline, London, UK) on a CFX 384 Bio-Rad thermal cycler (Bio-Rad, Veenendaal, NL). Cyclophilin, GADPH and/or 36B4 were used as housekeeping genes. Primer sequences can be found in table 6.1.

RESULTS

To investigate the molecular events occurring during exercise in non-exercising muscle, we carried out an acute exercise trial in which 12 human subjects performed moderate-high intensity cycling exercise with one leg, and muscle biopsies were taken before and

Table 6.1 Primer sequences

	Forward primer	Reverse primer
m36B4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG
Cyclophilin	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTGCAA
PGC1-a	AGACGGATTGCCCTCATTTGA	TGTAGCTGAGCTGAGTGTTGG
Cpt1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Lpl	CAGCTGGGCCTAACTTTGAG	GACCCCCTGGTAAATGTGTG
Angptl4	GTTTGCAGACTCAGCTCAAGG	CCAAGAGGTCTATCTGGCTCTG
Ppard	TTGAGCCCAAGTTCGAGTTTG	CGGTCTCCACACAGAATGATG
Cd36	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTCAGTGCAGAA
Plin2	GGATGTGGTGACGACTACCAT	ACAGACTTGGTCCTTTCCACG
Pdk4	TCTACAAACTCTGACAGGGCTTT	CCGCTTAGTGAACACTCCTTC
Ppara	TATTCGGCTGAAGCTGGTGTAC	CTGGCATTTGTTCCGGTTCT
Pparg	CACAATGCCATCAGGTTTGG	GCTGGTCGATATCACTGGAGATC
Prkaa1	TTCGGGAAAGTGAAGGTGGG	TCTTCTGCCGGTTGAGTATCT
Prkaa2	CAGGCCATAAAGTGGCAGTTA	AAAAGTCTGTCGGAGTGCTGA
Cs	GGACAATTTTCCAACCAATCTGC	TCGGTTCATTCCCTCTGCATA
Klf10	ATGCTCAACTTCGGCGCTT	CGCTTCCACCGCTTCAAAG
Angptl4	CACAGCCTGCAGACACAACTC	GGAGGCCAAACTGGCTTTGC
GAPDH	CATGTTCCAGTATGACTCCACTC	GGCCTCACCCCATTTGATGT

after exercise from the exercising and non-exercising (resting) leg. One legged cycling allows for direct analysis of the effects of acute exercise in exercising muscle, with the non-exercising leg serving as control leg. Microarray analysis was performed on all 4 muscle biopsies of 9 subjects [4]. Microarrays from two subjects failed to meet quality control criteria and were excluded from analysis, and one subjects refused to have biopsies taken. Surprisingly, the most significantly induced gene in the non-exercising leg was *Angptl4* (figure 6.1A), a sensitive target of the PPAR transcription factors that encodes a secreted

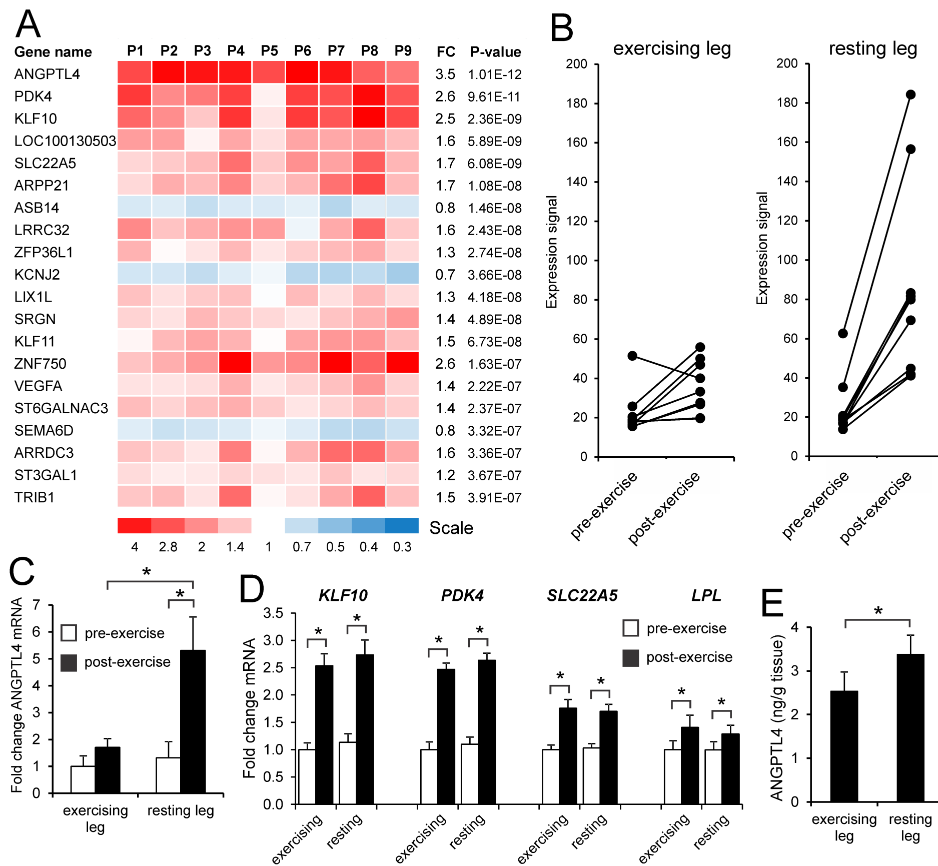

Figure 6.1 *Exercise induces Angptl4 gene expression in non-exercising human muscle.* ***(A)*** *Heatmap showing genes altered by exercise in the non-exercising leg muscle ranked according to statistical significance. Values are displayed per subject (P1 to P9). Fold-change (FC) in gene expression is indicated.* ***(B)*** *mRNA expression profile of* Angptl4 *in exercising and non-exercising leg according to microarray.* ***(C)*** *qPCR analysis of* Angptl4 *mRNA expression.* ***(D)*** *mRNA expression of PPARδ targets KLF10, PDK4, SLC22A5, as well as LPL.* ***(E)*** *Angptl4 protein levels in post-exercise muscle biopsies as determined by ELISA. Error bars represent SEM. *Significantly different according to paired Student's t test ($p < 0.01$).*

inhibitor of the enzyme lipoprotein lipase [23-25]. Lipoprotein lipase (LPL) catalyzes hydrolysis of circulating triglycerides and therefore plays a key role in uptake of fatty acids in skeletal muscle [26]. Paired individual gene expression profiles in muscle biopsies from both legs clearly showed that *Angptl4* was much more strongly induced in the non-exercising leg compared with the exercising leg (figure 6.1B), which was confirmed by qPCR analysis (figure 6.1C). In fact, the microarray analysis indicated that *Angptl4* was one of the very few genes that was more highly induced in the non-exercising leg compared with the exercising leg, whereas other PPAR targets such as *PDK4*, *SLC22A5* and *KLF10* were induced to the same extent in both legs (figure 6.1D). The same was true for *LPL* mRNA levels (figure 6.1D). In parallel with changes in *Angptl4* mRNA, the content of Angptl4 protein in muscle after exercise was significantly higher in the non-exercising leg compared to the exercising leg, as determined by ELISA (figure 6.1E).
To localize Angptl4 protein in human muscle, immunohistochemical staining was performed, which revealed substantial staining of Angptl4 in human muscle fibers, with a slight preference for type 1 slow oxidative fibers (figure 6.2). Consistent with the function of Angptl4 as inhibitor of endothelial-bound lipoprotein lipase, most intense Angptl4 protein staining was observed in the capillaries (figure 6.2).

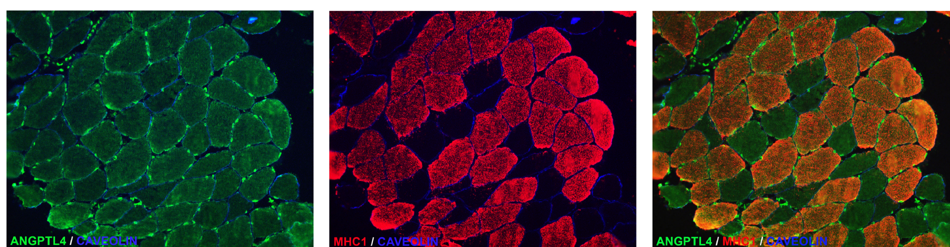

Figure 6.2 *Angptl4 protein is detected in human muscle in myocytes and endothelial cells. Immunofluorescent staining of Angptl4 in a biopsy from human vastus lateralis muscle. Angptl4 in green. Myosin heavy chain 1 (MHC1, marker type I fiber) in red. Caveolin in blue. Representative image is shown.*

Induction of *Angptl4* mRNA in skeletal muscle during one-legged exercise was associated with a significant increase in plasma Angptl4 concentration (figure 6.3A). Cycling with two legs for a more extended period (3 hours) caused an even more significant increase in plasma Angptl4 concentration (figure 6.3B). In contrast, the plasma Angptl4 concentration was not altered by two weeks of intense endurance exercise training (figure 6.3C), or by a twelve week endurance exercise program (figure 6.3D), indicating that Angptl4 is specifically induced by acute exercise.

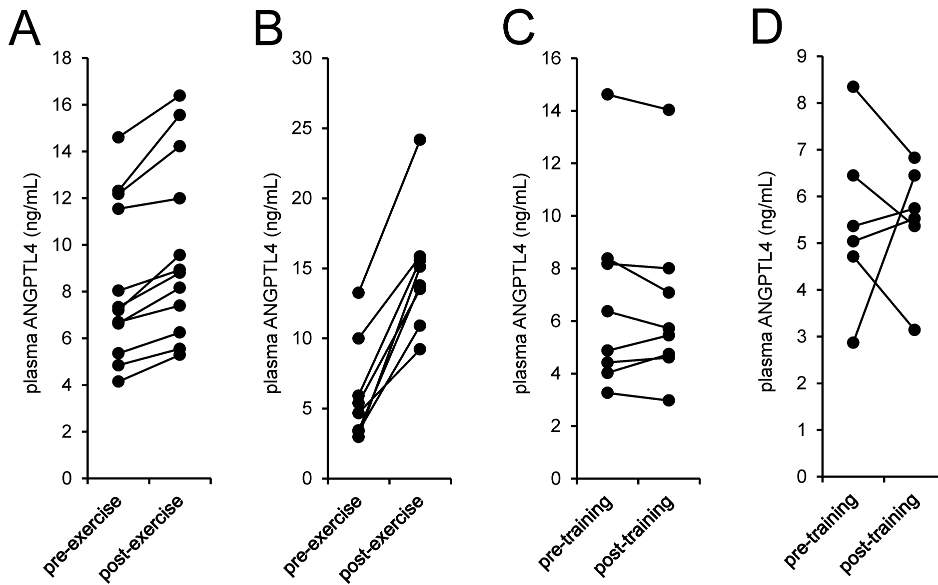

Figure 6.3 *Plasma Angptl4 are increased by acute exercise but not by exercise training.* ***(A)*** *Plasma Angptl4 levels before and after 1 hour of one-legged cycling exercise at 50% of one-legged* W_{max} *(Study A, n = 12).* ***(B)*** *Plasma Angptl4 levels before and after 3 hour cycling exercise at 40%* W_{max} *(Study B, n = 8).* ***(C)*** *Fasting plasma Angptl4 levels before and after an intense 2 week endurance training program on a cycling ergometer (Study C, n = 8).* ***(D)*** *Fasting plasma Angptl4 levels before and after a moderate intensity 12 week endurance training program on a cycling ergometer (Study D, n = 6).*

