Regulation of skeletal muscle and kidney metabolism by the PGC-1 family of transcriptional coactivators

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Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Svenia Schnyder

aus Basel (BS)

Basel, 2017

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

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Basel, den 17.10.2017

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Abstract

Metabolism is key for life and involves the interplay of anabolic and catabolic reactions within a cell to meet the required energy needs. Each cell, tissue and organ possesses a unique metabolic profile that all contribute to systemic energy homeostasis and disturbances at any level profoundly affect whole body metabolism. The complex integration of metabolism takes place at different levels including adjustments in gene transcription. Over the last 20 years, transcriptional coregulators have emerged as important players in the regulation of gene expression and the field is constantly expanding identifying new coregulator proteins and their metabolic functions. The peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) family includes the three family members termed PGC-1α, PGC-1β and PGC-related coactivator. They have been implicated to play important roles in oxidative metabolism and mitochondrial homeostasis in a variety of different tissues including skeletal muscle and kidney. Thus, during the course of this thesis, we studied the physiological and pathophysiological effects of PGC-1α- and PGC-1β-specific ablation in these two organs in four different projects.

In the first project, we assessed the role of PGC-1α in skeletal muscle in response to chronic ketogenic diet feeding. Ketogenic diets have gained more and more attention as therapeutic strategies in the treatment of metabolic diseases and other pathological disorders. However, the mode of action is still poorly understood, particularly upon chronic administration. Next to liver, brain, and heart, skeletal muscle is one of the main players involved in the regulation of physiological and pathophysiological ketosis. Thus, we studied the effects of 12 weeks of ketogenic diet feeding in wildtype (WT) and PGC-1α muscle-specific knockout mice (PGC-1α MKO). Importantly, muscle PGC-1α was essential to increase oxygen consumption and transcript levels of genes involved in fatty acid oxidation as well as to maintain exercise performance upon ketogenic diet feeding. Therefore, we elucidated a new role for muscle PGC-1α in the regulation of physiological adaptations to chronic ketogenic diet administration.

In the second project, we studied the PGC-1α-dependent transcriptional changes in skeletal muscle upon acute bouts of exercise and chronic exercise training. Skeletal muscle is a highly plastic organ with an enormous capacity to adapt its metabolism to different energy needs. Interestingly, many of these metabolic changes, especially in response to exercise, are known to be mediated by PGC-1α. Thus, we performed acute time-course and chronic exercise experiments with WT and PGC-1α MKO mice and defined the PGC-1α-dependent and -independent transcriptional changes. Thereby, we identified the WT time-course-specific and acute core exercise responses and could demonstrate that PGC-1α is substantially involved in the regulation of these adaptations in skeletal muscle. Furthermore, while the acute exercise response involved many transcriptional changes, chronic exercise training

exerted only minor adaptations in gene expression levels. Thus, we elucidated new important aspects of PGC-1α in the regulation of skeletal muscle exercise physiology.

The third project was aimed at determining the role of PGC-1β in skeletal muscle in response to fasting. Skeletal muscle constitutes the largest protein reservoir of the body and its catabolism is the main source of amino acids for hepatic gluconeogenesis during energy deprived conditions. Thus, skeletal muscle emerges as one of the key players in the whole body response to fasting, yet, the complex regulation of skeletal muscle metabolism upon energy deprivation is still poorly understood. Thus, we evaluated the involvement of PGC-1β in the control of fasting-induced skeletal muscle adaptations in WT and PGC-1β MKO mice. Interestingly, 24 h of fasting induced only a partial muscle mass loss in PGC-1β MKO animals, which was characterized by reduced myostatin mRNA levels, a blunted induction of atrophy markers gene expression and absent activation of AMP-dependent and cAMP-dependent protein kinases in comparison to WT animals. Furthermore, PGC-1β MKO mice exhibited increased transcriptional activity of the nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) and showed elevated PGC-1α expression levels. Thus, our data suggest that PGC-1β might inhibit Nfatc1 transcriptional activity during fasting-induced muscle atrophy. These data shed new light on the complex regulation of skeletal muscle metabolism under energy deprived conditions and revealed PGC-1β as an important player in the control of fasting.

The fourth project of this thesis assessed the function of PGC-1α in podocyte and kidney metabolism. Glomerular filtration is the first step in urine production and involves different types of cells including podocytes, which are part of the glomerular filtration barrier that contributes to the prevention of protein loss from the primary filtrate. Mitochondrial dysfunction has been implicated to trigger podocyte injury, which eventually progresses to the development of chronic kidney disease. However, mitochondrial function and its contribution to podocyte disorders are still poorly understood. Thus, we studied the role of PGC-1α in podocyte metabolism under basal and stress-induced conditions in WT and PGC-1α podocyte-specific knockout mice (PGC-1α PKO). The mild increase in glomerular basement membrane thickness in PGC-1α PKO animals did not result in any functional deficits and young and aged PGC-1α PKO mice showed unchanged kidney and podocyte function under basal and stress-induced conditions in comparison to WT animals. Therefore, we concluded that PGC-1α is not mandatory for normal podocyte function *in-vivo*.

In summary, this thesis describes new aspects of PGC-1α and PGC-1β in the regulation of skeletal muscle and kidney metabolism. Moreover, we identified new molecular pathways and mechanisms by which these two coactivators exert their biological functions. Finally, our results might serve as cornerstone in the development of future therapeutic strategies for the treatment of metabolic disorders and other disease conditions.

Abbreviations

AD	activation domain
AMPK	AMP-dependent protein kinase
ATF2	activating transcription factor 2
BAT	brown adipose tissue
β-OHB	β-hydroxybutyrate
CaMK	calcium/calmodulin-dependent protein kinase
CBP	CREB binding protein
CKD	chronic kidney disease
CnA	calcineurin A
CREB	cAMP-response element binding protein
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal trans-differentiation
EPO	erythropoietin
ER	estrogen receptor
ERK	extracellular-signal-regulated kinase
ERRα	estrogen-related receptor α
FFAR3	free fatty acid receptor 3
FoxO3	forkhead box O 3
GBM	glomerular basement membrane
GCN5	general control of amino-acid synthesis 5
GO	gene ontology
GPR	G-protein coupled receptor
HBM	HCF-binding motif
HCAR2	hydroxycarboxylic acid receptor 2
HCF	host cell factor
HDAC	histone deacetylase
HFD	high fat diet
HSF	heat shock transcription factor
Hsp	heat shock protein
IGF-1	insulin-like growth factor-1
ISMARA	integrated motif activity response analysis
KD	ketogenic diet
LXR	liver-X receptor
MAFbx	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MH	malignant hyperthermia
MKO	muscle-specific knockout mice
mTOR	mammalian target of rapamycin
MuRF-1	muscle RING finger-1
MyHC	myosin heavy chain
NAD	nicotinamide adenine dinucleotide

NAM	nicotinamide
Nfatc1	nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	nuclear factor-kappaB
PCr	phosphocreatine
PGC-1	PPARγ coactivator-1
PKA	cAMP-dependent protein kinase
PKO	podocyte-specific knockout mice
PPAR	peroxisome proliferator-activated receptor
PRC	PGC-related coactivator
RAAS	renin-angiotensin-aldosterone system
RRM	RNA-recognition motif
RS	Arg/Ser-rich domain
SIRT1	sirtuin 1
SRC-1	steroid receptor coactivator-1
SREBP	sterol regulatory element-binding protein
TF	transcription factor
TNF	tumor necrosis factor
TWEAK	TNF-like weak inducer of apoptosis
UCP1	uncoupling protein 1
VEGF	vascular endothelial growth factor
WT	wildtype

1. Introduction

1.1 Metabolism and energy homeostasis

Metabolism is key for life and describes a highly organized network of chemical reactions to provide a continual input of free energy in living organisms. This energy is derived from the environment and is required to meet three main purposes: a) the execution of mechanical work in muscle contractions and cellular movements, b) the active transport of molecules and ions, and c) the synthesis of biomolecules. Phototrophs like plants obtain this energy through photosynthesis in which they convert light energy into chemical energy. Animals are categorized as chemotrophs that obtain energy by the oxidation of molecules generated by photosynthetic organisms.

Metabolism consists of energy-yielding and energy-requiring reactions that are interdependent on each other to convert a particular molecule into another one. There are many defined metabolic pathways in a cell (Figure 1), which generally can be categorized into catabolic and anabolic reactions. Catabolism transforms fuels into cellular energy while anabolic reactions rely on energy produced during catabolism to build complex molecules or to generate high energy states. Thus, whole body metabolism is regulated by energy intake and energy expenditure and integrates all cells, tissues and organs to meet the required energy needs.

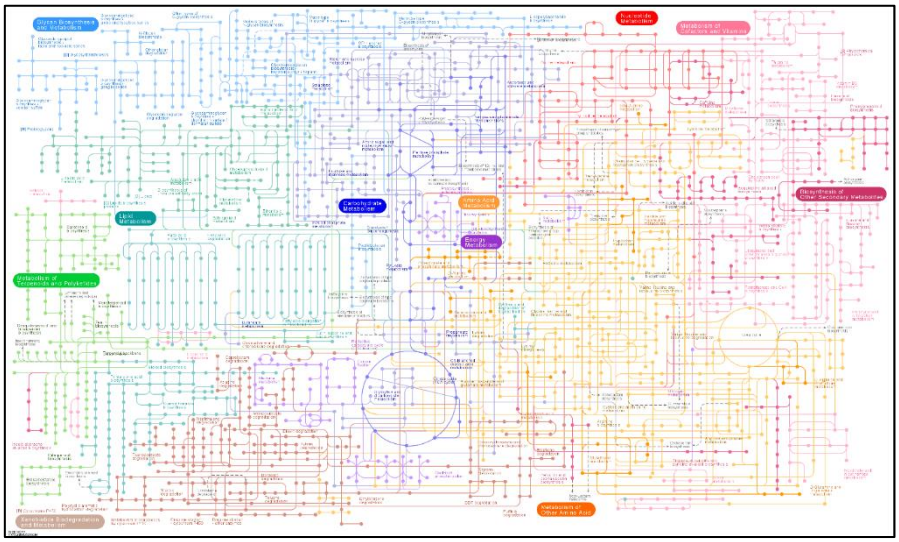

Figure 1 – The metabolic pathways *(from the Kyoto Encyclopedia of Genes and Genomes)*
Metabolism consists of a network of metabolic pathways, which convert molecule A into molecule B by linking series of chemical reactions.

1.2 Coregulators and their role in metabolism

The complex integration of internal and external cues takes place at different levels ranging from metabolic specializations of individual organs, to allosteric interactions and covalent modifications of proteins to adjustments of gene expression levels. Gene transcription is a highly regulated process in which the coordinated interaction between coregulators and transcription factors (TF) controls the expression of target genes. Coregulators thereby expand the complexity of the transcriptional regulation and function as metabolic sensors to ensure the fine-tuning of transcriptional events (Spiegelman and Heinrich 2004, Lonard and O'Malley B 2007, Mouchiroud, Eichner et al. 2014). Coregulators are not able to directly bind to the DNA themselves but interact with a variety of different TFs to induce expression of target genes. They can be broadly categorized into coactivators and corepressors promoting or inhibiting gene transcription, respectively, even though some coregulator proteins harbor both abilities (Lonard and O'Malley B 2007). In addition, some coregulators possess an intrinsic enzymatic activity to modify histones and thereby change the nature of the chromatin structure while others serve as recruiting platforms for different molecules to build large transcriptional complexes. Furthermore, coregulators are highly regulated and can be the primary target of hormonal control and signal transduction pathways (Spiegelman and Heinrich 2004). To date, over 450 different coregulator proteins have been identified showing pleiotropic functions in the control of tissue-specific and whole body metabolism (Malovannaya, Lanz et al. 2011).

1.2.1 The PGC-1 family of transcriptional coactivators

The peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1) family consists of the three family members PGC-1α, PGC-1β and PGC-related coactivator (PRC) (Figure 2) and over the last twenty years, their regulation and biological function in tissue-specific and whole body metabolism have been studied in greatest detail (Puigserver, Wu et al. 1998, Andersson and Scarpulla 2001, Kressler, Schreiber et al. 2002, Lin, Puigserver et al. 2002).

PGC-1α, the first family member described, was discovered as a cold-responsive protein interacting with PPARγ to induce uncoupling protein 1 (UCP1) expression and thus regulating adaptive thermogenesis in brown adipose tissue (BAT) (Puigserver, Wu et al. 1998). PGC-1α belongs to the class of coactivators without intrinsic enzymatic activity but it has been shown to bind several histone acetyltransferase-harboring proteins such as cAMP-response element binding protein (CREB) binding protein (CBP), p300 and steroid receptor coactivator-1 (SRC-1), which acetylate histones and thereby remodel chromatin structures to facilitate gene activation (Puigserver, Adelmant et al. 1999). In addition, the C-terminal region of PGC-1α serves as a docking platform for proteins of the mediator complex (Wallberg, Yamamura et al. 2003). PGC-1α is a master integrator of external stimuli such as

fasting and exercise and is especially important in metabolic organs like the liver, brain, BAT, kidney and skeletal muscle (Puigserver, Wu et al. 1998, Larrouy, Vidal et al. 1999). Furthermore, PGC-1α is essential for mitochondrial function and oxidative phosphorylation, which are crucial for fatty acid oxidation and cellular energy metabolism (Lin, Handschin et al. 2005). In addition, PGC-1α is also vitally for tissue-specific metabolic actions such as the above mentioned adaptive thermogenesis in BAT (Puigserver, Wu et al. 1998, Tiraby, Tavernier et al. 2003), hepatic gluconeogenesis and ketogenesis (Herzig, Long et al. 2001, Yoon, Puigserver et al. 2001, Rhee, Inoue et al. 2003) and the switch from glycolytic fast-twitch muscle fibers towards more oxidative slow-twitch fibers in skeletal muscle (Lin, Wu et al. 2002).

Even though PGC-1β shares extensive sequence identities with PGC-1α, especially in the N-terminal activation domain (AD) and the C-terminal RNA binding domain (RRM) (Figure 2), its role in regulating metabolism is less well studied and understood (Kressler, Schreiber et al. 2002, Lin, Puigserver et al. 2002). PGC-1β was originally identified as a protein interacting with nuclear receptors like PPARα and estrogen receptor α (ERα) as well as with host cell factor (HCF) and was thus implicated in the regulation of hepatic fasting, viral infection and cell proliferation (Kressler, Schreiber et al. 2002, Lin, Puigserver et al. 2002). Like PGC-1α, PGC-1β is involved in mitochondrial biogenesis and cellular respiration in different tissues and fasting induces its expression in the liver (Lin, Puigserver et al. 2002, Lin, Tarr et al. 2003, St-Pierre, Lin et al. 2003). Furthermore, hepatic PGC-1β expression is also induced upon short-term high fat diet (HFD) feeding to coactivate the sterol regulatory element-binding protein (SREBP) and liver-X receptor (LXR) families of transcription factors regulating *de novo* lipogenesis and lipoprotein secretion (Lin, Yang et al. 2005).

The third member of the PGC-1 family, PRC, is ubiquitously expressed and induced upon the initiation of cell proliferation. Furthermore, PRC is able to respond to metabolic stress by promoting the expression of genes involved in inflammation, proliferation and metabolic reprogramming and has been described as a mitochondrial biogenesis regulator (Andersson and Scarpulla 2001, Vercauteren, Pasko et al. 2006, Vercauteren, Gleyzer et al. 2009, Philp, Belew et al. 2011, Gleyzer and Scarpulla 2013).

As this thesis is aimed at elucidating the role of the PGC-1 family of transcriptional coactivators in skeletal muscle and kidney metabolism, the remainder of the introduction will focus on the regulation of PGC-1α and –β in these two tissues.

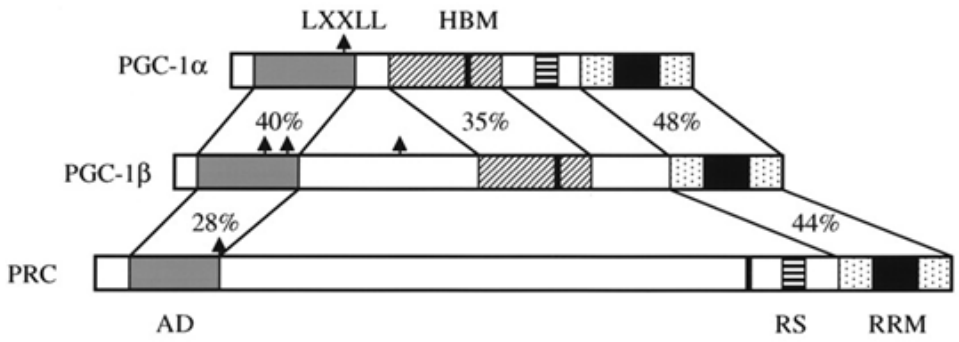

Figure 2 – The PGC-1 family of transcriptional coactivators

Gene structure of the three PGC-1 family members PGC-1α, -β and PRC. Conserved domains/motifs are indicated including the activation domain (AD), LXXLL motifs, host cell factor (HCF)-binding motif (HBM), Arg/Ser-rich domain (RS) and RNA-recognition motif (RRM) (Lin, Puigserver et al. 2002).

1.3 Skeletal muscle metabolism

Skeletal muscle is one of the most abundant and metabolically active tissues in mammals, accounting for approximately 40-50% of total body mass. Its main purpose is to generate force in order to facilitate movement of the body. Skeletal muscle is composed of myofibers with different functional and metabolic characteristics, e.g. slow-oxidative and fast-glycolytic fibers. Oxidative fibers appear red in color and are characterized by slow-twitch properties and a high mitochondrial number, oxidative capacity and resistance to fatigue. Slow fibers are also referred to as type I fibers, which mainly express the myosin heavy chain-I (MyHC-I) (Schiaffino and Reggiani 2011). The hallmarks of glycolytic fibers are a low mitochondrial number and endurance as well as fast-twitch contraction kinetics and high peak force. Fast fibers are distinguished as type IIA, IIB and IIX fibers specifically expressing MyHC-IIA, MyHC-IIB and MyHC-IIX, respectively, in rodents while MyHC-IIB is missing in human muscles (Schiaffino and Reggiani 2011). Moreover, type IIA fibers show intermediate properties between slow type I and fast type IIB/X fibers (Schiaffino and Reggiani 2011). These differences in fiber types facilitate a high degree of muscle plasticity to ensure the tight regulation of a variety of activities, from supporting the skeleton to stand upright, to performing explosive movements as during a sprint. Furthermore, external stimuli, such as the specific training of muscle tissue, can change the composition of fiber types, which is reflected by the fact that successful endurance athletes have relatively more slow-twitch fibers while the muscles of sprinters are predominantly composed of fast-twitch fibers (Costill, Daniels et al. 1976, Fink, Costill et al. 1977, Saltin, Henriksson et al. 1977).

Therefore, cellular metabolism within muscle tissue must be precisely regulated in order to ensure sufficient supply of energy for muscle movements. ATP is the main immediate free-energy donor during muscle contractions, however, intracellular ATP stores in human skeletal muscle are rather small (5-6 mM) (Sahlin, Tonkonogi et al. 1998). Accordingly, immediately available ATP is quickly depleted upon the onset of exercise and must continually be re-synthesized to ensure prolonged muscle contractions.

At high exercise intensities, also referred to as anaerobic metabolism, ATP is primarily regenerated through the breakdown of phosphocreatine (PCr) and muscle glycogen (Figure 3). Creatine kinase catalyzes the reaction in which PCr and ADP interact to yield creatine and ATP. Glycogen breakdown is much more complex and regulated by glycogen phosphorylase, which gets phosphorylated and activated by the action of phosphorylase kinase and dephosphorylated and inactivated by protein phosphatase 1 (Johnson 2009). The released glucose enters glycolysis and is converted to pyruvate, which ultimately is further oxidized to lactate by the action of lactate dehydrogenase (Westerblad, Bruton et al. 2010). The accumulation of lactate thus only occurs when oxygen availability is low and

the demand of ATP is high. The resulting lactate can then be converted back to glucose in the liver in a process known as the Cori cycle, which shifts the metabolic burden of the muscle to the liver.

In contrast to anaerobic metabolism, the generation of ATP through the complete oxidation of carbohydrates and lipids to CO_2 relies on sufficient oxygen supply and occurs at a much slower rate (Figure 3). In addition, amino acids from the breakdown of muscle proteins can be used for aerobic metabolism, however, this contributes only minor to the overall energy metabolism in skeletal muscle, especially, when carbohydrates are available (Lemon and Mullin 1980). Oxidative metabolism mainly uses glucose (from intramuscular glycogen stores but also from the circulation) and free fatty acids (from intramuscular and adipose tissue triglyceride stores) for the conversion into acetyl-CoA, which is then fed into the citric acid cycle inside the mitochondrial matrix. This cycle is important to generate NADH and $FADH_2$ in order to build a proton gradient across the inner mitochondrial membrane. The release of energy from the diffusion of protons back into the mitochondrial matrix is coupled to ATP production through the ATP synthase enzyme (Westerblad, Bruton et al. 2010). In contrast to anaerobic metabolism, which only yields 3 ATP molecules per glucose molecule, oxidative metabolism releases around 38 molecules of ATP per glucose molecule and is thus much more energy efficient (Egan, Hawley et al. 2016).

At rest, skeletal muscle only contributes to around 20-30% of the whole body energy expenditure, however, during maximal physical activity, oxygen consumption can dramatically increase up to 90% of the whole body oxygen uptake (Zurlo, Larson et al. 1990). Not surprisingly, muscle tissue thus shows an enormous capacity to adapt to different energy needs and substantially contributes to the regulation of whole body energy metabolism.

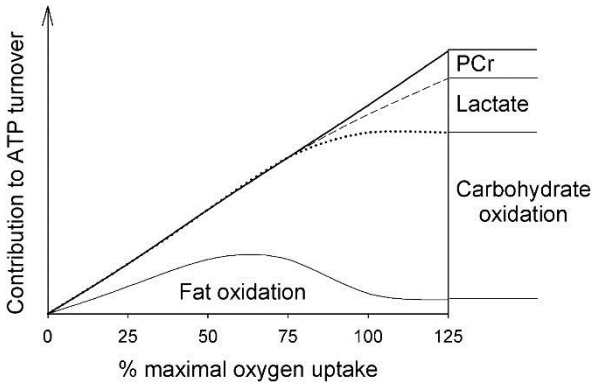

Figure 3 – Energy substrates and pathways as a function of exercise intensity in human leg muscles during upright cycling

At high exercise intensities phosphocreatine (PCr) and lactate are major contributors of ATP production while carbohydrate and fat oxidation become more important at moderate or low exercise intensities, respectively (Sahlin 1986, Westerblad, Bruton et al. 2010).

Skeletal muscle not only ensures movement and thus plays a major role in whole body energy expenditure but it also constitutes one of the largest energy stores in form of triglycerides, glycogen and proteins. These energy stores are of crucial importance during increased energy expenditure, as described above during exercise, but also during decreased energy intake, as during periods of fasting. If blood glucose and insulin concentrations drop, glucagon gets released from the pancreas to trigger hepatic glycogenolysis and gluconeogenesis to liberate *de-novo* produced glucose from the liver (Briant, Salehi et al. 2016). Consequently, the reduction in glucose availability and insulin release lead to an increase in lipolysis in white adipose tissue and fatty acid oxidation in skeletal muscle, respectively (Holness and Sugden 1990). If fasting is prolonged, liver glycogen stores get depleted and hepatic gluconeogenesis is solely fueled by glycerol from lipolysis in adipose tissue as well as from the catabolism of muscle proteins, ensuring whole body glucose homeostasis (Longo and Mattson 2014). Furthermore, the liver starts to produce ketone bodies like β-hydroxybutyrate (β-OHB) and acetoacetate in a process called ketogenesis (Krebs 1966). The use of circulating ketone bodies by extra-hepatic tissues, such as skeletal muscle and the brain, largely diminishes the need of glucose, which minimizes the loss of muscle protein and functionality and maintains cognitive function (Longo and Mattson 2014). Ketone bodies, as carbohydrates and fatty acids during oxidative metabolism, get metabolized to acetyl-CoA, which is fed into the citric acid cycle to generate energy for cellular processes.

Thus, skeletal muscle emerges as a tissue with pleiotropic functions in whole body metabolism, ranging from the production of force to generate movements, to substrate handling to adapt to a variety of different energy needs.

1.3.1 Skeletal muscle plasticity

Skeletal muscle is a highly plastic tissue and shows enormous potential to adapt its contractile and metabolic properties according to external stimuli. Different pathological conditions like cancer, heart, liver or renal failure, chronic obstructive pulmonary disease and AIDS profoundly contribute to the phenotype of skeletal muscle. Muscle wasting or atrophy is a common feature of these diseases, which also occurs during non-pathological conditions such as aging or fasting (Glass and Roubenoff 2010, Bonaldo and Sandri 2013). Atrophy is defined as a decrease in muscle size due to cellular shrinkage, which includes the loss of organelles, cytoplasm and proteins. Muscle mass strongly depends on protein and cell turnover and is the net result between protein synthesis and degradation. Accordingly, muscle atrophy occurs when the balance is shifted towards increased proteolysis, which is tightly regulated by different cell signaling cascades (Bonaldo and Sandri 2013, Egerman and Glass 2014). Importantly, the progression of the above mentioned diseases is strongly associated with excessive

muscle mass loss, which is a poor prognostic indicator ultimately increasing morbidity and mortality (Glass and Roubenoff 2010, Bonaldo and Sandri 2013). Thus, preservation of muscle mass, metabolism and aerobic fitness is fundamental for health and normal organ function and in fact, physical activity has been shown to lower the risk of different metabolic diseases including obesity or type 2 diabetes but also of cancer or skeletal muscle wasting (Haskell, Lee et al. 2007, Colberg, Sigal et al. 2010, Egan and Zierath 2013). Therefore, physical activity is another stimulus that largely contributes to skeletal muscle remodeling and whole body homeostasis.

Exercise-induced alterations in muscle phenotype include changes in contractile protein and function (e.g. MyHC composition), mitochondrial and metabolic adaptations as well as modifications of signal transduction pathways and transcriptional responses (Green, Helyar et al. 1992, Adams, Hather et al. 1993, Spina, Chi et al. 1996, Benziane, Burton et al. 2008, Kupr, Schnyder et al. 2017, Schnyder, Kupr et al. 2017). It is believed that each acute exercise bout elicits a transient peak in the expression level of certain genes, which over time cumulatively increases protein content and enzyme activity and finally results in improved exercise performance and whole body metabolism (Figure 4) (Egan and Zierath 2013). However, it is important to note that different exercise modalities, e.g. endurance versus resistance, but also the frequency, intensity and duration of the training lead to different metabolic and molecular skeletal muscle adaptations (Coffey, Shield et al. 2006, Coffey, Zhong et al. 2006).

Endurance training is characterized as a low-load exercise modality, which is performed during a long period of time and during which energy is derived through oxidative metabolism of carbohydrates and fatty acids. Thus, endurance training effectively modifies and increases muscle capillarisation, mitochondrial metabolism, oxidative function, lactate tolerance and anaerobic and endurance capacity (Egan and Zierath 2013). On the other hand, resistance training, also known as hypertrophy exercise, imposes a high-load demand on muscular contraction but only for a short period of time. This leads to a higher muscle mass with increased muscle fiber size, strength and power as well as to an elevation of the basal metabolic rate (Egan and Zierath 2013). Thus, resistance exercise is thought to be an effective therapeutic strategy for the treatment of muscle wasting (Glass and Roubenoff 2010, Egan and Zierath 2013). Furthermore, even though both exercise modalities exert health promoting effects, a combination of both, endurance and resistance training, emerges as the most effective in the treatment of metabolic diseases such as insulin resistance, obesity, the metabolic syndrome or type 2 diabetes (Sigal, Kenny et al. 2007, Davidson, Hudson et al. 2009).

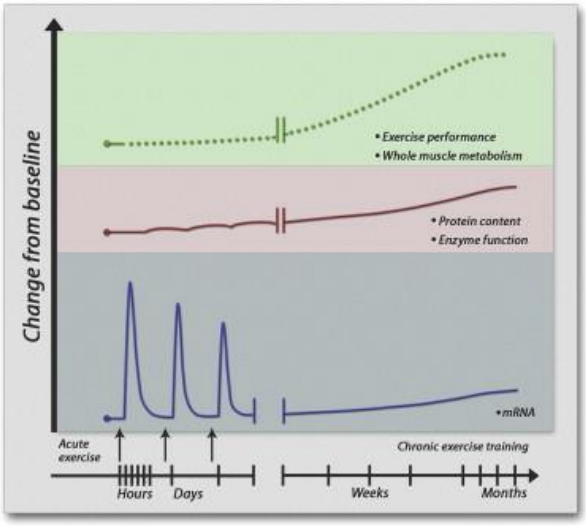

Figure 4 – Skeletal muscle adaptations to acute and chronic exercise training

Acute exercise bouts result in the transient increase in mRNA expression levels of exercise-responsive genes. Upon chronic exercise training these repeated peaks in expression changes cumulate and lead to a modest but gradual accumulation of protein, which over time results in improved exercise performance and whole body metabolism (Egan and Zierath 2013).

1.3.2 Regulation of PGC-1α/β expression and activity in skeletal muscle metabolism

Skeletal muscle adaptations to external stimuli are often coordinated at the gene expression level, in which the PGC-1 family plays a prominent role controlling skeletal muscle metabolism and plasticity (St-Pierre, Lin et al. 2003). Importantly, endurance and resistance exercise are able to stimulate the expression of the full-length PGC-1α1 and the PGC-1α4 isoform, respectively (Pilegaard, Saltin et al. 2003, Ruas, White et al. 2012), indicating that different exercise regimens can induce various PGC-1α isoforms with specific biological functions (Miura, Kai et al. 2008, Ruas, White et al. 2012, Wen, Wu et al. 2014). Interestingly, many of the exercise-induced pathways converge on PGC-1α to either increase its expression, protein stability or transcriptional activity, which makes it a master regulator of skeletal muscle plasticity (Correia, Ferreira et al. 2015). PGC-1α expression is regulated by calcium signaling via the action of the calcium/calmodulin-dependent protein kinase IV (CaMKIV) and calcineurin A (CnA), which involves the two transcription factors CREB and myocyte enhancer factor 2 (MEF2) (Wu, Kanatous et al. 2002, Handschin, Rhee et al. 2003). Furthermore, mRNA levels of PGC-1α are increased through the activation of the p38 mitogen-activated protein kinase (p38 MAPK) involving the downstream transcription factor activating transcription factor 2 (ATF2) and the AMP-dependent protein kinase (AMPK), which is largely dependent on the function of the PGC-1α protein (Akimoto, Pohnert et al. 2005, Jager, Handschin et al. 2007). In addition, exercise not only controls the expression of PGC-1α through different pathways but also its post-translational modifications. For example,

transcriptional activity of PGC-1α is enhanced by phosphorylation through p38 MAPK (Puigserver, Rhee et al. 2001) and AMPK (Jager, Handschin et al. 2007) and deacetylation through sirtuin 1 (SIRT1) (Canto, Gerhart-Hines et al. 2009), whereas the acetylase general control of amino-acid synthesis 5 (GCN5) counteracts the activation by SIRT1 (Lerin, Rodgers et al. 2006). Furthermore, acute exercise in human skeletal muscle has been shown to hypomethylate the promoters of PGC-1α and other exercise-responsive genes, indicating that dynamic changes in DNA methylation can also contribute to gene activation (Barres, Yan et al. 2012). Moreover, other upstream regulators of PGC-1α expression and protein activity have been identified, however, their role in exercise-mediated control of PGC-1α has not been studied so far (Fernandez-Marcos and Auwerx 2011, Kupr and Handschin 2015). In addition, PGC-1β expression is not affected or even reduced by exercise training, demonstrating that even though PGC-1α and –β share extensive sequence similarities, their role and biological function are clearly distinct (Koves, Li et al. 2005, Mortensen, Plomgaard et al. 2007).

Interestingly, while only PGC-1α is controlled by exercise-induced signaling cascades, both PGC-1 family members seem to contribute to the maintenance of skeletal muscle mass. Accordingly, disuse muscle atrophy in old individuals leads to marked reduction in the expression levels of PGC-1α and –β (Suetta, Frandsen et al. 2012). Even though the mechanisms that lead to this downregulation of PGC-1 expression are poorly understood, the tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) has been shown to significantly reduce the mRNA levels of PGC-1α by binding to Fn14, a member of the TNF-receptor superfamily (Hindi, Mishra et al. 2014). If this pathway also leads to the downregulation of PGC-1β expression remains to be elucidated by future studies.