Since the non-exercising leg was inactive, changes in gene expression in the resting leg muscle cannot be caused by local contractile activity but must be related to systemic factors, including circulating metabolites. To verify this notion, serum from fasted human subjects collected before and after 2h of cycling exercise was added to mouse C2C12 myotubes and *Angptl4* mRNA expression was determined by qPCR. Post-exercise serum markedly increased *Angptl4* mRNA compared to pre-exercise serum (figure 6.4A). Intriguingly, no such effect was observed using pre- and post-exercise plasma from subjects that received repeated glucose drinks during the cycling exercise (figure 6.4A). *Angptl4* expression in C2C12 myotubes was also more strongly induced by plasma collected from subjects in fasted state compared with fed state (figure 6.4B). In those subjects, skeletal muscle *Angptl4* expression was also markedly higher in the fasted state compared with the fed state (figure 6.4C). Together, these data suggest that *Angptl4* is induced by a circulating factor that is specifically induced by exercise in the fasted state but is not elevated when glucose is consumed during exercise, and is also enriched in

the fasted state compared with fed state. One such parameter is plasma free fatty acids (FFA) (figure 6.4A,B, right panel), levels of which also increased during one-legged cycling (figure 6.4D), and which were previously shown to potently induce *Angptl4* mRNA in

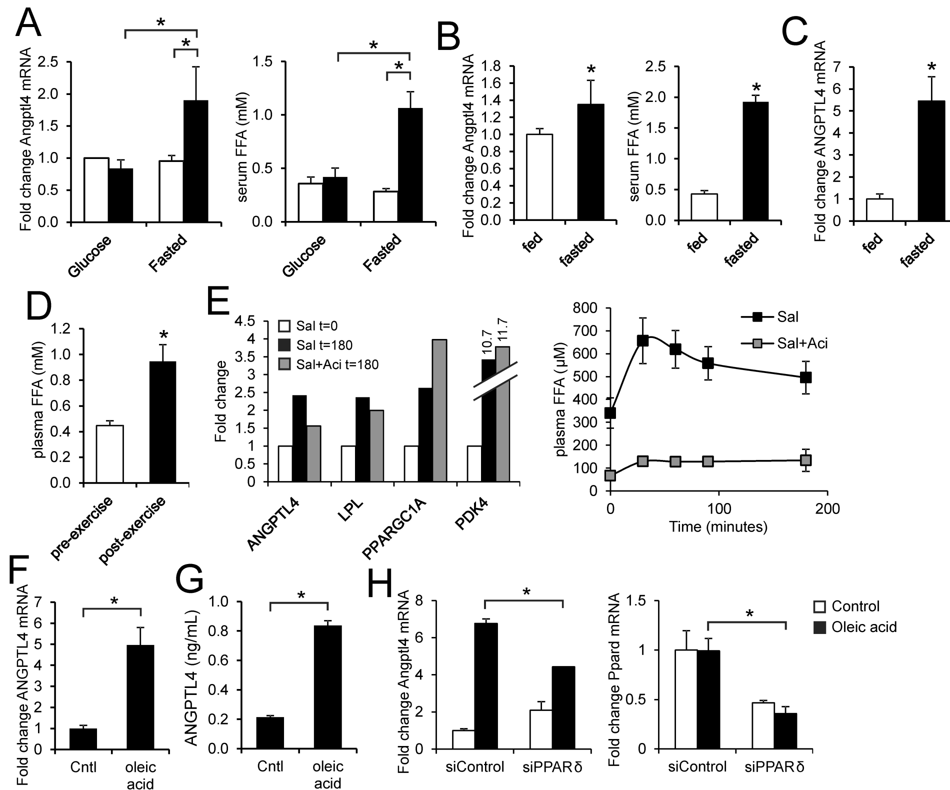

Figure 6.4 *Sensitive induction of Angptl4 gene by FFAs in human and mouse myocytes.* ***(A)*** *C2C12 myotubes were incubated for 6h with 10% serum from subjects (n = 5) before and after exercise performed in fasted state or with provision of glucose (Study E).* Angptl4 *mRNA in left panel. Serum FFA levels in right panel.* ***(B)*** *C2C12 myotubes were incubated for 3h with 10% serum from subjects (n = 12) at the end of 60h fast or a 60h normal fed condition (Study F).* Angptl4 *mRNA in left panel. Serum FFA levels in right panel.* ***(C)*** Angptl4 *mRNA in muscle biopsies collected at the end of 60h fast or a 60h normal fed condition (Study F).* ***(D)*** *Plasma FFA levels before and after one-legged exercise (n = 12).* ***(E)*** *Left panel: pooled mRNA expression of selected genes in muscle biopsies collected before and after Salbutamol infusion with and without prior Acipimox administration (Study G, n = 9). Right panel: Plasma FFA levels during salbutamol infusion. Error bars represent SEM.* Angptl4 *mRNA* ***(F)*** *and Angptl4 concentration in medium* ***(G)*** *in primary human myotubes treated with oleic acid.* ***(H)*** Angptl4 *and* Pppard *mRNA in C2C12 myotubes transfected with control (non-targeting) or PPARδ siRNA and treated with oleic acid. *Significantly different according to Student's t test ($p < 0.05$). Error bars represent SD unless indicated otherwise. Cells were treated for 12h unless indicated otherwise.*

various cell types, including in cultured myocytes [27-30]. In support of a role of plasma FFA in induction of *Angptl4* gene expression in human muscle, raising plasma FFA levels by salbutamol treatment markedly increased muscle *Angptl4* expression, which was largely blunted when salbutamol was co-administered with the lipolysis inhibitor Acipimox (figure 6.4E). This expression pattern was not found for other relevant genes such as *PPARGC1A* and *PDK4*.

To further study *Angptl4* gene regulation by fatty acids, we used cultured myocytes. Oleic acid markedly induced *Angptl4* mRNA in human primary myotubes (figure 6.4F), which was accompanied by a similar increase in Angptl4 protein secretion (figure 6.4G). Oleic acid also markedly induced *Angptl4* expression in C2C12 myotubes, which was significantly blunted upon siRNA-mediated knock-down of PPARδ (figure 6.4H). Consistent with high sensitivity of *Angptl4* gene regulation to stimulation by fatty acids, microarray analysis indicated that the (second) most highly induced gene by oleic acid in human primary myotubes and mouse C2C12 myotubes corresponded to *Angptl4* (supplemental figure S6.1). Taken together, these data strongly suggest that the marked induction of *Angptl4* mRNA in the non-exercising human muscle is caused by increased plasma FFA levels associated with exercise.

Despite being exposed to elevated plasma FFA as well, exercising human muscle shows only a very minor increase in *Angptl4* expression. Accordingly, we hypothesized that induction of *Angptl4* mRNA in exercising muscle by FFA is mitigated by a contraction-related factor. If that is true, expression of *Angptl4* should go up strongly after cessation of exercise when performed in the fasted state, since plasma FFA remain high for hours after such exercise. Indeed, *Angptl4* mRNA in exercising human muscle is increased 20-fold four hours post-exercise in the continued fasted state compared to baseline, concurrent with sustained elevation of plasma FFA (supplemental figure S6.2).

A candidate factor that may suppress *Angptl4* in exercising muscle is adenosine monophosphate-activated protein kinase (AMPK), which has been demonstrated to be activated specifically in exercising muscle but not non-exercising muscle during one-legged exercise [31,32]. In our study, despite the fact that muscle biopsies were collected approximately 10-15 minutes post-exercise, we found enhanced AMPK phosphorylation in some subjects (figure 6.5A), although not in all subjects. To study the impact of AMPK activation on *Angptl4* expression, we treated mouse C2C12 myotubes with the AMPK activator AICAR, leading to phosphorylation of AMPK (supplemental figure S6.3A). Strikingly, AICAR treatment markedly reduced *Angptl4* mRNA (figure 6.5B) and protein

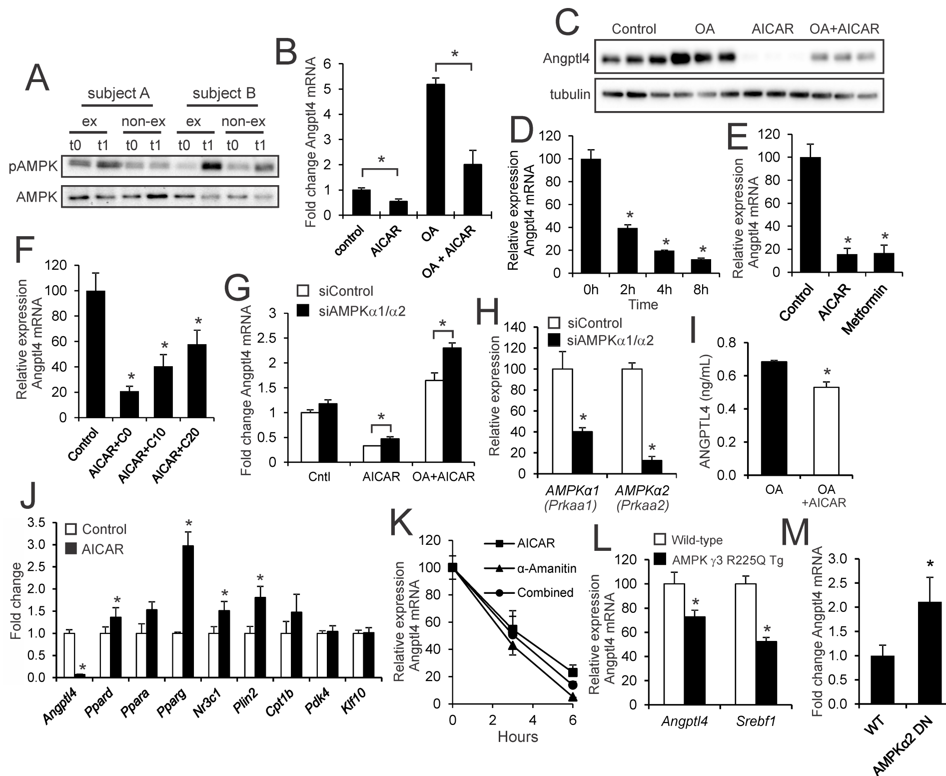

Figure 6.5 *AMPK activation suppresses* Angptl4 *mRNA.* ***(A)*** *Immunoblot for AMPK and phospo-AMPK in skeletal muscle biopsies from two selected subjects before (= t0) and after exercise (= t1)* ***(B)*** *Expression of* Angptl4 *mRNA in C2C12 myotubes treated with oleic acid (200 µM) and/or AICAR (1 mM) for 3 hours.* ***(C)*** *Immunoblot for Angptl4 in C2C12 myotubes treated with oleic acid and/or AICAR.* ***(D)*** *Time-course of the effect of AICAR on* Angptl4 *mRNA in C2C12 myotubes.* ***(E)*** *Comparison effect AICAR (1 mM) and metformin (0.5 mM) on* Angptl4 *mRNA in C2C12 myotubes.* ***(F)*** *Effect of AICAR (1 mM) and compound C (concentration indicated in mM) co-treatment on* Angptl4 *mRNA in C2C12 myotubes.* ***(G)*** Angptl4 *mRNA in C2C12 myotubes transfected with control (non-targeting) or AMPKα1/AMPKα2 siRNA and treated with AICAR.* ***(H)*** *Effective knock-down of AMPKα1 and AMPKα2 by AMPKα1/AMPKα2 siRNA.* ***(I)*** *Angptl4 levels in medium of human primary myotubes treated with oleic acid and AICAR.* ***(J)*** *Expression of PPARs and PPAR targets in C2C12 myotubes treated with AICAR.* ***(K)*** Angptl4 *mRNA in C2C12 myotubes pre-incubated with 50 µg/mL α-Amanitin for 1h and treated with AICAR for 3h and 6h.* ***(L)*** Angptl4 *mRNA in gastrocnemius of mice that overexpress an activating mutant of the muscle-specific isoform of the AMPKγ-subunit. Error bars represent SEM. Data were extracted from GSE4065 [34].* ***(M)*** Angptl4 *mRNA in gastrocnemius of mice that overexpress a dominant negative mutant of the AMPKα2 subunit. Cells were treated for 12h unless indicated otherwise. Error bars represent SEM. *Significantly different according to Student's t test ($p < 0.05$). Error bars represent SD unless indicated otherwise.*

(figure 6.5C) in C2C12 myotubes and blunted the induction of *Angptl4* expression by oleic acid. The suppressive effect of AICAR on *Angptl4* mRNA was very fast (figure 6.5D), was mimicked by the alternative AMPK activator metformin (figure 6.5E), could be partially abolished by the AMPK inhibitor compound C (figure 6.5F, supplemental figure S6.3B), and was modestly relieved upon combined knock-down of the AMPK α1 and α2 subunit (figure 6.5G,H). AICAR also modestly but significantly reduced the oleic acid-induced upregulation of *Angptl4* secretion in primary human myotubes (figure 6.5I). The reduction of *Angptl4* mRNA in C2C12 myotubes was not mediated by downregulation of PPARδ, PPARα, or PPARγ, as expression of all three PPARs was increased rather than decreased by AICAR treatment (figure 6.5J). Target genes of PPARδ, the main PPAR isotype in muscle, were also either increased or remained unchanged by AICAR including *Plin2*, *Pdk4*, *Klf10* and *Cpt1b*, as was the glucocorticoid receptor *Nr3c1*, another transcriptional inducer of *Angptl4* [33] (figure 6.5J). These data suggest that downregulation of *Angptl4* mRNA by AMPK activation is not mediated by any of the known transcriptional regulators of *Angptl4*. Time course studies in C2C12 myotubes indicated that AICAR reduces *Angptl4* gene expression with nearly the same speed as the transcriptional inhibitor α-amanitin, and no additive effect between the α-amanitin and AICAR was observed, suggesting that AMPK activation almost completely blocks *Angptl4* gene transcription (figure 6.5K). *In vivo* overexpression of an activating mutant of the muscle-specific isoform of the AMPKγ-subunit supported the suppressive effect of AMPK on *Angptl4* gene expression (figure 6.5L) [34]. Conversely, *in vivo* overexpression of a dominant negative mutant of the AMPKα2 subunit led a significant induction of *Angptl4* mRNA (figure 6.5M) [35]. The data suggest that the stimulatory effect of plasma FFA on skeletal muscle *Angptl4* mRNA is counteracted by AMPK activation in exercising muscle.

As previously observed for the PPARδ agonist GW501516 [29], induction of *Angptl4* mRNA in C2C12 myotubes by oleic acid was associated with a pronounced decrease in heparin releasable LPL activity (figure 6.6A), while inducing *Lpl* mRNA (figure 6.6B). To study the impact of *Angptl4* upregulation on skeletal muscle lipid uptake in vivo, we used *Angptl4*-transgenic mice characterized by overexpression of *Angptl4* mRNA and protein in a variety of tissues including skeletal muscle (figure 6.6C) [36]. Transgenic *Angptl4* overexpression did not affect muscle weights or percentage lean body mass (supplemental figure S6.4). To assess the functional effect of *Angptl4* overexpression during exercise, we subjected WT and *Angptl4*-Tg mice to an acute moderate exercise bout on a motorized treadmill. Total LPL protein in skeletal muscle (gastrocnemius) after exercise were not different

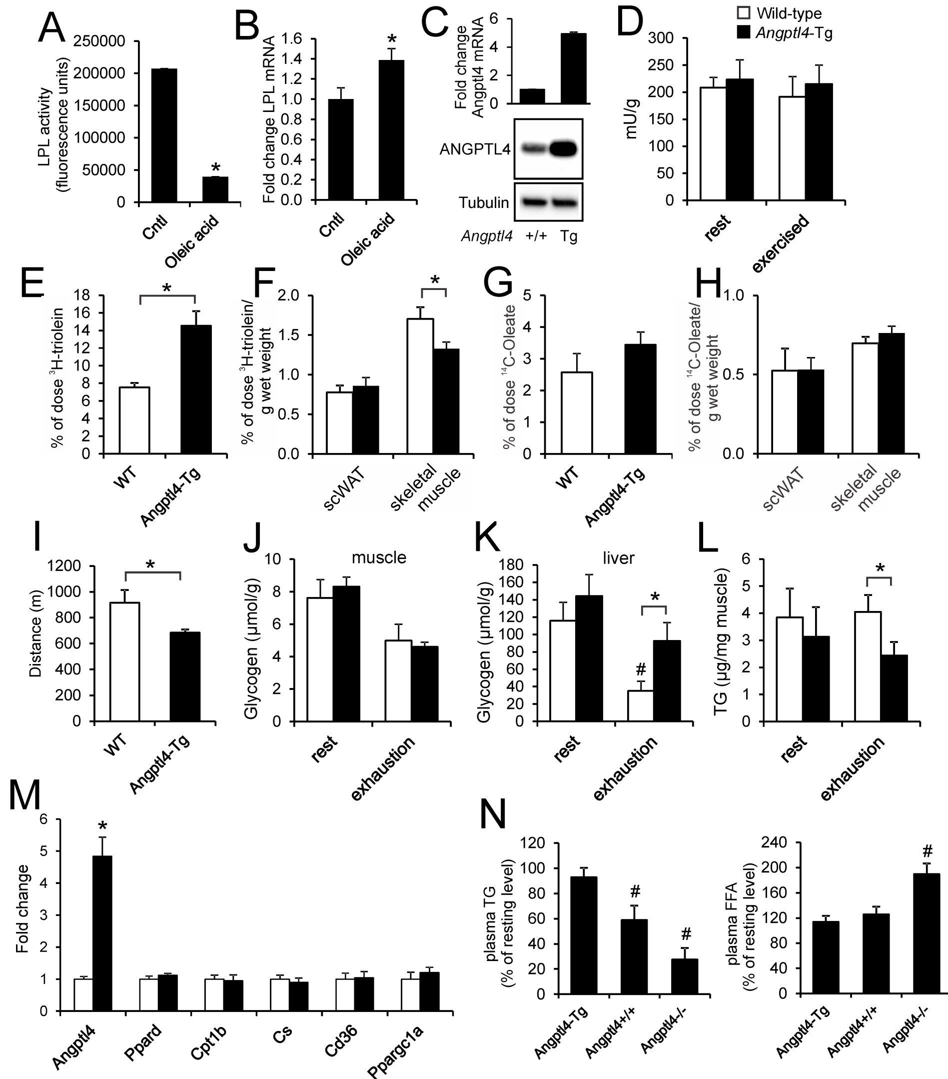

Figure 6.6 *Angptl4 upregulation impairs LPL activity and uptake of plasma TG-derived fatty acids in muscle. Heparin releasable LPL activity **(A)** and* LPL *mRNA **(B)** in mouse C2C12 myotubes treated with oleic acid (400 µM) for 24h. **(C)** Angptl4 protein abundance and mRNA expression in mouse skeletal muscle. **(D)** Total LPL activity in skeletal muscle (gastrocnemius) of WT and* Angptl4-*Tg mice at rest and after 90 minutes of moderate running exercise (12 m/min). **(E)** Serum ^{3}H-activity after 15 minutes of running in WT and* Angptl4-*Tg mice injected with [^{14}C]-oleate together with glycerol tri[^{3}H]oleate-labeled VLDL-like particles. **(F)** ^{3}H-activity in subcutaneous adipose tissue and gastrocnemius after 15 minutes of running. **(G)** Serum ^{14}C-activity after 15 minutes of running in WT and* Angptl4-*Tg mice injected with [^{14}C]-oleate together with glycerol tri[^{3}H]oleate-labeled VLDL-like particles. **(H)** ^{14}C-activity in subcutaneous adipose tissue and gastrocnemius after 15 minutes of running. **(I)** Distance covered excluding warm-up by WT and* Angptl4-*Tg mice subjected to an incremental exercise test to exhaustion.*

between WT and *Angptl4*-Tg mice (figure 6.6D). However, *Angptl4*-Tg mice showed markedly reduced plasma clearance of [^{3}H]triolein-labeled very-low-density lipoprotein (VLDL)-like particles during exercise (figure 6.6E) and reduced fatty acid uptake from the labeled VLDL-like particles into skeletal muscle, but not into subcutaneous adipose tissue (figure 6.6F). Plasma clearance of [^{14}C]-oleic acid and uptake into skeletal muscle and subcutaneous adipose tissue was unaffected in *Angptl4*-Tg mice (figure 6.6G,H). Plasma levels of FFA, glucose, glycerol and β-hydroxybutyrate were not different between WT and *Angptl4*-Tg mice in exercised or resting state (supplemental figure S6.5A). To assess whether reduced muscle uptake of plasma TG-derived fatty acids in *Angptl4*-Tg mice had any influence on muscle performance, we determined the maximal endurance capacity using an incremental treadmill protocol until exhaustion, characterized by a gradual increase in speed and slope of the treadmill. Strikingly, *Angptl4*-Tg mice ran significantly less far compared to WT mice (figure 6.6I). Strength as determined using the horizontal wire test was not different between the two sets of animals (supplemental figure S6.5B). Depletion of muscle glycogen stores during exhaustive exercise was comparable between WT and *Angptl4*-Tg mice (figure 6.6J), whereas liver glycogen levels remained higher in *Angptl4*-Tg mice after exhaustive exercise, which reached statistical significance (figure 6.6K). Intramuscular triglycerides were lower in *Angptl4*-Tg mice, which also reached significance after exhaustive exercise (figure 6.6L). No differences in baseline expression of markers of oxidative capacity were observed between the two genotypes suggesting that Angptl4 does not influence oxidative capacity (figure 6.6M). Finally, we determined the relative decrease in plasma TG in exercised state compared to resting state in WT, *Angptl4*-Tg and *Angptl4-/-* mice, as a measure of relative plasma TG utilization. The relative decrease in plasma TG in exercised vs. resting state was much more pronounced in *Angptl4-/-* compared to WT and especially *Angptl4*-Tg mice (figure 6.6N), likely due to preferential use of plasma TG in *Angptl4-/-* mice. Conversely, the relative increase in plasma FFA in exercised vs. resting state was more pronounced in *Angptl4-/-* mice, likely

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*Muscle glycogen **(J)**, liver glycogen **(K)** and muscle triglyceride **(L)** levels in WT and* Angptl4-*Tg mice at rest or after exhaustive running exercise. **(M)** mRNA expression of selected genes in skeletal muscle (gastrocnemius) of WT and* Angptl4-*Tg mice at rest. **(N)** the relative level of plasma TG (left panel) and FFA (right panel) in exercised state (90 minutes of moderate running exercise) compared to resting state (90 minutes rest) in WT,* Angptl4-*Tg and* Angptl4-/- *mice. *Significantly different from WT mice according to Student's t test ($p < 0.05$). [#]Significantly different from resting mice according to Student's t test ($p < 0.05$). Error bars represent SEM. N = 6-10 mice per group.*

due to sparing of plasma FFA in favor of use of TG-derived fatty acids. Overall, these data indicate that upregulation of Angptl4 impairs skeletal muscle uptake of fatty acids from circulating TG-rich lipoproteins and reduces liver glycogen utilization, leading to decreased exhaustive exercise performance.