1.3.3 PGC-1α/β in the regulation of skeletal muscle plasticity

As described, regulation of PGC-1 expression and activity is tightly controlled by a variety of pathways that profoundly contribute to skeletal muscle plasticity. Both coactivators have been implicated in regulating skeletal muscle oxidative metabolism, e.g. overexpression of PGC-1α and PGC-1β drives the formation of slow-twitch MyHC type I and IIA or MyHC type IIX fibers, respectively (Lin, Wu et al. 2002, Arany, Lebrasseur et al. 2007), while the knockout of either PGC-1 family member results in a shift towards more fast-twitch glycolytic fibers (Handschin, Chin et al. 2007, Rowe, Patten et al. 2013, Gali Ramamoorthy, Laverny et al. 2015). In addition, both PGC-1 coactivators are of crucial importance for the expression of genes involved in oxidative phosphorylation and single as well as total ablation of PGC-1α/β in skeletal muscle drastically diminishes mitochondrial respiration resulting in severe defects in exercise capacity (Handschin, Chin et al. 2007, Rowe, Patten et al. 2013, Gali Ramamoorthy, Laverny et al. 2015). On the other hand, overexpression of either PGC-1 family member results in improved oxidative capacity and increased exercise performance (Lin, Wu et al. 2002, Arany, Lebrasseur et al.

2007, Lee, Leone et al. 2017). Furthermore, PGC-1α and –β are involved in the regulation of angiogenesis, through coactivation of estrogen-related receptor α (ERRα), inducing the expression of vascular endothelial growth factor (VEGF) (Arany, Foo et al. 2008, Chinsomboon, Ruas et al. 2009, Rowe, Jang et al. 2011). Thus, even though only PGC-1α seems to be controlled by exercise, both coactivators profoundly contribute to skeletal muscle oxidative capacity and endurance performance. In addition, PGC-1α has been described to be involved in a variety of other processes regulating skeletal muscle plasticity, such as calcium and lipid handling (Summermatter, Baum et al. 2010, Summermatter, Thurnheer et al. 2012) as well as glycogen, lactate and ketone body homeostasis (Wende, Schaeffer et al. 2007, Summermatter, Santos et al. 2013, Svensson, Albert et al. 2016), while the role of PGC-1β in these contexts has not been studied so far.

PGC-1 coactivators are not only important for mitochondrial and oxidative metabolism but also contribute to the maintenance of skeletal muscle mass. Muscle-specific overexpression of PGC-1α4, a recently identified isoform of the PGC-1α family, leads to hypertrophy and increased muscle strength while it reduces muscle mass loss caused by hindlimb suspension or cancer cachexia (Ruas, White et al. 2012). It does so by activating the expression of insulin-like growth factor-1 (IGF-1) and suppressing myostatin gene transcription (Ruas, White et al. 2012). Moreover, it also stimulates the expression of G protein-coupled receptor 56 (GPR56) and its ligand collagen III, inducing mammalian target of rapamycin (mTOR) signaling, which promotes cellular hypertrophy in cultured myotubes (White, Wrann et al. 2014). Furthermore, overexpression of PGC-1α or –β in skeletal muscle has been shown to protect from fiber atrophy and proteolysis mainly by reducing forkhead box O 3 (FoxO3) and nuclear factor-kappaB (NF-κB)-dependent transcription of target genes (Sandri, Lin et al. 2006, Brault, Jespersen et al. 2010). Importantly, FoxO transcription factors activate the expression of two essential E3 ubiquitin ligases, muscle atrophy F-box (MAFbx) and muscle RING finger-1 (MuRF-1), so called atrogenes, that have been identified as key regulators of muscle atrophy (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001, Sandri, Sandri et al. 2004, Stitt, Drujan et al. 2004). Even though overexpression of PGC-1α and PGC-1β protects against muscle wasting, the loss of either PGC-1 family member does not increase the propensity for muscle atrophy. For example, muscle-specific PGC-1α knockout mice are not more susceptible to denervation-induced fiber atrophy showing unchanged muscle weights and atrogenes expression compared to control mice (Handschin, Chin et al. 2007). Also, ablation of PGC-1β specifically in muscle tissue does not affect muscle mass or myofiber structure and cross-sectional area (Gali Ramamoorthy, Laverny et al. 2015).

To conclude, PGC-1 coactivators are versatile proteins that substantially contribute to skeletal muscle plasticity being involved in mitochondrial and oxidative metabolism as well as protein degradation through transcriptional activation and repression.

1.4 Kidney metabolism

The kidneys are two bean-shaped organs lying in the abdominal cavity at each side of the spine. They are essential for whole body metabolism regulating blood pressure, acid-base balance, electrolyte homeostasis and the excretion of waste products such as urea. The renal cortex is the outer zone of the kidney and the renal medulla is the inner zone. Each kidney is composed of around 900'000 – 1 Mio. of nephrons, the functional unit of the kidney (Bertram, Douglas-Denton et al. 2011). A nephron consists of the glomerulus and the associated tubule that connects to the collecting duct. The glomerulus is the basic filtering unit of the nephron, which is composed of a network of capillaries that is surrounded by the Bowman's capsule into which the blood plasma is filtered (Figure 5). Blood enters the glomerular capillaries from an afferent arteriole and leaves through an efferent arteriole. Capillaries are lined by fenestrated endothelial cells that allow the free filtration of plasma and solutes while larger molecules, such as red blood cells, cannot pass through. The endothelium is followed by the glomerular basement membrane (GBM), a specialized extracellular matrix, that represents another filtration barrier for bigger proteins like albumin (Miner 2012, Suh and Miner 2013). Podocytes sit on the opposite side of the GBM forming large projections, the so called foot processes, connected by the slit diaphragm. These three-layered structure facilitates the efficient flow of water and small solutes into the Bowman's space while the retention of bigger molecules and proteins is ensured. The glomerulus also contains mesangial cells that have contractile and phagocytic properties.

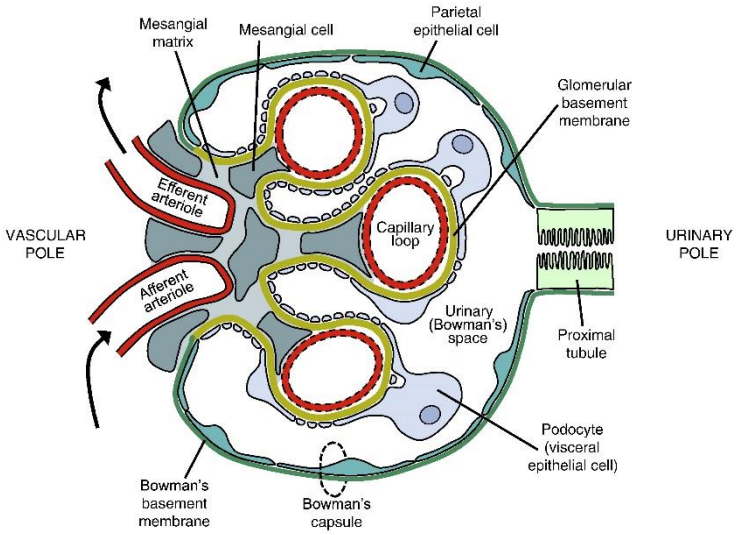

Figure 5 – The glomerulus and the composition of the glomerular filtration barrier

Schematic representation of the glomerulus and the glomerular filtration barrier. Blood enters the glomerulus through an affarent arteriole and leaves through an efferent arteriole. It is filtered through a barrier composed of three layers: the fenestrated endothelium, the glomerular basement membrane (GBM) and the podocytes with foot processes (Leeuwis, Nguyen et al. 2010).

The ultrafiltrate, produced by the glomerulus, passes into the tubules where its volume and content is altered by reabsorption or secretion. Most solute and water reabsorption occurs in the proximal tubule consisting of the proximal convoluted and the proximal straight tubule that connect the Bowman's capsule to the loop of Henle (Curthoys and Moe 2014). Sodium transport at the apical membrane is passive and often coupled to chloride, glucose or amino acid symport as well as to hydrogen ion antiport (Aronson and Sacktor 1975, Bobulescu and Moe 2006). Importantly, apical H^+-secretion generates bicarbonate that exits the basolateral membrane by a sodium-bicarbonate cotransport, which substantially contributes to the whole body acid-base balance (Boron and Boulpaep 1983, Schmitt, Biemesderfer et al. 1999). At the basolateral membrane, the export of sodium is actively driven by Na^+-K^+-ATPases while water moves osmotically with the reabsorbed solutes so that the proximal fluid remains almost isoosmotic to plasma (Curthoys and Moe 2014). Potassium is reabsorbed para- and transcellularly by the activity of a luminal K^+-pump (Giebisch 1998). The straight proximal tubule connects to the loop of Henle composed of the thin descending limb, the thin ascending limb and the thick ascending limb. The thin descending limb is permeable to water but not to sodiumchloride while the thin ascending limb is permeable to sodiumchloride but not to water. These differences in permeability allow the generation of a concentrated medullary interstitium and produce a diluted fluid. The final reabsorption of sodium and potassium takes place in the thick ascending limb of the loop of Henle as well as in the distal tubule and the collecting ducts. Thus, excreted urine is formed by three main processes: glomerular filtration, tubular reabsorption and tubular secretion.

In total, about 99% of the filtered sodium and water is being reabsorbed, mostly by active transport, which requires a lot of energy in form of ATP (O'Connor 2006). Thus, it is not surprising that the kidneys are second to the heart in terms of oxygen consumption and mitochondrial number (O'Connor 2006, Parikh, Yang et al. 2015). Especially, segments of the nephron with a high abundance of Na^+-K^+-ATPases, such as the proximale tubule and the thick ascending limb of Henle's loop, are densely packed with mitochondria (Parikh, Yang et al. 2015). Furthermore, the proximal tubule mainly relies on oxidative phosphorylation to generate energy and emerges as the only place where gluconeogenesis occurs as evidenced by the presence of gluconeogenic enzymes (Vandewalle, Wirthensohn et al. 1981). On the other hand, more distal nephron segments have been shown to produce large amounts of lactate suggesting that anaerobic glycolysis may contribute substantially to the production of ATP in these parts of the kidney (Bagnasco, Good et al. 1985, Soltoff 1986). Thus, kidney is an important player in the regulation of whole body glucose homeostasis by using glucose as an energy substrate for aerobic and anaerobic metabolism, by reabsorbing glucose from the primary filtrate and by releasing *de-novo* produced glucose from renal gluconeogenesis (Gerich 2010).

Next to its role in fluid, salt and glucose homeostasis, the kidney is an important endocrine organ and secretes various hormones such as renin, erythropoietin (EPO), 1,25 dihydroxy vitamin D3, kallikrein

and eicosanoids. Furthermore, the kidney is also the primary target of various hormones like aldosterone, angiotensin and vasopressin (Sahay, Kalra et al. 2012). The renin-angiotensin-aldosterone system (RAAS) is essential in the control of blood pressure and begins with the release of renin from the juxtaglomerular apparatus in the kidney, whenever there is a drop in blood pressure or body fluid volume. Renin leads to the cleavage of angiotensinogen to angiotensin I and via the angiotensin converting enzyme to angiotensin II. The latter leads to vasoconstriction of capillaries and the release of aldosterone from the adrenal cortex, which increases sodium and water retention in the nephron (Brewster and Perazella 2004). Furthermore, angiotensin II promotes the feeling of thirst and leads to the liberation of vasopressin from the brain, which increases the rate of water reabsorption by the distal tubule and the collecting duct through exocytosis of the water channel molecule aquaporin (Nielsen, DiGiovanni et al. 1993). All of these pathways of the RAAS finally lead to an increase in blood pressure and thereby contribute to the regulation of whole body homeostasis.

EPO is another important hormone that is mainly synthesized in the kidney, which controls erythrocyte differentiation (Jacobson, Goldwasser et al. 2000, Foley 2008). Because of its stimulating effect on red blood cell production, recombinant human EPO has been produced for the use in therapeutic settings, however, it has also been misused in sports in the form of doping (Salamin, Kuuranne et al. 2017).

Thus, the kidney is a highly active organ that substantially contributes to whole body metabolism and homeostasis.

1.4.1 Kidney plasticity

The kidney is a highly complex organ with a huge number of different cell types and disturbances in metabolic function and cellular homeostasis in either of them can contribute to the development and progression of a variety of pathological conditions. Regardless of the cause, kidney diseases are characterized by a loss of kidney function and are generally classified into acute, which develop within seven days, and chronic kidney diseases (CKD), which develop over three or more months (Levey, Coresh et al. 2003, Mehta, Kellum et al. 2007). CKD is an emerging health problem in the Western World and several risk factors including race, gender, age, family history but also diabetes and hypertension can contribute to the development of CKD (Snyder and Pendergraph 2005, Kazancioglu 2013). Nowadays, it is believed that in response to injury, renal cells can undergo an epithelial–mesenchymal trans-differentiation (EMT), meaning, that they regress from an adult, mature phenotype to an embryonic one. This phenotypic change, together with inflammatory processes and remodeling of the extracellular matrix, is involved in tissue scarring and the progression of CKD (El-Nahas 2003, El Nahas 2003). Furthermore, oxidative stress and mitochondrial dysfunction have been observed in patients with CKD suggesting that mitochondria are also involved in the development and

progression of CKD (Granata, Zaza et al. 2009, Small, Coombes et al. 2012). In fact, mitochondrial dysfunction, due to a decrease in mitochondrial DNA copy number, loss of mitochondrial membrane potential and reduced ATP production, is an early event in aldosterone-induced podocyte injury (Su, Dhoopun et al. 2013). The latter is known to induce proteinuria that eventually induces tubular epithelial cell apoptosis and EMT, which finally progresses to CKD (Nangaku 2004). Emerging evidence suggest that mitochondrial dysfunction, for example through increased oxidative stress, might be involved in many other renal diseases as in diabetic nephropathy, a CKD initiated by diabetes mellitus (Kdoqi 2007, Che, Yuan et al. 2014, Higgins and Coughlan 2014, Granata, Dalla Gassa et al. 2015). However, future studies are needed to determine the impact of mitochondrial dysfunction on renal physiology and pathophysiology. To conclude, CKD develops and progresses through different mechanisms involving phenotypic changes in renal cells, inflammation, extracellular matrix remodeling and mitochondrial dysfunction.

1.4.2 Regulation of PGC-1α expression and activity in kidney metabolism

Mitochondrial function and oxidative respiration are essential for kidney metabolism, which is also reflected by the high basal expression of PGC-1α (Puigserver, Wu et al. 1998, Larrouy, Vidal et al. 1999). As in many other pathological conditions in different organs, PGC-1α expression is downregulated, for example in patients with CKD, which seems to negatively correlate with plasma levels of malondialdehyde, a product of oxidative stress-induced lipid damage (Zaza, Granata et al. 2013, Elsayed, Nassra et al. 2017). Furthermore, aldosterone-induced podocyte injury decreases PGC-1α mRNA levels and induces mitochondrial damage (Yuan, Huang et al. 2012). Sepsis-associated acute kidney injury promotes renal dysfunction and suppresses PGC-1α leading to the downregulation of oxidative phosphorylation genes (Tran, Tam et al. 2011). In addition, transient local induction of ischemia impairs renal function, induces renal steatosis and reduces PGC-1α expression (Tran, Zsengeller et al. 2016). As in skeletal muscle, the inflammatory cytokine TWEAK has been shown to decrease renal PGC-1α expression through NF-κB activation and histone deacetylation leading to impaired mitochondrial function (Ruiz-Andres, Suarez-Alvarez et al. 2016). Furthermore, PGC-1α expression also seems to be regulated by extracellular-signal-regulated kinase 1/2 (ERK1/2) that phosphorylates FoxO3a/1 inactivating and preventing it from increasing PGC-1α expression (Collier, Whitaker et al. 2016). Interestingly, ERK1/2 is stimulated by epidermal growth factor receptor (EGFR), which itself has been shown to be transactivated by TWEAK (Rayego-Mateos, Morgado-Pascual et al. 2013). Thus, TWEAK seems to be a potent negative regulator of PGC-1α expression in different tissues including skeletal muscle and kidney.

As downregulation of PGC-1α expression and reduced protein activity are evident in many renal diseases, numerous studies focused on the prevention of this deregulation to improve mitochondrial and kidney functions. Fenofibrate treatment ameliorates diabetic nephropathy through activation of the AMPK-PGC-1α axis in *db/db* mice and renal lipotoxicity and hypertension through stimulation of the PPARα-FoxO3a-PGC-1α pathway in HFD fed rats, respectively (Chung, Lim et al. 2012, Hong, Lim et al. 2014). Moreover, resveratrol, a proposed SIRT1 activator, has been shown to protect from aldosterone-induced podocyte injury, renal lipotoxicity and mesangial cell glucotoxicity by activating PGC-1α (Yuan, Huang et al. 2012, Kim, Lim et al. 2013). Thus, PGC-1α is an essential player in mitochondrial homeostasis and oxidative metabolism in renal physiology and stimulation of PGC-1α activity and expression shows potential in the treatment of different kidney pathologies.

1.4.3 PGC-1α in the regulation of kidney plasticity

Even though PGC-1α is important for kidney metabolism and homeostasis, it seems to be dispensable for basal renal function, however, necessary for the recovery from different kidney disorders. For example, global– and proximal tubule-specific PGC-1α knockout mice show an impaired ability to recover from sepsis-induced acute kidney injury (Tran, Tam et al. 2011). Furthermore, overexpression of PGC-1α or SIRT1 in podocytes protects from aldosterone-induced cell damage, depletion and mitochondrial dysfunction (Yuan, Huang et al. 2012, Zhao, Yuan et al. 2016). In addition, inducible nephron-specific PGC-1α knockout mice show a mild salt-losing phenotype with elevated urinary sodium excretion and display impaired renal steatosis upon HFD treatment (Svensson, Schnyder et al. 2016). Recently, PGC-1α has been shown to regulate renal nicotinamide adenine dinucleotide (NAD) biosynthesis (Tran, Zsengeller et al. 2016). Following ischemia, global PGC-1α knockout mice exhibited exacerbated renal function, lipid accumulation and tubular injury, which could be prevented by the supplementation with nicotinamide (NAM), a precursor for the synthesis of NAD (Tran, Zsengeller et al. 2016). Furthermore, overexpression of PGC-1α in tubular epithelial cells blunted the response to ischemia and better preserved kidney function (Tran, Zsengeller et al. 2016). These results clearly demonstrate that PGC-1α is a potent mediator of renal stress resistance and strongly suggest that PGC-1α might be a valuable target in the treatment of renal diseases.

2. Aims of the thesis

Coregulator proteins have emerged as important players in the control of gene transcription and exert essential cellular functions as metabolic sensor and effector proteins. During this thesis, we particularly focused on the regulation of the PGC-1 family of transcriptional coactivators and their role in tissue-specific metabolism and plasticity. Using different mouse knockout models, molecular biology techniques including mRNA sequencing and cell culture approaches, we elucidated the role of PGC-1α and PGC-1β in skeletal muscle metabolism under various stress conditions. Furthermore, we determined the function of PGC-1α in podocyte biology and its contribution to renal physiology. Thus, during the course of my thesis, we tried to achieve the following aims:

1. Define the role of PGC-1α in the regulation of muscle and whole body metabolism in response to long-term ketogenic diet feeding.

Ketogenic diets have emerged as potent therapeutic interventions in the treatment of epilepsy as well as of metabolic disorders such as obesity or type 2 diabetes. Furthermore, PGC-1α has recently been identified as an important modulator of systemic ketone body homeostasis through the regulation of ketolytic gene expression in skeletal muscle. This prompted us to conduct a follow-up study in order to elucidate the role of muscle PGC-1α in the regulation of the physiological adaptations to a long-term ketogenic diet, which is described in chapter 3 of this thesis.

2. Elucidate the acute time-course specific and chronic transcriptional changes upon exercise and their dependence on skeletal muscle PGC-1α.

PGC-1α is an important key player in mediating exercise-induced skeletal muscle adaptations. As a transcriptional coactivator, PGC-1α is thus required for the expression of target genes. In chapter 4 of this thesis, we defined the acute time-course and chronic transcriptional changes induced by exercise and the involvement of PGC-1α in these contexts.

3. Determine if PGC-1β is involved in the fasting response in skeletal muscle and thereby elucidate how PGC-1β expression is regulated. Furthermore, define the PGC-1β-dependent and –independent fasting response in skeletal muscle and whole body metabolism.

PGC-1β is known to be an essential regulator of mitochondrial metabolism in different tissues including skeletal muscle. Contrary to PGC-1α, PGC-1β expression is largely unaffected by different stimuli such as exercise or cold exposure. Nevertheless, fasting and short-term high fat feeding induce hepatic PGC-1β mRNA levels. Thus, we evaluated if PGC-1β expression in skeletal muscle is also affected by fasting

and if so, how. Furthermore, we defined the PGC-1β-dependent fasting response in skeletal muscle and whole body metabolism, which is described in chapter 5 of this thesis.

4. Define the role of PGC-1α in podocyte physiology and its function in stress-induced kidney metabolism.

Podocytes are highly specialized kidney cells participating in the renal filtration process. Recently, mitochondrial dysfunction in podocytes and other kidney cells has been implicated to contribute to the development and progression of kidney malfunction and diseases. Since PGC-1α is an essential regulator of oxidative metabolism and mitochondrial homeostasis in different tissues including the kidney, we determined its role in podocyte metabolism under basal and stress-induced conditions. The results of this study can be found in chapter 6 of this thesis.

3. Muscle PGC-1α is required for long-term systemic and local adaptations to a ketogenic diet in mice

Am J Physiol Endocrinol Metab 312: E437–E446, 2017.
First published February 21, 2017; doi:10.1152/ajpendo.00361.2016.

RESEARCH ARTICLE

Muscle PGC-1α is required for long-term systemic and local adaptations to a ketogenic diet in mice

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Submitted 6 October 2016; accepted in final form 18 February 2017

Schnyder S, Svensson K, Cardel B, Handschin C. Muscle PGC-1α is required for long-term systemic and local adaptations to a ketogenic diet in mice. *Am J Physiol Endocrinol Metab* 312: E437–E446, 2017. First published February 21, 2017; doi:10.1152/ajpendo.00361.2016.—Low-carbohydrate/high-fat (LCHF) diets are increasingly popular dietary interventions for body weight control and as treatment for different pathological conditions. However, the mechanisms of action are still poorly understood, in particular, in long-term administration. Besides liver, brain, and heart, skeletal muscle is one of the major organs involved in the regulation of physiological and pathophysiological ketosis. We assessed the role of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) in skeletal muscle of male wild-type control and PGC-1α muscle-specific knockout mice upon 12 wk of LCHF diet feeding. Interestingly, LCHF diet administration increased oxygen consumption in a muscle PGC-1α-dependent manner, concomitant with a blunted transcriptional induction of genes involved in fatty acid oxidation and impairment in exercise performance. These data reveal a new role for muscle PGC-1α in regulating the physiological adaptation to long-term LCHF diet administration.

skeletal muscle; ketogenic diet; PGC-1α; exercise; ketone bodies

IN RECENT YEARS, ketogenic diets have emerged as potent therapeutic strategies for numerous diseases (27). In contrast to classical high-fat diets, ketogenic diets are characterized by a lower content of carbohydrates and proteins and will promote a dietary state reminiscent of fasting, diametrically opposite of the fed-like phenotype evoked by high-fat diets. Historically, low-carbohydrate/high-fat (LCHF) diets have been developed for and successfully used in the treatment of epilepsy, in particular, to reduce seizures in children who are nonresponders to pharmacological interventions (19). Increasing evidence has expanded the use of LCHF diets to metabolic disorders, such as obesity, cardiovascular diseases, or type 2 diabetes, but also to certain types of cancer (6, 7, 9, 12, 30, 37). LCHF diets induce a state known as ketosis, which also occurs physiologically after prolonged fasting periods, exercise, or other contexts of low-carbohydrate availability (20). Ketosis is characterized by the increased production of ketone bodies, such as β-hydroxybutyrate (β-OHB) and acetoacetate, in a process called ketogenesis in the liver (14). Circulating ketone bodies are then used by extrahepatic tissues as energy substrates in the Krebs cycle and oxidative phosphorylation, in particular, in the brain, skeletal, and heart muscles. The exact mechanisms by which LCHF diets exert their actions are still poorly understood. However, increased fatty acid oxidation (25, 34), mitochondrial biogenesis, and ATP production (8) have been proposed to be important pathways mediating the positive effects of ketogenic diets.

The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) functions as an essential transcriptional coactivator for target genes in all of these metabolic processes (4). Furthermore, PGC-1α regulates ketolytic gene expression in skeletal muscle and thereby, potently affects systemic ketosis (33). Strikingly, high-muscle PGC-1α reduced postexercise ketosis in mice, as previously observed in trained vs. untrained individuals (1, 33), and thus constitutes a major regulator of ketone body homeostasis in exercise. Moreover, skeletal muscle emerges as the key tissue to modulate ketone body homeostasis actively and voluntarily. Importantly, the beneficial and detrimental effects of long-term administration of LCHF diets are still debated, and the compatibility with exercise training is unclear. Therefore, we now tested whether muscle PGC-1α, the regulatory nexus in endurance training, also contributes to the local and systemic effects of long-term LCHF diet feeding and thus evaluated whole-body homeostasis and skeletal muscle metabolism in wild-type control (CTRL) and PGC-1α muscle-specific knockout (mKO) mice fed an LCHF diet for 12 wk. Indeed, we demonstrate that PGC-1α in skeletal muscle is not only essential for basal ketolytic gene expression but also affects exercise performance and whole-body oxygen consumption ($\dot{V}O_2$) upon LCHF diet feeding. These findings reveal a new role for PGC-1α in systemic ketone body metabolism and shed new light onto the mechanisms through which LCHF diets exert their effects.

MATERIALS AND METHODS

Mice and diets. Male mice, at the age of 15 wk, were housed in a conventional facility with a 12-h:12-h light-dark cycle, with free access to food and water. Experiments were performed in accordance with Swiss federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt, Switzerland. The C57BL/6 PGC-1α mKO mice used in this study were generated as described in Svensson et al. (33). A chow diet (AIN-93G; 7% fat, 58.5% carbohydrates, and 18% protein) and a ketogenic diet (XL75:XP10; 74.4% fat, 3% carbohydrates, and 9.9% protein) were purchased from Provimi Kliba AG (Kaiseraugst, Switzerland). After 12 wk of chow or LCHF diet feeding ad libitum, mice were not fed for 2 h in the morning and euthanized by carbon dioxide inhalation, and tissue samples were collected.

Body composition and indirect calorimetry. Body weight was monitored weekly, and body composition was determined using an

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Table 1. *qRT-PCR primer list*

Gene Name	Forward Primer	Reverse Primer
18S	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Acadl	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Acadvl	GTAGCCTCCATCCGAAGCTC	CAGGCCCCCATTACTGATCC
Acat1	GTGAAGGAAGTCTACATGGGCA	TGTGGTGCATGGAGTGGAAATA
Bdh1	TTTGCTGGCTGTTTGATGAAGG	TTGAGCTGGATGGTTCTCAGTC
CD36	GGCAAAGAACAGCAGCAAAAT	TGGCTAGATAACGAACTCTGTATGTGT
Cpt1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Cs	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
ERRα	ACTGCAGAGTGTGTGGATGG	GCCCCCTCTTCATCTAGGAC
Glut 4	GATGAGAAACGGAAGTTGGAGAGA	GCACCACTGCGATGATCAGA
HKII	AAAACCAAGTGCAGAAGGTTGAC	GAACCGCCTAGAAATCTCCAGAA
Mct1	TGCAACGACCAGTGAAGTATCA	ACAACCACCAGCGATCATTACT
Oxct1	CCCATACCCACTGAAAGACGAA	CTGGAGAAGAAAGAGGCTCCTG
Pdk4	AAAATTTCCAGGCCAACCAA	CGAAGAGCATGTGGTGAAGGT
Pfkm	GGGGATCACCAATCTGTGTGT	ATCATTCAGCAAGTCGCTCCA
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
Pkm1	CATTATCGTGCTCACCAAGTCTG	GATTTCGAGTCACGGCAATGATA
PPARα	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACAG
PPARδ	GCAAGCCCTTCAGTGACATCA	CCAGCGCATTGAACTTGACA
Sdhb	TGACGTCAGGAGCCAAAATGG	CCTCGACAGGCCTGAAACTG
Uqcrc2	CCCATCTTGCTTTGCTGTCTG	AATAAAATCTCGAGAAGGACCCG

EchoMRI-100 analyzer (EchoMRI, Houston, TX) at the end of the treatment period.

Mice were placed in a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH) to assess $\dot{V}O_2$, carbon dioxide production, the respiratory exchange ratio (RER), as well as food intake and spontaneous locomotion (number of breaks of infrared beams in *x,y,z* dimensions).

Exercise tests. Animals were acclimatized to an open treadmill (Columbus Instruments) for 2 days before the start of the experiment, for 5 min at 0 m/min, followed by 5 min at 8 m/min and 5 min at 10 m/min, with an incline of 5°. The endurance exercise trial started at 5 m/min for 5 min with a 5° incline, followed by 8 m/min for 10 min. The speed of the treadmill was subsequently increased by 2 m/min every 15 min until exhaustion. Basal blood glucose and lactate levels were assessed in tail-vein blood before and after exercise. For indirect calorimetry assessments, mice were acclimatized to treadmill running, as described above. Mice were placed in a closed treadmill (Columbus Instruments), where they first sat for 5 min at 0 m/min at a 5° incline. Subsequently, the test started at 8 m/min for 5 min, and the speed was increased every 5 min for 2 m/min until exhaustion.

Blood analysis. Blood glucose, lactate, and β-OHB were measured in tail-vein blood with a glucose meter (Accu-Chek; Roche, Mannheim, Germany), a Lactate Plus meter (Nova Biomedical, Waltham, MA), or a β-OHB meter (Precision Xtra; Abbott Laboratories, Abbott Park, IL). For plasma analysis, whole tail-vein blood was collected in Microvette tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 2,000 *g* for 5 min. Total cholesterol, aspartate transaminase (ASAT), and alanine transaminase (ALAT) levels were analyzed with a Cobas c 111 system (Roche Diagnostics AG, Rotkreuz, Switzerland). Nonesterified fatty acids (NEFAs) were measured in plasma using a NEFA kit, according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA).

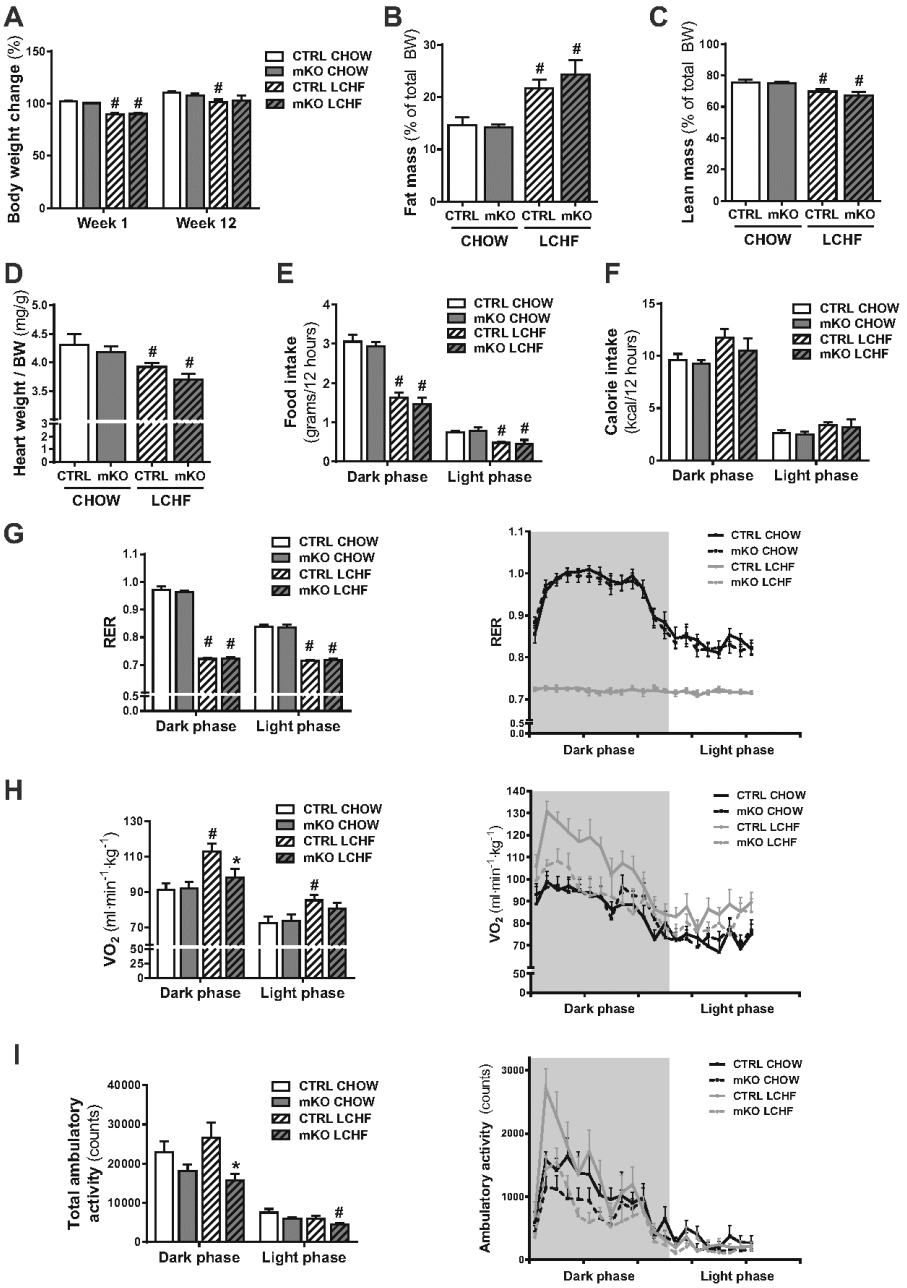
Glycogen measurement. Frozen tissue (10 mg) was homogenized in 200 μl water using a motorized pestle. To inactivate enzymes, samples were boiled at 95°C in a water bath for 10 min before centrifugation at 18,000 *g*. Supernatant was assayed for glycogen using a glycogen assay kit, according to the manufacturer's instructions (Abcam, Cambridge, UK).