DISCUSSION

The activity of LPL and associated tissue uptake of plasma TG-derived fatty acids is under control of different physiological stimuli in different tissues [26]. In white adipose tissue LPL activity is decreased by fasting, which was unequivocally demonstrated to be mediated by upregulation of *Angptl4* [37]. In brown adipose tissue LPL activity is increased by cold exposure, which is associated with a decrease in *Angptl4* mRNA [38], hinting at a potential role of Angptl4. Our data suggest that Angptl4 also plays an important role in LPL-dependent plasma TG clearance in skeletal muscle and particularly during acute exercise by coordinating lipid uptake between exercising and non-exercising muscles.
Acute exercise increases adipose tissue lipolysis and raises plasma levels of FFA. Whereas the abundant plasma FFA are efficiently oxidized in exercising muscle, their increase leads to elevated intramuscular fat storage in non-exercising muscle, possibly leading to lipotoxicity [39]. Previously, we found that Angptl4 functions as a fatty acid-inducible anti-lipotoxic factor in cardiomyocytes and macrophages [27,40]. The present paper suggests that the exercise-induced increase in plasma FFA stimulates Angptl4 synthesis in non-exercising human muscle, leading to local inhibition of LPL activity and diminished uptake of plasma TG-derived fatty acids in compensation for elevated uptake of plasma FFA, presumably to mitigate lipid overload and associated lipotoxicity in non-exercising muscle during prolonged exercise. In contrast, in exercising muscle the stimulatory effect of FFA on Angptl4 is counteracted by AMPK-mediated suppression of *Angptl4* mRNA, thereby maintaining LPL activity and supporting the use of plasma TG as fuel for the exercising muscle (figure 6.7).
Previously, in vivo AMPK activation by AICAR was found to lower plasma TG levels [41,42]. Suggesting a role for LPL in plasma TG lowering by AMPK, AMPK activation by AICAR or metformin increased intralipid clearance and increased heparin-releasable LPL activity in rat hearts, without causing any change in *LPL* mRNA [43,44]. Furthermore, AICAR and metformin enhanced LPL activity in rat L6 muscle cells [45]. Based on the data presented here, it is plausible that the stimulatory effect of AICAR and metformin on LPL activity and

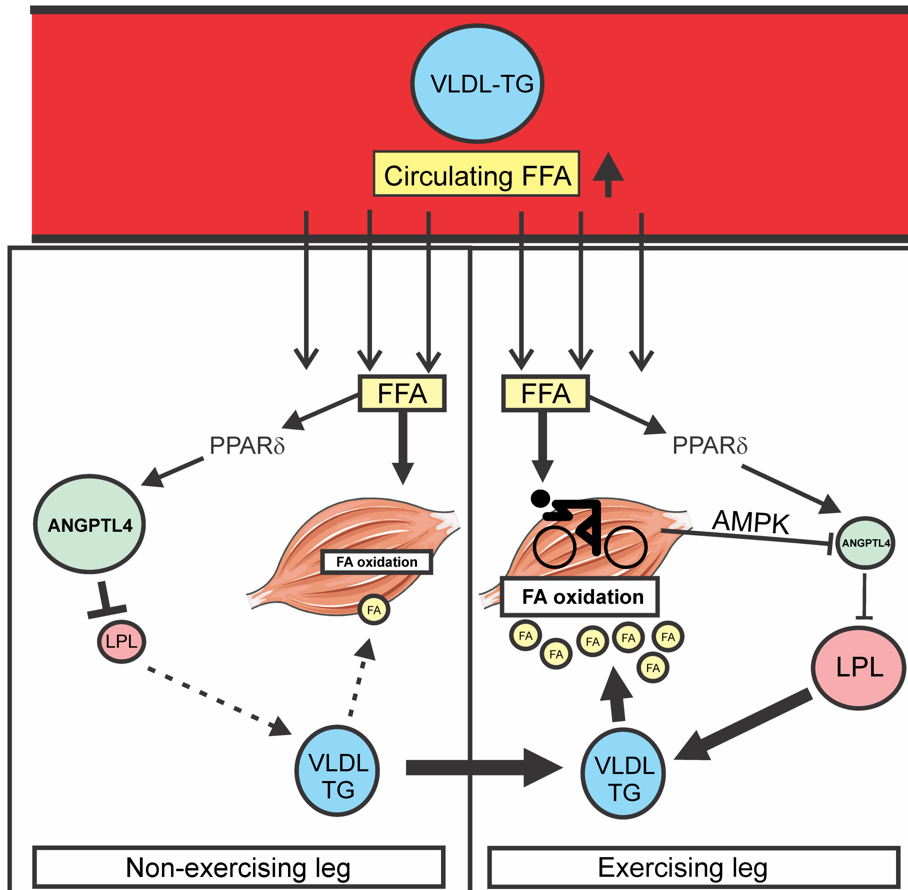

Figure 6.7 *Schematic representation of the proposed role of Angptl4 in lipid provision to exercising muscle. During exercise, circulating FFAs and VLDL particles are directed to exercising and non-exercising muscle. In the non-exercising leg, increased FFA levels provoke an increase in Angptl4 expression via PPARδ, leading to inhibition of LPL activity and consequent reduction in uptake of fatty acids derived from VLDL, which is likely aimed at preventing lipid overload. In contrast, in the exercising leg the stimulatory effect of FFA on* Angptl4 *mRNA is counteracted by AMPK-mediated suppression of* Angptl4 *mRNA. As a result, LPL activity remains high, allowing full exploitation of fatty acids derived from VLDL as substrate for fatty acid oxidation to meet the energetic needs of exercising muscle.*

concomitant lowering of plasma TG is mediated by suppression of Angptl4 in muscle and possibly other tissues. Future studies will have to address this question in more detail. Our data indicate that overexpression of *Angptl4* reduces maximal endurance exercise performance, most likely by limiting the provision of plasma TG-derived fatty acids to the muscle and by limiting utilization of liver glycogen, which is an important fuel for

exercising mice. Until recently, due to a number of methodological issues, the role of plasma TG as source of fatty acids for oxidation in exercising muscle and thereby its overall importance as fuel during endurance exercise has likely been underestimated (reviewed in [1]). Indeed, during leg exercise plasma TG uptake and clearance are increased manifold compared to the resting situation [46]. The relevance of plasma TG during exercise is further suggested by the adaptive increase in muscle *LPL* mRNA and activity in response to an acute exercise bout and following exercise training [47,48]. In contrast to *Angptl4*, expression of *LPL* is induced to the same extent in exercising and non-exercising muscle. Enhanced activity of LPL in skeletal muscle is believed to account for the low plasma TG concentrations in trained individuals [49,50].

Angptl4 adds to a growing list of secreted proteins whose production in muscle is increased by acute exercise [51,52]. For many of these proteins it is unclear whether the change in production mainly impacts the muscle locally, or whether the proteins also exert an endocrine effect and thus function as a myokine. With respect to Angptl4, even though acute exercise leads to elevated Angptl4 levels in the circulation, it is unclear whether this increase mainly stems from increased mRNA and production of Angptl4 in skeletal muscle, or whether other tissues contribute as well. Overall, the co-expression of *Angptl4* with *LPL* in tissues such as (cardiac) muscle and adipose tissue combined with the tissue-specific regulation of *Angptl4* expression suggests that Angptl4 may mainly act via local inhibition of LPL, perhaps already in the sub-endothelial space via co-secretion with LPL [29,53].

In conclusion, our data indicate that in addition to the responses in exercising muscle, molecular changes in non-exercising muscle likely play a key role in regulation of fuel supply during exercise. It can be speculated that the beneficial effects of exercise on various health parameters are conveyed by adaptive changes in non-exercising muscles.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasma and tissue metabolites

Plasma metabolites and liver and muscle glycogen were determined as previously described [1]. Skeletal muscle total lipids were extracted by Folch and triglyceride levels were measured using colorimetric assay kit (INstruchemie) [2].

LPL activity

Oleic acid (400 μM) was added to cells complexed with BSA. After 24h, the medium was aspirated and the cells were washed twice with PBS. LPL was released from the myotubes by addition of 0.5mL PBS per well containing 100IU/ml heparin (LEO, Ballerup, Denmark). Heparin releasable LPL was collected after 5 minutes of incubation. LPL activity was measured with an LPL activity assay kit (Roar Biomedical, New York, USA) on a Fluoroskan Ascent FL Microplate Fluorometer (Thermo Fisher Scientific, Massachusetts, USA). LPL activities in homogenates of mouse skeletal muscle was measured as previously described [3].

Western blot

Protein lysates (20-30 μg protein/lane) were loaded on a denaturing gel and separated by SDS gel electrophoresis. Protein were transferred to a PDVF membrane. The primary antibody (rabbit anti-Phospho-AMPKα antibody (Thr172) (40H9), rabbit anti-AMPKα antibody, Cell Signalling; rat anti mouse Angptl4 antibody (Kairos 142-2), AG-20A-0054-C100 Adipogen) was used at a 1:1000 and 1:2000 ratio, and the secondary antibody (HRP-labelled goat anti-rabbit or anti-rat IgG, Sigma) at 1:5000. All incubations were performed in Tris-buffered saline, pH 7.5, with 0.1% Tween-20 and 5% dry milk, except for the final washings when dry milk was omitted.

Angptl4 elisa

Angptl4 levels in cell culture medium and in muscle homogenates were measured by ELISA as detailed previously [4]. Briefly, 96-well plates were coated with anti-human Angptl4 polyclonal goat IgG antibody (AF3485, R&D Systems) and incubated overnight at 4°C. Plates were washed extensively between each step. After blocking, 100 μL of medium of cells was applied, followed by 2 hour incubation at room temperature. A standard curve was prepared using recombinant human Angptl4 (3485-AN, R&D Systems) at 0.3 to 2.1 ng/well. Next, 100 μL of diluted biotinylated anti-human Angptl4 polyclonal goat

IgG antibody (BAF3485, R&D Systems) was added for 2 hour, followed by addition of streptavidin-conjugated horseradish peroxidase for 20 min, and tetramethyl benzidine substrate reagent for 6 min. The reaction was stopped by addition of 50 µL of 10% H_2SO_4, and the absorbance was measured at 450 nm.

Microarray processing

Total RNA (100 ng) was labeled using an Ambion WT expression kit (Life Technologies, Bleiswijk, NL) and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays coding 19,732 genes (Affymetrix ,Santa Clara, CA).

Microarray analysis was performed using MADMAX pipeline for statistical analysis of microarray data [5]. Quality control was performed and all arrays met our criteria. For further analysis a custom annotation was used [6] and expression values were calculated using robust multichip average (RMA) method [7]. Microarray data was filtered, probe sets with expression values > 20, located at > 5 arrays and with an Inter Quartile Range (IQR) value > 0.2 were stated to be expressed and selected for further statistical analysis. Significant differences in expression were assessed using Intensity-Based Moderated T-statistic (IBMT) [8]. Genes were defined as significantly changed when the p value was < 0.01. The microarray dataset has been deposited to the NCBI Gene expression omnibus, accession number GSE41769.

Microarray analysis of human myotubes from three subjects treated with oleic acid has been previously described [9]. Microarray analysis of C2C12 myotubes treated with oleic acid has also been previously described [10]. The microarray datasets have been deposited to the NCBI Gene expression omnibus, accession numbers GSE18589 and GSE38590.

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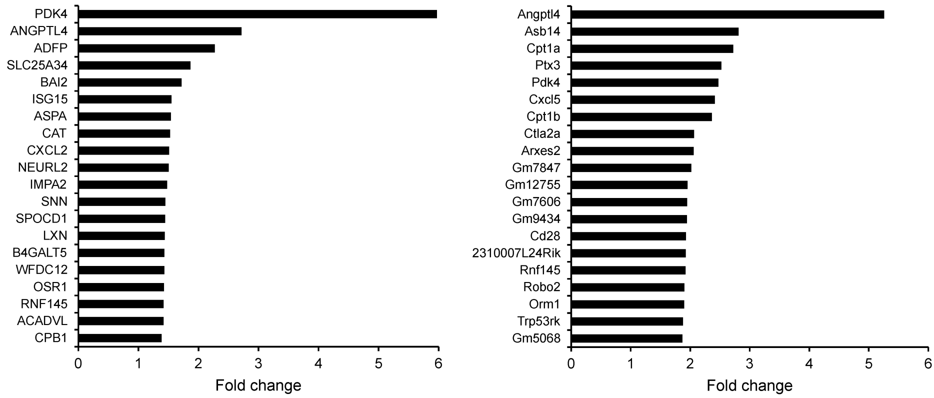
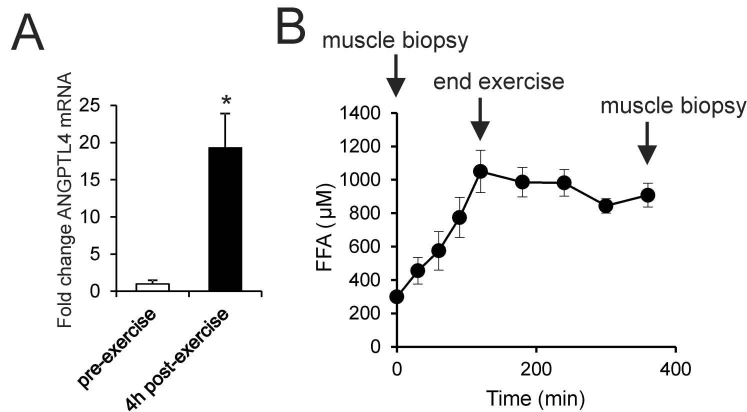
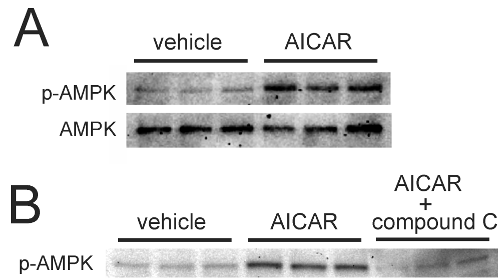


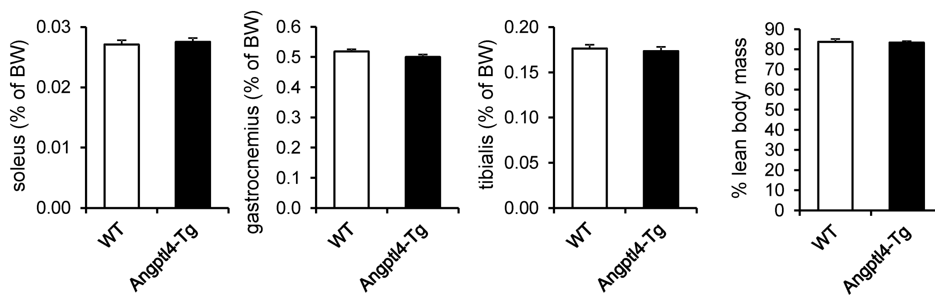
Supplemental figure S6.1 *The top 20 of genes most highly induced by oleic acid in primary human myotubes (left panel) or mouse C2C12 myotubes (right panel) ranked according to fold-change, as determined by Affymetrix microarray.*



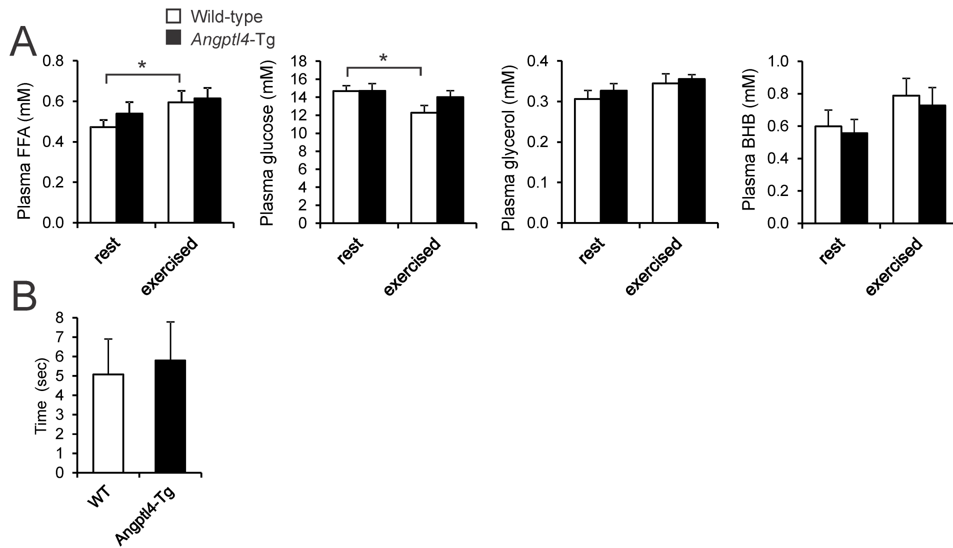
Supplemental figure S6.2 (***A)*** *Angptl4 mRNA in human vastus lateralis muscle collected before a 2h cycling exercise bout and after 4 hours of post-exercise recovery, all in fasted state.* ***(B****) Plasma free fatty acid concentrations during the entire exercise trial [11]. Error bars represent SEM.*



Supplemental figure S6.3 (***A)*** *Immunblot for AMPK and phospho-AMPK in C2C12 myotubes treated with AICAR (1 mM) for 12h.* ***(B)*** *Immunoblot for phospho-AMPK in C2C12 myotubes treated with AICAR (0.5 mM) and compound C (20 μM) for 12h.*



Supplemental figure S6.4 *Weight of soleus, gastrocnemius, tibialis anterior and lean body mass in WT and* Angptl4-*Tg mice.*



Supplemental figure S6.5 ***(A)*** *plasma metabolic parameters in WT and* Angptl4-*Tg mice at rest or after 90 minutes of moderate running exercise (12 m/min).* ***(B)*** *Horizontal wire test: average time until mice grasped wire with both hind legs. *Significantly different according to Student's t test (p < 0.05). Error bars represent SEM.*

General discussion

The primary aim of this thesis was to study the signalling role of skeletal muscle during exercise. To accomplish this, first an overview was given of the current knowledge on myokines produced by skeletal muscle. Second, the local effect of exercise on gene expression changes in skeletal muscle was assessed using high through-put transcriptomics tools. Third, the effect of exercise on gene expression in a non-active organ, in this case non-exercising skeletal muscle, was assessed. Finally, we identified myokines produced during acute exercise and exercise training using secretome analysis and assessed the role of one of the newly identified myokines Angptl4 in skeletal muscle.

ACUTE EXERCISE VERSUS EXERCISE TRAINING

The effects on gene expression of acute exercise and exercise training are studied in chapter 3 and 4. In chapter 4 we show that acute exercise has a large impact on gene expression of active skeletal muscle, illustrated by the number of genes changed and the large fold changes. The effect of exercise training on gene expression as studied in chapter 3 is more modest. The fold changes are smaller and fewer genes changed. The results of chapter 3 and 4 illustrate the different effects of acute exercise and exercise training. The existing literature supports the differential effects of acute exercise and exercise training, since it is shown that acute exercise and exercise training change different sets of genes and that genes change in an opposite manner [1]. In theory, exercise training is nothing more than a series of acute exercise sessions resulting in adaptation. In practice, the interaction between the acute exercise response and the adaptation induced by exercise training is complex and sophisticated. Therefore in the following paragraphs the interaction between acute exercise and exercise training will be discussed.

Stress-like response during acute exercise

Every single acute exercise bout increases the demand for ATP, oxygen and fuel substrates. Also the clearance of lactate, CO_2 and other compounds is increased. This remodelling and restructuring is accompanied by the activation of many biological pathways. Many of the remodelling and restructuring in response to acute exercise occurs at the protein and enzymatic level. In chapter 4 we show that changes in gene expression also play a role in this response. Other studies show that responses to acute exercise are further characterized by increased binding activity of several transcription factors and DNA hypomethylation [2-5].

The instant remodelling and restructuring in biological pathways illustrates that acute exercise can be characterized as a stress-like response, that is often accompanied by muscle damage [6,7]. Up to now it remains unclear whether this damage is favourable or unfavourable for recovery and adaptation. In the past muscle damage was seen as an unfavourable side effect of strenuous exercise, causing an very pronounced stress response which interfered with the adaptive response [8]. Since the 1980s evidence is growing that muscle damage plays a crucial role in the adaptive response during exercise training and might even be essential for adaptation [6]. Examples of stress responses during exercise-induced muscle damage that are important for adaptation are infiltration of immune cells and reactive oxygen species production in skeletal muscle. After muscle damage in mice, immune cells such as macrophages and neutrophils infiltrate the muscle tissue [9]. This infiltration is shown to play an important role in hypertrophy, since hypertrophy is blunted when there is impaired infiltration [10]. Production of reactive oxygen species is the another example of the stress response induced by acute exercise [11]. Production of reactive oxygen species was shown to be crucial in the metabolic signalling pathways that result in adaptation after exercise [12,13]. Recent studies indicate that antioxidant supplementation blunts the adaptive response to exercise training, supporting the importance of the stress response upon acute exercise in the adaptive response of exercise training [14,15].

Immune cell infiltration might not only play a role in hypertrophy during the adaptive response of exercise training. Chapter 5 shows that CCL2 and CX3CL1 increase mRNA and plasma levels after acute exercise. Both myokines are well known attractors of immune cells and are extensively linked to metabolism [9,16-18]. These links to metabolism and immune cell infiltration suggest that the stress-like response of acute exercise in the form of immune cell infiltration can play a role in metabolic remodelling of skeletal muscle. In future research this question could be partially addressed via a human study in which participants are administered with a placebo or non-steroidal anti-inflammatory drug such as ibuprofen shortly before an endurance exercise session. If inflammation and associated immune cel infiltration is important in the metabolic remodelling after acute exercise, the metabolic remodelling will be blunted in the group receiving ibuprofen.

Adaptive response during exercise training

As stated before, in theory exercise training is nothing more than a series of acute exercise sessions resulting in adaptation. In practice, the training effect of exercise is complicated

and not straightforward at all. During acute exercise the majority of the changes occur via allosteric regulation and covalent modification of rate-limiting enzymes. In exercise training the role of gene expression changes is very important [19]. In the next paragraphs we will therefore focus on the responses in gene expression changes induced by exercise training.