RNA extraction and qRT-PCR. Frozen tissue was homogenized, and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Zug, Switzerland), according to the manufacturer's protocol. cDNA synthesis was done using 1 μg total RNA. Semiquantitative real-time PCR (semi-qRT-PCR) analysis was performed with Fast SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (both from Thermo Fisher Scientific, Waltham, MA). Relative expression levels for each gene of interest were calculated with the comparative threshold (ΔΔCt) method, using 18S rRNA as the normalization control. The primer sequences are listed in Table 1.

Immunoblot analysis. Tissues were homogenized in radioimmunoprecipitation assay buffer, and equal amounts of proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Whatman; Sigma-Aldrich, St. Louis, MO). The proteins of interest were detected with the following antibodies: succinyl-CoA: 3-ketoacid-CoA transferase 1 (OXCT1; ab105320; Abcam), acetyl-CoA acetyltransferase 1 (ACAT1; HPA004428; Sigma-Aldrich), eukaryotic elongation factor 2 (eEF2; 2332; Cell Signaling Technology, Danvers, MA), mitoprofile (MS604; Mitosciences, Eugene, OR), and polyclonal swine anti-rabbit Igs/horseradish peroxidase or polyclonal rabbit anti-mouse Igs/horseradish peroxidase, respectively (P0399 and P0260; Dako, Kyoto, Japan). Densitometric analysis of immunoblots

Fig. 1. LCHF diet feeding increases fat mass and oxygen consumption ($\dot{V}O_2$) while lowering the respiratory exchange ratio (RER). *A*: body weight (BW) curve of mice with an initial weight of 28 g fed a chow or an LCHF diet for 1 or 12 wk (n = 13–16). *B*: fat mass in percent of total body weight measured by EchoMRI in mice fed a chow or LCHF diet for 12 wk (n = 7–8). *C*: lean mass in percent of total body weight measured by EchoMRI in mice fed a chow or LCHF diet for 12 wk (n = 7–8). *D*: relative heart weight of mice fed a chow or LCHF diet for 12 wk (n = 7–8). *E*: average food intake, measured over a 48-h period, in mice fed a chow or LCHF diet for 8 wk (n = 6–8). *F–I*: average calorie intake (*F*), RER (*G*), $\dot{V}O_2$ rate (*H*), and total ambulatory activity (*I*) measured by indirect calorimetry over a 48-h period in mice fed a chow or LCHF diet for 8 wk (n = 7–8). Error bars represent SE. *Significant differences between genotypes: chow-fed CTRL and mKO mice and LCHF diet-fed CTRL and mKO mice ($P < 0.05$), respectively; #significant differences between conditions: chow and LCHF diet-fed CTRL and chow and LCHF diet-fed mKO mice ($P < 0.05$), respectively.

3. Muscle PGC-1α is required for long-term systemic and local adaptations to a ketogenic diet in mice



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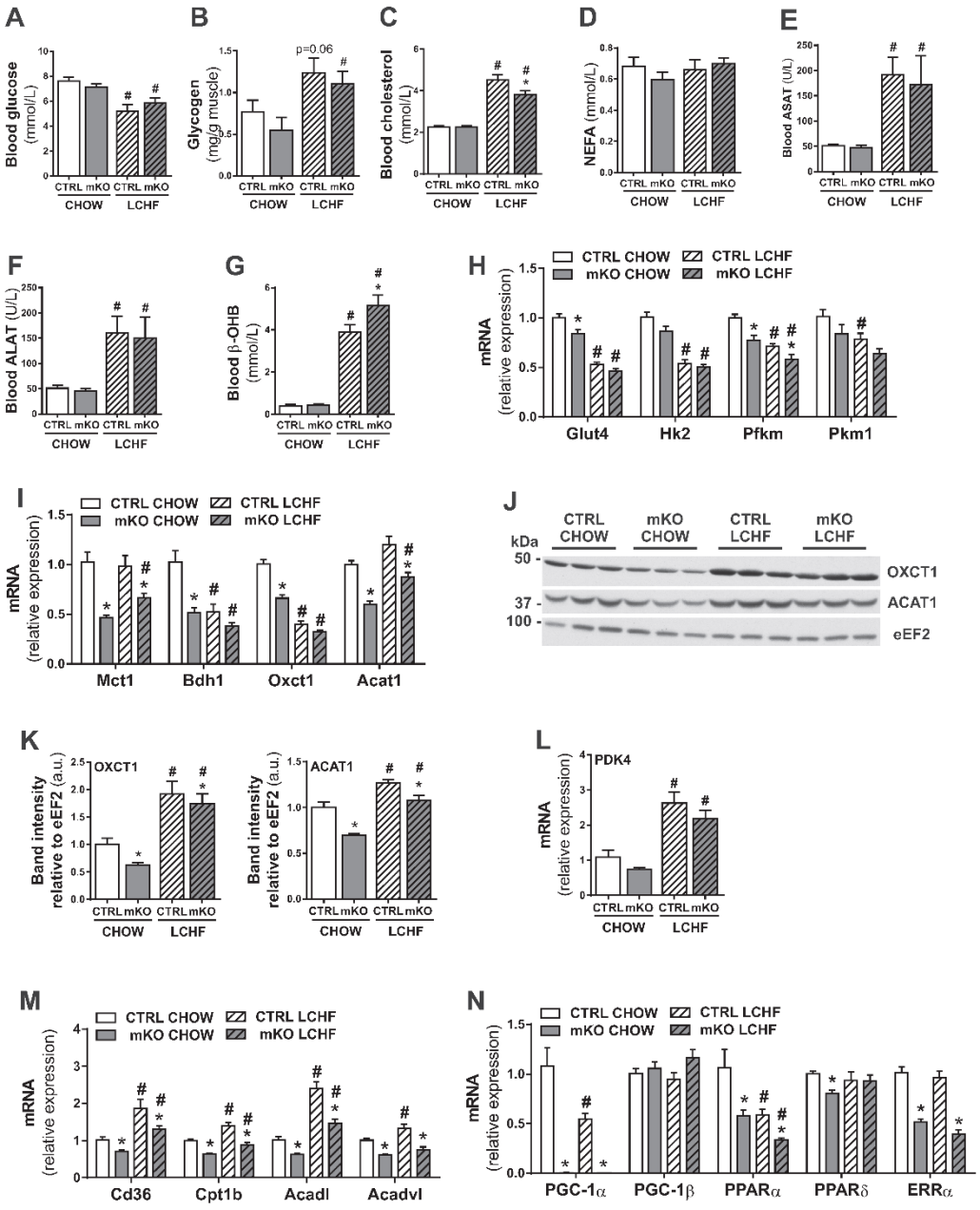

was performed on six individual samples with ImageJ software (National Institutes of Health, Bethesda, MD); a representative selection from this group is presented in the respective figures.

Seahorse assay. Total mitochondria were isolated from fresh quadriceps muscle using gradual centrifugation. Minced muscle was homogenized with a motorized pestle and centrifuged at 700 *g* for 10 min. Supernatant was recentrifuged at 10,500 *g* for 10 min to obtain a crude mitochondrial pellet. Equal amounts of protein were plated on a 96-well Seahorse plate, and mitochondrial respiration was measured using the Seahorse XF Cell Mito Stress Test kit (103015-100; Agilent Technologies, Santa Clara, CA) on an XF^{e}96 Extracellular Flux Analyzer (Agilent Technologies). The assay buffer was supplemented with either 10 mM malate/10 mM pyruvate or 20 mM succinate/2 μM rotenone, respectively, to assess complex I or complex II activity. The amount of ADP used was 4 mM, and ATP production was estimated by subtracting ADP-induced $\dot{V}O_2$ rate (OCR) values from oligomycin-induced OCR values.

Statistical analysis. Data are presented as means ± SE. The unpaired two-tailed Student's *t*-test was used to determine differences between groups. Significance was set at $P < 0.05$, and significant differences between the genotypes (CTRL CHOW vs. mKO CHOW and CTRL LCHF vs. mKO LCHF diets) and between the conditions (CTRL CHOW vs. CTRL LCHF and mKO CHOW vs. mKO LCHF diets) were marked.

RESULTS

PGC-1α mKO mice fail to increase $\dot{V}O_2$ on an LCHF diet. PGC-1α mKO and CTRL mice were fed a normal chow diet or an LCHF diet for 12 wk. Both LCHF diet-fed CTRL and PGC-1α mKO mice showed a reduction in body weight after 1 wk compared with the chow-fed cohorts (Fig. 1*A*). After 12 wk, only the LCHF diet-fed CTRL mice were significantly lighter than their chow-fed counterparts (Fig. 1*A*). At the end of the 12 wk of LCHF diet feeding, CTRL and PGC-1α mKO mice displayed a significant increase in fat mass (Fig. 1*B*), as well as reduced lean mass (Fig. 1*C*), compared with the chow-fed cohorts. This was reflected further in the relative decrease in heart weight (Fig. 1*D*) in LCHF diet-fed compared with chow-fed mice. LCHF diet feeding resulted in reduced food intake by weight (Fig. 1*E*) but importantly, not by caloric content (Fig. 1*F*). LCHF diet-fed mice showed a significant decrease in RER compared with chow-fed mice (Fig. 1*G*), which reflected the high-fat content of the LCHF diet. Interestingly, LCHF diet feeding increased the OCR only in CTRL mice, whereas PGC-1α mKO mice displayed no increase with LCHF diet feeding (Fig. 1*H*). LCHF diet-fed PGC-1α mKO mice also showed a significantly reduced ambulatory activity compared with LCHF diet-fed CTRL mice (Fig. 1*I*). These findings indicate that PGC-1α mKO mice exhibit a blunted adaptation to long-term LCHF diet feeding.

PGC-1α mKO mice show a reduced induction of genes encoding proteins involved in fatty acid metabolism in skeletal muscle. LCHF diets affect both glucose and cholesterol metabolism (6, 7, 9, 12, 30, 37). In our study, LCHF diet feeding led to reduced circulating glucose levels and increased muscle glycogen content in CTRL and PGC-1α mKO mice (Fig. 2, *A* and *B*) compared with the chow-fed counterparts. Circulating cholesterol levels were increased in both genotypes (Fig. 2*C*). However, blood cholesterol was significantly lower in LCHF diet-fed PGC-1α mKO mice compared with LCHF diet-fed CTRL mice (Fig. 2*C*). Circulating NEFAs were not different between the groups (Fig. 2*D*). In line with previous studies (15, 17), significantly increased circulating levels of ASAT and ALAT were observed in LCHF diet-fed CTRL and PGC-1α mKO mice (Fig. 2, *E* and *F*), indicative of liver stress caused by LCHF diet feeding. Furthermore, LCHF diet feeding elevated circulating β-OHB levels in both cohorts, even though PGC-1α mKO mice depicted a significant hyperketonemia compared with CTRL mice (Fig. 2*G*), similar to our previous observations (33). Next, we assessed the impact of LCHF diet feeding on metabolic pathways in skeletal muscle of CTRL and PGC-1α mKO mice. In line with the reduced circulating glucose levels with LCHF diet feeding, there was a significant reduction in the expression of genes involved in glucose uptake [glucose transporter 4 (*Glut4*)] and glycolysis [hexokinase II (*HKII*); muscle phosphofructokinase (*Pfkm*); pyruvate kinase muscle 1 (*Pkm1*)] in skeletal muscle from LCHF diet-fed CTRL and PGC-1α mKO mice (Fig. 2*H*). Surprisingly, the transcription of ketolytic genes [3-OHB dehydrogenase type 1 (*Bdh1*) and *Oxct1*] was reduced significantly upon LCHF diet feeding (Fig. 2*I*). In stark contrast, protein levels of OXCT1 and ACAT1 were increased significantly (Fig. 2, *J* and *K*). The transcript levels of *Glut4*, *Pfkm*, monocarboxylate-transporter 1 (*Mct1*), *Bdh1*, *Oxct1*, and *Acat1* (Fig. 2, *H* and *I*) were lower in PGC-1α mKO mice, even when compared with LCHF diet-fed CTRL animals. The increased levels of pyruvate dehydrogenase lipoamide kinase isozyme 4 (*Pdk4*) with LCHF diet feeding (Fig. 2*L*) and various genes encoding proteins involved in fatty acid uptake [cluster of differentiation 36 (*Cd36*)] and oxidation [carnitine palmitoyltransferase 1b (*Cpt1b*); CoA dehydrogenase long chain (*Acadl*); CoA dehydrogenase very long chain (*Acadvl*)] indicate a substrate shift toward fatty acid metabolism in CTRL mice (Fig. 2*M*). Importantly, the induction of these genes was blunted in PGC-1α mKO mice (Fig. 2*M*). Interestingly, despite the central role of PGC-1α and peroxisome proliferator-activated receptor α (*PPARα*) for the transcriptional control of fatty acid metabolism in skeletal muscle (35), gene expression of both of these regulators was reduced in muscle with LCHF diet feeding (Fig. 2*N*). Further-

Fig. 2. LCHF diet-fed mice show a PGC-1α-dependent switch from glucose to fatty acid oxidation in skeletal muscle. *A*: plasma glucose levels of mice fed a chow or LCHF diet for 12 wk ($n = 7–8$). *B*: relative glycogen levels in gastrocnemius muscle of mice fed a chow or LCHF diet for 12 wk ($n = 6–8$). *C–G*: plasma total cholesterol (*C*), nonesterified fatty acids (NEFA; *D*), ASAT (*E*), ALAT (*F*), and β-hydroxybutyrate (β-OHB; *G*) levels of mice fed a chow or LCHF diet for 12 wk ($n = 7–9$). *H* and *I*: gene expression in gastrocnemius muscle relative to 18S of genes involved in glucose metabolism (*H*) and ketolysis (*I*; $n = 6–8$). Hk2, HKII. *J* and *K*: representative immunoblots (*J*) and protein levels (*K*) of OXCT1 and ACAT1 in gastrocnemius muscle relative to eukaryotic elongation factor 2 (eEF2; $n = 6$). *L–N*: gene expression in gastrocnemius muscle relative to 18S of PDK4 (*L*) and genes involved in fatty acid uptake and oxidation (*M*) and transcriptional regulation (*N*; $n = 6–8$). Error bars represent SE. *Significant differences between genotypes: chow-fed CTRL and mKO mice and LCHF diet-fed CTRL and mKO mice ($P < 0.05$), respectively; #significant differences between conditions: chow and LCHF diet-fed CTRL and chow and LCHF diet-fed mKO mice ($P < 0.05$), respectively.

more, the expression levels of PGC-1β, PPARδ, and estrogen-related receptor α (*ERRα*) were not changed upon LCHF diet feeding, but transcript levels of PPARδ and ERRα were reduced significantly in PGC-1α mKO mice (Fig. 2*N*).

LCHF diet feeding leads to impaired exercise performance, specifically in PGC-1α mKO mice. Since LCHF diet-fed PGC-1α mKO mice showed a blunted induction of fatty acid metabolism in skeletal muscle, we were interested if this would affect exercise performance and substrate use during endurance exercise. In line with previous findings (16), PGC-1α mKO mice displayed reduced endurance exercise performance compared with CTRL mice (Fig. 3*A*). LCHF diet feeding did not affect the endurance capacity of CTRL mice (Fig. 3*A*). Strikingly however, this diet specifically impaired the exercise performance of PGC-1α mKO mice (Fig. 3*A*). This phenotype was not associated with any impairment in the ability of PGC-1α mKO mice to increase circulating glucose levels with exercise (Fig. 3*B*). Moreover, whereas PGC-1α mKO mice showed elevated blood lactate levels upon exhaustion, as previously published (32), this effect was compared between chow-fed and LCHF diet-fed PGC-1α mKO mice (Fig. 3*C*). In closed treadmills, LCHF diet-fed mice displayed elevated $\dot{V}O_2$ during the exercise compared with chow-fed mice (Fig. 3*D*). CTRL mice were able to maintain this elevated $\dot{V}O_2$ during the entire exercise period, except for the last time point of measurement (Fig. 3*D*). In contrast, $\dot{V}O_2$ levels dropped rapidly in PGC-1α mKO animals as exercise intensity increased (Fig. 3*D*). Similarly, PGC-1α mKO animals could not maintain the low RER observed in LCHF diet-fed CTRL mice and displayed an earlier shift to carbohydrate metabolism, indicated by the sharp increase in RER (Fig. 3*E*). These differences were, however, diet independent, since chow-fed mKO mice also performed significantly worse than their CTRL littermates. Collectively, these findings suggest that LCHF diet-fed PGC-1α mKO mice have difficulties in keeping up with the increased energy demand in endurance exercise and are unable to properly cope with the metabolic changes elicited by LCHF diet feeding, in particular, in exercise.

LCHF diet feeding does not lead to increased mitochondrial biogenesis or ATP levels in skeletal muscle. Ketogenic diet feeding has been proposed to increase mitochondrial biogenesis and ATP levels in the context of neurological diseases (8). Thus to test whether LCHF diet feeding also leads to an induction of mitochondrial biogenesis in skeletal muscle, we measured the levels of mitochondrial gene expression [mitochondrial succinate dehydrogenase iron-sulfur subunit (*Sdhb*); citrate synthase (*Cs*); mitochondrial cytochrome *b*-c1 complex subunit 2 (*Uqcrc2*)], as well as mitochondrial proteins (ATP synthase 5 alpha; UQCRC2; mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 8; Fig. 4, *A* and *B*). As expected, PGC-1α mKO mice exhibited reduced mitochondrial gene expression and protein content (22, 23). However, in contrast to studies in neurological tissues (8), LCHF diet feeding did not lead to increased mitochondrial transcript or protein levels in skeletal muscle (Fig. 4, *A* and *B*). Furthermore, mitochondria isolated from quadriceps muscles of LCHF diet-fed mice showed a drop in ADP-induced complex I respiration and concomitant complex I ATP production (Fig. 4, *C* and *D*), whereas complex II respiration was not affected by LCHF diet feeding (Fig. 4, *E* and *F*).

DISCUSSION

Besides physical activity, dietary interventions are a mainstay of prevention and therapy of many diseases. LCHF diets have been increasingly studied in the past decades due to their therapeutic potential, not only in the treatment of epilepsy and other brain-related disorders but also in other pathologies that are associated with peripheral organs (27). Endogenous ketone body levels are, in part, controlled by hepatic ketogenesis. Dietary ketosis is, however, largely determined by the ketone body metabolism in the brain, heart, and skeletal muscle. Of these three main consumers, only skeletal muscle can be directly and voluntarily affected, and indeed, training can reduce postexercise ketosis (1). Moreover, we have demonstrated previously that muscle PGC-1α can modulate systemic ketosis in numerous acute physiological and pathophysiological contexts (33). Here, we show that muscle PGC-1α likewise contributes to the local and systemic adaptations of long-term LCHF diet feeding. In particular, LCHF diet-induced $\dot{V}O_2$ was severely blunted in PGC-1α mKO mice. Even more dramatic, LCHF diet-fed PGC-1α mKO mice displayed a marked impairment in running performance already at moderate exercise intensities, and the initial increased OCR quickly dropped to the same level as that of chow-fed PGC-1α mKO mice. In contrast, LCHF diet-fed CTRL mice were able to run the same amount of time as their chow-fed counterparts, despite their reduced lean mass, assuming that the efficiency of consuming energy from fats is higher upon LCHF diet feeding, as suggested by the study of Paoli et al. (25). The analysis of skeletal muscle samples revealed that transcript levels of genes involved in fatty acid uptake and oxidation were elevated in LCHF diet-fed CTRL mice, whereas the upregulation of these genes in PGC-1α mKO animals was blunted. It is conceivable that the difference in OCRs between LCHF diet-fed PGC-1α mKO and CTRL mice is, in part, due to this reduced induction of the respective genes in skeletal muscle in PGC-1α mKO animals. Thus PGC-1α seems to participate in the LCHF diet-controlled metabolic switch from glucose to ketone body and fatty acid use. Furthermore, the decrease in activity levels in LCHF diet-fed mKO mice could also contribute to the reduced $\dot{V}O_2$. Thus muscle PGC-1α might thereby influence whole-body metabolism in LCHF diet feeding.

Given the important role of PGC-1α in systemic ketone body metabolism (33) and exercise (28), these findings raise questions about the compatibility of LCHF diets and training. Studies, so far, have been inconclusive as to whether LCHF diets improve or hinder training adaptations (24). For example, in the recent study of Zajac et al. (38), maximal $\dot{V}O_2$ and the lactate threshold were increased significantly in off-road cyclists treated with an LCHF diet. In competitive gymnasts, LCHF diets do not negatively impact explosive and strength performance, only when an adequate amount of protein is provided (26). Thus administration of LCHF diets might differ in endurance compared with resistance training, since LCHF diet feeding induces a "fasting-like" state that could hinder the buildup of muscle mass. The inherent problems of the LCHF diet could be circumvented by direct administration of ketone bodies, e.g., in the form of transesterified β-OHB precursor metabolites without the massive acid/salt load associated with intake of β-OHB in acid or salt form (11). The nutritional ketosis elicited by such metabolites promoted an improvement

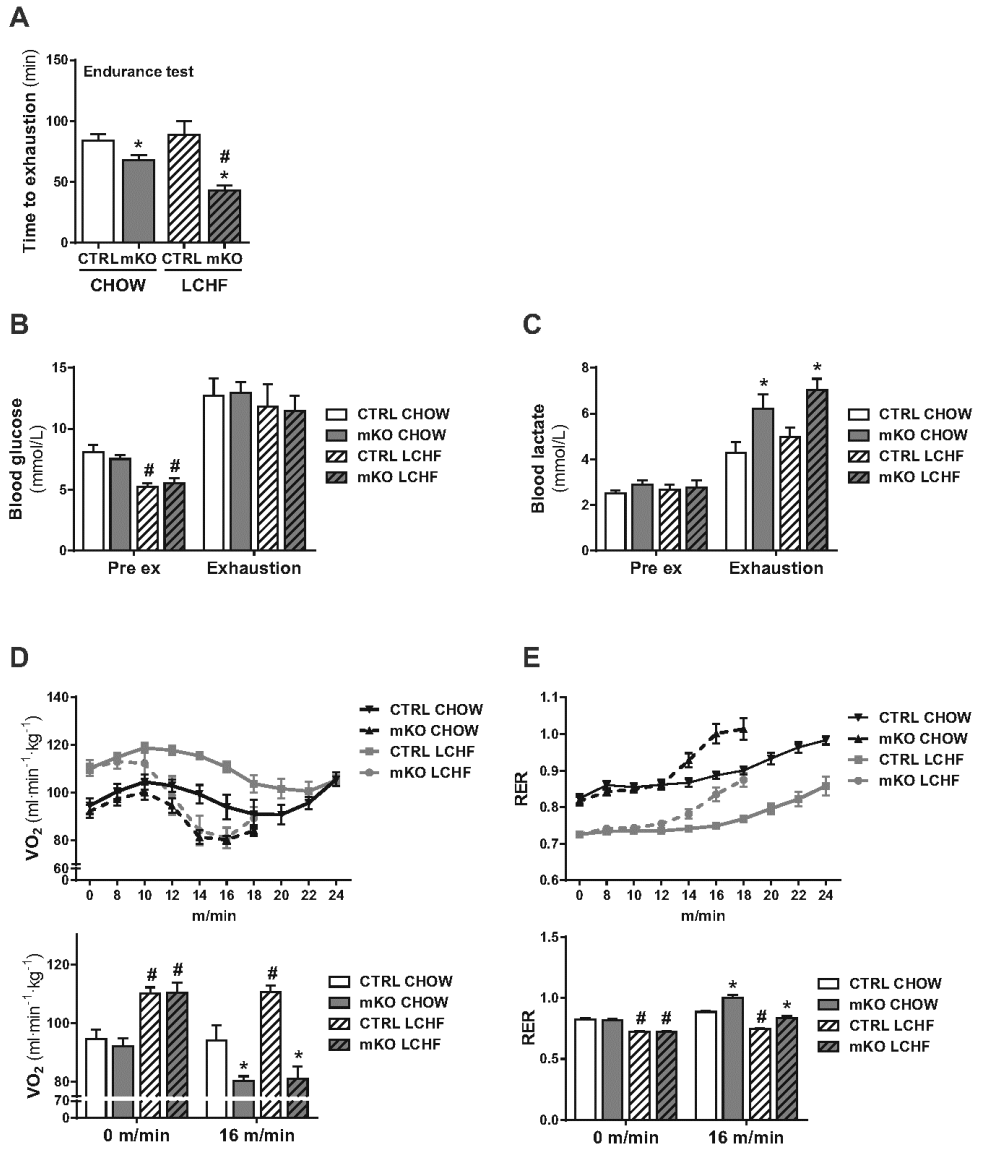

Fig. 3. PGC-1α in skeletal muscle is essential to maintain adequate energy levels during exercise upon LCHF diet feeding. *A*: endurance exercise test of mice fed a chow or LCHF diet for 10 wk (n = 7–8). *B* and *C*: blood glucose (*B*) and lactate (*C*) levels before (Pre ex) and after (Exhaustion) exhaustive endurance exercise test of mice fed a chow or LCHF diet for 10 wk (n = 7–8). *D* and *E*: average oxygen consumption rate ($\dot{V}O_2$; *D*) and respiratory exchange ratio (RER; *E*) measured by indirect calorimetry in a closed treadmill of mice fed a chow or LCHF diet for 11 wk and corresponding bar graphs (n = 6–8). Error bars represent SE. *Significant differences between genotypes: chow-fed CTRL and mKO mice and LCHF diet-fed CTRL and mKO mice ($P < 0.05$), respectively; #significant differences between conditions: chow and LCHF diet-fed CTRL and chow and LCHF diet-fed mKO mice ($P < 0.05$), respectively.

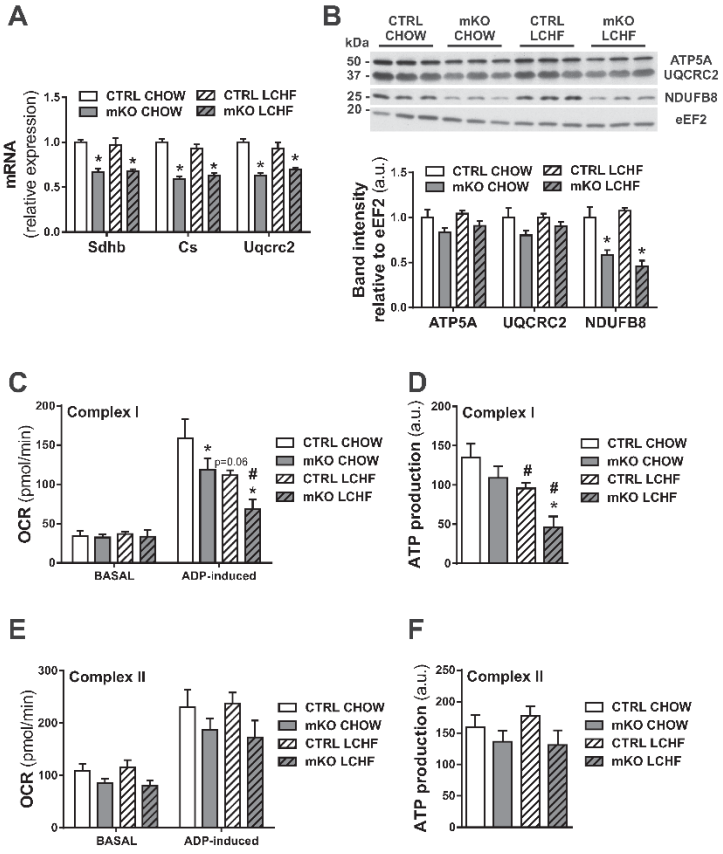

Fig. 4. LCHF diet feeding does not affect mitochondrial biogenesis and lowers ATP production in skeletal muscle. *A*: gene expression in gastrocnemius muscle relative to 18S of genes involved in mitochondrial homeostasis (n = 6–8). *B*: protein levels of different mitochondrial chain complexes in gastrocnemius muscle relative to eukaryotic elongation factor 2 (eEF2) and representative immunoblots (n = 6). ATP5A, ATP synthase 5 alpha; NDUFB8, mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 8. *C* and *D*: complex I-induced $\dot{V}O_2$ rate (OCR; *C*) and estimated ATP production (*D*) of isolated mitochondria from quadriceps muscle (n = 4–6). *E* and *F*: complex II-induced OCR (*E*) and estimated ATP production (*F*) of isolated mitochondria from quadriceps muscle (n = 4–6). Error bars represent SE. *Significant differences between genotypes: chow-fed CTRL and mKO mice and LCHF diet-fed CTRL and mKO mice ($P < 0.05$), respectively; #significant differences between conditions: chow and LCHF diet-fed CTRL and chow and LCHF diet-fed mKO mice ($P < 0.05$), respectively.

in endurance performance in cyclists, even in the presence of normal muscle glycogen, elevated insulin levels, or coadministrated carbohydrates (11).

The "Atkins diet," a particular form of the LCHF diet, has popularized LCHF diet interventions for weight loss (3). However, despite the widespread use of the Atkins and related diets, the molecular mechanisms and potential detrimental effects are still largely unknown. Indeed, in our study, LCHF diet-fed mice displayed some negative effects on whole-body metabolism. Even though LCHF diet feeding led to an initial weight loss after 1 wk of treatment, which has also been shown in other rodent studies (5, 17, 18), the difference in body weight after 12 wk of LCHF diet feeding was only minor. Second, LCHF diet-fed mice displayed an increase in fat mass and a concomitant decrease in lean mass (10, 36). Even more alarmingly, LCHF diet-fed animals showed increased circulating levels of cholesterol, ASAT, and ALAT, indicative of dyslipidemia, and a certain degree of liver stress, in line with other studies in mice and humans (13, 21, 31, 39). In fact, long-term administration of LCHF diets in rodents, in most cases, leads to the development of hepatic steatosis and nonalcoholic fatty liver disease (29). Thus even though the effect of such diets on hepatic lipid levels in humans is less clear, caution is advised, in particular, in patients with nonalcoholic fatty liver disease (2). It is possible that administration of transesterified ketone body precursor metabolites could act therapeutically without the potential side effects of an LCHF diet (11). Furthermore, whereas the reason for the reduced cholesterol levels in LCHF diet-fed mKO compared with CTRL mice is unclear, this change might be a consequence of the hyperketonemia in mKO animals. Thus our previous (33) and present findings would suggest that physical activity and thereby elevation of muscle PGC-1α are important adjuvant interventions to manage the pathological consequences of ketosis.

In the brain, the therapeutic effect of LCHF diets on seizures and other pathologies has been linked to increased mitochondrial biogenesis or ATP levels (8). Surprisingly, even though the elevated $\dot{V}O_2$ and the lower RER values of LCHF diet-fed mice indicate an overall increase in oxidative metabolism, we did not find any change in mitochondrial gene expression and

protein levels in skeletal muscle. Intriguingly, ATP production in isolated mitochondria from LCHF diet-fed mice was even lower than in chow-fed mice. Thus the observed increase in oxidative metabolism upon LCHF diet feeding is most likely due to the availability of energy substrates, which are mainly ketone bodies and other kind of fats. Furthermore, these data indicate that LCHF diet feeding predominantly acts on fatty acid oxidation rather than on mitochondrial biogenesis or ATP production in skeletal muscle. Moreover, a recent study in mitochondrial myopathy patients showed short-term adverse and long-term beneficial effects of LCHF diet feeding on skeletal muscle health. Acute treatment of patients with a modified Atkins diet resulted in muscle damage, especially in ragged-red fibers, indicating that nutrition can modify mitochondrial disease progression (1). Surprisingly, in the 2.5-yr follow-up study, patients showed improvements in muscle strength, suggesting that the initial fiber degeneration promoted subsequent fiber regeneration, resulting in increased muscle force. Thus care must be taken when administering LCHF diets to patients with mitochondrial-associated diseases and in evaluating responses to short-term treatment.

Taken together, our results clearly demonstrate that PGC-1α in skeletal muscle is essential for maintaining sufficient energy levels during prolonged muscle contractions, especially when carbohydrate availability is low, with important implications for whole-body metabolism and energy homeostasis. Finally, it is important to note that even though an LCHF diet induces beneficial health effects by increasing systemic oxidative metabolism, such interventions also exert potentially detrimental effects, including increasing total blood cholesterol levels—a known risk factor for cardiovascular diseases—or impaired liver function. Therefore, future studies should aim at elucidating the potential of non-LCHF diet-based interventions to modulate ketone body levels, such as nutritional ketosis. Alternatively, physiological, e.g., by adjuvant physical activity, or pharmacological modulation of muscle PGC-1α should be considered to mitigate the unwanted side effects of such interventions.

ACKNOWLEDGMENTS

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GRANTS

This project was funded by the Swiss National Science Foundation, European Research Council (ERC) Consolidator Grant 616830-MUSCLE_NET, Swiss Cancer Research Grant KFS-3733-08-2015, Swiss Society for Research on Muscle Diseases (SSEM), SystemsX.ch, "Novartis Stiftung für Medizinisch-Biologische Forschung," and University of Basel.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.S. conceived and designed research; S.S., K.S., and B.C. performed experiments; S.S., K.S., B.C., and C.H. analyzed data; S.S., K.S., B.C., and C.H. interpreted results of experiments; S.S. and K.S. prepared figures; S.S. and C.H. drafted manuscript; S.S. and C.H. edited and revised manuscript; S.S., K.S., B.C., and C.H. approved final version of manuscript.

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4. PGC-1α is important for the exercise-induced acute time-course and heat-stress response in skeletal muscle

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Abstract

Skeletal muscle adaptations to acute bouts of exercise or chronic exercise training are complex and involve profound changes in muscle and whole body metabolism. One of the known key players in mediating the transcriptional responses to exercise in muscle tissue is the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). In this study, we highlight the PGC-1α-dependent and -independent time-course transcriptional changes upon an acute bout of exercise using control (WT) and muscle-specific PGC-1α knockout (MKO) mice. We identified the WT time-course-specific and acute core exercise responses using RNA sequencing and showed that PGC-1α is important for the exercise-induced acute- and heat-stress response in skeletal muscle. Furthermore, the comparison of the acute and chronic exercise-induced transcriptional changes revealed only little overlap between the two conditions suggesting that the acute and chronic transcriptional responses to exercise are clearly distinct from each other. Thus, our study adds new aspects to the complex regulation of skeletal muscle exercise physiology and the important role of PGC-1α in this context.

Abbreviations

AP-1, activator protein 1 complex; ATF, activating transcription factor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; DE, differentially expressed; Egr1, early growth response 1; FC, fold change; FDR, false discovery rate; GO, gene ontology; HS, horse serum; HSF, heat shock factor; Hsp, heat shock protein; ISMARA, integrated motif activity response analysis; Maf, proto-oncogene c-Maf; MAPK, mitogen-activated protein kinase; MHC, myosin heavy chain; MKO, muscle-specific PGC-1α knockout mice; Nr4a3, nuclear receptor subfamily 4 group A member 3; PCA, principal component analysis; PGC-

1α, peroxisome proliferator-activated receptor γ coactivator-1α; SEM, standard errors of the means; SRF, serum response factor; WT, control mice

Introduction

Skeletal muscle is a highly plastic organ and substantially contributes to whole body energy metabolism, especially during strenuous exercise. Nowadays, the latter is considered to be part of a healthy lifestyle and is widely accepted as a therapeutic strategy to counteract different metabolic diseases such as obesity or type 2 diabetes (Haskell, Lee et al. 2007, Colberg, Sigal et al. 2010). Furthermore, regular exercise has been shown to exert beneficial effects in the treatment of cancer or other muscle wasting diseases like sarcopenia (Haskell, Lee et al. 2007, Egan and Zierath 2013). Multiple pathways coordinate the complex metabolic changes in response to exercise and numerous molecular regulators involved in skeletal muscle exercise physiology have been elucidated (Gabriel and Zierath 2017). Interestingly, many of the exercise-induced molecular changes converge on the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis and oxidative metabolism (Lin, Handschin et al. 2005), to increase its expression (Wu, Kanatous et al. 2002, Handschin, Rhee et al. 2003, Akimoto, Pohnert et al. 2005, Jager, Handschin et al. 2007), protein stability (Puigserver, Rhee et al. 2001, Jager, Handschin et al. 2007, Canto, Gerhart-Hines et al. 2009) or hypomethylate its promotor (Barres, Yan et al. 2012). PGC-1α itself interacts with a variety of different transcription factors to promote many of the exercise-induced transcriptional changes in mitochondrial metabolism, angiogenesis and β-oxidation (Catoire, Mensink et al. 2012, Egan and Zierath 2013). Even though skeletal muscle exercise metabolism and the role of PGC-1α in this context have been largely explored, the complete picture of exercise-induced skeletal muscle changes is still unclear. Nevertheless, it is believed that each bout of acute exercise elicits alterations in mRNA levels, which over time accumulate and lead to the increased abundance of proteins, finally resulting in improved exercise performance and skeletal muscle metabolic functions (Yang, Creer et al. 2005, Gabriel and Zierath 2017, Robinson, Dasari et al. 2017). Yet, the time-course of the acute exercise-induced transcriptional changes and their contributions to the chronic training-induced skeletal muscle adaptations have not been explored so far. Thus, we performed a time-course study with control (WT) and muscle-specific PGC-1α knockout mice (MKO) using mRNA sequencing. Only a small fraction of differentially expressed (DE) genes overlapped between all of the different time points in WT animals, which we identified as the acute core exercise response. Interestingly, MKO animals showed a different pattern of DE genes during the time-course and a much smaller core exercise response. Importantly, stress-induced transcripts seemed to be dependent on skeletal muscle PGC-1α, especially genes belonging to the family of heat shock proteins. Thus, our data demonstrate

that PGC-1α is important for the induction and perpetuation of the acute time-course and heat-stress response in skeletal muscle. Furthermore, chronic exercise training of WT and MKO mice only resulted in a small amount of DE genes with a minimal overlap with the acute time-course. These results suggest that chronic exercise training might lead to an increase in protein rather than transcript levels and demonstrate that the acute and chronic transcriptional exercise responses are clearly distinct from each other. Thus, for the first time, we characterize the acute and chronic core exercise responses and the dependence of skeletal muscle PGC-1α and thereby solidify its key role in exercise and muscle physiology.

Material and Methods

Mice and training protocols

Experiments were performed with the approval of the Swiss authorities on adult male mice (15-20 weeks old). Mice had free access to food and water and were housed in a conventional facility with a 12 h light/12 h dark cycle. The PGC-1α muscle-specific knockout (MKO) mice used in this study were generated as described previously (Svensson, Albert et al. 2016) and floxed littermates were used as controls (WT).

For the time-course study mice were acclimatized to treadmill running (Columbus Instruments) as described in Table 1 of the Supplemental Material. Two days after acclimatization, the test started at 0 m/min for 5 min, 5 m/min for 5 min and 8 m/min for 5 min with a 5° incline and the speed was increased 2 m/min every 15 min until exhaustion. Blood lactate and glucose were measured from tail blood with a lactate plus meter (Nova Biomedical, Labor-Systeme Flükiger AG) or glucose meter (Accu-Chek, Roche), respectively, before and after the treadmill test. Immediately, 4 h 6 h and 8 h after the test mice were killed by CO_2 and tissues collected. Sedentary mice were not exposed to any treadmill running.

For chronic training mice were acclimatized to treadmill running and treadmill training was carried out five times a week for four weeks as described in Table 2a and b of the Supplemental Material. Endurance capacity of the mice was determined on an open treadmill as described above. VO_2max was measured in a closed treadmill (Columbus Instruments) and the test started at 0 m/min for 5 min and 10 m/min for 3 min with a 15° incline and the speed was increased 2 m/min every 2 min until exhaustion. Chronically trained mice were killed by CO_2 18 h after the last training session and organs were removed.

mRNA sequencing and analysis

Total RNA was isolated from *Quadriceps* muscle with TRI reagent (T9424, Sigma) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop OneC spectrophotometer (Thermo Scientific). 7500 ng RNA was further purified with the direct-zol RNA MiniPrep Kit (R2050, Zymo Research) according to the manufacturer's instructions. For RNAseq library preparation 1 µg of purified RNA was used and libraries prepared with the TruSeq RNA library Prep Kit (Illumina) according to the manufacturer's instructions. Single read sequencing was performed with a HighSeq 2500 machine (50 cycles, Illumina). Fastq files were mapped to the mouse genome (mm10) and RNAseq and statistical analysis performed with the CLC Genomics Workbench Software (Qiagen). Differentially expressed genes were pictured in a Venn diagram with the use of the interactiVenn web-based tool (Heberle, Meirelles et al. 2015). Gene ontology (GO) analysis was executed by the usage of GeneCodis (Carmona-Saez, Chagoyen et al. 2007, Nogales-Cadenas, Carmona-Saez et al. 2009, Tabas-Madrid, Nogales-Cadenas et al. 2012). Only GO terms with at least five mapped genes were considered to be enriched and GO list was summarized using REVIGO with an allowed similarity of 0.5 (Supek, Bosnjak et al. 2011). Enriched GO terms were furthermore sorted by enrichment (calculated by: (Support/List Size)/(Reference Support/Reference size)). Integrated motif activity response analysis (ISMARA) was used to predict enriched transcription factor binding motifs (Balwierz, Pachkov et al. 2014).

Cell culture

C2C12 myoblasts were grown in proliferation medium (DMEM, 10% FetalClone Serum [FCS, SH30066.03, GE Healthcare Life Sciences], 1% Penicillin/Streptomycin [15140122, Thermo Scientific]) until confluency and then medium was switched to differentiation medium (DMEM, 2% horse serum [HS, 16050122, Thermo Scientific]) for 4 days. Myotubes were infected with si-Scr or si-PGC-1α for 24 h. For serum shock experiments cells were treated with DMEM containing 50% HS for 15 min, 30 min, 1 h, 2 h or 4 h and RNA was harvested as described below. For heat shock experiments cells were put at 42 °C for 15 min, 30 min, 1 h, 2 h or 4 h and RNA was harvested as described below.

RNA isolation and real-time qPCR

Total RNA was isolated from C2C12 mytubes with 1 mL TRI reagent (T9424, Sigma) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop OneC spectrophotometer (Thermo Scientific), treated with DNase I (18068015, Thermo Scientific) and then reverse transcribed using hexanucleotide mix (11277081001, Sigma) and SuperScript II reverse transcriptase (18064022, Thermo Scientific). The level of relative mRNA was quantified by real-time PCR on a Light Cycler 480 II system (Roche) using Fast Start Essential DNA Green Master mix

(06924204001, Roche). The analysis of the mRNA was performed by the comparative CT method using TATA binding protein (TBP) as endogenous control. Primer sequences are listed in Table 3 of the Supplemental Material.

Statistical analysis

Values are expressed as means ± standard errors of the means (SEM) and statistical significance was determined with unpaired two tailed t-tests using Excel software. P values <0.05 were considered statistically significant. Significant differences between WT and MKO mice or between untreated (Ctrl) and treated cells, respectively, are indicated by an asterisk (*). Significant differences between pre- and post-acute exercise, si-Scr and si-PGC-1α treated cells and sedentary and chronically trained mice, respectively, are indicated by a hashtag (#).

Results

PGC-1α substantially contributes to gene expression changes after acute treadmill running

To elucidate transcriptional changes after an acute bout of exercise a time-course study was performed. Thus, control (WT) and muscle-specific PGC-1α knockout mice (MKO) were run on an open treadmill until exhaustion and then killed 0 h, 4 h, 6 h or 8 h after the exercise test. As expected, MKO animals ran significantly less (Fig. 1A) and showed higher post-lactate levels (Fig. 1B) than WT mice, which is in accordance with the current literature (Handschin, Chin et al. 2007, Summermatter, Santos et al. 2013). Furthermore, treadmill running increased blood lactate (Fig. 1B) and glucose (Fig. 1C) levels in WT and MKO animals compared to sedentary animals indicative that the mice indeed reached exhaustion.

Quadriceps muscles were used for RNAseq analysis and principal component analysis (PCA) was conducted. WT and MKO animals were clearly distinct from each other clustering at different points on the PC-3 axis (Fig. 1D). Furthermore, the different time points of the acute exercise time-course distributed along the axes of PC-1 and -2, being more dispersed in WT than in MKO animals (Fig. 1D). Interestingly, the 6 h time point clustered the farthest away from the sedentary mice, which was also reflected in the highest total number of differentially expressed (DE) genes over the time-course in WT and MKO animals (Fig. 1E). Moreover, MKO animals showed substantially less DE genes than WT animals (854 versus 1339) suggesting that PGC-1α contributes to many of the gene expression changes induced after acute treadmill running (Fig. 1E-G). Venn diagram representation of DE genes clearly revealed 56 and 25 overlapping genes in WT (Fig. 1F) and MKO (Fig. 1G) animals, respectively (Supp. Fig. 1). The overlapping 56 DE genes in WT animals were thus defined as the acute core exercise response. These data suggest that PGC-1α is not only important for the determination of the amount

of DE genes after acute treadmill running but might also be essential for the acute core exercise response in skeletal muscle.

PGC-1α is involved in the acute core exercise response in skeletal muscle

To characterize the acute core exercise response, we performed gene ontology (GO) analysis on the 56 and 25 DE genes, respectively, and identified five overrepresented categories in WT animals (Fig. 2A) while we could not find any enriched GO category in MKO mice. The top overrepresented category in WT animals was "cellular response to calcium ion" with an enrichment of over 200 and associated genes of the Fos and Jun gene families (Fig. 2A and B). Interestingly, these genes associated also to other GO terms of the five identified categories related to the control of gene transcription and apoptotic processes (Fig. 2B). Furthermore, the second top overrepresented category, "response to stress", involved genes of the heat shock protein (Hsp) family, of which some also associated to another category "negative regulation of apoptotic processes" (Fig. 2B). Thus, the acute core exercise response in WT animals mainly involves the transcriptional regulation of target genes due to calcium ions and stress. Moreover, PGC-1α seems to contribute to this stress-induced transcriptional regulation indicated by the reduced number of DE genes associated to the acute core exercise response in MKO animals.

We also assessed the time point specific GO categories of WT and MKO animals excluding the core exercise response. Immediately after the exercise test (0 h), DE genes of WT animals clustered into categories involved in immunity, hypoxia, inflammation, transcription and signal transduction (Fig. 2C and Supp. Fig. 2A), while DE genes of MKO animals clustered into categories related to the WT core exercise response like "cellular response to calcium", "regulation of apoptotic process" or "response to stress" next to categories involved in immunity, inflammation and transcription (Fig. 2D and Supp. Fig. 2B). These results suggest that PGC-1α might not be important for the induction of the exercise-induced acute stress response but rather for the perpetuation of gene transcription over time. At the 4 h time point of the time-course, DE genes in WT animals associated to different categories than at the 0 h time point like "glucose metabolic process", "angiogenesis", "fatty acid metabolic process" or "actin cytoskeleton organization" (Fig. 2E and Supp. Fig. 2A). Interestingly, "response to stress", one of the GO terms of the acute core exercise response, also appeared in the overrepresented categories of the 4 h time point suggesting that certain members of the Hsp family seem to be important throughout the time-course while others cluster at a given time point (Fig. 2E and Supp. Fig. 2A). The two top GO terms of MKO animals were related to glucose metabolism and angiogenesis, as it was also seen in WT mice, followed by "protein homooligomerization" and transcriptional regulation (Fig. 2F Supp. Fig. 2B). These categories again differed from the ones of WT animals, which was also the case at the 6 h time point. WT animals showed enriched GO terms related to cell growth, inflammation and wound healing

while MKO animals showed “heart development”, “regulation of cell proliferation” and “regulation of ion transmembrane transport” as top overrepresented categories with very low enrichments (Fig. 2G, H and Supp. Fig. 2A, B). At the 8 h time point only one GO term could be identified for both genotypes, “regulation of transcription, DNA-templated” (Supp. Fig. 2A and B). These data demonstrate that the time-courses of WT and MKO animals are clearly distinct from each other and suggest that PGC-1α might be important for the acute core exercise response as well as for the timely regulation of transcription of target genes.

MKO animals show different expression patterns of genes associated to the acute core exercise response

We next assessed the time-course gene expression patterns of genes associated to the acute core exercise response. Fos (Fig. 3A) and Jun (Fig. 3B) of the category “cellular response to calcium ion” showed very similar patterns between WT and MKO animals, however, Fos was significantly lower expressed at the 6 h time point in MKO compared to WT animals (Fig. 3A). Candidates of the second top GO term of the acute core exercise response, “response to stress”, depicted a blunted induction of gene expression in MKO animals. Hsp90aa1 was significantly lower expressed at time points 0 h, 6 h and 8 h (Fig. 3C) while Hspa1b showed reduced transcript levels at time points 6 h and 8 h (Fig. 3D) in MKO compared to WT animals. The same pattern was observed for the early growth factor 1 (Egr1), a gene that is involved in transcriptional regulation (Fig. 3E). On the other hand, nuclear receptor subfamily 4 group A member 3 (Nr4a3) seemed to be negatively regulated by PGC-1α in sedentary mice and at the 6 h time point, even though its transcript levels peaked at the same point in WT and MKO animals (Fig. 3F). Taken together, these results demonstrate that PGC-1α is indeed important for the perpetuation of transcription of genes belonging to the acute core exercise response.

Integrated motif activity response analysis (ISMARA) was conducted to identify enriched transcription factor binding motifs. Serum response factor (SRF) was one of the top candidates of predicted transcription factors, which showed increased transcriptional activity immediately after the exercise test, however, there was no difference between WT and MKO animals (Fig. 3G) (Rose, Kiens et al. 2006). Interestingly, Egr1, Fos and Hsp90aa1 are predicted to be target genes of SRF (data not shown) (Chai and Tarnawski 2002), which would correlate with our gene expression data and suggest that SRF is one of the first transcription factors to be activated after an acute bout of exercise to regulate the stress response. Furthermore, also Jun (Fig. 3H) and Fos (3I) were predicted to have increased transcriptional activity immediately after and at the 4 h time point, respectively, but again independently of PGC-1α. Thus, Fos and Jun of the acute core exercise response not only show induced mRNA levels but also increased transcriptional activity at target gene promoters.

Serum and heat shock experiments in C2C12 myotubes cannot mimic the complex acute core exercise response

As we identified the acute core exercise response to be a stress response and SRF as one of the early induced predicted transcription factors, we tried to mimic this response in differentiated C2C12 myotubes. SRF is known to be induced by a variety of different factors including serum (Chai and Tarnawski 2002). Thus, we treated C2C12 myotubes with 50% horse serum (HS) for different periods of time. Serum shock significantly induced mRNA levels of Fos, Jun, Egr1, Nr4a3 and PGC-1α at different time points, while the transcript levels of Hsp90aa1 were not affected and the ones of Hspa1b were even reduced (Fig. 4A). Thus, serum shock of cells can only limited mimic transcriptional changes observed after acute exercise *in-vivo*. We further assessed the dependence on PGC-1α in this context and serum shocked cells for 15 min to analyze the time-course expression of the SRF target genes. Si-RNA treatment against PGC-1α led to a 50% reduction in PGC-1α mRNA levels, however, this downregulation had no effect on any of the SRF target genes like Fos (Fig. 4B and data not shown).

As we did not observe an upregulation of Hsp after serum shock we tried to mimic the exercise-induced heat-stress response with another stimulus. As the name of the Hsp already suggests, their expression is induced upon heat-stress (Salo, Donovan et al. 1991). Thus, we put C2C12 myotubes at 42 °C for different periods of time and analyzed gene expression changes. Fos, Jun, Nr4a3 and PGC-1α were mildly induced after prolonged heat-stress while the mRNA levels of Egr1 continually decreased over time (Fig. 4C). However, Hsp90aa1 and Hspa1b were nicely induced already after 30 min and 1 h, respectively, of heat-stress (Fig. 4C). We again performed a knockdown experiment of PGC-1α to evaluate its role in the heat-stress response (Xu, Ma et al. 2016). As upon serum shock, si-RNA treatment against PGC-1α had no effect on the induction of Hspa1b or other target genes of the acute core exercise response (Fig. 4D and data not shown). Nevertheless, mRNA levels of Hspa1b were significantly lower in si-PGC-1α compared to si-Scr treated C2C12 myotubes in control conditions (Ctrl) and at the 4 h time point suggesting that PGC-1α might be important for basal Hsp expression (Fig. 4D). These data demonstrate that the acute core exercise response *in-vivo* is a combination of different external triggers and stimuli that is too complex to be mimicked by one single factor in a system *in-vitro*.

Chronic exercise training shows little overlap with the acute-induced time-course transcriptional changes

As described in the introduction, chronic exercise adaptations are believed to be the cumulative result of changes caused by acute bouts of exercise (Egan and Zierath 2013). Thus, we chronically trained WT and MKO mice on a treadmill five times a week for a total of five weeks to analyze and compare the transcriptome to the acute time-course transcriptional changes. Both, WT and MKO animals

significantly improved their endurance exercise capacity (Fig. 5A) as well as their maximal oxygen consumption (VO_{2max}) (Fig. 5B) in a PGC-1α-independent manner. RNAseq analysis revealed only 138 DE genes in WT and 41 DE genes in MKO animals, respectively, upon chronic exercise training compared to 1339 DE genes in WT and 854 DE genes in MKO animals, respectively, upon an acute bout of exercise (Fig. 5D and E, Fig. 1F and G). The three top GO terms in chronically trained WT animals were all related to peptide metabolism and included genes of the myosin heavy chain (MHC) class II antigen family as well as of the serine protease inhibitor family (Fig. 5C) while for chronically trained MKO animals no enriched GO terms were found. Also, only a small fraction of the chronically-induced DE genes overlapped with any of the acute time point transcriptional changes in both WT and MKO animals (Fig. 5D and E). GO analysis of these overlapping genes identified only one enriched category in WT and MKO animals related to transcriptional regulation (Fig. 5F and G). Interestingly, Nr4a3 and Fos of the acute core exercise response also seemed to be chronically regulated, however, in different directions (Fig. 5F). While both genes were significantly induced after an acute bout of exercise, their expression upon chronic exercise training was reduced compared to sedentary mice (data not shown). Thus, these data demonstrate that the acute and chronic transcriptional exercise responses are very distinct from each other and show little overlap in their transcriptome.

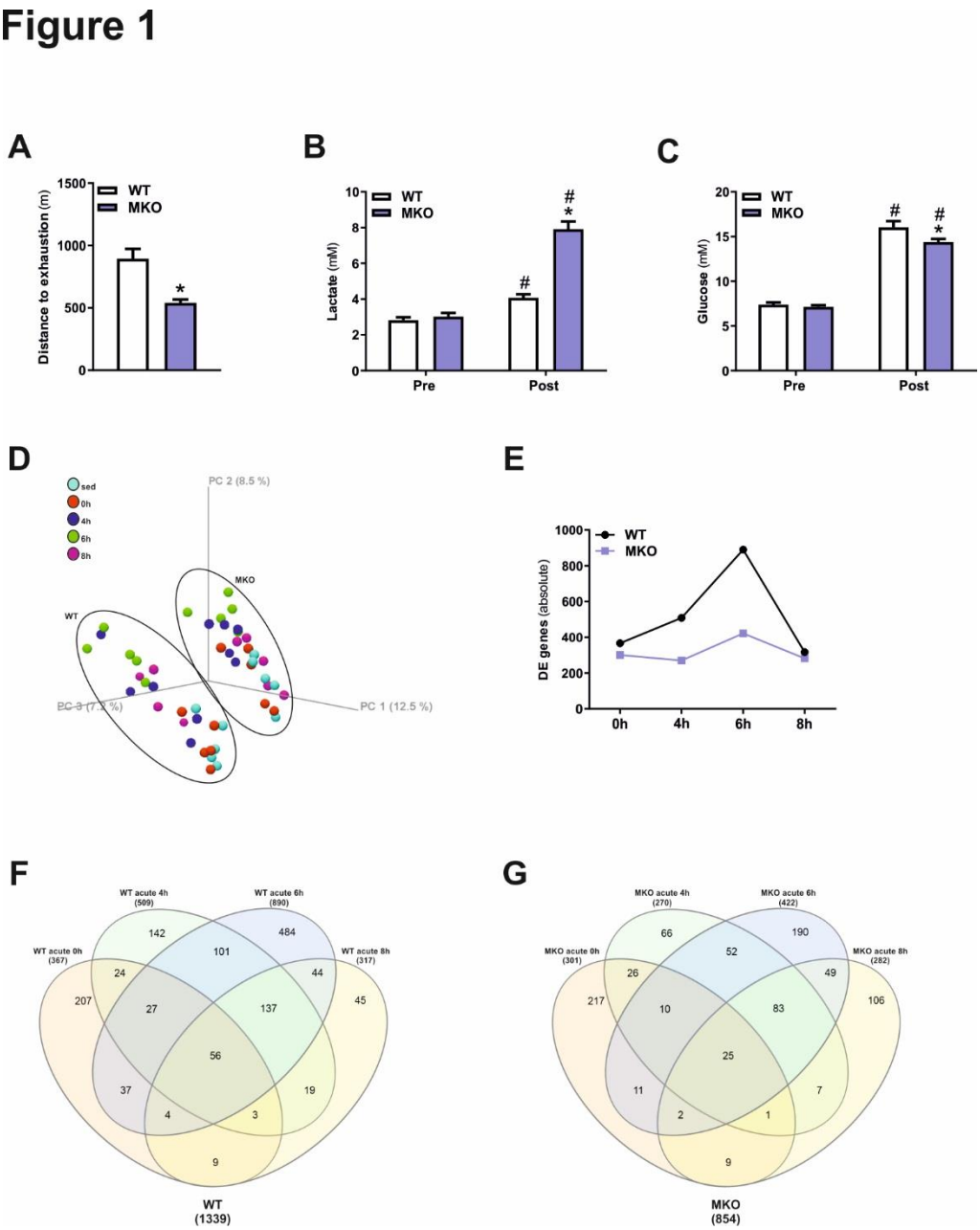


Figure 1. PGC-1α substantially contributes to gene expression changes after acute treadmill running

A) Distance until exhaustion of acutely treadmill run WT and MKO mice (n=24).

B-C) Pre- and post-lactate (B) and –glucose (C) blood levels of acutely treadmill run WT and MKO mice (n=24).

D) Principal component analysis in *Quadriceps* muscle of WT and MKO mice killed 0 h, 4 h, 6 h, or 8 h after the acute treadmill test (n=5).

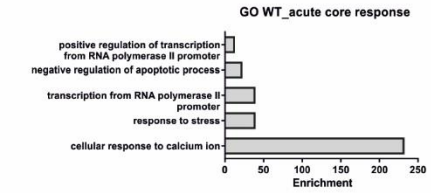
E) Total differentially expressed (DE) genes in *Quadriceps* muscle of WT and MKO mice killed 0 h, 4 h, 6 h, or 8 h after the acute treadmill test compared to sedentary mice with a false discovery rate (FDR) < 0.01 and a $\log_2$ fold change (FC) < -0.6 and > 0.6 (n=5).

F-G) Venn diagram of DE genes in *Quadriceps* muscle of WT (F) and MKO (G) mice killed 0 h, 4 h, 6 h, or 8 h after the acute treadmill test compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

* indicating significant differences between WT and MKO mice; # indicating significant differences between pre- and post-acute exercise.

Figure 2

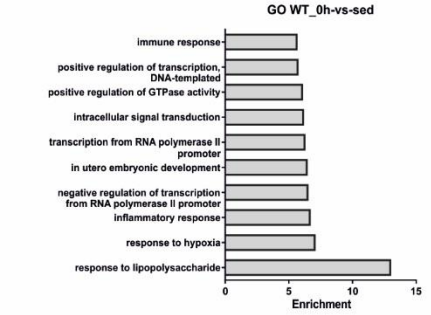
A



B

GO term	Associated genes
cellular response to calcium ion	Jund,Jun,Fosb,Junb,Fos
response to stress	Hsp90aa1,Ppp1r15a,Zfp36,Hspa1a,Hspb1,Dnajb1,Hspa1b
transcription from RNA polymerase II promoter	Egr1,Jund,Jun,Fosb,Junb,Fos
negative regulation of apoptotic process	Nr4a3,Cdkn1a,Jun,Serpine1,Hspb1,Sox9,Sphk1,Plk3,Cyr61,Btg2,Hspa1b
positive regulation of transcription from RNA polymerase II promoter	Nr4a3,Egr1,Abra,Jund,Jun,Gdnf,Csrnp1,Sox9,Cebpd,Cyr61,Fos,Btg2

C



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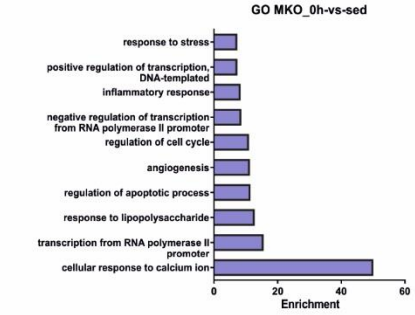

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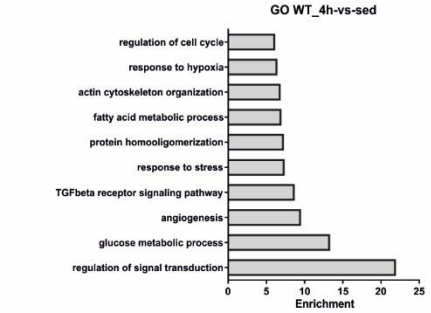

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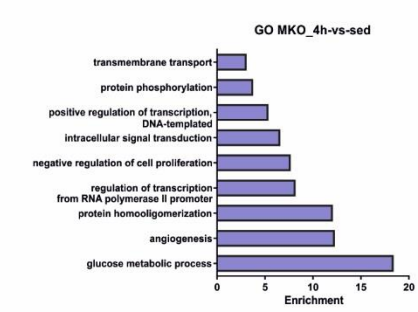

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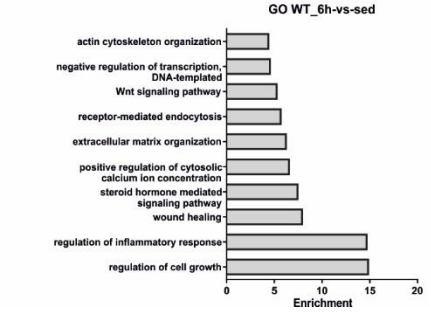

H

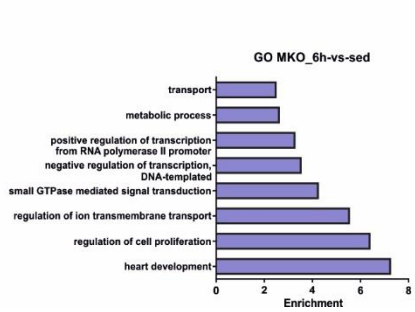

Figure 2. PGC-1α is involved in the acute core exercise response in skeletal muscle

A-B) Gene ontology (GO) analysis of the acute core exercise response and associated genes (B) in *Quadriceps* muscle of WT animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

C-H) GO analysis of differentially expressed (DE) genes in *Quadriceps* muscle of WT and MKO animals 0 h (C, D), 4 h (E, F) and 6 h (G, H) after the treadmill test compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

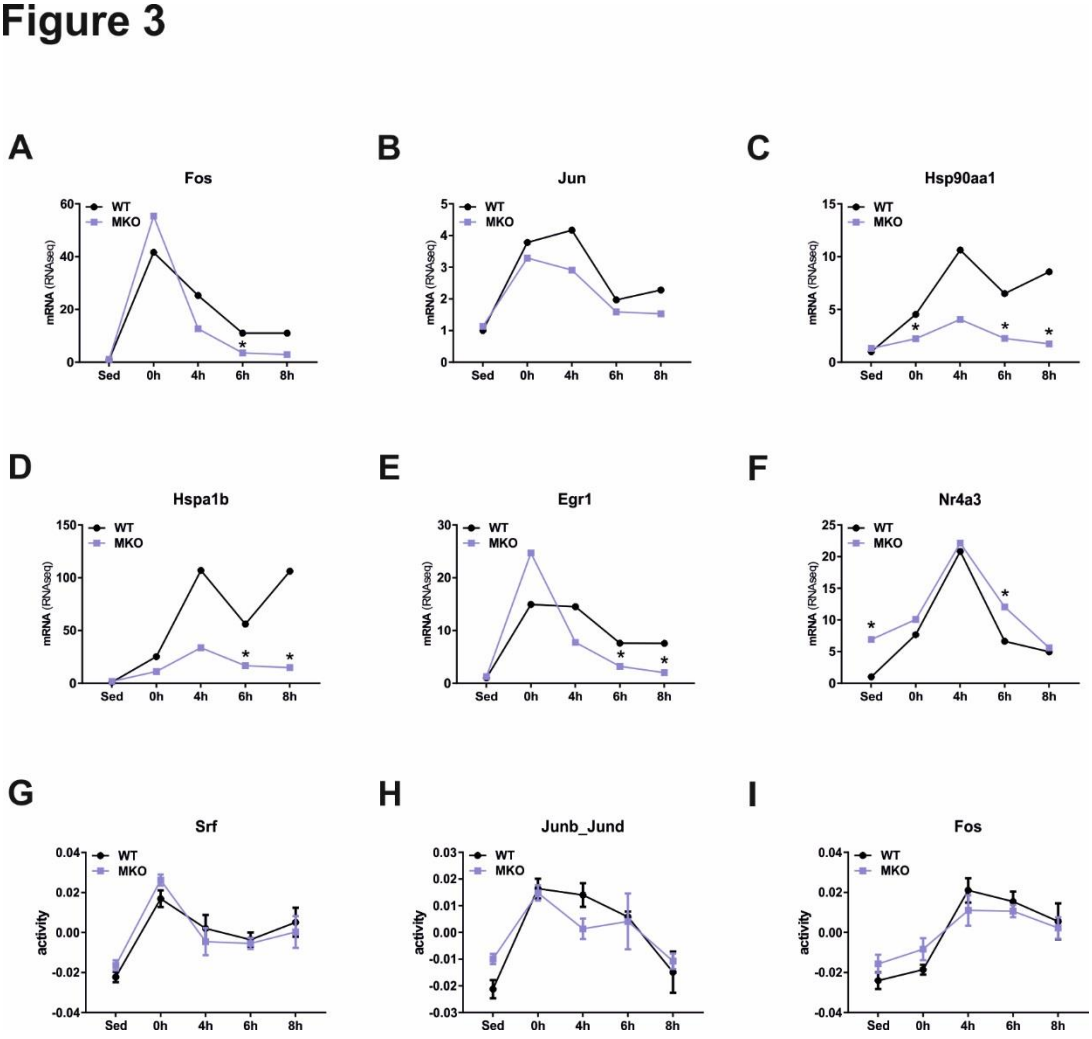

Figure 3. MKO animals show different expression patterns of genes associated to the acute core exercise response

A-F) mRNAseq time-course expression levels of Fos (A), Jun (B), heat shock protein 90aa1 (Hsp90aa1) (C), Hspa1b (D), early growth response 1 (Egr1) (E) and nuclear receptor subfamily 4 group A member 3 (Nr4a3) (F) in *Quadriceps* muscle of WT and MKO animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

G-I) ISMARA predictions of enriched transcription factor binding motifs like serum response factor (SRF) (G), Junb_Jund (H) and Fos (I) in *Quadriceps* muscle of WT and MKO animals (n=5).