Gene expression response curves

Each exercise-reactive gene has a unique response curve during acute exercise. These response curves are the cornerstone of the adaptive training effect on gene expression. Expression of all induced genes eventually returns back to baseline [20,21]. The speed of this decline (or increase) to baseline varies per gene and the form of the response curve varies per gene [21]. Some genes are characterized as early response genes and their expression changes shortly after onset of exercise and returns back to baseline shortly after termination [21]. Other genes change expression after termination of exercise and are restored very slowly during recovery. Some genes do not return to baseline levels until 48 hours into recovery. Since every gene has an unique response curve, the integration of acute gene expression response curves into the chronic exercise training adaptation response is complicated and different for every gene.

Chronic adaptation

Consecutive gene expression response curves as described in the previous paragraph will result in chronic adaptation. The adaptive gene expression response during exercise training can be simplified by dividing it into two types of adaptation outcomes. The first type of adaptation outcome is chronically changed mRNA levels of genes important in the adaptive response. This first type of adaptation outcome of exercise training is illustrated in chapter 3. The second type of adaptation outcome of exercise training is characterized by changed response sizes after exercise. Specifically, the amplitude of the response curves of genes can be increased or decreased in amplitude by exercise training. Whether acute gene expression changes result in chronic adaptation depends on many factors, such as timing, intensity and duration of consecutive exercise sessions [20]. Also the nutritional status before, during and after exercise is an important factor in the adaptive response after exercise [20,22]. Since acute exercise is a stress-like response, multiple acute exercise bouts performed with very limited recovery between each bout might not result in actual adaptation, since during repair and adaptation new damage

is induced. On the other hand, when exercise sessions are performed with a very long intervals of rest, adaptations might also not occur. Too much time between sessions will results in discontinuation of adaptations initiated by exercise. Besides timing, the intensity of the exercise and the amount of muscle damage induced is important for which and how much adaptation occurs.

Types of adaptive responses

The adaptations during exercise training can be characterized by four different adaptive responses (see also figure 7.1). The responses illustrated in figure 7.1 depict genes showing increased expression. A similar but opposite figure can be made for genes showing decreased expression. The first response (A) represents consecutive bouts of acute exercise separated by a long recovery period. These exercise sessions only elicit an

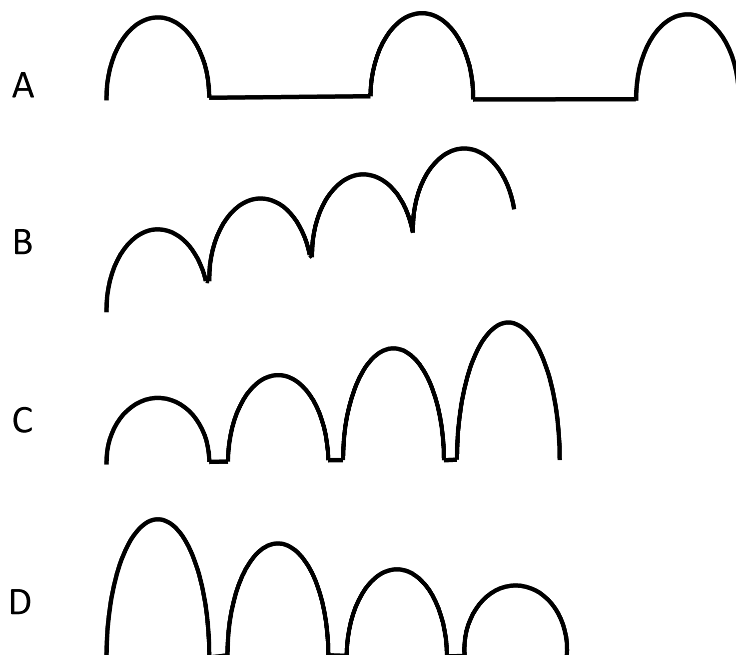


Figure 7.1 *Adaptive response on exercise training. Response A represents consecutive bouts of acute exercise that only elicit an acute effect, but do not result in any training effect, since recovery periods are too long. Response B represents consecutive bouts of exercise that are separated by appropriate recovery, which will result in overall increased basal levels. Response C represents consecutive bouts that are also separated by appropriate recovery and result in a higher response. Response D represent the opposite of response C, every bout results in a lower response. Based on Haskell [20].*

acute effect, but do not result in any training effect. The second response (B) represents consecutive bouts of exercise that are separated by appropriate recovery time. Such a response will induce an overall improvement after the training period: the basal levels will be increased, as is shown in this thesis. The third response (C) represents consecutive bouts that are also separated by appropriate recovery, but results in a higher response and not in higher baseline levels. Finally, the opposite of effect C can occur, where every exercise bout will give a lower response than the previous one (D). Response D can result in overtraining, but also beneficial adaptations can be characterized by a lower response. In chapter 3 it is likely that for some genes not (only) response B occurred, but response C. The limiting factor here is the designs used, which do not allow us to show differences in response. It is important to keep in mind that every response will result in levelling off of the training effect, since the adapted muscle will experience less stress with every training session. Every adaptation will reach a plateau at a certain point in time. Another important remark is that the net training effect can differ between genes, proteins and activity levels. Appropriate recovery for one factor might be not optimal for another factor.

How does acute exercise contribute to the exercise training effect?

Acute exercise can directly or indirectly contribute to the adaptations observed after exercise training. Acute exercise contributes directly to adaptation via gene expression changes induced by acute exercise bouts. Those gene expression changes result in adaptation in several ways as depicted in figure 7.1. Final adaptation outcomes will be changed basal levels or changed response curves of genes. The acute changes in mRNA levels direct the gene expression adaptations seen at the end of an exercise training period.

An indirect contribution is via the stress induced by increased demands for ATP, oxygen and fuel substrates during acute exercise. This stress causes a lot of changes and disturbances and results in for example immune cells infiltration and reactive oxygen species production. These changes are shown to be crucial for adaptation and therefore characterize the indirect influence of acute exercise on the adaption during exercise training.

In conclusion, acute exercise and the stress-like response characterizing acute exercise are important in the adaptations induced by exercise training. Furthermore acute exercise determines the magnitude of the adaptations induced by exercise training. Timing,

intensity, duration and the amount of stress induced are important factors influencing the adaptations.

MYOKINES: WHAT IS IN IT FOR THE FUTURE?

The background and motivation for the research described in this thesis was the growing interest in myokines and the impact of the identification of IL-6 as the first myokine. IL-6 produced during contraction has a positive effect on insulin resistance [18]. These findings are in contrast with earlier findings pinpointing IL-6 as a pro-inflammatory cytokine that induces insulin resistance [23,24]. Based on the identification of IL-6 as a myokine and the systemic role of IL-6, myokines are classified as the long-searched 'exercise factors'. Exercise factors circulate in the blood and are produced during exercise to communicate with organs not directly affected by exercise. Muscle contractions cause changes in skeletal muscle and these changes also demand changes in other organs of the body. Myokines are potentially the missing link between contracting muscle and other organs such as adipose tissue. Furthermore, there is a potential large impact of myokines on health, since they are the link that explains the positive health effects of exercise on relatively passive organs.

The evolvement of the myokine field

In 1998 IL-6 was identified as the first myokine, and surprisingly 16 years later IL-6 is still the best characterized myokine. The field did not evolve much further, despite that many studies were performed to identify new myokines and to assess their role during exercise. This conclusion can also be drawn from the review in chapter 2. A myokine that was recently discovered and received a lot of attention was irisin [25]. Irisin is discussed in detail in chapter 2 of this thesis. In short, a lot of controversy surrounds this myokine, since the induction during exercise cannot be replicated in humans and functionalityof irisin in humans is questionable [26]. Irisin is one of the few myokines for which a potential systemic role has been studied. Another example is SPARC [27]. For many other newly identified myokines data on their production by skeletal muscle and induction by exercise are limited [28,29]. Studies on the function of myokines and the mechanisms behind their induction are scarce. If the mechanism was assessed in previous studies, often the focus was on the local role. An example of a study assessing the local role of a myokine in exercise is presented in chapter 6. Also in this chapter the systemic role of the

myokine remains unrevealed. For the myokines identified in chapter 5 it was shown that their levels increase during exercise, but the function of this increase was not explored. From chapter 2 it can be concluded that myokines have received a lot of attention in the past years. Many studies only identified new myokines and only a small subset of those studies generated more knowledge on the systemic role of myokines. This knowledge gap raises questions on the systemic relevance of myokines. Two explanations can be given for the knowledge gap in the myokine field. On the one hand it is possible that the systemic role of myokine is complicated and therefore relatively hard to reveal. On the other hand, there is the possibility that the systemic role of myokines is limited. If the last explanation is true, it implies that myokines potentially only serve a local role during exercise. This has no implications for the importance of myokines locally, but it could mean that systemically those myokines are not important in the adaptations observed in other tissues such as adipose tissue or the liver.

Only a local role for myokines?

Not only the current status of literature raises questions on the systemic relevance of myokines, also several findings presented in this thesis contribute to the doubt regarding the systemic role of myokines. We show that CCL2, CX3CL1 and Angptl4 plasma increase levels during exercise, but that these increases are relatively modest. The increased plasma levels can just be a spill-over from the increased local levels. Furthermore it can be imagined that to have an impact on other organs via the circulation considerable increases in plasma levels are needed, which are clearly not observed for these myokines. It is important to note that we used one-legged cycling, a model in which the muscle mass used is relatively limited.

Furthermore we show that there is an important role for circulating free fatty acids. In chapter 4 we show that free fatty acids induce gene expression changes in non-exercising skeletal muscle. This role for free fatty acids indicates that not only myokines but also other circulating factors can induce gene expression changes in the non-exercising muscle. Exercise factors might therefore not only be myokines but also other circulating factors whose plasma levels change during exercise. The identification of BAIBA as an exercise factor is very interesting [30], since this confirms that metabolite can indeed serve a signalling role upon exercise. The limited role for myokines in gene expression changes in the non-exercising leg is somewhat contradicted by our analysis in chapter 5 that shows a strong similarity in genes correlating with CCL2 and CX3CL1 in the exercising

and non-exercising skeletal muscle, which do support some role for myokines in the gene expression changes in the non-exercising muscle. It remains unclear whether the overlap in genes in the exercising and non-exercise skeletal muscle is systemically or locally driven.

Role for immune cells in production myokines

In chapter 2 the current myokine field is extensively reviewed and several myokines are described. For IL-6 the literature was searched for studies assessing IL-6 levels during exercise. After careful examination it appears that to achieve increased IL-6 mRNA and/or plasma levels intensive exercise was needed. The type of exercise used was weight-baring, a type of exercise that often induces muscle damage [11]. Based on these findings it appears that some degree of muscle damage is needed to increase IL-6 plasma levels. This role for muscle damage in IL-6 production is confirmed in the literature [31]. In chapter 5 IL-6 only increases at the mRNA level, but not at the plasma level. The type of exercise used in chapter 5 is very modest and does not induce muscle damage, since inflammatory gene expression is only moderately induced. Interestingly, the myokines IL-8 and IL-15 also need (very) intense exercise to increase plasma levels [32,33]. Based on these conclusions and findings, the role of muscle damage in myokine production becomes very interesting. The role of muscle damage in the adaptations after acute exercise is previously discussed in this discussion, and there it is concluded that infiltration of immune cells induced by exercise-induced muscle damage likely plays an important role in the adaptations after endurance and resistance exercise training [34-37]. Given that intense exercise is needed to produce myokines, exercise-induced immune cell infiltration might play an important role in the production of myokines.