* indicating significant differences between WT and MKO mice.

Figure 4

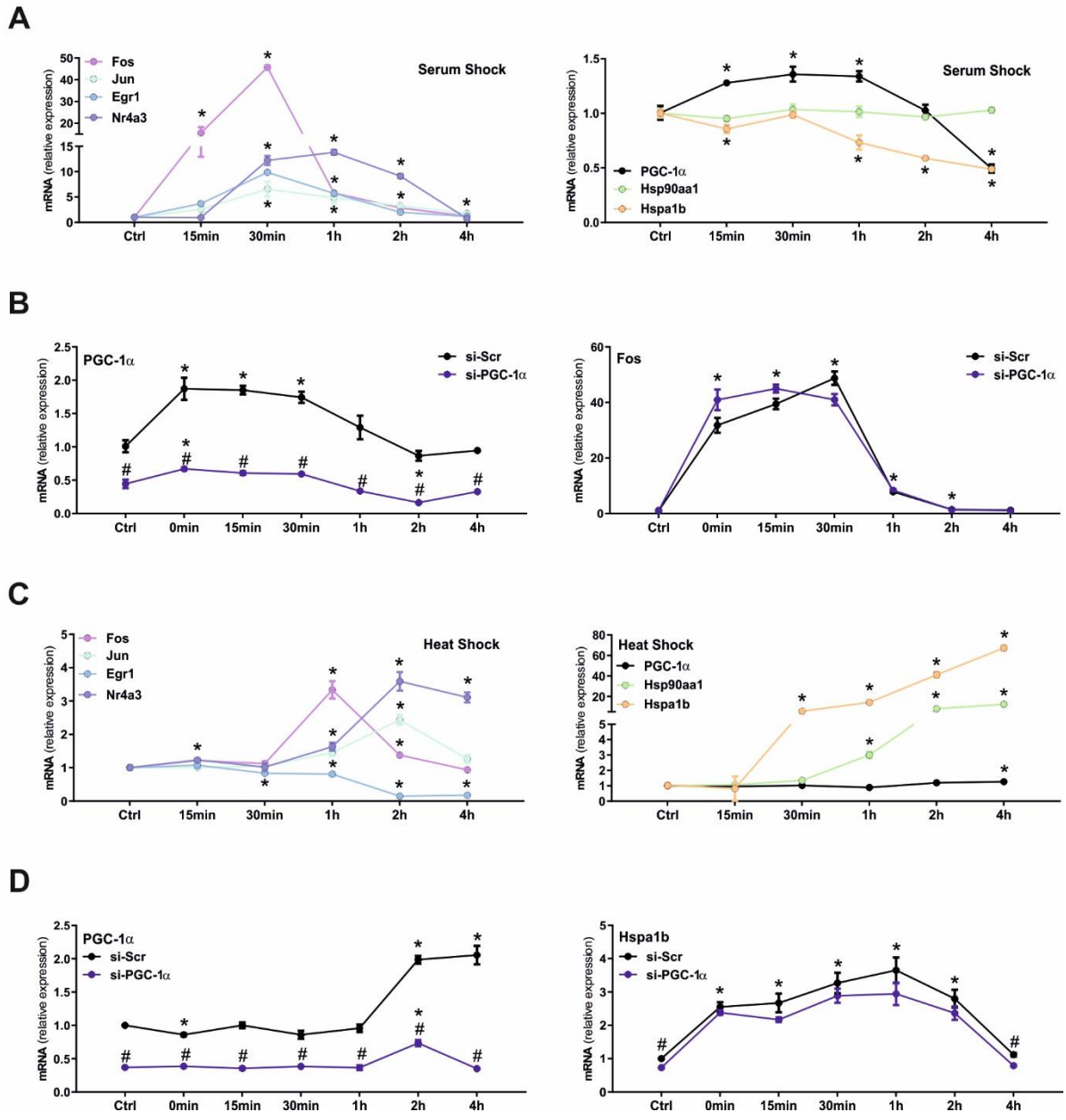

Figure 4. Serum and heat shock experiments in C2C12 myotubes cannot mimic the complex acute core exercise response

A) Gene expression of Fos, Jun, early growth response 1 (Egr1), nuclear receptor subfamily 4 group A member 3 (Nr4a3), PGC-1α, heat shock protein 90aa1 (Hsp90aa1) and Hspa1b relative to TATA binding protein (TBP) in C2C12 myotubes after serum shock for 15 min, 30 min, 1 h, 2 h or 4 h (n=3).

B) Time-course gene expression of PGC-1α and Fos after 15 min of serum shock in C2C12 myotubes treated with control si-RNA (si-Scr) or si-RNA targeting PGC-1α (si-PGC-1α) (n=3).

C) Gene expression of Fos, Jun, early growth response 1 (Egr1), nuclear receptor subfamily 4 group A member 3 (Nr4a3), PGC-1α, heat shock protein 90aa1 (Hsp90aa1) and Hspa1b relative to TATA binding protein (TBP) in C2C12 myotubes after heat shock for 15 min, 30 min, 1 h, 2 h or 4 h (n=3).
D) Time-course gene expression of PGC-1α and Hspa1b after 30 min of heat shock in C2C12 myotubes treated with control si-RNA (si-Scr) or si-RNA targeting PGC-1α (si-PGC-1α) (n=3).
* indicating significant differences between untreated (Ctrl) and treated cells; # indicating significant differences between si-Scr and si-PGC-1α treated cells.

Figure 5

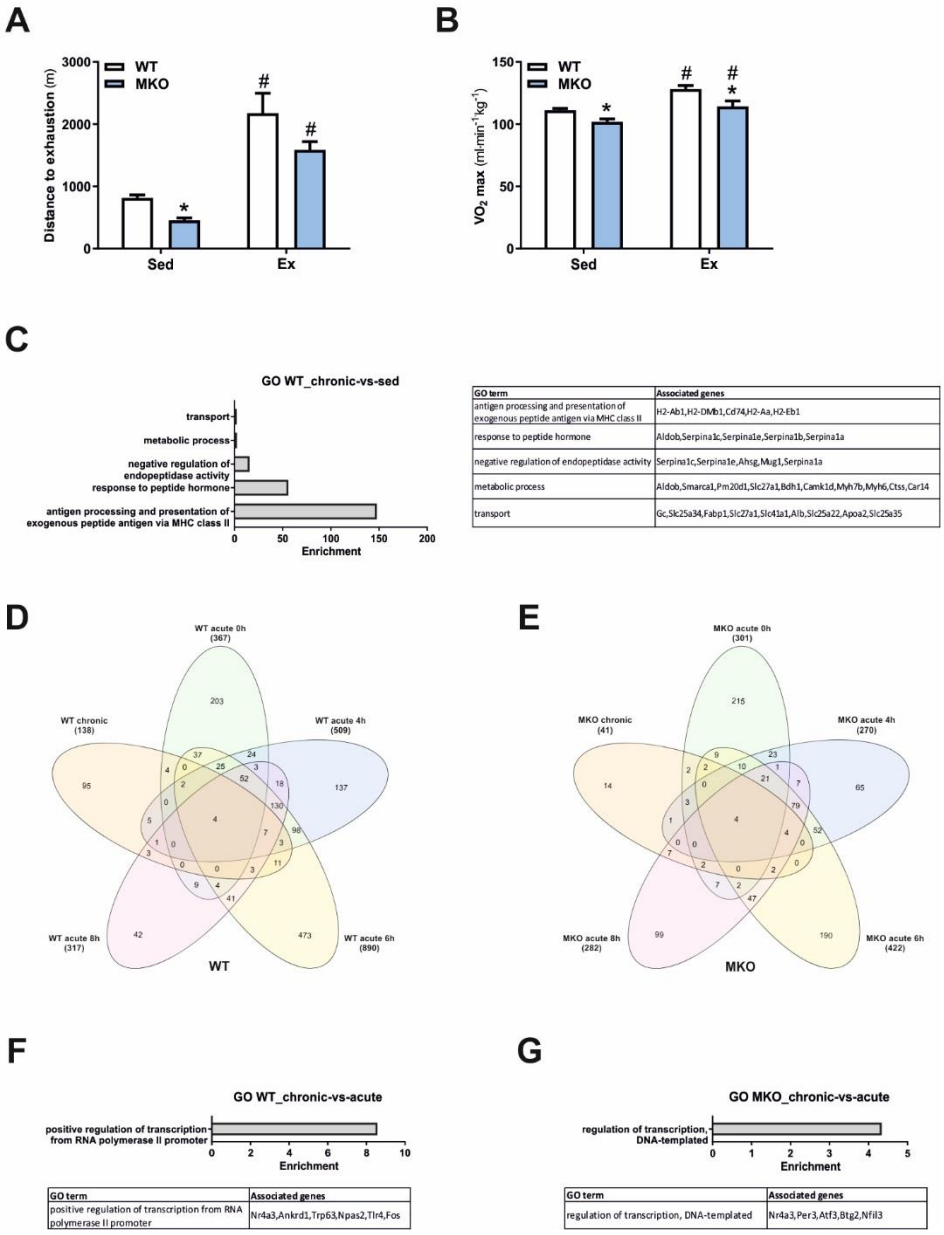

Figure 5. Chronic exercise training shows little overlap with the acute-induced time-course transcriptional changes.

A) Distance until exhaustion of sedentary and chronically trained WT and MKO mice (n=6).

B) Maximal oxygen consumption (VO_{2max}) of sedentary and chronically trained WT and MKO mice (n=6).

C) Gene ontology (GO) analysis of the chronic exercise response with associated genes in *Quadriceps* muscle of WT animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

D-E) Venn diagram of differentially expressed (DE) genes in *Quadriceps* muscle of WT (D) and MKO (E) mice chronically trained or killed 0 h, 4 h, 6 h, or 8 h after the acute treadmill test compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

F-G) Gene ontology (GO) analysis of the overlap of DE genes of chronically and acutely run WT (F) or MKO (G) mice in *Quadriceps* muscle with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

* indicating significant differences between WT and MKO mice; # indicating significant differences between sedentary and chronically trained mice.

Supplemental Figure 1

WT_56 genes					
Pde4b	Ifrd1	Glul	Pdlim1	Crem	Dnajb4
Trib1	Abra	Phlda1	Ppp1r15a	Jund	Cnn1
Midn	Dusp5	Map3k6	Arid5b	Gdnf	Sbk3
Ier5	Rhob	Cebpd	Sox9	Jun	Ctgf
Nle1	Mt2	Hsp90aa1	Cbarp	Mt1	Ptprn
Cyr61	Gadd45g	Zfp36	Nr4a3	Csrnp1	Arc
Plk3	Habp2	Maff	Egr1	Hspa1a	Junb
Atf3	Btg2	Hspa1b	Dnajb1	Fos	Ddit4
Hspb1	Itga5	Dnaja1	Cdkn1a	Serpine1	Sphk1
Otud1	Fosb				

MKO_25 genes					
Ppara	Tuba1c	Nudt18	Emd	Ifi30	Ctla2a
Slc20a1	Gm13889	Itga5	Serpine1	Dusp5	Gadd45b
Hspa1a	Mt2	Chrna9	Cyr61	Mt1	Hspa1b
Slc25a25	Cbarp	Otud1	Atf3	Arc	Dnajb1
Btg2					

Supplemental Figure 1. PGC-1α substantially contributes to gene expression changes after acute treadmill running

List of genes associated to the acute core exercise responses in *Quadriceps* muscle of WT and MKO animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

Supplemental Figure 2

A

WT_0h-vs-sed

GO term	Associated genes	Enrichment
response to lipopolysaccharide	Ccr5,F2r,Ccr1,Cebpb,Ticam1,Nfkbia,Nod2,Ptgs2	13.0
response to hypoxia	Adora1,Fosl2,Cited2,Bmp2,Nr4a2	7.1
inflammatory response	F2r,Cd14,Ccr1,Ticam1,Thbs1,Cxcl1,Nod2,Ptgs2,Ccr2	6.7
negative regulation of transcription from RNA polymerase II promoter	Jdp2,Efna1,Ppp1r13l,Skil,Zbtb10,Hes1,Bcl6b,Klf11,Tsc22d3,Sik1,Klf4, Tgif1,Tbx3,Per1,Sox17,Cited2,Nrarp,Bmp2,Rybp,Id1,Fgfr3,Irf2bp2,Vdr,Ahr	6.5
in utero embryonic development	Hcn4,Hes1,Tbx3,Dll1,Bcl2l1,Cited2,Nos3,Bmp2,Klf2,Notch1,Bcl2l11	6.5
transcription from RNA polymerase II promoter	Egr2,Klf11,Fosl2,Klf4,Ahr	6.3
intracellular signal transduction	Dgke,Adcy4,Rapgef3,Pag1,Wsb1,Nod2,Gucy1a3,Spsb1,Prkd2,Cish,Dusp1	6.2
positive regulation of GTPase activity	Rin3,Rin2,Rgs3,Rgs2,Tbc1d10a,Stard13,Arhgap25,Rgs4	6.1
positive regulation of transcription, DNA-templated	Hes1,Nfe2l2,Elf4,F2r,Egr2,Rel,Klf7,Fosl2,Cebpb,Klf4,Tbx3,Sox17,Nfkbia, Sertad1,Cited2,Bmp2,Klf2,Notch1,Myog,Nr4a1,Ahr,Nr4a2	5.8
immune response	Tinagl1,Tnfsf10,Ccr1,Cxcl1,Pnp,Ccr2	5.7

WT_4h-vs-sed

GO term	Associated genes	Enrichment
regulation of signal transduction	Cnksr3,Runx1,Spry2,Acvr1b,Ptpn1	22.0
glucose metabolic process	Pdk4,Igfbp5,Hk2,Cryab,Myc,Gck,Cpt1a	13.3
angiogenesis	Col4a1,Cspg4,Egfl7,Hmox1,Fgf1,Adam15,Myh9,Col4a2,Klf5,Hbegf,Eng, Fzd5,Ccl2,Tnfrsf12a,Srpk2	9.5
transforming growth factor beta receptor signaling pathway	Pdgfb,Bmpr1b,Smad1,Eng,Ccl2	8.7
response to stress	Hsf2bp,Hspb7,Hspa1l,Hspe1,Hspb8,Hspa8,Rps6ka5,Hspb3,Hsf2	7.4
protein homooligomerization	Ehd4,Tgm2,Hmox1,Gch1,Cryab,Cpt1a,P2rx5	7.3
fatty acid metabolic process	Acot11,Aacs,Lpin3,Acsl3,Cpt1a,Ucp3	7.0
actin cytoskeleton organization	Pdgfb,Csrp1,Myh10,Nrap,Inf2,Daam1,Enah	6.9
response to hypoxia	Acot2,Cryab,Eng,Sox4,Egln3,Ucp3	6.4
regulation of cell cycle	Zfp703,Gadd45a,Ppp2ca,Dtl,Hspa8	6.2

WT_6h-vs-sed

GO term	Associated genes	Enrichment
regulation of cell growth	Htra3,Hrasls,Hsd3b7,Cd44,Htra1,Wisp2,Crim1	14.9
regulation of inflammatory response	Sema7a,Sbno2,Clcf1,Bcl6,Tlr9	14.8
wound healing	Lox,Tgfb1,Cx3cl1,Plau,Erbb2	8.0
steroid hormone mediated signaling pathway	Nr2c2,Nr3c2,Esrrg,Thrb,Rarg	7.5
positive regulation of cytosolic calcium ion concentration	Itpr1,Itpr3,Lrp6,Slc8a1,Adra1a,Pth1r,F2rl1,Fzd2,P2ry2	6.6
extracellular matrix organization	Spock2,Postn,Atp7a,Fbln1,Tnxb,Elf3,Col18a1	6.3
receptor-mediated endocytosis	Scarf2,Prg4,Lrp6,Lrp1,Ldlrap1,Stab1	5.8
Wnt signaling pathway	Hic1,Tax1bp3,Slc9a3r1,Lrp6,Wnt11,Cd44,Fzd10,Sfrp5,Tnks,Fzd2,Pygo1, Col18a1	5.4
negative regulation of transcription, DNA-templated	Glis2,Trps1,Hdac4,Ncoa2,Zhx1,Sox8,Tgfb1,Sbno2,Wnt11,Kctd1,Elf3,Sox10, Zgpat,Snai1,Tbx2,Id4,Fabp4,Thrb,Jarid2,Eid1,Cbx4,Bcl6,Cebpa,Heyl,Foxn3	4.6
actin cytoskeleton organization	Slc9a3r1,Agap2,Shroom3,Sh2b2,Bcl6,Cdc42ep2	4.5

WT_8h-vs-sed

GO term	Associated genes	Enrichment
regulation of transcription, DNA-templated	Pdcd7,Clock,Npas2,Ccdc85b,Esr1,Irf5	3.3

B

MKO_0h-vs-sed

GO term	Associated genes	Enrichment
cellular response to calcium ion	Jund,Jun,Fosb,Junb,Fos	50.0
transcription from RNA polymerase II promoter	Egr1,Egr2,Klf11,Fosl2,Klf4,Jund,Jun,Fosb,Junb,Fos,Ahr	15.6
response to lipopolysaccharide	F2r,Cebpb,Nfkbia,Nod2,Gch1,Ptgs2,Ptger4	12.8
regulation of apoptotic process	Skil,Card10,Mnt,Tnfrsf10b,Nod2,Gas1,Sox9,Irak2,Fgfr3,Dusp1,Bcl2l11, Dusp2	11.5
angiogenesis	Rhob,Zc3h12a,Epha2,Tal1,Sox17,Jun,Ctgf,Sox18,Nos3,Adra2b,Ptgs2,S1pr1	11.3
regulation of cell cycle	Cdkn1a,Gadd45g,Mnt,Sgk1,Jun,Junb	11.0
negative regulation of transcription from RNA polymerase II promoter	Jdp2,Hic1,Skil,Zbtb10,Hes1,Egr1,Bcl6b,Tal1,Per3,Klf11,Tsc22d3,Sik1,Klf4,Tgif1,Per1,Sox17,Zfp36,Nrarp,Cbx6,Smad7,Id1,Fgfr3,Irf2bp2,Pik3,Kdm6b, Bcl6,Vdr,Ahr	8.6
inflammatory response	Ccrl2,F2r,Cd14,Thbs1,Cxcl1,Nod2,Ptgs2,Nfkbiz,Sphk1,Kdm6b	8.4
positive regulation of transcription, DNA-templated	Ppargc1a,Hes1,Nfe2l2,Egr1,F2r,Egr2,Tal1,Klf7,Fosl2,Cebpb,Klf4,Sox17, Nfkbia,Sertad1,Jun,Sox18,Sox9,Irf8,Klf2,Myog,Irf1,Nr4a1,Fos,Ahr,Nr4a2	7.4
response to stress	Hsp90aa1,Ppp1r15a,Sgk1,Zfp36,Ahr,Nr4a2	7.3

MKO_4h-vs-sed

GO term	Associated genes	Enrichment
glucose metabolic process	Hk2,Npy1r,Cryab,Myc,Gck	18.4
angiogenesis	Cspg4,Angpt2,Hmox1,Fgf1,Klf5,Hspb1,Hbegf,Fzd5,Tnfrsf12a,Srpk2	12.3
protein homooligomerization	Lgi1,Ehd4,Hmox1,Angptl4,Cryab,P2rx5	12.1
regulation of transcription from RNA polymerase II promoter	Frzb,Nr4a3,Ankrd1,Fzd4,Myod1,Rarb,Esrrg,Smad1,Fzd5,Myf6,Heyl	8.2
negative regulation of cell proliferation	Frzb,Nox4,Hmox1,Spry2,Xirp1,Fosl1,Rarb,Agt,Smad1,Fzd5,Hmga1	7.7
intracellular signal transduction	Cspg4,Hmox1,Spsb2,Tiam2,Kalrn,Asb5,Twf2,Cblb	6.6
positive regulation of transcription, DNA-templated	Fzd4,Runx1,Fgf1,Trp63,Myod1,Abra,Pdgfb,Ets2,Klf5,Agt,Esrrg,Myc,Myf6, Hmga1	5.4
protein phosphorylation	Plk2,Map3k8,Cdk5r1,Trib2,Mknk2,Kalrn,Pdgfb,Musk,Smad1,Srpk2,Trib1	3.8
transmembrane transport	Slc12a6,Slc22a4,Slc10a7,Kcnf1,Slc7a5,Slc43a1,Slc22a3	3.1

MKO_6h-vs-sed

GO term	Associated genes	Enrichment
heart development	Bmp4,Tead1,Pbrm1,Ece1,Itga4,Kcnj8,Nfatc4	7.3
regulation of cell proliferation	Tead1,Plau,Tbx3,Nr3c1,B4galt1	6.4
regulation of ion transmembrane transport	Clcn2,Kcng4,Kcnj8,Cacna1h,Clcn3,Scn7a	5.6
small GTPase mediated signal transduction	Lrrk2,Tiam1,Rasl12,Rasd2,Arhgap5	4.3
negative regulation of transcription, DNA-templated	Trps1,Bmp4,Tbx3,Rps6ka5,Tbx2,Thrb,Eid1,Cbx4,Foxn3	3.6
positive regulation of transcription from RNA polymerase II promoter	Gli1,Bmp4,Tead1,Bmp6,Npas2,Cytl1,Esr1,Rps6ka5,Ldb2,Thrb,Plscr1, Nfatc4,Nr3c1	3.3
metabolic process	Zc3h12c,Gstt2,Nat6,Acot11,Pde10a,Dpm1,B3galt1,Tnfaip3,Dcp2,Eno2,Atp 2a3,Ddah1,Atp7a,Gcnt2,Pign,Alg10b,Ece1,Edem3,Pck2,Pggt1b,Alpl,Alkbh8, Car14,Aldh9a1,Chd6,Gla,Fktn	2.6
transport	Clcn2,Slc45a4,Kcnk5,Kcng4,Cygb,Syt7,Ano1,Slc6a19,Atp2a3,Chrna4,Atp7a, Abcb1a,Fads3,Lcn2,Ap4e1,Kcnj8,Slc16a10,Cacna1h,Clcn3,Syt12,Xpo4, Cpt1a,Scn7a,Osbpl6,Pitpnc1	2.5

MKO_8h-vs-sed

GO term	Associated genes	Enrichment
regulation of transcription, DNA-templated	Nr4a3,Per3,Atf3,Btg2,Nfil3	4.3

Supplemental Figure 2. PGC-1α is involved in the acute core exercise response in skeletal muscle

A) Gene ontology (GO) analysis of the acute time-course exercise response with associated genes in *Quadriceps* muscle of WT animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

B) Gene ontology (GO) analysis of the acute time-course exercise response with associated genes in *Quadriceps* muscle of MKO animals compared to sedentary mice with a FDR < 0.01 and a $\log_2$FC < -0.6 and > 0.6 (n=5).

Discussion

Regular exercise is widely accepted as a strategy in the prevention, management and treatment of many metabolic and chronic diseases such as hypertension, cardiovascular diseases, type 2 diabetes and sarcopenia (Haskell, Lee et al. 2007, Colberg, Sigal et al. 2010). Many of the exercise-induced benefits in whole body metabolism are mediated by profound metabolic and molecular changes in skeletal muscle (Egan and Zierath 2013). These adaptations include changes in the contractile apparatus (Adams, Hather et al. 1993, Widrick, Stelzer et al. 2002), mitochondrial and metabolic function (Green, Helyar et al. 1992, Spina, Chi et al. 1996), signaling cascades (Benziane, Burton et al. 2008) and transcriptional responses (Pilegaard, Saltin et al. 2003). One of the most important mediator of the transcriptional adaptations to exercise is PGC-1α (Catoire, Mensink et al. 2012, Egan and Zierath 2013). For the first time, we now defined the transcriptional acute core exercise response and identified PGC-1α as an essential player in the perpetuation of this response over time. Furthermore, the acute and chronic transcriptional responses to exercise are clearly distinct from each other suggesting that chronic exercise training leads to changes in protein rather than mRNA expression levels.

It is well established that elevated cytoplasmic calcium concentrations after neuronal stimulation activate different signaling cascades such as the Ca^{2+}/calmodulin-dependent protein kinases (CaMK) (Gehlert, Bloch et al. 2015). Thus, it is not surprising that the top GO term of the acute core exercise response was identified to be “cellular response to calcium ion”. Also, the associated genes of the Fos and Jun gene family have been shown to be induced after exercise in human skeletal muscle (Puntschart, Wey et al. 1998), most likely through the activation of CaMKs and mitogen-activated protein kinases (MAPK) (Carrasco, Riveros et al. 2003). Interestingly, PGC-1α expression is also known to be induced by CaMKIV and the p38 MAPK (Wu, Kanatous et al. 2002, Akimoto, Pohnert et al. 2005) and seems to be involved in the perpetuation of Fos gene expression over time. Furthermore, PGC-1α has been shown to interact with the activator protein 1 complex (AP-1), a transcription factor composed of different proteins belonging to the Fos, Jun, proto-oncogene c-Maf (Maf) and activating transcription factor (ATF) gene families (Shaulian and Karin 2002), to induce transcription of target genes (Baresic, Salatino et al. 2014). It is conceivable that some of the exercise-induced gene expression changes are regulated by the AP-1 complex, which could explain why MKO animals show a different time-course expression pattern and a reduced number of DE genes. Thus, it would be interesting to perform knockdown studies of the two AP-1 complex members Fos and Jun to identify PGC-1α/AP-1-dependent target genes that are involved in the acute response to exercise.

SRF was predicted to have increased transcriptional activity immediately after the exercise bout (Rose, Kiens et al. 2006), which could also be due to increased cytoplasmic calcium concentrations and

activated CaMK. At least *in-vitro* it was shown that SRF is a substrate of CaMKII, which phosphorylates it at Ser^{103} and Thr^{160} leading to its translocation to the nucleus (Fluck, Booth et al. 2000). These *in-vitro* data suggest that the predicted increased activity of SRF could be also caused by an elevation of cytoplasmic calcium concentrations and would indicate that SRF activation upon exercise is calcium-dependent, not only *in-vitro* but also *in-vivo*. Therefore, future studies could elucidate the acute exercise response and the activation of SRF under conditions in which calcium release from the sarcoplasmic reticulum is blocked (e.g. by blocking the ryanodine receptor) or in which CaMK activity is disabled. Furthermore, SRF could be a potential new transcriptional partner of PGC-1α and thus, as for Fos and Jun, SRF knockdown studies would help in identifying SRF/PGC-1α-dependent target genes. The acute core exercise response also included the GO term "response to stress" in which Hsp family members clustered. Hsp, which have been shown to be upregulated upon an acute bout of exercise in different tissues including skeletal muscle, are chaperones that help to correctly fold newly synthesized proteins or to prevent protein aggregation (Henstridge, Febbraio et al. 2016). Interestingly, Hsp are not only regulated by heat-stress but also by other physiological stressors such as energy depletion, hypoxia or acidosis, all of which are induced by exercise (Kregel 2002). Moreover, it cannot be excluded that increased cytoplasmic calcium concentrations could also lead to an induction of Hsp, even though experimental data for this hypothesis are missing. Thus, it is difficult to dissect if upon an acute bout of exercise Hsp are only induced due to heat-stress caused by the mechanical contraction or due to a combination of the different stressors mentioned above. However, one could perform exercise training in a temperature controlled environment to prevent overheating of the muscle and thereby elucidate if Hsp are only induced by heat-stress or other factors. Nevertheless, PGC-1α seems to be substantially involved in the Hsp response upon exercise given the reduced induction and perpetuation of Hsp90aa1 and Hspa1b expression in MKO animals. Recently, PGC-1α has been shown to interact with heat shock factor-1 (HSF-1) to induce transcription of Hsp genes (Xu, Ma et al. 2016), which could explain the different response in MKO animals. However, knockdown of PGC-1α in C2C12 myotubes did not lead to a defect in Hsp transcriptional induction. These data suggest that either the induced heat-stress was to weak, even though Hsp expression was significantly induced, or that the C2C12 myotubes system was not chosen wisely because basal mRNA expression of PGC-1α is too low. Therefore, either primary cells or *in-situ* experiments would be more suitable systems to study the heat-stress response.

Finally, the acute and chronic transcriptional responses were clearly distinct from each other, even though theoretically it is believed that induced transcript levels after each bout of acute exercise would result in a sustained increase in mRNA expression after chronic training (Egan and Zierath 2013). However, it is possible that the chronic exercise groups would have already shown changes in protein rather than transcript levels, which we did not assess in this study. Thus, mass spectrometry analysis

of the chronically trained animals should be conducted and compared to the transcriptional changes of the acute time-course. Furthermore, it would be interesting to perform a time-course analysis of chronically trained mice and compare that to the acute time-course. These data would give insights in how close the acute and chronic time-course responses are to each other and whether chronic exercise training leads to adaptations that would blunt the response to each acute exercise bout in order to reduce the exercise-induced stress.

To conclude, our data highlight the acute and chronic exercise responses and the dependency of PGC-1α in these contexts, especially its function in the acute core exercise response, which solidifies its key role in exercise and muscle metabolism. Furthermore, new exciting questions regarding the acute and chronic exercise time-courses arose, which will hopefully be answered in future studies to contribute to the elucidation of the complex biology of exercise.

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Supplemental Material

Table 1. Acclimatization protocol for treadmill running of the acute time course study at 5° inclination.

Day 1	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	10 min 10 m/min		
Day 2	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	5 min 12 m/min	
Day 3	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	5 min 12 m/min	2 min 14 m/min
Day 4	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	10 min 12 m/min	2 min 14 m/min
Day 5	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	10 min 12 m/min	5 min 14 m/min

Table 2a. Acclimatization protocol for treadmill running of chronically trained mice.

Day 1	0° inclination	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	5 min 10 m/min
Day 2	5° inclination	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	5 min 10 m/min

Table 2b. Training protocol for chronic treadmill exercise.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Week 1	10 m/min	10.5 m/min	11 m/min	11.5 m/min	12 m/min
Week 2	12.5 m/min	13 m/min	13.5 m/min	14 m/min	14.5 m/min
Week 3	15 m/min	15.5 m/min	16 m/min	16.5 m/min	17 m/min
Week 4	17.5 m/min	18 m/min	18.5 m/min	Rest	Exercise test
Week 5	Exercise test	18.5 m/min	18.5 m/min	Sacrifice	

Table 3. qPCR primer sequences.

Gene Name	**Forward primer**	**Reverse primer**
Egr1	GAGCACCTGACCACAGAGTC	CGAGTCGTTTGGCTGGGATA
Fos	TACTACCATTCCCCAGCCGA	GCTGTCACCGTGGGGATAAA
Hsp90aa1	CGACGATGAGCAGTATGCCT	TCCACGACCCATTGGTTCAC
Hspa1b	GTGCACTGTACCAGGGGATTAT	CCCAGGCTACTGGAACACTG
Jun	TGGGCACATCACCACTACAC	TCTGGCTATGCAGTTCAGCC
Nr4a3	CTGCTGCTTCGCCTATCCC	GCAGGGCATATCTGGAGGGT
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
TBP	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT

5. PGC-1β is involved in the response to fasting-induced skeletal muscle atrophy by inhibiting Nfatc1 activity

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Abstract

Skeletal muscle accounts for around 40-50% of total body weight and serves as the largest protein reservoir of the body. As such, protein catabolism from muscle tissue is of crucial importance when energy supply is restricted, as during fasting conditions, to maintain *de-novo* production of glucose in the liver. Thus, skeletal muscle emerges as one of the key players in the whole body response to energy deprivation. However, the complex regulation of skeletal muscle metabolism upon fasting is still poorly understood. We now assessed the role of the peroxisome proliferator-activated receptor γ coactivator-1β (PGC-1β) in skeletal muscle of male wild type control (WT) and PGC-1β muscle-specific knockout (MKO) mice upon 24 h of fasting. Interestingly, MKO animals are partially protected from the fasting-induced muscle atrophy even though PGC-1β expression is downregulated in muscle of WT mice after a 24 h energy deprivation phase. Moreover, MKO animals show significantly reduced myostatin mRNA levels, a blunted induction of atrophy markers gene expression and absent activation of AMP-dependent and cAMP-dependent protein kinases. The increased transcriptional activity of the nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) and the concomitant elevation of PGC-1α mRNA levels provide evidence for the blunted fasting response in MKO animals and suggest that PGC-1β acts as an inhibitor for Nfatc1 activity. Thus, these data highlight the complex regulation of muscle metabolism under fasting conditions and reveal PGC-1β as an important regulator in the control of fasting.

Abbreviations

AMPK, AMP-dependent protein kinase; ATP5A, ATP synthase 5 alpha; BSA, bovine serum albumin; β-OHB, β-hydroxybutyrate; BW, body weight; CaMK, Ca2+/calmodulin-dependent protein kinase; CEE, chicken embryo extract; COX, cytochrome oxidase; Cox4i1, cytochrome C oxidase subunit 4 isoform 1; Cox5B, cytochrome C oxidase subunit 5B; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate; CRE, cAMP response element; Creb, cAMP response element binding protein; CSA, cross sectional area; CytC, cytochrome C; DE, differentially expressed; DMSO, dimethyl sulfoxide; EDL, *Extensor digitorum longus* muscle; FC, fold change; FDR, false discovery rate; FoxO, forkhead box O; GO, gene ontology; HS, horse serum; HSA, human skeletal actin; IBMX, 3-isobutyl-1-methylxanthine; ISMARA, integrated motif activity response analysis; MAFbx, muscle atrophy F-box; March1, membrane associated ring-CH-type finger 1; Mettl11b, methyltransferase like 11B; minFeret, minimal fiber feret; MKO, PGC-1β-specific muscle knockout; Mstn, myostatin; MuRF-1, muscle RING finger 1; NDUFB8, NADH dehydrogenase 1 beta subcomplex subunit 8; NEFA, non-esterified fatty acid; Nfatc1, nuclear factor of activated T-cells, cytoplasmic 1; Nos1, nitric oxide synthase 1; PGC-1, peroxisome proliferator-activated receptor γ coactivator-1; PKA, cyclic AMP-dependent protein kinase; Pomk, protein-O-mannose kinase; RER, respiratory exchange ratio; SDH, succinate dehydrogenase; SDHB, mitochondrial succinate dehydrogenase iron-sulfur subunit; SEM, standard errors of the means; Sh3kbp1, SH3 domain containing kinase binding protein 1; TA, *Tibialis anterior* muscle; TBP, TATA-binding protein; TGF, transforming growth factor; UQCRC2, mitochondrial cytochrome b-c1 complex subunit 2; WT, wild type control

Introduction

Sufficient energy supply is key for life and multiple mechanisms have evolved over billions of years to ensure energy homeostasis even during fasting or starvation. Lately, different forms of fasting (e.g. intermittent or periodic) have gained more and more attention as approaches to counteract weight gain and obesity (Varady and Hellerstein 2007, Harvie, Pegington et al. 2011). Furthermore, emerging evidence suggests that fasting could even be protective against a variety of different pathological conditions such as diabetes, cancer or neurodegeneration, at least in rodent models (Pedersen, Hagemann et al. 1999, Halagappa, Guo et al. 2007, Lee, Raffaghello et al. 2012). As an adaptive hypometabolic response to energy deprivation, fasting involves the complex interplay between all organs of the body in a temporal highly organized manner. Initially, blood glucose concentration drops consequently leading to the release of glucagon from the pancreas, which mainly acts on the liver to release glucose into the circulation by triggering glycogenolysis and gluconeogenesis (Briant, Salehi et al. 2016). Furthermore, the reduced blood insulin levels lower the entry of glucose into adipose tissue

and muscle resulting in increased lipolysis and fatty acid oxidation, respectively (Holness and Sugden 1990). These early metabolic changes all contribute to the maintenance of blood glucose levels and ensure the proper functioning of organs mainly relying on glucose as an energy fuel like red blood cells or the brain. If fasting is prolonged, liver glycogen stores get depleted so that the only source of glucose comes from gluconeogenesis, which is fueled by glycerol from lipolysis in adipose tissue as well as from the catabolism of muscle proteins (Longo and Mattson 2014). Hence, the fasting-induced muscle breakdown will necessitate a loss of function resembling other catabolic conditions such as denervation or cancer cachexia (Mitch and Goldberg 1996). However, during fasting, muscle loss is limited to a minimum by the production of ketone bodies in a process called ketogenesis in the liver (Krebs 1966). Released ketone bodies can be used by extra-hepatic tissues such as skeletal muscle and brain, which largely diminishes the need of glucose and consequently minimizes the loss of muscle protein and functionality. Thus, skeletal muscle is one of the most important players in the regulation of whole body energy homeostasis during fasting. Even though the fasting response in skeletal muscle has been extensively studied at the level of protein modifications and post-translational changes (de Lange, Farina et al. 2006, de Lange, Moreno et al. 2007) only very few studies focused on the transcriptional regulation, especially on a transcriptome-wide level (Fong, Moldawer et al. 1989, Wu, Inskeep et al. 1999, Hildebrandt and Neufer 2000, Jagoe, Lecker et al. 2002). Moreover, the involvement of coregulator proteins, which are able to control the transcription of target genes by interacting with different transcription factors without possessing an intrinsic DNA binding side themselves (Schnyder, Kupr et al. 2017), is still unknown. The peroxisome proliferator-activated receptor γ coactivator-1β (PGC-1β) belongs to the PGC-1 family of transcriptional coactivators (Kressler, Schreiber et al. 2002, Lin, Puigserver et al. 2002) and has been implicated to play a role in skeletal muscle mitochondrial homeostasis, angiogenesis and fiber type determination (Arany, Lebrasseur et al. 2007, Zechner, Lai et al. 2010, Rowe, Jang et al. 2011, Gali Ramamoorthy, Laverny et al. 2015). Furthermore, hepatic PGC-1β mRNA levels are induced upon fasting (Lin, Tarr et al. 2003) and liver-specific PGC-1β knockout mice show a blunted fasting-refeeding response (Chambers, Chen et al. 2012). Given the important role of skeletal muscle in the whole body response to energy deprivation and the fact that PGC-1β is an important regulator of skeletal muscle metabolism, we wondered if muscle PGC-1β might be also involved in the fasting response. Thus, the aim of our study was to elucidate the role of PGC-1β in the regulation of fasting on a transcriptome-wide level using wild type control (WT) and PGC-1β muscle-specific knockout (MKO) mice. Our data show that PGC-1β is substantially involved in the response to energy deprivation even though its expression in WT mice is downregulated upon fasting most likely through cAMP-inducing pathways. Surprisingly, MKO mice were partially protected from the fasting response and showed a blunted induction of myostatin (Mstn) and atrophy markers gene expression and reduced activation of AMP-dependent protein kinase

(AMPK) and cAMP-dependent protein kinase (PKA). We identified increased transcriptional activity of the nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) in MKO animals leading to increased PGC-1α expression, which resulted in the blunted induction of the fasting-induced muscle atrophy. Thus, our results shed new light on the complex transcriptional network after energy deprivation and identified PGC-1β and Nfatc1 as novel players in the fasting response in skeletal muscle.

Material and Methods

Animal housing and PGC-1β muscle-specific knockout mouse generation

Mice had free access to food and water and were housed in a conventional facility with a 12 h light/12 h dark cycle. Experiments were performed with the approval of the Swiss authorities on adult male mice (10 weeks or older). For the knockout confirmation (Fig. 1E and F) 3-4 weeks old male and female mice were used. PGC-1β muscle-specific knockout (MKO) animals were generated as follows. PGC-$1\beta^{loxP/loxP}$ animals were purchased from the Jackson Laboratory (B6.129X1-Ppargc1b$^{tm1.1Dpk}$/J, strain number 012378; (LAI, LEONE ET AL. 2008)) and crossed with human skeletal actin (HSA)-Cre transgenic mice to generate MKO mice. PGC-$1\beta^{loxP/loxP}$ animals without Cre expression were used as wild type control (WT) mice. Genotyping was performed from tail biopsies by PCR using specific primer pairs to detect the presence of the loxP sites, which resulted in amplicons of ~500 bp (WT allele, 318 bp). Specific primer pairs to detect Cre recombinase resulted in amplicons of 100 bp in MKO mice.

In vivo analysis

To determine PGC-1 gene expression after maximal endurance performance mice were acclimatized to treadmill running (Columbus Instruments) as described in Table 1 of the Supplemental Experimental Procedures. Two days after acclimatization, the test started at 5 m/min for 5 min and 8 m/min for 5 min with a 5° incline and the speed was increased 2 m/min every 15 min until exhaustion. 4 h after the exercise test mice were killed by CO_2 and *Quadriceps* muscles removed.

All mice were fed a normal chow diet. For fasting experiments food was withdrawn from the mice in the morning and 24 h later mice were killed by CO_2 and organs removed

Body composition of the mice was determined using an EchoMRI-100^{TM} analyzer (EchoMRI Medical Systems).

For indirect calorimetry mice were placed in a CLAMS system (Columbus Instruments) for four days (2 days acclimatization, 2 days measurement) to assess their VO_2 consumption and respiratory exchange ratio (RER). For body temperature measurements anipills (Phymep S.a.r.l.; REF: 01101, LOT: 15-03,1 (DL 01-2017)) were implanted into the intraperitoneal cavity two weeks before CLAMS measurements.

Blood analysis

Blood analysis was carried out in the morning of *ad-libitum* fed or 24 h fasted animals. Blood glucose and ketone bodies were measured from tail blood with a glucose meter (Accu-Chek, Roche) or a ketone body meter (Precision Xtra, Abbott Laboratories), respectively. Plasma was obtained from whole blood, which was collected in microvette tubes (Sarstedt) and centrifuged at 2000 g for 10 min. Non-esterified fatty acids (NEFA) were measured in plasma using a NEFA-Kit (HR Series NEFA-HR(2), Wako Diagnostics) according to the manufacturer's instructions.

Primary cell culture

For the establishment of primary cell cultures, single fibers of 3 weeks old male WT mice were isolated as described in the Supplemental Experimental Procedures. Primary myoblasts were differentiated at around 60-70% confluency using differentiation medium (DMEM Glutamax, 4% HS, 1% P/S, 1% CEE) for 3 days. The next day, cells were serum starved with low glucose medium (D6046, Sigma) for 16 h before treatment of the different compounds for 6 h. Compounds used were forskolin (100 µM, F3917, Sigma), 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP, 100 µM, C3912, Sigma), 3-isobutyl-1-methylxanthine (IBMX, 1 mM, I5879, Sigma). All compounds were diluted in dimethyl sulfoxid (DMSO, 1%, D2650, Sigma).

Skeletal muscle stainings

Freshly isolated *Gastrocnemius* muscles were placed in 8% tragacanth (G1128, Sigma) and frozen in liquid nitrogen cooled isopentane before cutting 10 µm cryo-cross-sections.

Succinate dehydrogenase (SDH) staining: sections were exposed to 50 mM sodium succinate (S2378, Sigma) in 0.1 M phosphate buffer in the presence of 0.5 mg/ml nitroblue tetrazolium (N5514, Sigma) for 30 min at 37 °C. Then, sections were washed with ddH_2O, dehydrated with ethanol and mounted with histomount (008030, Thermo Scientific).

Cytochrome oxidase (COX) staining: slides were exposed to 0.5 mg/ml 3,3'-Diaminobenzidine tetrahydrochloride hydrate (D5637, Sigma), 0.2 mg/ml cytochrome c (C2506, Sigma) and 0.125 mg/ml catalase (C40, Sigma) in PBS for 1h at 37 °C. Then slides were washed with ddH_2O, dehydrated with ethanol and mounted with histomount (008030, Thermo Scientific).

Fiber typing was carried out as described in the Supplemental Experimental Procedures. Whole sections were pictured using a slide scanner (Axio Scan.Z1, Zeiss). For minFeret measurements and fiber type counting, square pictures from total sections were cropped out (mean of two pictures in the oxidative part of the muscle, one in the glycolytic part of the muscle). For minFeret determination a Fiji script was used as described in the Supplemental Experimental Procedures.

mRNA sequencing and analysis

mRNA sequencing library preparation was carried out as described in the Supplemental Experimental Procedures. Fastq files were mapped to the mouse genome (mm10) and RNAseq and statistical analysis performed with the CLC Genomics Workbench Software (Qiagen).

Differentially expressed (DE) genes were pictured in a Venn diagram with the use of the eulerAPE drawing tool (Micallef and Rodgers 2014). Gene ontology (GO) analysis was executed by the usage of GeneCodis (Carmona-Saez, Chagoyen et al. 2007, Nogales-Cadenas, Carmona-Saez et al. 2009, Tabas-Madrid, Nogales-Cadenas et al. 2012). Only GO terms with at least 5 mapped genes were considered to be enriched and GO list was sorted according to corrected hypergeometric p-value. The top ten of enriched GO terms (Table 3-7 in the Supplemental Experimental Procedures) were furthermore sorted by enrichment (calculated by: (Support/List Size)/(Reference Support/Reference size)). Integrated motif activity response analysis (ISMARA) was used to predict enriched transcription factor binding motifs (Balwierz, Pachkov et al. 2014).

RNA isolation and real-time qPCR

Total RNA was isolated from powdered *Quadriceps* and *Gastrocnemius* muscles or two pieces of liver with FastPrep tubes (MP Biomedicals) and TRI reagent (T9424, Sigma) according to the manufacturer's instructions. Total RNA from primary myotubes was isolated using the RNeasy Micro Kit (74004, Qiagen) according to the manufacturer's instructions (without DNase treatment). RNA concentration was measured with a NanoDrop OneC spectrophotometer (Thermo Scientific). RNA was treated with DNase I (18068015, Thermo Scientific) and then reverse transcribed using hexanucleotide mix (11277081001, Sigma) and SuperScript II reverse transcriptase (18064022, Thermo Scientific). The level of relative mRNA was quantified by real-time PCR on a StepOnePlus system (Applied Biosystems) using Fast SYBR green PCR master mix (4385612, Thermo Scientific) or on a Light Cycler 480 II system (Roche) using Fast Start Essential DNA Green Master mix (06924204001, Roche), respectively. The analysis of the mRNA was performed by the comparative CT method using TATA binding protein (TBP) and 18S as endogenous controls. Primer sequences are listed in Table 2 of the Supplemental Experimental Procedures.

Protein isolation and Western blotting

Powdered tissue samples were homogenized with a polytron device in 300 µl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% NP-40 substitute, 150 mM NaCl, 0.2% Na-deoxycholate, 1 mM DTT, fresh protease and phosphatase inhibitor cocktail, 10 mM nicotinamide). Samples were then shaken at 1300 rpm for 30 min at 4 °C, subsequently centrifuged at 13000 g for 10 min at 4 °C, and the protein concentration of the supernatant was determined by the Bradford assay

(5000006, Bio-Rad). Equal aliquots of protein were boiled for 5 min in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue, and 20% β-mercaptoethanol). Samples were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked for 1 h in 5% bovine serum albumin (BSA) in Tris-buffered saline and Tween 20 (TBST) before overnight incubation at 4 °C with the appropriate primary antibody diluted in TBST (1:1000 dilution). Primary antibodies used are listed in the Supplemental Experimental Procedures. Following incubation, membranes were washed with TBST before incubation with an appropriate peroxidase-conjugated secondary antibody diluted in TBST (1:10'000 dilution). Antibody binding was detected using the enhanced chemiluminescence horseradish peroxidase (HRP) substrate detection kit (32106, Pierce). Quantification of Western blots was performed with the ImageJ software.

Glycogen isolation

Around 10 mg of powdered *Gastrocnemius* muscle were homogenized on ice in 200 µl water using a polytron device. Then samples were boiled for 5 min in order to inactivate enzymes. After centrifugation for 5 min at 13000 rpm supernatant was moved to new tube and glycogen content measured using a glycogen assay kit (ab65620, Abcam) according to the manufacturer's instructions.

Statistical analysis

Values are expressed as means ± standard errors of the means (SEM). Statistical significance was determined with unpaired two tailed t-tests using Excel software and $p < 0.05$ was considered as significant. Significant differences between fed WT and fed MKO mice and fasted WT and fasted MKO mice, respectively, are indicated by an asterisk (*). Significant differences between fed and fasted WT and fed and fasted MKO mice, respectively, are indicated by a hashtag (#).

Results

PGC-1β expression is downregulated in skeletal muscle upon fasting

The regulation of peroxisome proliferator-activated receptor γ coactivator-1 β (PGC-1β) expression in skeletal muscle is still largely unexplored. While PGC-1α mRNA levels are known to increase after an acute exercise bout, PGC-1β expression levels seem to be unaffected by exercise (Meirhaeghe, Crowley et al. 2003, Koves, Li et al. 2005), which we confirmed in our study (Fig. 1A). Moreover, hepatic PGC-1α and –β expression levels have been shown to increase upon fasting (Lin, Puigserver et al. 2002) (Fig. 1B) whereas in skeletal muscle, we observed unchanged PGC-1α mRNA levels contrary to significantly reduced PGC-1β levels after a 24 h fasting stimulus (Fig. 1C). These results demonstrate that PGC-1

expression levels upon fasting are regulated differently in liver and skeletal muscle and suggest that PGC-1β might indeed be involved in the fasting response in skeletal muscle.

PGC-1α expression in liver and skeletal muscle is known to be controlled by cAMP-dependent pathways (Herzig, Long et al. 2001, Handschin, Rhee et al. 2003). To evaluated if PGC-1β mRNA levels are also controlled by cAMP signaling, we treated primary myotubes with forskolin, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) or 3-isobutyl-1-methylxanthine (IBMX) to increase intracellular cAMP levels (Totsuka, Ferdows et al. 1983, Parsons, Ramkumar et al. 1988, Bartsch, Zorn-Kruppa et al. 2003). Interestingly, all three compounds suppressed PGC-1β mRNA levels by around 50% while PGC-1α expression, as expected, was highly induced (Fig. 1D). These results indicate that PGC-1β expression seems to be controlled and downregulated by cAMP-inducing pathways and highlight that PGC-1α and –β might be controlled by the same pathway in a different way.

To further explore the role of muscle PGC-1β in the regulation of fasting, we generated PGC-1β muscle-specific knockout mice (MKO) by crossing HSA-Cre transgenic mice with floxed PGC-1β mice (Lai, Leone et al. 2008), which deleted exons 4-6 of the PGC-1β gene specifically in skeletal muscle while other tissues were unaffected by the knockout (Fig. 1E). Furthermore, there was no compensatory increase in PGC-1α expression detected and various mitochondrial target genes like cytochrome C (CytC), cytochrome C oxidase subunit 4 isoform 1 (Cox4i1) and cytochrome C oxidase subunit 5B (Cox5B) were downregulated in the muscles of MKO animals (Fig. 1F). PGC-1β ablation resulted only in a minor reduction in the protein levels of the mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 8 (NDUFB8) while the other mitochondrial complexes like ATP synthase 5 alpha (ATP5A), mitochondrial cytochrome b-c1 complex subunit 2 (UQCRC2) and mitochondrial succinate dehydrogenase iron-sulfur subunit (SDHB) were not differently expressed between MKO and WT mice (Fig. 1G and H). Nevertheless, MKO mice showed a reduction in oxidative capacity as measured by the activity of the two mitochondrial enzymes succinate dehydrogenase (SDH) and cytochrome oxidase (COX) (Fig. 1I and J), which has already been shown by other studies (Zechner, Lai et al. 2010, Gali Ramamoorthy, Laverny et al. 2015).

Taken together, our data demonstrate that muscle PGC-1β is an important regulator of mitochondrial gene expression and oxidative capacity and confirm the results obtained by other groups (Zechner, Lai et al. 2010, Gali Ramamoorthy, Laverny et al. 2015).

PGC-1β MKO animals preserve body weight and lean mass upon 24 h of fasting

Since we were interested in the fasting response and the role of PGC-1β in this context we fasted WT and MKO mice for 24 h and measured different blood parameters. As expected, glucose plasma levels dropped after 24 h of fasting (Longo and Mattson 2014) but no differences between MKO and WT mice

were observed (Fig. 2A). Concomitantly, non-esterified fatty acid (NEFA) (Fig. 2B) and ketone body, specifically β-hydroxybutyrate (β-OHB) (Fig. 2C), plasma levels were significantly elevated after 24 h of fasting but again no genotype differences could be detected. Furthermore, mice were put in a CLAMS system to monitor the oxygen consumption rate (VO_2) (Fig. 2D), respiratory exchange ratio (RER) (Fig. 2E) and body temperature (Fig. 2F). Fasting significantly reduced all of these three parameters independently of skeletal muscle PGC-1β. Next, body composition of *ad-libitum* fed and 24 h fasted mice was evaluated by EchoMRI. There was no difference between MKO and WT mice under normal feeding conditions (Fig. 2G), however, after 24 h of fasting MKO animals showed an increased body weight (BW), due to increased lean mass and total water content in comparison to their WT fasted counterparts (Fig. 2H). These results demonstrate that the whole body response to energy deprivation was not different between MKO and WT mice while the fasting induced muscle atrophy seemed to be dependent on skeletal muscle PGC-1β.

Muscle PGC-1β is necessary for the fasting-induced fiber atrophy

To further explore the fasting-induced muscle atrophy we measured individual muscle weights. *Gastrocnemius* (Fig. 3A) and *Soleus* (Fig. 3B) muscles of fasted MKO animals were significantly heavier compared to fasted WT mice. Furthermore, WT animals showed a fasting-induced decrease in muscle mass, which was only observed in *Tibialis anterior* (TA) muscle but not in *Gastrocnemius* or *Soleus* muscles in the fasted MKO animals (Fig. 3A-C). To evaluate if the preserved muscle mass in fasted MKO animals also correlated with an increase in cross sectional area (CSA) of single fibers we analyzed the minimal fiber ferets (minFeret) in *Gastrocnemius* muscle cross-sections. Oxidative fibers (in the center of the muscle) were not different between the genotypes in fed (Fig. 3D) and fasted (Fig. 3F) conditions. However, energy deprivation induced a slight right shift of the minFeret curve in WT animals (Supp. Fig. 1A) leading to a lower percentage of very small fibers and to a higher percentage of middle sized fibers. This effect was completely absent when comparing fasted MKO with fed MKO animals (Supp. Fig. 1B). Glycolytic fibers (at the edge of the muscle) of fed MKO and fed WT mice were not different (Fig. 3G) but fibers between fasted MKO and fasted WT animals (Fig. 3H). MKOs showed a lower percentage of small to middle sized fibers, however, a higher percentage of big fibers (Fig. 3H). When we compared fasted WT and fed WT animals (Supp. Fig. 1C), we actually observed a left shift of the minFeret curve towards smaller fibers in fasted animals, which we did not detect between fasted MKO and fed MKO mice (Supp. Fig. 1D). We also analyzed the proportion of fiber types between the different groups but did not detect any differences (Supp. Fig. 1E). These data demonstrate that fasted WT animals showed a shift in fiber CSA with a higher percentage of middle sized oxidative fibers and a lower percentage of big glycolytic fibers, which was not the case in fasted MKO animals. Together with the conserved muscle mass in fasted MKO mice these results suggest that muscle PGC-1β might be

necessary for the fasting-induced muscle atrophy and could thus explain why MKO animals show a reduced fasting response in skeletal muscle regarding lean mass.

Fasted MKO mice show a reduced induction of myostatin and atrophy markers gene expression

Since PGC-1β is an important transcriptional coactivator we investigated the transcriptional changes between 24 h fasted WT and MKO animals by RNA sequencing (RNAseq) and subjected all differentially expressed (DE) genes with a false discovery rate (FDR) < 0.05 and a fold change (FC) ± 1.2 to gene ontology (GO) analysis. The top overrepresented GO categories were “electron transport chain”, “transforming growth factor (TGF)-β receptor signaling pathway” and “cell differentiation” (Fig. 4A, Supp. Fig. 2). Interestingly, TGF-β signaling and in particular the associated DE gene myostatin (Mstn) have been implicated to induce muscle atrophy by activating the ubiquitin proteolytic system (McFarlane, Plummer et al. 2006, Mendias, Gumucio et al. 2012). Furthermore, Mstn is a well characterized negative regulator of skeletal muscle mass (McPherron, Lawler et al. 1997). In our RNAseq data Mstn was significantly reduced in fasted MKO compared to fasted WT mice suggesting a downregulation of Mstn induced TGF-β signaling. Indeed, we could confirm by qPCR that Mstn expression was significantly lower in fasted MKO mice while being upregulated in fasted WT compared to fed WT animals (Fig. 4B). Moreover, the induction of two important ubiquitin ligases involved in muscle atrophy (Bodine, Latres et al. 2001), muscle RING finger 1 (MuRF-1) and muscle atrophy F-box (MAFbx), was blunted in fasted MKO animals (Fig. 4B). Furthermore, the lower expression in MuRF-1 and MAFbx correlated with a reduced ubiquitination of proteins in fasted MKO animals (Fig. 4C and D). Transcriptional induction of MuRF-1 and MAFbx is known to be controlled by forkhead box O (FoxO) transcription factors (Sandri, Sandri et al. 2004), which includes the three family members FoxO1, FoxO3, and FoxO4 (Ho, Myatt et al. 2008). Moreover, dephosphorylation of FoxO transcription factors leads to their nuclear entry and the transcriptional induction of target genes (Ramaswamy, Nakamura et al. 2002). mRNA levels of all three FoxO members were upregulated upon fasting independently of skeletal muscle PGC-1β (Fig. 4E). Nevertheless, the phosphorylation of FoxO3a was significantly higher in fed and fasted MKO animals, however, also total FoxO3a protein levels were induced in fasted MKO compared to fasted WT mice (Fig. 4F and G). Thus, the ratio of phosphorylated to total FoxO3a protein was significantly lower upon fasting but not different between the genotypes (Fig. 4G). These data suggest that the reduced fasting-induced muscle atrophy might be a consequence of the absent and blunted induction of Mstn, MuRF-1 and MAFbx gene expression, respectively.

MKO mice show reduced activation of AMPK and PKA upon fasting

Recently, Mstn has been shown to activate the AMP-dependent protein kinase (AMPK), an important cell energy sensor, to regulate glucose and glycogen homeostasis in skeletal muscle (Chen, Ye et al.

2010). AMPK is sensitive to the intracellular AMP:ATP ratio and upon its phosphorylation the catalytic subunit gets activated to switch on catabolic pathways generating ATP while turning off anabolic pathways consuming ATP, respectively (Hardie 2007). Thus, we evaluated AMPK activity in fed and fasted WT and MKO animals. Total AMPK protein levels were increased in fed MKO mice while being significantly lower in fasted MKO animals in relation to their fed counterparts (Fig. 5A and B). Moreover, the phosphorylation of AMPK was higher in fasted WT compared to fed WT mice resulting in a significantly increased phosphorylated to total AMPK ratio (Fig. 5A and B). This increase was completely absent with the ablation of PGC-1β demonstrating that fasted MKO animals suffer less from energy stress than their fasted WT counterparts. Furthermore, the absent activation of AMPK provides another possible explanation for the reduced muscle atrophy in fasted MKO animals.

As described before, Mstn regulates glucose and glycogen homeostasis by promoting glycolysis and inhibiting glycogen synthesis through the activation of AMPK in muscle cells (Chen, Ye et al. 2010). Furthermore, the AMPK complex has been shown to bind glycogen (Hudson, Pan et al. 2003, Polekhina, Gupta et al. 2003), which also regulates its activity, being repressed when muscle glycogen is high (Wojtaszewski, Jorgensen et al. 2002, Wojtaszewski, MacDonald et al. 2003). Since AMPK activation was absent in fasted MKO animals we evaluated if glycogen levels were also different between the groups. Indeed, fasted MKO animals showed significantly higher total glycogen levels in *Gastrocnemius* muscle compared to fasted WT animals (Fig. 5C). These results suggest that in addition to the regulation by Mstn, the absent activation of AMPK might furthermore be due to the increased glycogen content in fasted mice with ablated muscle PGC-1β expression.

An important regulator of glycogen content in skeletal muscle is the cAMP-dependent protein kinase (PKA) (Bishop and Larner 1969). Total PKA protein levels were significantly increased in fasted mice independent of PGC-1β and the phosphorylation of PKA in fed MKO and fasted WT mice was induced (Fig. 5A and D). Fasted MKO animals showed a reduced phosphorylation of PKA resulting in a lower ratio of phosphorylated to total PKA protein levels, which in fasted WT mice was increased compared to their fed counterparts (Fig. 5A and D). These data indicate that besides AMPK also activation of PKA was absent in fasted MKO animals providing a possible explanation for the observed increased glycogen content.

We further investigated the effects of reduced PKA signaling in fasted MKO animals by analyzing the activity of a cAMP-PKA target protein, cAMP response element binding protein (Creb) (Shaywitz and Greenberg 1999). Creb is being phosphorylated at a particular site (Ser^{133}), which is required for transcriptional induction of target genes (Shaywitz and Greenberg 1999). Total Creb protein levels were increased in fed MKO and fasted WT animals and surprisingly, phosphorylation of Creb was significantly higher in fasted MKO animals compared to their fed MKO and fasted WT counterparts (Fig. 5A and E). Accordingly, the ratio of phosphorylated to total Creb protein tended to be induced in

fasted MKO mice (Fig. 5A and E). These data suggest that the increase in Creb phosphorylation is most likely due to the action of another kinase, since PKA signaling was reduced in fasted MKO animals.

Nfatc1 activity is increased in fasted MKO animals

Creb is known to be phosphorylated by a variety of other kinases including the Ca^{2+}/calmodulin-dependent protein kinases (CaMK) (Shaywitz and Greenberg 1999). Interestingly, the activity of CaMKIIα seemed to be higher in fasted MKO compared to fasted WT mice (Fig. 6A and B). Total CaMKIIα protein levels were significantly lower in MKO animals in relation to their WT controls and fasting decreased the protein content of CaMKIIα in WT and MKO animals (Fig. 6A and B). However, phosphorylation of CaMKIIα was strongly induced in fasted MKO animals, which resulted in a massive increase in the ratio of phosphorylated to total CaMKIIα protein levels (Fig. 6A and B). These results indicate that the increase in Creb signaling could be due to the higher activity of CaMKIIα and suggest that Ca^{2+}/calmodulin-dependent signaling might be increased in fasted MKO animals.

To assess if other downstream targets of Ca^{2+}/calmodulin-dependent signaling were induced in fasted MKO animals, we performed integrated motif activity response analysis (ISMARA) with our RNAseq data. Indeed, we identified the nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1), a known target protein of the phosphatase calcineurin (Semsarian, Wu et al. 1999), as top transcription factor with predicted increased activity in fasted MKO compared to fasted WT animals (Fig. 6C). Interestingly, PGC-1α belonged to the predicted ISMARA Nfatc1 target genes, next to protein-O-mannose kinase (Pomk), membrane associated ring-CH-type finger 1 (March1), SH3 domain containing kinase binding protein 1 (Sh3kbp1), methyltransferase like 11B (Mettl11b) and nitric oxide synthase 1 (Nos1). Except for March1, we could confirm that the predicted Nfatc1 target genes indeed showed increased expression in fasted MKO animals (Fig. 6D). These data suggest that PGC-1β might act as a inhibitor for Nfatc1 activity leading to muscle atrophy upon energy deprivation.