During injury immune cell infiltration follows a specific time course [9]. Whether the order of infiltration is identical in acute exercise in unclear. During injury first neutrophils infiltrate that promote more damage in skeletal muscle [9,38]. Next, pro-inflammatory M1-like macrophages and anti-inflammatory M2-like macrophages infiltrate in skeletal muscle [9,37]. Macrophages have a clear role in repair, especially M2-like macrophages that have as main function repair and regeneration [37,39,40]. Interestingly, macrophages are known to secrete a large subset of pro- and anti-inflammatory cytokines during an inflammatory response to initiate other immune cells and influence surrounding tissues [9,39]. Many of the cytokines produced by macrophages are similar to the known myokines. Immune cells could therefore contribute to the increased myokine levels measured.

Since most macrophages infiltrate after the initial infiltration of neutrophils, these macrophages cannot play a role in the initial secretion of inflammatory-linked myokines. Secretion of these myokines starts during exercise, while macrophages infiltrate during recovery [37]. Interestingly, a group of macrophages resides permanently in skeletal muscle near the connective tissue [41]. This group of macrophages can be activated immediately when damage is induced by for example exercise. Exercise could thus cause secretion of pro- and anti-inflammatory cytokines by those residing macrophages. It is interesting that many of the chemokines involved in macrophage infiltration after injury are also increased after acute exercise, as described in chapter 5 [9]. Also peritendonous tissue can produce cytokines during exercise as was shown by Langsberg et al. [42]. The cytokines produced are similar to the known myokines. This production suggests that also connective tissue plays a role in the increased myokine levels measured in plasma after exercise.

In conclusion, the myokine field is currently characterized by an expanding list of myokines, and large knowledge gaps remain concerning the systemic relevance of the known myokines. Furthermore, we conclude from the literature and findings presented in this thesis that other cell types residing in skeletal muscle, such as immune cells and connective tissue, can have a role in the myokine production during exercise.

ANGPTL4 AND SKELETAL MUSCLE

In a review by Raschke et al. [43], Angptl4 is for the first time classified as a myokine. Raschke et al. base the classification of Angptl4 as a myokine on previously shown increases in Angptl4 plasma levels during acute exercise and production of Angptl4 by skeletal muscle cells in vitro [44,45]. In chapter 6 we show that Angptl4 is not only increased in plasma during exercise, but that local mRNA and protein levels in skeletal muscle increase as well during acute exercise. Furthermore we show in chapter 6 a role for PPARs in the induction of Angptl4. This finding is supported by the findings of a study that used an in vitro skeletal muscle model and shows that PPARδ is responsible for regulation of Angptl4 levels [46]. The most important finding presented in chapter 6 is that Angptl4 is crucial in the direction of triglyceride-derived free fatty acids to active skeletal muscle. Angptl4 prevents via lipoprotein lipase (LPL) inhibition a lipid overload in the passive skeletal muscle and assures supply of free fatty acids to active skeletal muscle. It is interesting to compare this novel role of Angptl4 in skeletal muscle to the roles

of Angptl4 in other tissues. Angptl4 is generally known as a crucial regulator of lipid metabolism. In adipose tissue, Angptl4 is thought to play a local key role in increasing fat liberation and decreasing fat storage [47]. In liver, the role of Angptl4 is thought to be more systemically, since Angptl4 produced by the liver is secreted into the circulation. Liver-derived Angptl4 serves as an endocrine factor that inhibits LPL in peripheral tissues [48]. In heart, Angptl4 serves an important role by preventing a lipid overload in the heart that can have an toxic effect [49]. In intestine, Angptl4 not only regulates LPL activity, but also pancreatic lipase activity. Functionally, Angptl4 in intestine reduces fat absorption [50]. Finally, in macrophages Angptl4 reduces excessive fat uptake from chylomicrons to prevent an uncontrolled fat-induced inflammation [51]. The role of Angptl4 in skeletal muscle matches very well with the role of Angptl4 in all other tissues. In muscle Angptl4 prevents for lipid overload in the resting skeletal muscle, which is comparable to the functioning of Angptl4 in heart and macrophages.

What is interesting is that hepatic Angptl4 serves a systemic role and Angptl4 is thus released into the circulation [52]. Also for intestinal Angptl4 a systemic role was suggested [53]. We showed that during exercise Angptl4 plasma levels increased together with increased local Angptl4 levels, suggesting that these increased levels originate from the increased skeletal muscle Angptl4 levels. Norheim et al. [54] showed that during exercise Angptl4 levels also increased in liver and adipose tissue in mice. They suggest that the increased Angptl4 levels therefore mainly originate from liver and adipose tissue, and not from skeletal muscle [54]. If increased plasma levels do not originate from skeletal muscle, this implies that Angptl4 as a myokine does not have a systemic role. This conclusion does not change the importance of Angptl4 as a local myokine, but it does hold an important message for other myokines that exhibit increaseedg plasma levels during exercise, which is that other tissues can contribute to the increased plasma levels. Norheim et al. also show that gene expression levels of Angptl4 are relatively low compared to adipose tissue and liver gene expression levels [54]. Also the increases during exercise are larger in liver compared to skeletal muscle. In chapter 6 we do not compare Angptl4 skeletal muscle gene expression levels to other tissue gene expression levels. Furthermore, western blot comparison between tissues shows that Angptl4 protein levels in skeletal muscle in mice are comparable to protein levels in liver and brown adipose tissue (data not shown). Hence despite lower gene expression levels in skeletal muscle, protein levels are comparable. This observation suggests that Angptl4 gene expression levels are a relatively poor predictor of Angptl4 protein levels.

In conclusion, Angptl4 is likely a myokine that serves a local role in skeletal muscle. This local role is partly comparable to the role of Angptl4 in other tissues. A major novel finding is that induction of Angptl4 directs triglyceride-derived fatty acids to tissues where the fatty acids are needed.

FUTURE DIRECTIONS

In the past 15 years many myokines were identified using several approaches. Most studies identifying myokines used an untargeted approach, resulting in an extensive list of myokines. As stated previously in this discussion, these untargeted approaches did not result in knowledge about the physiological and systemic function of these myokines. Therefore in future research it is important that the myokine field shifts from the now popular untargeted approach to a targeted approach. In this targeted approach an in-depth analysis of one identified myokine should be performed to gain more insight in the mechanisms that induced the myokine during exercise and to determine the local and systemic function during exercise. A good example of a study using such an approach is chapter 6, where the mechanism behind the induction of new myokine Angptl4 is assessed and the local functional role is addressed. To apply such a targeted approach on other myokines, use of cell culture and animal models is preferred and is even compulsory. Cell culture models can give more insight in the local function and mechanisms behind the induction, for example by treatment with agonists and antagonists of specific pathways and transcriptional regulators. Also administration of the recombinant myokine to cell culture systems can provide valuable insights into the functioning of a myokine. Knockout and (muscle-specific) transgenic mouse models can give very relevant information about the local function of a specific myokine. More importantly knockout and transgenic mouse models can give very valuable information about the systemic function of a specific myokine. Regular exercise is partly comparable with a muscle-specific transgenic model, since they both result in chronically elevated systemic levels. Therefore such an model provides insight in the phenotype induced by increased levels of that specific myokine. Knockout mice models can provide insight in the role of a myokine during exercise (training). If the myokine plays an important role in the exercise response, the lack of that specific myokine in a (tissue specific) knockout model will influence the exercise response.

Once the mechanism and the local and systemic function for several myokines are clear, research can shift to the effect of those myokines in the context of health. Resolving the

function of myokines will furthermore provide more insight into one of the concerns raised in the discussion, namely whether myokines are systemically relevant. If myokines turn out to serve a metabolically relevant systemic role, the role of myokines in metabolic disease and insulin signalling would be a very interesting topic for future research.

FINAL CONCLUSIONS

In this thesis we studied the signalling role of skeletal muscle during exercise. Taking all the chapters together, we conclude that skeletal muscle has an important signalling role during exercise. However, it remains unclear how important this signalling role is systemically.

This conclusion is based on several observations. We show that exercise not only elicits molecular changes in active or trained skeletal muscle, but also in non-active organs (non-exercising skeletal muscle). After providing an overview of literature, we conclude that a subset of 14 myokines is currently identified in humans, playing a role in the (local) signalling function of skeletal muscle. Furthermore, we are able to identify several myokines produced by skeletal muscle during exercise in our studies, of which some are known and some are new myokines. CX3CL1 and Angptl4 are added to the list of myokines. We confirm CCL2 as a myokine with a potential systemic function, while other known myokines (IL-6, IL-8, IL-15, FGF-21) cannot or can only partly be confirmed as myokines. For Angptl4 we are able to provide more insight in the mechanism and functioning during exercise, concluding that Angptl4 is important in the substrate distribution during exercise. CCL2, CX3CL1 and Angptl4 all increase plasma levels during acute exercise, but based on the research presented in this thesis we cannot conclude that skeletal muscle serves a significant systemic signalling role.

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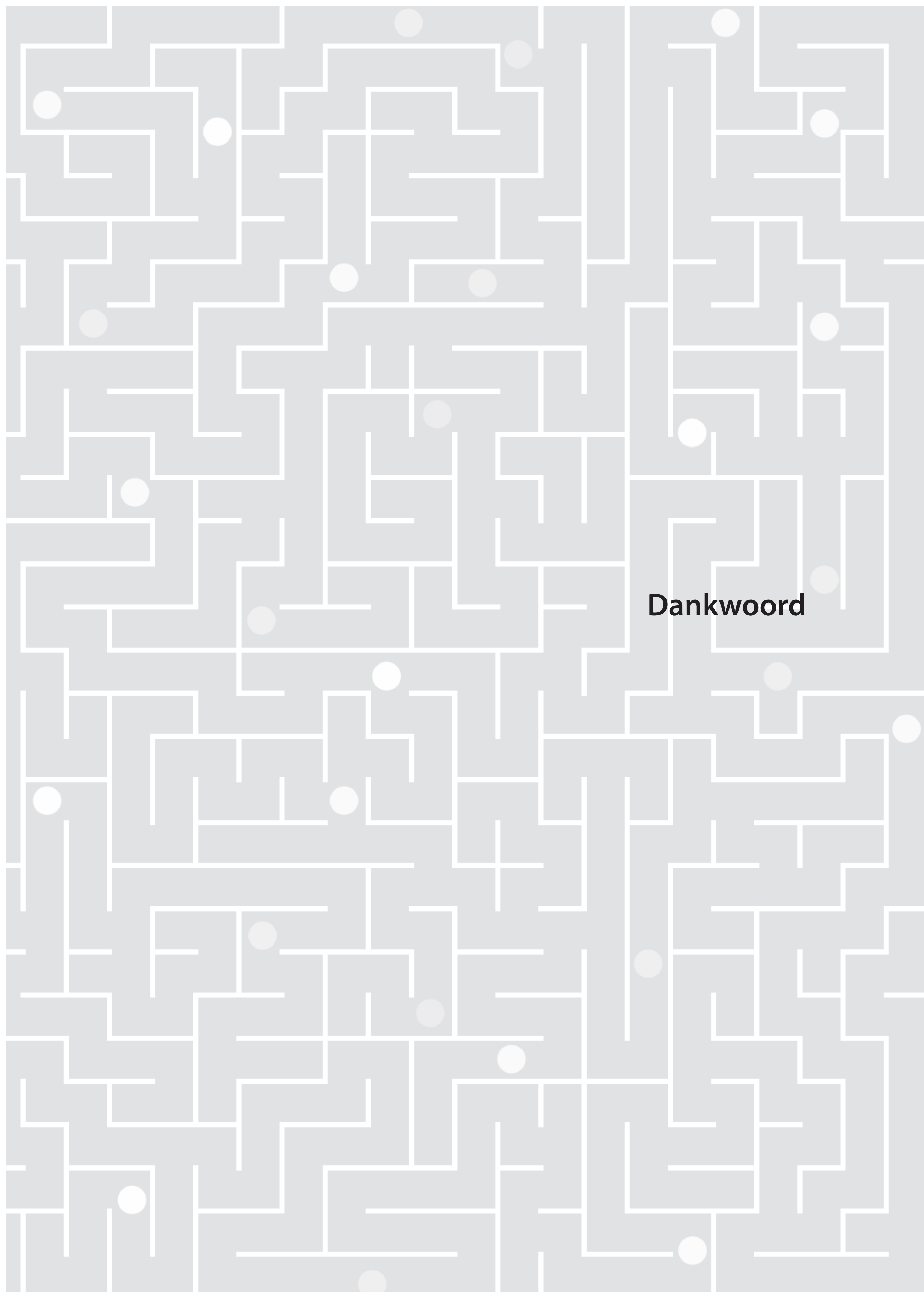
Nederlandse samenvatting