5. PGC-1β is involved in the response to fasting-induced skeletal muscle atrophy by inhibiting Nfatc1 activity

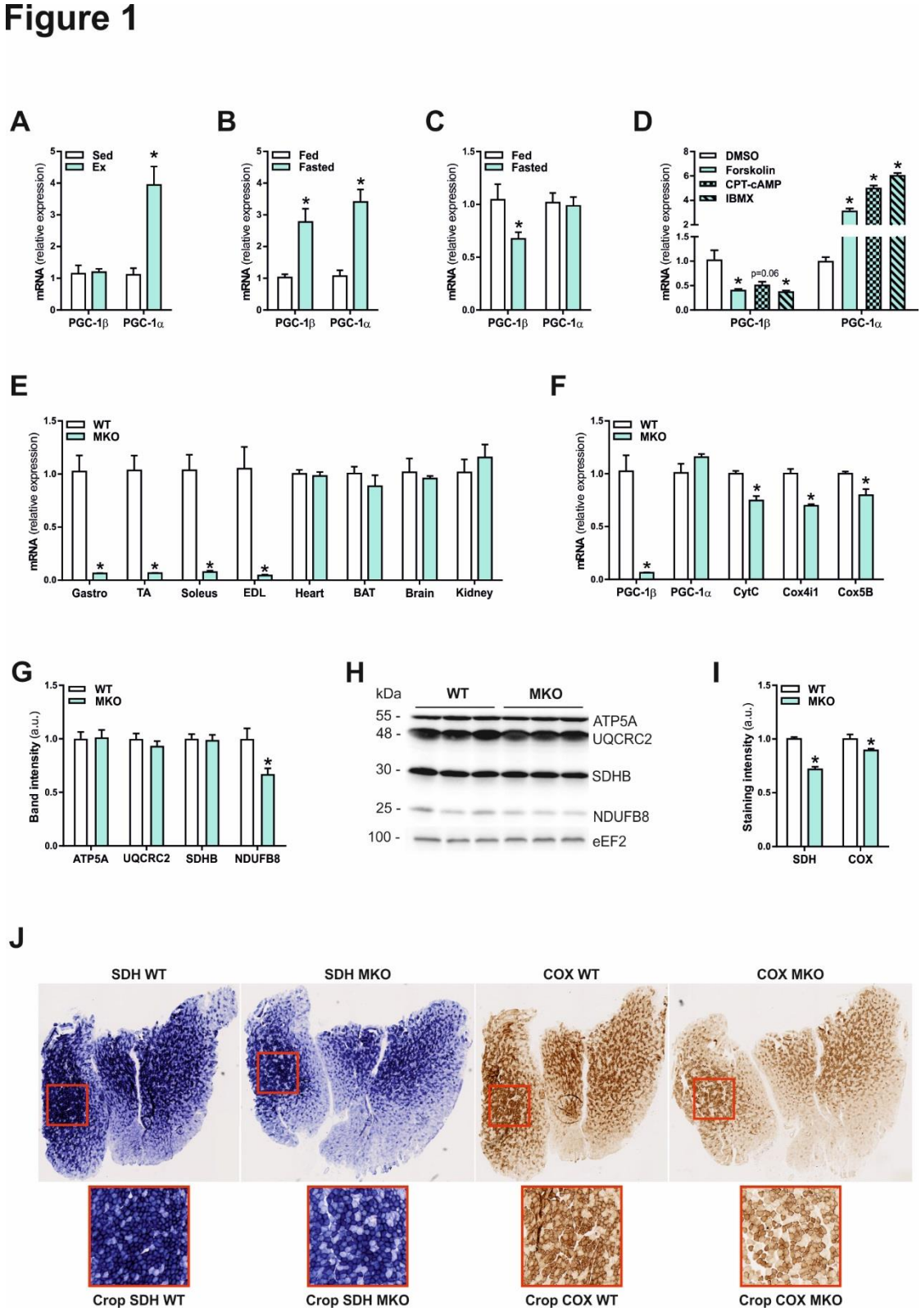


Figure 1. PGC-1β expression is downregulated in skeletal muscle upon fasting

A) Gene expression of PGC-1β and PGC-1α relative to TATA-box binding protein (TBP) in *Quadriceps* muscle of sedentary or exercised mice (n=5-6).

B-C) Gene expression of PGC-1β and PGC-1α relative to 18S in liver (B) and *Gastrocnemius* muscle (C) of *ad-libitum* fed or 24 h fasted mice (n=5-6).

D) Gene expression of PGC-1β and PGC-1α relative to 18S in primary myotubes of WT mice treated with forskolin, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) or 3-isobutyl-1-methylxanthine (IBMX) for 6 h (n=3).

E) Gene expression of PGC-1β relative to TATA-box binding protein (TBP) or 18S in different muscles and other tissues of WT and PGC-1β MKO mice (n=3-4).

F) Gene expression of PGC-1β, mitochondrial target genes and PGC-1α relative to TATA-box binding protein (TBP) or 18S in *Gastrocnemius* muscle of WT and MKO mice (n=3-6).

G-H) Protein levels of different mitochondrial chain complexes (F) in *Gastrocnemius* muscle of WT and MKO mice and representative immunoblots (G) (n=5-6). As a loading control eukaryotic elongation factor 2 (eEF2) was used.

I-J) Representative succinate dehydrogenase (SDH) and cytochrome oxidase (COX) stainings of *Gastrocnemius* muscle cross-sections (I) of WT and MKO mice and corresponding quantification (H) (n=4-5).

* indicating significant differences between sedentary and exercised mice, fed and fasted mice, untreated (DMSO) and treated cells and WT and MKO mice, respectively.

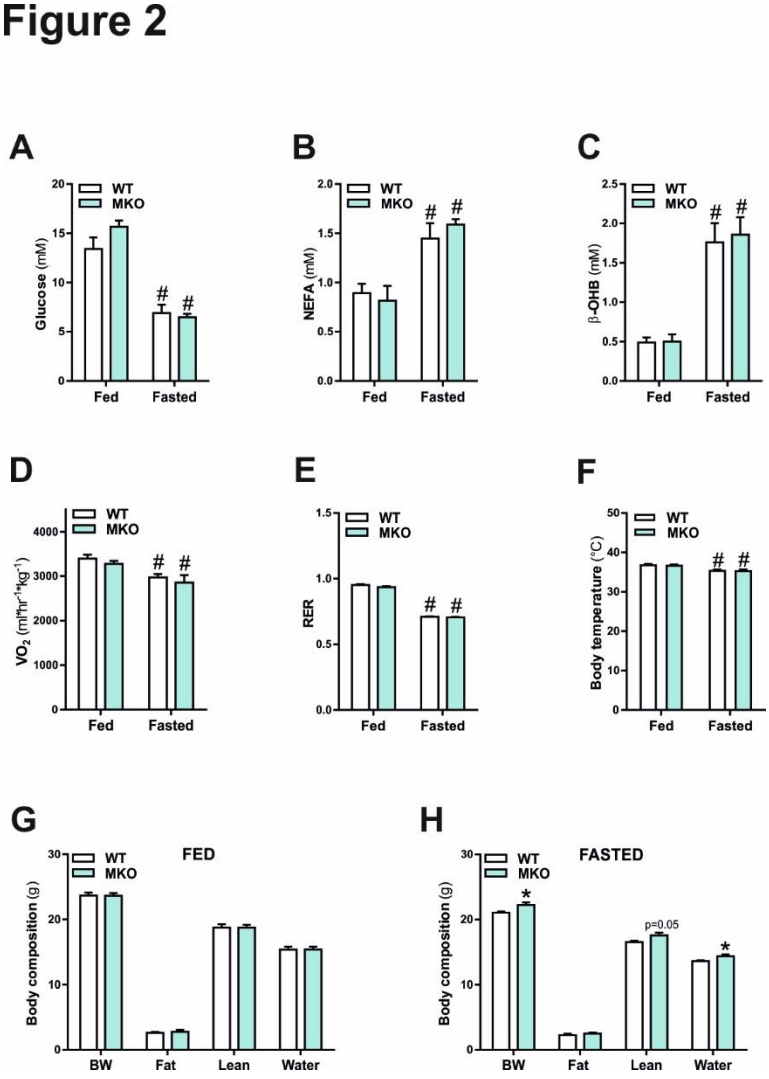


Figure 2. MKO animals preserve body weight and lean mass upon 24h fasting

A-C) Plasma glucose (A), non-esterified fatty acids (NEFA) (B) and β-hydroxybutyrate (β-OHB) (C) levels of *ad-libitum* fed or 24 h fasted mice (n=5-8).

D-F) Average oxygen consumption rate (VO_2) (D), respiratory exchange ratio (RER) (E) and body temperature (F) measured during nighttime by indirect calorimetry over a 48 h period in *ad-libitum* fed or 24 h fasted mice (n=7-8).

H-I) Body composition measured by EchoMRI of *ad-libitum* fed or 24 h fasted mice with an initially identical body weight (n=6-7).

* indicating significant differences between WT and MKO mice; # indicating significant differences between fed and fasted conditions.

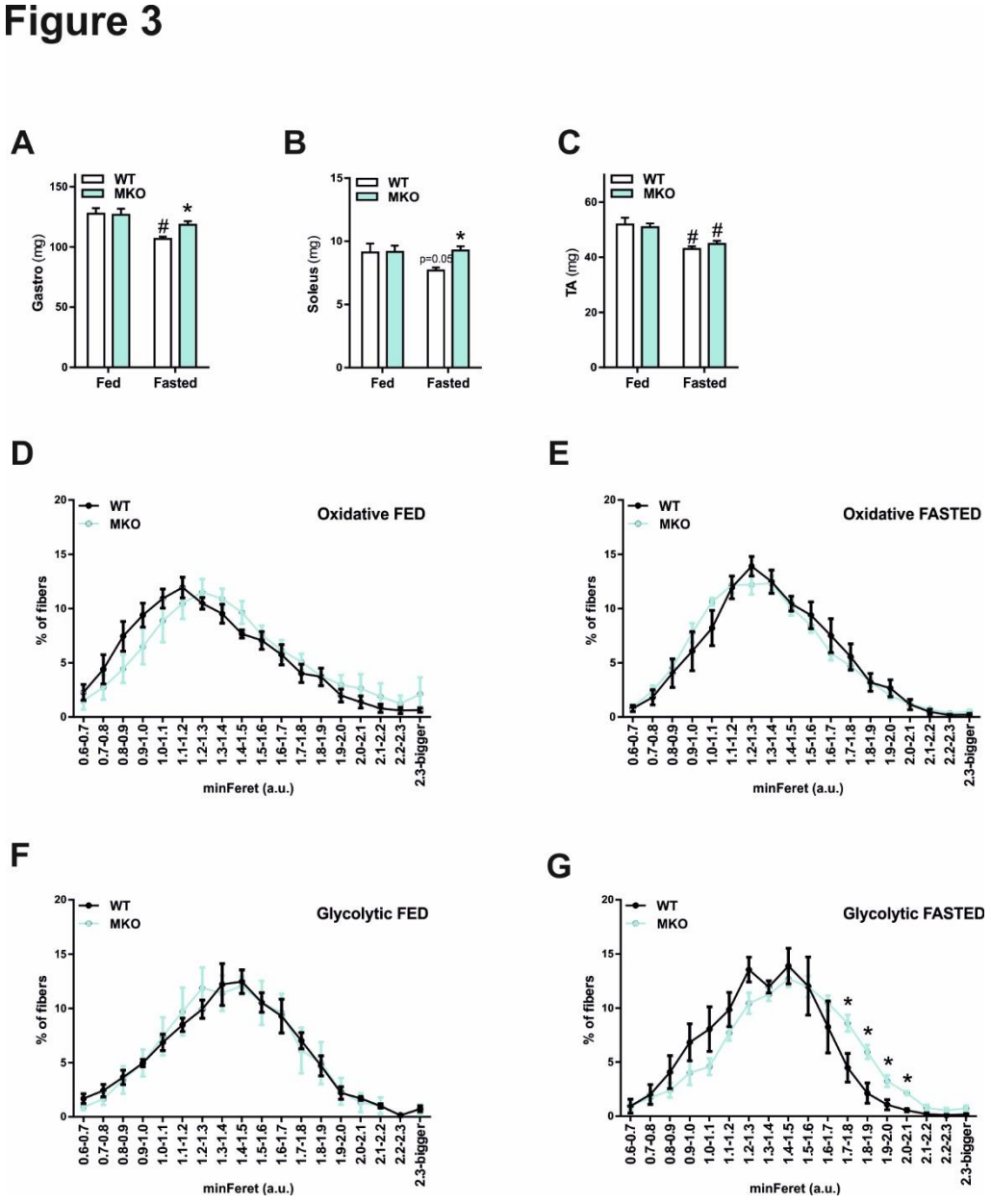


Figure 3. PGC-1β is necessary for the fasting-induced fiber atrophy

A-C) *Gastrocnemius* (A), *Soleus* (B) and *Tibialis anterior* (TA) (C) muscle weights of *ad-libitum* fed or 24 h fasted mice (n=4-6).

D-G) Minimal fiber ferrets (minFerret) of oxidative (D and E) and glycolytic (F and G) *Gastrocnemius* muscle cross-sections of *ad-libitum* fed (D and F) or 24 h fasted (E and G) mice (n=4-6).

* indicating significant differences between WT and MKO mice; # indicating significant differences between fed and fasted conditions.

Figure 4

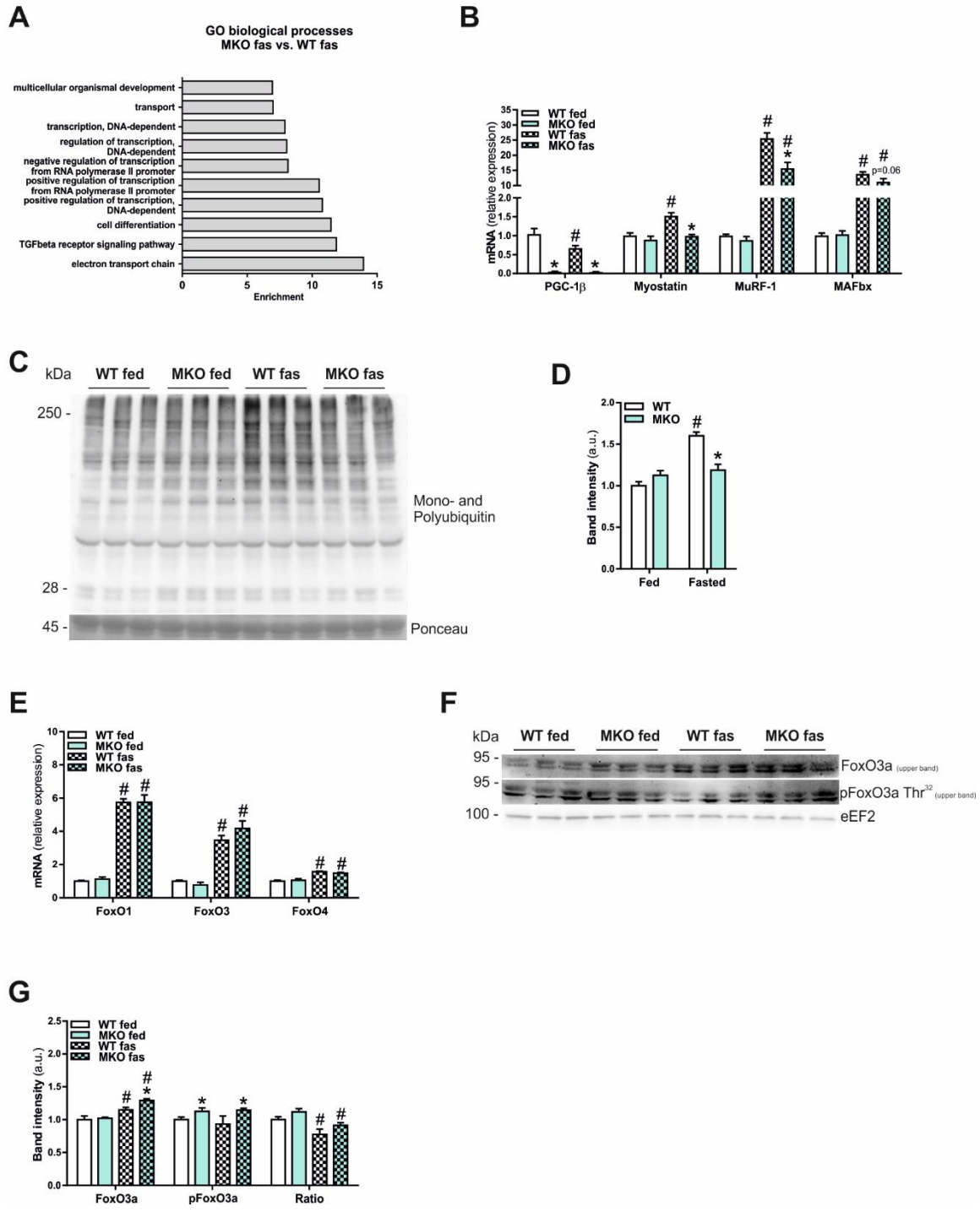


Figure 4. Fasted MKO mice show reduced induction of myostatin and atrophy markers gene expression

A) Gene ontology (GO) analysis of differentially expressed (DE) genes between fasted MKO vs. fasted WT mice (n=3-5).

B) Gene expression of PGC-1β, myostatin (Mstn), muscle RING finger 1 (MuRF-1) and muscle atrophy F-box (MAFbx) relative to 18S in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice (n=5-6).
C-D) Representative immunoblot of ubiquitinylated proteins (C) in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice and corresponding quantification (D) (n=5-6). Equal loading was verified by ponceau staining of the membranes.
E) Gene expression of forkhead box O (FoxO) transcription factors 1, 3 and 4 relative to 18S in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice (n=5-6).
F-G) Representative immunoblots of total and phosphorylated forkhead box O 3a (FoxO3a) proteins (F) in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice and corresponding quantifications (G) (n=3-6). As a loading control eukaryotic elongation factor 2 (eEF2) was used.
* indicating significant differences between WT and MKO mice; # indicating significant differences between fed and fasted conditions.

Figure 5

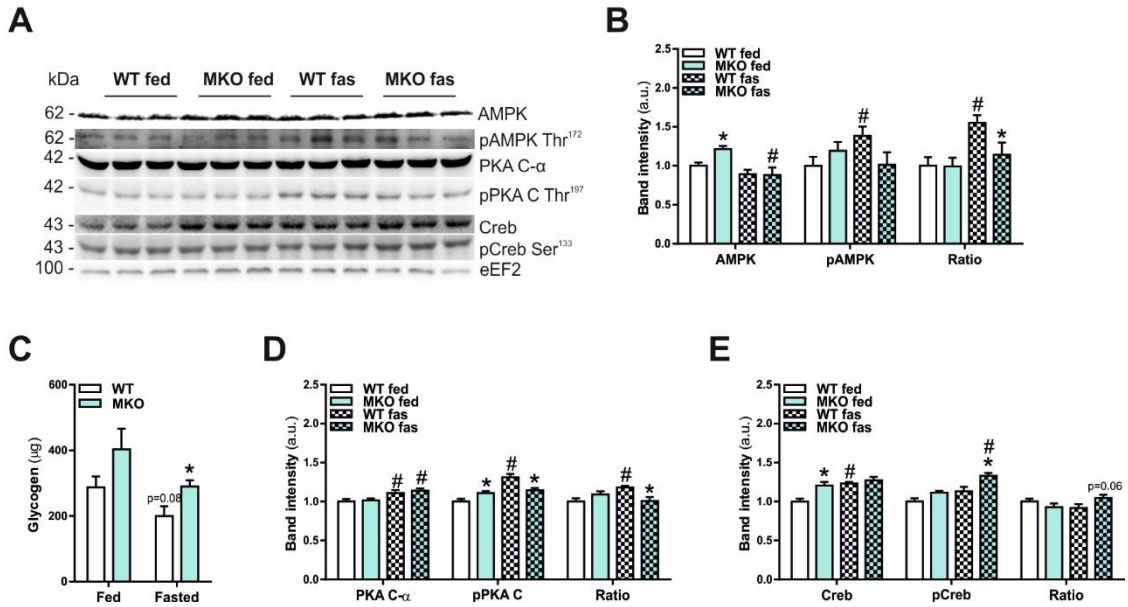


Figure 5. MKO mice show reduced activation of AMPK and PKA upon fasting

A-B and D-E) Representative immunoblots of total and phosphorylated AMP-dependent protein kinase (AMPK), cAMP-dependent protein kinase (PKA) and cAMP response element binding protein (Creb) protein levels (A) in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice and corresponding quantifications (B, D and E) (n=5-6). As a loading control eukaryotic elongation factor 2 (eEF2) was used.

C) Total glycogen content in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice (n=5-6).

* indicating significant differences between WT and MKO mice; # indicating significant differences between fed and fasted conditions.

Figure 6

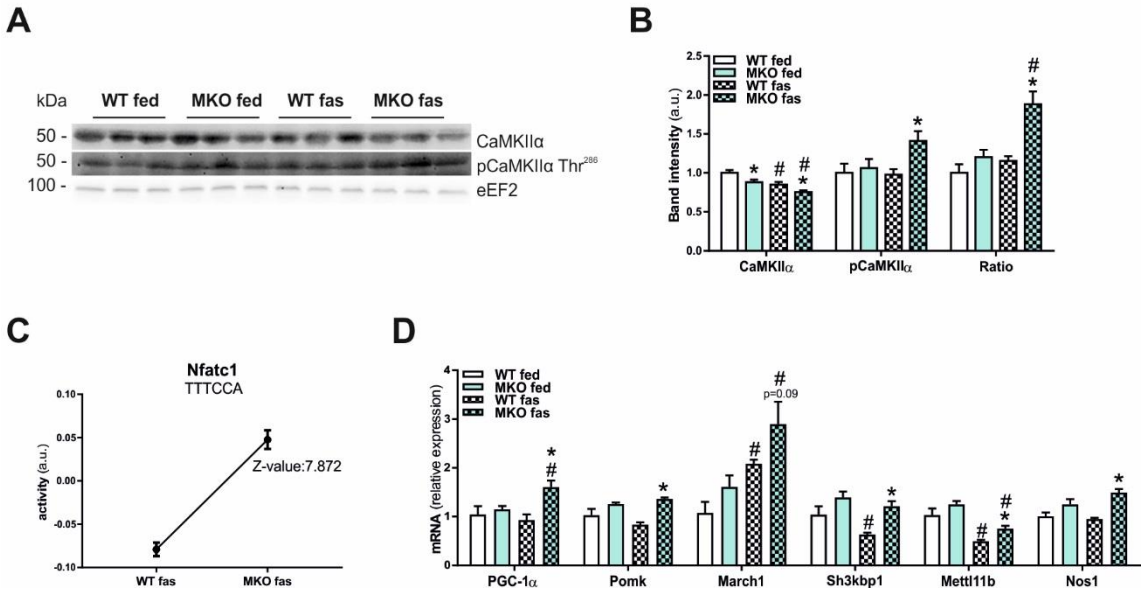


Figure 6. Nfatc1 activity is increased in fasted MKO animals

A-B) Representative immunoblots of total and phosphorylated Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) protein levels (A) in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice and corresponding quantifications (B) (n=5-6). As a loading control eukaryotic elongation factor 2 (eEF2) was used.

C) ISMARA predictions of enriched transcription factor binding motifs like the top target nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) in *Gastrocnemius* muscle of 24 h fasted WT and MKO mice (n=3-5).

D) Gene expression of predicted Nfatc1 target genes PGC-1α, protein-O-mannose kinase (Pomk), membrane associated ring-CH-type finger 1 (March1), SH3 domain containing kinase binding protein 1 (Sh3kbp1), methyltransferase like 11B (Mettl11b) and nitric oxide synthase 1 (Nos1) relative to 18S in *Gastrocnemius* muscle of *ad-libitum* fed or 24 h fasted mice (n=5-6).

* indicating significant differences between WT and MKO mice; # indicating significant differences between fed and fasted conditions.

Figure 7

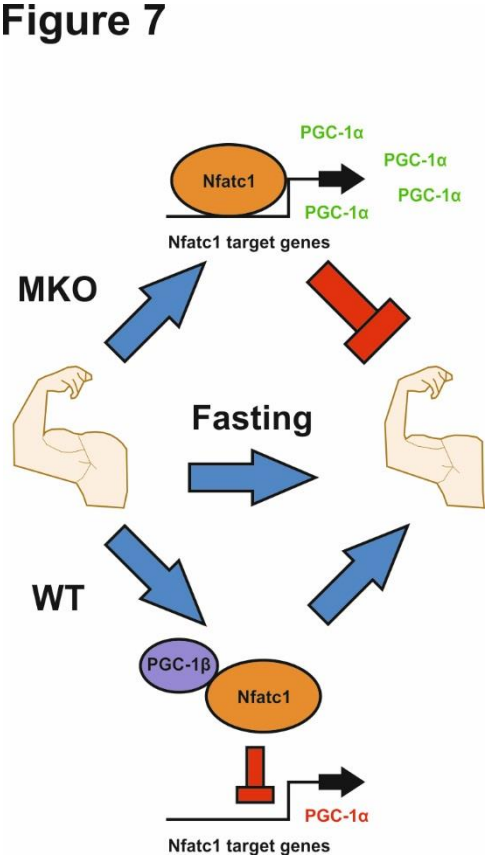
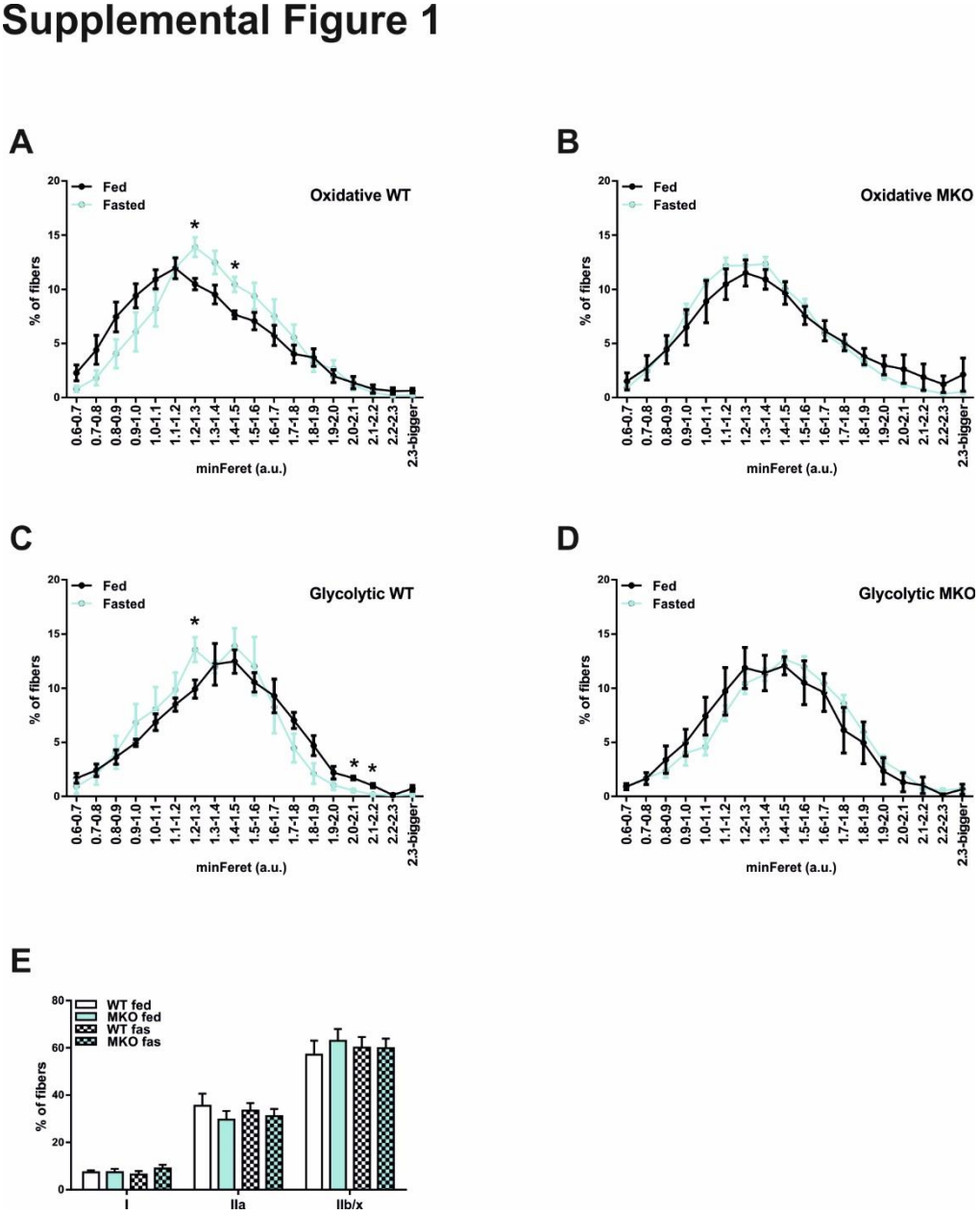


Figure 7. Proposed working model

In a WT setting, PGC-1β functions as an inhibitor for Nfatc1 transcriptional activity, which prevents expression of target genes such as PGC-1α. Ablation of PGC-1β in skeletal muscle results in the translocation of Nfatc1 into the nucleus leading to the induction of target genes and the blunted response to energy deprivation.



Supplemental Figure 1. PGC-1β is necessary for the fasting-induced fiber atrophy

A-D) Minimal fiber ferrets (minFerret) of oxidative (A and B) and glycolytic (C and D) *Gastrocnemius* muscle cross-sections of *ad-libitum* fed or 24 h fasted WT (A and C) and MKO (B and D) mice (n=4-6).

E) Quantification of type I, IIa and IIb/x fibers in *Gastrocnemius* muscle cross-sections of *ad-libitum* fed or 24 h fasted mice (n=4-6).

* indicating significant differences between fed and fasted conditions.

Supplemental Figure 2

GO category	Hyp_c	Genes	Enrichment
electron transport chain	1.92E-07	Ndufv1,Cyc1,Uqcrfs1,Uqcrq,Ndufs4,Ndufa10,Enox2,Ndufs2,Sdhc,Ndufs1,mt-Nd5,Uqcr11	11.86429471
transforming growth factor beta receptor signaling pathway	0.000370987	Hipk2,Smad3,Col1a2,Bmpr1b,Fos,Tgfbr3,Mstn	11.4551811
cell differentiation	8.98E-07	Gadd45b,Lrrc8a,Bex1,Peg10,Trp63,Aimp2,Gsk3b,Arnt,Npnt,Hspa1l,Mlf1,Ostn,Bmpr1b,Notch4,Chrdl1,Fgfr3,Gna13,Sema4d,Ttll7,Figf,Pkdcc,Mitf,Eda2r,Myf6,Ifrd1	4.162910425
positive regulation of transcription, DNA-dependent	6.24E-06	Col1a1,Brpf1,Ing1,Ppargc1a,Hipk2,Nos1,Egr1,Trp63,Esr2,Arnt,Egf,Smad3,Sertad2,Mecp2,Zbtb16,Sp1,Mitf,Nfia,Myf6,Spen,Fos,Mstn	4.094344841
positive regulation of transcription from RNA polymerase II promoter	6.43E-06	Nr4a3,Ppargc1a,Hipk2,Pfn1,Nos1,Cd28,Egr1,Tceb2,Bex1,Trp63,Esr2,Gsk3b,Maf,Arnt,Mllt10,Arrb1,Smad3,Acvr1b,Sdpr,Sp1,Mitf,Nfia,Fos,Mafb,Sox12	3.690293845
negative regulation of transcription from RNA polymerase II promoter	0.000232742	Ptch1,Sin3a,Hipk2,Egr1,Trp63,Tsc22d3,Esr2,Tle4,Zfhx3,Cry2,Maf,Smad3,Nr1d1,Mecp2,Bhlhe40,Fgfr3,Sema4d,Kdm6b,Foxn2	3.680352645
regulation of transcription, DNA-dependent	3.06E-12	Runx1t1,Nr4a3,Ppargc1a,Tceal5,Zfp703,Sin3a,Hipk2,Vgll4,Taf1d,Zfp263,Ifi205,Egr1,Tceb2,Klf10,Trp63,Zfp322a,Tsc22d3,Esr2,Ikbkg,Zbtb20,Mnt,Tle4,Cry2,Maf,Arnt,Dmd,Arrb1,Smad3,Acvr1b,Sertad2,Gatad2a,Med7,Zkscan1,Arid5a,Nr1d1,Mecp2,Txlng,Bhlhe40,Notch4,Ell2,Arid3a,Zbtb16,Zfp397,Thrsp,Musk,Elk4,Ssbp2,Sp1,Mitf,Nfia,Myf6,Tsc22d1,Spen,Zfp280d,Fos,Hsf2,Foxn2,Mafb,Sox12	3.222063926
transcription, DNA-dependent	1.75E-10	Gtf3c4,Runx1t1,Nr4a3,Ppargc1a,Tceal5,Zfp703,Sin3a,Hipk2,Vgll4,Taf1d,Zfp263,Ifi205,Egr1,Tceb2,Klf10,Trp63,Zfp322a,Esr2,Ikbkg,Zbtb20,Mnt,Tle4,Cry2,Maf,Arnt,Arrb1,Smad3,Sertad2,Mlf1,Gatad2a,Med7,Zkscan1,Arid5a,Nr1d1,Mecp2,Txlng,Bhlhe40,Notch4,Ell2,Arid3a,Zfp397,Thrsp,Ssbp2,Sp1,Mitf,Nfia,Tsc22d1,Spen,Zfp280d,Hsf2,Foxn2,Mafb,Sox12	3.116766392
transport	1.02E-06	Chrna9,Slc4a10,Ndufv1,Vldlr,Rhbdf2,Slc38a4,Tmem38b,Slc2a1,Slc6a6,Slc25a25,Scn3b,Arrb1,Kcnj2,Smad3,Scn1b,Kcnv2,Slc9a1,Cyc1,Uqcrfs1,Uqcrq,Atp5k,Ndufs4,Abca1,Kcnq5,Cacnb4,2610002M06Rik,Ndufa10,Slc35f5,Alb,Enox2,Ndufs2,Sdhc,Ndufs1,Atp5g3,mt-Nd5,Sfxn3,Osbpl1a,Slc39a14,Plin3,Pkdcc,Slc12a2,Slc43a1,Vps45,Slc40a1,Uqcr11	2.636509936
multicellular organismal development	0.0029047	Gadd45b,Bex1,Trp63,Aimp2,Gsk3b,Fzd9,Npnt,Hspa1l,Mlf1,Wls,Ostn,Etl4,Notch4,Chrdl1,Shisa2,Sema4d,Ttll7,Figf,Pkdcc,Mitf,Eda2r,Myf6,Ifrd1,Tapt1	2.612321771

Supplemental Figure 2. Fasted MKO mice show reduced induction of myostatin and atrophy markers gene expression

Gene ontology (GO) analysis of differentially expressed (DE) genes between fasted MKO vs. fasted WT mice and associated genes (n=3-5).