Tijdens inspanning wordt er veel gevraagd van de skeletspieren. De spieren zijn verantwoordelijk voor de uitvoering van de bewegingen die worden gemaakt tijdens inspanning. Om dit te bewerkstelligen vinden er veel lokale veranderingen en aanpassingen plaats in de spieren. Andere organen zoals de lever worden minder beïnvloed door inspanning, maar laten desondanks na inspanning ook lokale veranderingen en aanpassingen zien. De veranderingen in deze 'passieve' organen suggereren dat de spieren misschien met andere organen kunnen communiceren. In het afgelopen decennia zijn er vele onderzoeken uitgevoerd die hebben onderzocht hoe spieren met andere organen zouden kunnen communiceren. Eind jaren '90 werd er ontdekt dat spieren hormoonachtige stoffen kunnen uitscheiden, deze stoffen werden myokines genoemd. De myokines kunnen via de bloedbaan naar andere (passieve) organen reizen en hiermee de veranderingen initiëren die worden geobserveerd tijdens inspanning. Er is nog maar weinig bekend over welke myokines spieren precies aanmaken en wat de functies van deze myokines zijn. Daarom hebben we in dit proefschrift onderzocht welke myokines aangemaakt worden door de spieren tijdens inspanning en wat de rol zou kunnen zijn van deze myokines. Verder hebben we ook gekeken naar de lokale veranderingen die plaatsvinden in de spier tijdens inspanning, zodat we de aanmaak van myokines beter kunnen begrijpen.
In **hoofdstuk 2** hebben we een overzicht gemaakt van de gepubliceerde literatuur over myokines. Daaruit bleek dat er relatief veel myokines bekend zijn, maar dat er nog maar weinig bekend is over hun functie. Verder zijn er weinig myokines waarvan is aangetoond dat ze daadwerkelijk tijdens inspanning aangemaakt worden. Vervolgens hebben we in **hoofdstuk 3** gekeken naar de effecten van training op genexpressieveranderingen in de spier. Er zijn drie verschillende types training met elkaar vergeleken, namelijk duurtraining, krachtraining en een combinatie van kracht- en duurtraining. Bij de proefpersonen werd een spierbiopt afgenomen voor en na de trainingsperiode. Met een microarray-analyse is vervolgens een compleet profiel gemaakt van de genexpressies van alle genen. Uit de microarray-analyse bleek dat de genexpressieprofielen van de verschillende trainingtypes op elkaar leken, maar dat er ook een aantal belangrijke verschillen waren. Het belangrijkste verschil was dat vergeleken met de genexpressieveranderingen veroorzaakt door duurtraining de genexpressieveranderingen veroorzaakt door gecombineerde training minder gerelateerd zijn aan het aanmaken van energie met behulp van zuurstof of vetten. Veranderingen in de expressie van deze genen spelen een belangrijke rol in de trainingseffecten veroorzaakt door inspanning en zijn daarom een

zeer belangrijk onderdeel van de aanpassingen veroorzaakt door training. In **hoofdstuk 4** is er gekeken naar de genexpressieveranderingen veroorzaakt door acute inspanning. Proefpersonen hebben een uur met één been gefietst. Vooraf en direct na afloop van het uur fietsen zijn er spierbiopten afgenomen. Met deze spierbiopten is er een microarray-analyse gedaan om een compleet beeld te krijgen van de genexpressieveranderingen in de actieve en rustende spier. Hieruit bleek dat inspanning genexpressieverandering induceert in zowel de rustende als de actieve spier, al zijn de veranderingen in de rustende spier wat kleiner dan de veranderingen in de actieve spier. Deze bevinding bewijst dat spieren tijdens inspanning met passieve organen kunnen communiceren: de rustende spier wordt immers niet gebruikt, maar een relatief groot aantal genen in de rustende spier veranderen toch in expressie. Verder bleek uit de resultaten dat de veranderingen in de rustende spier deels worden geïnduceerd door vrije vetzuren uit het bloed.

In **hoofdstuk 5** hebben we gekeken welke myokines door de spier worden aangemaakt tijdens acute inspanning. Om dit te onderzoeken zijn de resultaten van de eenbenige fietsstudie gebruikt die al eerder in hoofdstuk 4 beschreven zijn. De microarray-analyse is verder geanalyseerd door middel van een secretoomanalyse, wat inhoudt dat bij alle significant veranderde genen werd gekeken of deze uitgescheiden kunnen worden en of ze dus mogelijke myokines kunnen zijn. Uit deze analyse kwam een lijst met mogelijke myokines en van deze lijst is er een selectie in het bloed gemeten. Twee van de gemeten myokines gingen omhoog in het bloed, namelijk de eiwitten CCL2 en CX3CL1. Omdat deze twee eiwitten in het bloed te meten zijn, spelen zij mogelijk een rol in de effecten van inspanning op de passieve organen. In **hoofdstuk 6** is een ander eiwit dat in genexpressie omhoog ging tijdens acute inspanning eruit gelicht. Dit eiwit Angptl4 had als enige gen een veel hogere expressie in de rustende spier vergeleken met de fietsende spier. Om uit te vinden wat de rol van dit verschil in expressie was, hebben wij verschillende muis- en celkweekexperimenten uitgevoerd. Uit de experimenten kwam dat de vrije vetzuren uit het bloed de verhoogde Angptl4-expressie in de rustende spier veroorzaakten. In de fietsende spier hadden de vrije vetzuren dit effect ook, maar werd het effect geremd door activatie van het enzym AMPK. Dit enzym is heel belangrijk in het aansturen van alle effecten die worden geobserveerd tijdens inspanning. Uit dit onderzoek bleek dat AMPK tevens de Angptl4-genexpressie remt. Het verschil in Angptl4-expressie tussen de benen reguleert de instroom van vetzuren, omdat deze vetzuren een belangrijke energiebron zijn voor de actieve spier tijdens inspanning. Angptl4 zorgt ervoor dat de vetzuren de fietsende spier ingaan en niet de rustende spier waar ze toch niet nodig zijn.

Uiteindelijk kunnen we concluderen dat de skeletspieren een belangrijke signaal-overbrengende rol hebben tijdens inspanning. Kanttekeningen zijn echter dat er nog steeds slechts beperkte kennis is over de mechanismen achter de werking van de myokines. Van veel myokines is bijvoorbeeld niet bekend wat de exacte functie is van het eiwit.



Dankwoord

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About the author

CURRICULUM VITAE

Milène Catoire was born on May 2, 1987 in Woerden, the Netherlands. She completed secondary school at the Minkema College in Woerden. After completion of secondary school she started in 2005 her BSc and MSc Human Movement Sciences at the VU University in Amsterdam. During her studies she specialized in movement systems. During her bachelor she performed a research project studying the effects of water temperature in swimming performance. During her master she performed her master thesis at the laboratory of Clinical Cardiovascular Physiology of the AMC hospital in Amsterdam. During this thesis she studied the cerebral and systemic vascular responses during resistance exercise in type 2 diabetes. In 2009 she obtained her MSc degree cum laude, after which she travelled around the world for 6 months. In august 2010 she started her PhD project within a consortium funded by the Diabetes Foundation with the title 'Optimizing the beneficial health effects of exercise for diabetes: focus on the liver!'. Milène performed her research at the Nutrition, Metabolism and Genomics group of Wageningen University and focussed on the signalling role of skeletal muscle during exercise. She was supervised by prof. dr. Sander Kersten, prof. dr. Patrick Schrauwen and dr. Marco Mensink. During her PhD Milène was involved in teaching and supervised master students during their master thesis. During her studies in Amsterdam and period as a PhD student in Wageningen Milène worked as a swim coach at several swimming clubs. Currently she is active as swimming coach in Nijmegen at the Talent Centre/AquaNovio '94 Nijmegen and is employed as a teacher at the Royal Dutch Swimming Association (KNZB). Since September 2014 Milène is appointed as a lecturer at the department of Physiology of the Radboud University Medical Center in Nijmegen.

LIST OF PUBLICATIONS

This thesis

Catoire, M., Mensink, M., Boekschoten, M.V., Hangelbroek, R., Müller, M., Schrauwen, P., Kersten, S. *Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle.* PLoS One 2012;7(11):e51066.

Catoire, M.*, Alex, S.*, Paraskevopulos, N., Mattijssen, F., Schaart, G., Kneppers, A., van Gogh, I., Mensink, M., Voshols, P., Olivecrona, G., Hesselink, M., Berbee, J., Müller, M., Rensen, P., Kalkhoven, E., Schrauwen, P., Kersten, S. *Fatty acid-inducible ANGPTL4 governs lipid metabolic response to acute exercise*. PNAS 2014 Mar 18;111(11):E1043-52.
*Authors equally contributed to this paper.

Catoire, M., Mensink, M., Kalkhoven, E., Schrauwen, P., Kersten, S. *Identification of human exercise-induced myokines using secretome analysis*. Physiological Genomics 2014 Apr 1;46(7):256-67.

Catoire, M., Mensink, M., Hooiveld, G., Schrauwen, P., Kersten, S. *Combined exercise training is less efficient in inducing health beneficial gene expression changes compared to endurance training alone*. In preparation.

Catoire, M., Kersten, S. *The search for exercise factors in humans*. In preparation.

Other

Wijnen, A., Steennis, J., Catoire, M., Wardenaar, F., Mensink, M. *Post-exercise rehydration: effect of beer consumption on fluid balance*. Submitted.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

NWO voedingsdagen, Deurne (2010, 2011, 2012)
NVDO annual meeting, Oosterbeek (2010, 2011, 2012, 2013)
NVDO jonge onderzoekersdag, Soesterberg (2011)
Nutrigenomics in clinical interventions, Kuopio (2011)
Masterclass Nutrigenomics, Wageningen (2011)
NuGo week, Wageningen, Friesing (2011, 2013)
Advanced visualization, integration and biological interpretation of -omics data, Wageningen (2011)
Masterclass "Analysis using R", Wageningen (2012)
European congress of Sport Science, Bruges (2012)
Annual meeting of EASD, Berlin (2012)

General

VLAG PhD week, Baarlo (2010)
PhD assesment (2011)
Project & time management (2011)
Scientific Writing (2011)
Writing and presenting of a scientific paper (2012)
Career perspectives (2013)

Optionals

Preparation of a research proposal (2010)
Advanced metabolic aspects of nutrition (2011)
PhD tour & committee, US and Mexico (2011)
Visit to the Technical University Munich (2011)
Journal club/weekly meeting TSK(2010-2014)
Weekly NMG meeting (2010-2014)

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