Discussion

Skeletal muscle atrophy can be induced by a variety of different stimuli such as disuse, aging, starvation or diseases (e.g. cancer cachexia). Regardless of the primary cause, muscle atrophy is always characterized by protein catabolism and reduced fiber diameter, which results in a loss of mass and functionality. Indeed, 24 h of fasting resulted in a substantial loss of muscle mass characterized by significantly elevated Mstn, MuRF-1 and MAFbx mRNA levels (McPherron, Lawler et al. 1997, Bodine, Latres et al. 2001, McFarlane, Plummer et al. 2006, Mendias, Gumucio et al. 2012) and increased activities of AMPK and PKA in WT animals. Importantly, all of these changes were blunted or even absent in MKO animals suggesting that PGC-1β substantially contributes to muscle atrophy at least under fasting conditions. Thus, the model we propose is that in a WT setting PGC-1β acts as an inhibitor for Nfatc1 transcriptional activity, which prevents it from binding to target promoters to induce transcription of target genes such as PGC-1α (Fig. 7). However, in the MKO animals, Nfatc1 activity cannot be repressed by PGC-1β leading to the induction of target genes including PGC-1α (Fig. 7). It is important to note that the increased PGC-1α levels in MKO animals were not just due to compensation of PGC-1β, but rather a result of the increased Nfatc1 activity specifically in fasted MKO animals, since PGC-1α mRNA levels were not different between fed WT and MKO animals. Interestingly, PGC-1α has been implicated to protect from muscle atrophy by decreasing FoxO3 transcriptional activity leading to a reduction in atrophy-specific gene transcription (Sandri, Sandri et al. 2004, Sandri, Lin et al. 2006). The increased PGC-1α mRNA levels in MKO animals could thus explain the blunted induction of MuRF-1 and MAFbx expression. Furthermore, elevated levels of PGC-1α are known to contribute to increased glycogen stores suggesting that MKO animals displayed higher muscle glycogen due to increased PGC-1α transcription (Wende, Schaeffer et al. 2007). This provides further evidence that the repressed activity of AMPK might have been caused by the increase in muscle glycogen levels (Wojtaszewski, Jorgensen et al. 2002, Wojtaszewski, MacDonald et al. 2003). Finally, all of these changes possibly contributed to the protection of muscle mass suggesting that the reduced Mstn expression levels were a cumulative, secondary result of the blunted fasting response in MKO animals.

Our results suggest that PGC-1β possibly acts as a transcriptional inhibitor for Nfatc1 activity under fasting conditions, probably as PGC-1α for FoxO3 (Sandri, Lin et al. 2006). At first sight this seems to be paradoxical, since PGC-1β expression in WT animals is itself reduced upon fasting and overexpression of PGC-1β has been shown to protect from protein degradation, induction of ubiquitin ligases and disuse atrophy (Brault, Jespersen et al. 2010). However, it is feasible that the reduction in PGC-1β expression in WT animals might serve as a protective mechanism to reduce its inhibitory effect on Nfatc1 and would therefore explain why MKO mice suffer less from an actual fasting stimulus. Interestingly, increased transcriptional activity of the calcineurin target protein Nfatc1 has previously

been implicated to mediate hypertrophy in skeletal muscle (Semsarian, Wu et al. 1999). Our data would thus indicate that Nfatc1 is not only involved in muscle hypertrophy but also in muscle atrophy, which could be limited or even prevented by increasing Nfatc1 transcriptional activity, for example by reducing PGC-1β expression. As we showed in the beginning, PGC-1β expression could be targeted by increasing intracellular cAMP levels. However, we did not investigate how cAMP signaling reduces PGC-1β mRNA levels. So far, no cAMP response element (CRE) has been identified on the PGC-1β gene as it is the case for PGC-1α, which is known to harbor a CRE around 100 base pairs upstream of its transcriptional start site (Herzig, Long et al. 2001, Handschin, Rhee et al. 2003). Thus, it is conceivable that PGC-1β is indirectly regulated by cAMP signaling contrary to PGC-1α, which might also explain the different expression profiles of the two PGC-1 family members upon increased cAMP levels.

To conclude, we identified PGC-1β as a new important player in the response to fasting-induced muscle atrophy. Muscle-specific ablation of PGC-1β resulted in a Nfatc1-dependent increase in PGC-1α expression, which resulted in a blunted fasting response and reduced muscle mass loss. Thus, our results shed new light on the complex regulation of muscle metabolism after energy deprivation and revealed PGC-1β and Nfatc1 as new possible target candidates for the treatment of different muscle wasting diseases.

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Supplemental Experimental Procedures

Table 1. Acclimatization protocol for treadmill running at an incline of 5°.

Day 1	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	10 min 10 m/min		
Day 2	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	5 min 12 m/min	
Day 3	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	5 min 12 m/min	2 min 14 m/min
Day 4	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	10 min 12 m/min	2 min 14 m/min
Day 5	5 min 0 m/min	5 min 5 m/min	5 min 8 m/min	15 min 10 m/min	10 min 12 m/min	5 min 14 m/min

Single fiber isolation and primary cell culture

Before the isolation of single fibers, all dishes were coated with horse serum (HS, 16050122, Thermo Scientific) in order to prevent sticking of the fibers. Single fibers were isolated from *Extensor digitorum longus* (EDL) muscles of 3 weeks old male WT mice by microdissection. Muscles were digested in 0.2% collagenase A (10103586001, Roche) in DMEM Glutamax (31966021, Thermo Scientific) supplemented with 1% Penicillin/Streptomycin (P/S, 15140122, Thermo Scientific) for 1.5 h at 37 °C. Then EDLs were transferred to a new dish without collagenase for another 30 min. Single fibers were isolated by triturating the muscles with fire polished glass pipettes (pre-coated with HS) and distributed on matrigel coated plates (354234, BD Biosience). After incubation for 1 h at 37 °C fresh seeding medium was slowly added (DMEM Glutamax, 10% HS, 1% P/S, 0.5% chicken embryo extract (CEE, C3999, US Biological Life Sciences), 0.004% FGF-Basic Recombinant Human Protein (PHG0024, Thermo Scientific)).

After two days of incubation, the medium was changed to proliferation medium (DMEM Glutamax, 20% FetalClone Serum (FCS, SH30066.03, GE Healthcare Life Sciences), 10% HS, 1% P/S, 1% CEE, 0.005% FGF). Two days after proliferation, cells were washed with PBS and trypsinized (25300054, Thermo Scientific) for 5-10 min at 37°C. The reaction was stopped by the addition of HyClone medium (HyClone (SH30262.01, Thermo Scientific), 20% FetalClone Serum (FCS, SH30066.03, GE Healthcare Life Sciences), 10% HS, 1% P/S, 1% CEE, 1% L-glutamine (G7513, Sigma), 0.005% FGF). All cells were put on a non-coated dish for 1 h at 37 °C to let fibroblasts adhere. After incubation, floating primary myoblasts were carefully removed, counted and re-plated on new matrigel coated plates in HyClone medium.

Fiber Typing and minFeret determination

Sections were blocked in blocking solution (PBS + 0.4% Triton X-100 (93426, Sigma) + 10% goat serum (G9023, Sigma)) for 30 min. After washing with PBS sections were exposed to primary antibodies for 1 h at room temperature. Primary antibodies used were mouse IgG b2 MHC type 1 (BA-F8, DSHB), mouse IgG 1 MHC type 2a (SC-71, DSHB) and laminin (ab11575, Abcam). After washing with PBS slides were incubated with the appropriate secondary antibodies AF647 IgG 2b goat-anti-mouse (A-21242, Life Technologies), AF568 IgG1 goat-anti-mouse (A-21124, Life Technologies) and AF488 IgG goat-anti-rabbit (A-11008, Life Technologies) for 1 h at room temperature. Finally, sections were washed with PBS, dehydrated with ethanol and mounted with ProLong Gold Antifade reagent (P36930, Life Technologies).

Fiji script for minFeret determination:

- run("3D Hysteresis Thresholding", "high=5 low=5");
- run("Analyze Particles...", "size=150-Infinity pixel circularity=0-1 show=Masks");
- run("Invert LUT");
- run("Dilate");
- run("Dilate");
- run("Dilate");
- run("Dilate");
- run("Skeletonize");
- run("Dilate");
- run("Invert LUT");
- run("Analyze Particles...", "size=500-Infinity pixel circularity=0.30-1.00 show=Overlay display exclude clear record add");
- drawAllFeretsDiameters();
- function drawAllFeretsDiameters() {for (i=0; i<nResults; i++) {x = getResult('XStart', i); y = getResult('YStart', i); doWand(x,y); drawFeretsDiameter(); if (i%5==0) showProgress(i/nResults); } run("Select None"); } function drawFeretsDiameter() {requires("1.29n"); run("Line Width...", "line=1"); diameter = 0.0; getSelectionCoordinates(xCoordinates, yCoordinates); n = xCoordinates.length; for (i=0; i<n; i++) {for (j=i; j<n; j++) {dx = xCoordinates[i] - xCoordinates[j]; dy = yCoordinates[i] - yCoordinates[j]; d = sqrt(dx*dx + dy*dy); if (d>diameter) {diameter = d; i1 = i; i2 = j; }}} setForegroundColor(255,0,0); drawLine(xCoordinates[i1], yCoordinates[i1], xCoordinates[i2],yCoordinates[i2]);}

mRNAseq library preparation and sequencing

Polyadenylated mRNA was isolated from around 10 mg of powdered *Gastrocnemius* muscle using the dynabeads mRNA direct kit (61012, Thermo Scientific). Fragmentation of mRNA was carried out by alkaline hydrolysis (50 mM sodium carbonate pH 9.2, 1 mM EDTA) at 95 °C for 5 min. mRNA was cleaned and purified using the RNeasy MinElute Cleanup kit (74204, Qiagen). 3'- and 5'-ends were dephosphorylated by alkaline phosphatase treatment (FastAP, EF0651, Thermo Scientific) to prevent re-ligation of the mRNA fragments. Before mRNA was cleaned and purified as described above, 5'-OH-ends were re-phosphorylated by the usage of a T4 polynucleotide kinase (EK0032, Thermo Scientific) for 1 h at 37 °C. Addition of the 3'-adapter (5'-(5rApp)-TGGAATTCTCGGGTGCCAAGG-(3SpC3)-3', 1 µmol, Integrated DNA Technologies) was carried out using a truncated and mutated T4 RNA ligase (M0351L, BioLabs) overnight at 4 °C. mRNA was cleaned and purified as described above before addition of the 5'-adapter (5'-GUUCAGAGUUCUACAGUCCGACGAUC, 1 µmol, Microsynth) by the action of a T4 RNA ligase (AM2141, Thermo Scientific) overnight at 4 °C. mRNA was cleaned and purified as described above before reverse transcription using a reverse transcription primer (5'-GCCTTGGCACCCGAGAATTCCA, 1 µmol, Microsynth) and SuperScript II reverse transcriptase (18064022, Thermo Scientific). cDNA was amplified and labelled by the addition of a 5'-RNA PCR primer (5'-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA, 1 µmol, Microsynth) and 3'-indexed RNA PCR primers (TruSeq Small RNA PCR primer sequences from Illumina, 0.2 µmol, Microsynth) using Taq DNA polymerase (D1806, Sigma). Finally, PCR product was purified by the Agencourt AMPure XP system (A63880, Beckman Coulter) and send for sequencing. Single read sequencing was performed with a GFB NextSeq 500 (R2D2) machine (81 cycles, Illumina RTA Version 2.4.6).

Primary antibodies for Western blot analysis

MitoProfile (ab110413, Mitosciences, Abcam), mono- and polyubiquitinylated conjugates monoclonal antibody (BML-PW8810, Enzo Life Sciences), FoxO3a (9467, Cell Signaling), p-FoxO3a (9464, Cell Signaling), AMPKα (2532, Cell Signaling), p-AMPKα (2531, Cell Signaling), PKA-Cα (4782, Cell Signaling), p-PKA C (4781, Cell Signaling), Creb (9104, Cell Signaling), p-Creb (9191, Cell Signaling), CaMKIIα (3362, Cell Signaling) and pCaMKIIα (12716, Cell Signaling). As a loading control eEF2 (2332, Cell Signaling) was used.

Table 2. qPCR primer sequences

Gene Name	Forward primer	Reverse primer
18S	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Cox4i1	TACTTCGGTGTGCCTTCGA	TGACATGGGCCACATCAG
Cox5B	CTTCAGGCACCAAGGAAGAC	TTCACAGATGCAGCCCACTA
CytC	TGCCCAGTGCCACACTGT	CTGTCTTCCGCCCGAACA
FoxO1	AATCCAGCATGAGCCCTTTG	CGTAACTTGATTTGCTGTCCTGAA
FoxO3	CCGGACAAACGGCTCACT	GGCACACAGCGCACCAT
FoxO4	CCACGAAGCAGTTCAAATGCT	TCAGACTCCGGCCTCATTG
MAFbx	CCAAAACTCAGTACTTCCATCAAG	CTATCAGCTCCAACAGCCTTAC
March1	GGCCTACAACCGTGTGATCT	GCATCCTTGATTTCCGTGTT
Mettl11b	CCAGACAAGCGTCCTTTCTC	CAACGGGATCTAAAGGCAAA
Mstn	GCTGGCCCAGTGGATCTAAA	GCCCCTCTTTTTCCACATTTT
MuRF-1	AGGCAGCCACCCGATGT	TCACACGTGAGACAGTAGATGTTGA
Nos1	CAAGCCAAAGGGTAGCAGAG	ACCTCAGACCCAGCTCAAGA
PGC-1α	TGATGTGAATGACTTGGATACAGACA	GCTCATTGTTGTACTGGTTGGATATG
PGC-1β	ATGCTTCCCTCACACCTCAG	GCTTTTGCCTTGTAGGCTTG
Pomk	CTCCAGAGGCCAGAAGACAC	CAGTGACCTGCTTGGTTCAA
Sh3kbp1	AGCACAGAAGGAAAGCCAAA	AGCTCACGGACTTGCATCTT
TBP	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT

6. PGC-1α is dispensable for normal and stress-induced podocyte function in-vivo

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Abstract

Podocytes are highly specialized kidney cells contributing to the glomerular filtration barrier, which prevents the loss of proteins from the filtered blood. Emerging evidence suggests that mitochondrial dysfunction might lead to podocyte injury contributing to the development of chronic kidney disease. However, the complex regulation of mitochondrial homeostasis in podocyte function is still poorly understood. Thus, we assessed the role of the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) in podocytes of male wild type control (WT) and PGC-1α podocyte specific knockout (PKO) mice. Interestingly, only the glomerular basement membrane thickness mildly increased upon the ablation of PGC-1α, which did not result in any functional abnormalities. Furthermore, young and aged PKO animals showed normal kidney and podocyte function under basal and stress-induced conditions. These results clearly demonstrate that PGC-1α is not required for normal podocyte function and question the role of PGC-1α in podocyte metabolism in this context.

Abbreviations

ACR, albumin to creatinine ratio; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; GBM, glomerular basement membrane; HBSS, Hank's balanced salt solution; HFD, high fat diet; PAS, periodic acid-Schiff; PBS, phosphate buffered saline; PC-R, protein to creatinine ratio; PGC-1,

peroxisome proliferator-activated receptor γ coactivator 1; PKO, PGC-1α podocyte-specific knockout mice; ROS, reactive oxygen species; SEM, standard errors of the means; TBP, TATA binding protein; WT, wild type; WT1, Wilms tumor 1

Introduction

The kidneys are bean-shaped organs at the back of the abdominal cavity filtering waste products from the blood and controlling body fluid and salt as well as blood pressure homeostasis. The filtration of blood takes place in the glomerulus, which is composed of a ball of capillaries surrounded by the Bowman's capsule into which urine is filtered. Glomerular epithelial cells, also called podocytes, are highly specialized kidney cells lining the outer aspect of the glomerular basement membrane (GBM). They are composed of a cell body, major processes and foot processes. Neighboring foot processes are connected by the slit diaphragm, a continuous membrane-like structure. Podocytes form the final barrier to protein loss, which explains why podocyte injury leads to proteinuria and occurs in many glomerular diseases that finally progress to chronic kidney diseases (Shankland 2006, Cheng and Harris 2010, Imasawa and Rossignol 2013). Recent studies have shown that mitochondrial dysfunction is associated with many types of kidney diseases (Hagiwara, Yamagata et al. 2006, Hall and Unwin 2007, Schiffer and Friederich-Persson 2017). Moreover, podocytes also contribute to the progression of the diseases by showing abnormalities in their mitochondria as well as reduced expression of mtDNA and its encoded proteins (Gucer, Talim et al. 2005, Hagiwara, Yamagata et al. 2006, Reidy, Kang et al. 2014). The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a major regulator of mitochondrial biogenesis and oxidative metabolism (Lin, Handschin et al. 2005, Schnyder, Kupr et al. 2017). PGC-1α muscle-specific transgenic mice show increased exercise performance and mitochondrial density while PGC-1α muscle-specific knockout mice suffer from impaired mitochondrial respiratory function and reduced exercise capacity (Lin, Wu et al. 2002, Handschin, Choi et al. 2007, Wende, Schaeffer et al. 2007, Calvo, Daniels et al. 2008). Even though PGC-1α is highly expressed in kidneys, its role in renal physiology and podocyte function is largely unexplored (Puigserver, Wu et al. 1998). Nevertheless, PGC-1α in renal tubules has been shown to be important for age-related sodium excretion and transcriptional regulation of mitochondrial metabolism under high fat diet (HFD) conditions (Svensson, Schnyder et al. 2016). Moreover, endogenous PGC-1α levels seem to be essential for podocyte maintenance and mitochondrial function under basal conditions. Aldosterone-induced podocyte injury suppressed PGC-1α and caused mitochondrial damage in podocytes, which could be attenuated by a SIRT1 activator by increasing PGC-1α expression (Yuan, Huang et al. 2012). Furthermore, overexpression of PGC-1α protects from aldosterone- and adriamycin-induced podocyte injury and mitochondrial dysfunction (Zhu, Xuan et al. 2014, Zhao, Yuan et al. 2016). Thus,

mitochondrial energy metabolism by PGC-1α may play an important role not only in renal tubular but also in podocyte function. Our study was designed to elucidate if endogenous PGC-1α is important and required for proper podocyte metabolism. For that purpose, PGC-1α podocyte-specific knockout mice (PKO) were generated and characterized. PKO animals showed a minor increase in the GBM width, which had no effect on normal podocyte function. Furthermore, bovine serum albumin (BSA) overload and HFD treatment did not lead to any functional differences between wild type (WT) and PKO mice. These results demonstrate that PGC-1α is dispensable for normal and stress-induced podocyte function *in-vivo* and suggest that PGC-1α might not be that important in podocyte metabolism as previously believed.

Material and Methods

Animal housing and PGC-1α podocyte-specific knockout mouse generation

Experiments were performed with the approval of the Swiss authorities on adult male mice. Animals were housed in a conventional facility with a 12 h light/12 h dark cycle and had free access to food and water. Mice were killed by CO_2 inhalation.

PKO mice were generated by crossing PGC-1α$^{loxP/loxP}$ mice (Lin, Wu et al. 2004) with Podocin-Cre transgenic mice (Moeller, Sanden et al. 2003, Huber, Hartleben et al. 2009), generously provided by Prof. Dr. Tobias Huber. PGC-1α$^{loxP/loxP}$ animals without Cre expression were used as wild type control (WT) mice. Specific primer pairs were used to detect the presence of the loxP sites, which resulted in amplicons of ~400 bp (WT allele, 650 bp). Specific primer pairs to detect Cre recombinase resulted in amplicons of 100 bp in PKO mice.

In-vivo and urine analysis

All mice were fed a normal chow diet. For HFD treatment, mice were fed a 60% kcal % fat HFD for two months (2127, Provimi Kliba AG).

For urine collection, mice were housed in single mouse metabolic cages (3600M021, Tecniplast) for 24 h. Water consumption was recorded and urine was collected for further analysis. Levels of creatinine (enzymatic method) in urine were determined using an automated biochemical analyzer (Cobas c111 analyzer, Roche). Protein content in the urine was determined with the Bradford assay (Bio-Rad) and albumin levels were measured with a mouse albumin ELISA Kit (ab108792, Abcam) according to the manufacturer's instructions.

Body composition of the mice was determined before and after HFD treatment using an EchoMRI-100^{TM} analyzer (EchoMRI Medical Systems).

Glucose tolerance tests were performed before and after the HFD treatment. Mice were fasted overnight for 16 h and blood glucose was measured from tail blood using a glucose meter (ACCU-CHEK Aviva). Subsequently, mice were injected intraperitoneally with a 20% glucose solution normalized to their body weight (2 g glucose/kg body weight) and blood glucose was measured at 0', 15', 30', 45', 60', 90', 120' and 180' after the injection from tail blood.

Fatty acid free BSA (A8806, Sigma) in 0.9% sodium chloride solution (B. Braun) was injected intraperitoneally for 7 consecutive days with increasing dosages (2, 4, 6, 8 and 3x10 g/kg body weight). Mice were put in metabolic cages before and after the treatment period for 24 h.

Primary glomeruli isolation and podocyte culture

Mice were anesthetized with 450 mg/kg pentobarbital (50 mg/mL Ketalar, Parke-Davis) and perfused through the aorta. First, 10 mL Hank's balanced salt solution (HBSS) was administered before adding 35 mL of dynabeads M-450 tosylactivated in HBSS (14013, Thermo Scientific). Kidneys were removed, minced and treated with 1 mg/mL collagenase A (10103586001, Roche) and 100 U/mL DNase I (18047019, Thermo Scientific) at 37 °C for 30 min with gentle agitation. Digested tissue was filtered through a 100 µm cell strainer (352360, BD Falcon) and centrifuged at 1700 rpm for 5 min. Pellet was re-suspended in 4 mL of HBSS and distributed in four Eppendorf tubes for magnetic concentration (A13346, Thermo Scientific) of the dynabeads. Primary glomeruli were either used for RNA extraction as described below or primary podocyte culture. For this purpose, glomeruli were plated on collagen coated plates (354400, Becton Dickinson) in proliferation medium (RPMI-1640 (R0883, Sigma), 5% FetalClone Serum (SH30066.03, GE Healthcare Life Sciences) and 1% Penicillin/Streptomycin (15140122, Thermo Scientific)) for 10 days. Medium was changed every second day. Primary podocytes were trypsinized (25300054, Thermo Scientific) for 5 min at 37 °C, passed through a 40 µm cell strainer (352340, BD Falcon) and spun down at 1200 rpm for 5 min. Pellet was used for RNA isolation as described below.

For the knockout confirmation, mice were anesthetized as described above and both kidneys, still connected to the vasculature, removed and placed in HBSS. First, kidneys were perfused with a mixture of dynabeads (M-450 tosylactivated (14013, Thermo Scientific) and M-450 Epoxy (14011, Thermo Scientific)) in HBSS and then with a digest solution containing dynabeads mixture, 300 U/mL collagenase type II (17101015, Thermo Scientific), 5 U/mL pronase E (7433, EM Millipore) and 100 U/mL DNase I (18047019, Thermo Scientific). Kidneys were minced and digested in digest solution for 20 min at 37 °C on a rotor (100 rpm). Primary glomeruli were isolated as described above. Single cells were obtained by digesting primary glomeruli for 40 min at 37 °C in a thermomixer (1400 rpm). Beads were removed by magnetic concentration and cell suspension centrifuged for 5 min at 2000 rpm at 4 °C. Single cells were re-suspended in 4% paraformaldehyde, washed and permeabilized with 0.5%

triton (X100, Sigma) for 10 min. After washing, cells were blocked in 5% BSA and incubated with an anti-podocin antibody (P0372, Sigma) coupled to Alexa Fluor 488 (Z25302, Thermo Scientific) for 1 h in the dark. Finally, cells were washed and sorted by fluorescence-activated cell sorting (FACS) in FACS buffer (0.1% BSA in phosphate buffered saline (PBS)).

DNA isolation and PCR reaction

Total DNA was isolated from two pooled samples of FACS sorted podocytes by phenol chloroform DNA purification using phenol:chloroform:isoamyl alcohol (15593031, Thermo Scientific) and glycogen (10814010, Thermo Scientific) according to the manufacturer's instructions. The knockout was confirmed by PCR amplification using specific primer pairs to detect the knockout or WT band. Primers used are listed in Table 1.

Table 1. PCR primers for knockout confirmation.

Gene Name	**Forward primer**	**Reverse primer**
PGC-1α WT	ACCTGTCTTTGCCTATGATTC	CCAGTTTCTTCATTGGTGTG
PGC-1α PKO	TCCAGTAGGCAGAGATTTATGAC	CCAACTGTCTATAATTCCAGTTC

RNA isolation and real-time qPCR

Total RNA was isolated from crushed whole kidney with FastPrep tubes (116913500, MP Biomedicals) and TRI reagent (T9424, Sigma) according to the manufacturer's instructions. Total RNA from primary glomeruli and podocytes was isolated using the RNeasy Micro Kit (74004, Qiagen) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop OneC spectrophotometer (Thermo Scientific). RNA from whole kidney was treated with DNase I (18068015, Thermo Scientific) and then reverse transcribed using hexanucleotide mix (11277081001, Sigma) and SuperScript II reverse transcriptase (18064022, Thermo Scientific). RNA from primary glomeruli and podocytes was reverse transcribed using hexanucleotide mix (11277081001, Sigma) and SuperScript III reverse transcriptase (18080093, Thermo Scientific). The level of relative mRNA was quantified by real-time qPCR on a StepOnePlus system (Applied Biosystems) using Fast SYBR green PCR master mix (4385612, Thermo Scientific). The analysis of the mRNA was performed by the comparative CT method using TATA binding protein (TBP) as endogenous control. Table 2 lists the primer sequences used.

Table 2. qPCR primer list

Gene Name	**Forward primer**	**Reverse primer**
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Podocin	GTGAGGAGGGCACGGAAG	AGGGAGGCGAGGACAAGA
TBP	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT
WT1	ATCCTCTGTGGTGCCCAGTA	GTTGGGGCCACTCCAGATAC

Kidney staining

Freshly isolated kidney was cut in half and fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Fixed tissue was washed twice in water, processed and dehydrated for 25 h (Shandon Pathcentre, Thermo Scientific). Then paraffin blocks (107337, Merck Millipore) were made (Microm EC 350-1, Histocom-AG) and 5 µm sections cut using a microtom (Microm cool cut HM360, Histocom-AG). Slides were dried overnight at 37 °C.

For periodic acid-Schiff (PAS) staining slides were de-paraffinized and stained with periodic acid solution (3951, Sigma) for 5 min. After washing with water slides were stained with Schiff's reagent (3952, Sigma) for 15 min, again washed and counter-stained with hematoxylin solution, Gill No. 3 (GHS3, Sigma) for 1.5 min. Then, slides were dehydrated and mounted with histomount (008030, Thermo Scientific). Per sample 20-30 glomeruli were quantified and glomerular area was measured with the ImageJ software.

Transmission electron microscopy

Two small pieces of the cortex region of a freshly isolated kidney were fixed in 3% paraformaldehyde and 0.5% glutaraldehyde for 4 h. Then, tissue was further fixed in 1% osmium tetroxide and embedded in Epon. 60–70 nm sections were cut, and pictures were taken with a Morgagni 268(D) transmission electron microscope (FEI). Per sample, 10 independent measurements of the GBM thickness were quantified in 5 different pictures with the ImageJ software.

Statistical analysis

Values are expressed as means ± standard errors of the means (SEM). Statistical significance was determined with unpaired two tailed t-tests using Excel software. Significant differences ($p < 0.05$) between WT and PKO mice are indicated by an asterisk (*). Significant differences between young and old or before and after BSA or HFD treatments, respectively, are indicated by a hashtag (#).

Results

Confirmation of the podocyte-specific PGC-1α knockout

PKO mice were generated by crossing PGC-1α$^{loxP/loxP}$ mice (Lin, Wu et al. 2004) with Podocin-Cre transgenic mice (Moeller, Sanden et al. 2003, Huber, Hartleben et al. 2009). Gross kidney morphology and relative kidney weights did not differ between the genotypes (Fig. 1A). In order to confirm the successful and specific knockout of PGC-1α in podocytes, we performed qPCR on whole kidney, primary glomeruli and primary podocytes samples to determine the expression levels of PGC-1α. On whole kidney level we could not detect any differences in PGC-1α expression between WT and PKO

animals indicating that the knockout was presumably podocyte-specific (Fig. 1B). However, also in primary glomeruli and podocytes mRNA levels of PGC-1α did not differ comparing WT and PKO animals (Fig. 1C and D). Furthermore, expression levels of the podocyte-specific markers, podocin and Wilms tumor 1 (WT1), were not different between WT and PKO animals in all of the tissue fractions (Fig. 1B-D). These results indicate that either the recombination of the floxed construct was not successful or that the basal expression of PGC-1α was so low that no differences between WT and PKO animals could be observed. Thus, we verified the recombination of the floxed construct by PCR with specific primer pairs detecting the presence of either the WT or the PKO PCR product. As expected, we only observed the WT band in WT animals, however, in the PKO samples we detected the knockout but also the WT band (Fig. 1E). In whole kidney and primary glomeruli samples this result could be expected since both samples contained a lot of other cell types with intact PGC-1α expression. Furthermore, primary podocyte cultures seemed to be a mixture of WT and PKO cells suggesting some contamination from other glomerular cell types, which explains why we saw no differences in gene expression levels between WT an PKO animals (Fig. 1D). Thus, we pooled two mice of each genotype together and isolated primary podocytes by FACS to obtain a pure podocyte culture. With this approach we were finally able to confirm the successful recombination of the floxed construct in PKO mice (Fig. 1F).

Basal characterization of young and old PKO mice

After the confirmation of the PGC-1α knockout in podocytes, we characterized the phenotype of young (3 months of age) and old (22 months of age) WT and PKO mice. First, animals were put into metabolic cages to monitor their drinking behavior and to collect urine samples. We did not observe any genotype differences in young and old mice, however, drinking volume was significantly reduced and urine volume increased in old WT and PKO mice (Fig. 2A and B). Albuminuria and proteinuria are markers of kidney function and injury and are measured by the ratio of albumin or protein to the amount of creatinine in the urine (Biljak, Honovic et al. 2017). The loss of PGC-1α in podocytes did not result in any differences in the albumin to creatinine (ACR) or protein to creatinine ratios (PC-R) between WT and PKO animals at either age (Fig. 2C and D). Old mice showed an increase in the ACR and a decrease in the PC-R suggesting reduced kidney function with age. Histological analysis of kidney cross-sections was carried out by periodic acid-Schiff (PAS) stainings and revealed a significant increase in the glomerular area with age but no differences between genotypes (Fig. 2E). Moreover, podocyte structure was examined by transmission electron microscopy of kidney cortex regions. Basal lamina width was significantly increased in young PKO mice compared to their WT counterparts (Fig. 2F). Furthermore, old animals showed a massive increase in the width of the basal lamina in relation to young mice (Fig. 2F). These results suggest that young PKO animals show a mild pre-aging phenotype with a small but significant increase in the basal lamina width.

BSA-induced podocyte stress leads to albuminuria and proteinuria independently of PGC-1α

Since PKO animals showed only a very mild pre-aging phenotype under basal conditions we examined podocyte function under stress conditions. Thus, we induced albuminuria and proteinuria with increasing dosages of intraperitoneal BSA injections in WT and PKO animals (Chen, Wei et al. 1995). BSA injections led to no differences in drinking volume (Fig. 3A) while urine volume (Fig. 3B) was significantly higher after the treatment but not different between the genotypes. Furthermore, total albumin (Fig. 3C) and ACR (Fig. 3D) as well as total protein (Fig. 3E) and PC-R (Fig. 3F) levels all significantly increased after BSA treatment, which was independent of PGC-1α. These results indicate that PKO animals are able to cope with an increased protein loading to the same extend as WT mice and suggest that PGC-1α might not be required for proper podocyte function under basal and stress-induced conditions *in-vivo*.

HFD treatment leads to metabolic syndrome and albuminuria in WT mice

To further test if PGC-1α is indeed dispensable for stress-induced podocyte function, we induced proteinuria by HFD treatment, which has been shown to provoke the metabolic syndrome as well as renal injury and albuminuria (Deji, Kume et al. 2009). Body weight (Fig. 4A) and fat mass (Fig. 4B) were significantly increased while lean mass (Fig. 4C) was reduced after two months of HFD treatment independently of PGC-1α. Furthermore, glucose tolerance was lower (Fig. 4D) and concomitantly the area under the curve (Fig. 4E) increased in HFD-treated mice. These results demonstrate that the HFD treatment successfully induced hallmarks of the metabolic syndrome like increased fat mass and glucose intolerance. Surprisingly, WT animals showed an induction of the ACR indicative of albuminuria, which we did not observe in PKO mice (Fig. 4F). However, the excretion of proteins in the urine was not different between the conditions or the genotypes (Fig. 4G). These data suggest that the ablation of PGC-1α in podocytes protected mice from developing albuminuria after HFD treatment and confirms that PGC-1α is dispensable for BSA and HFD stress-induced podocyte function.

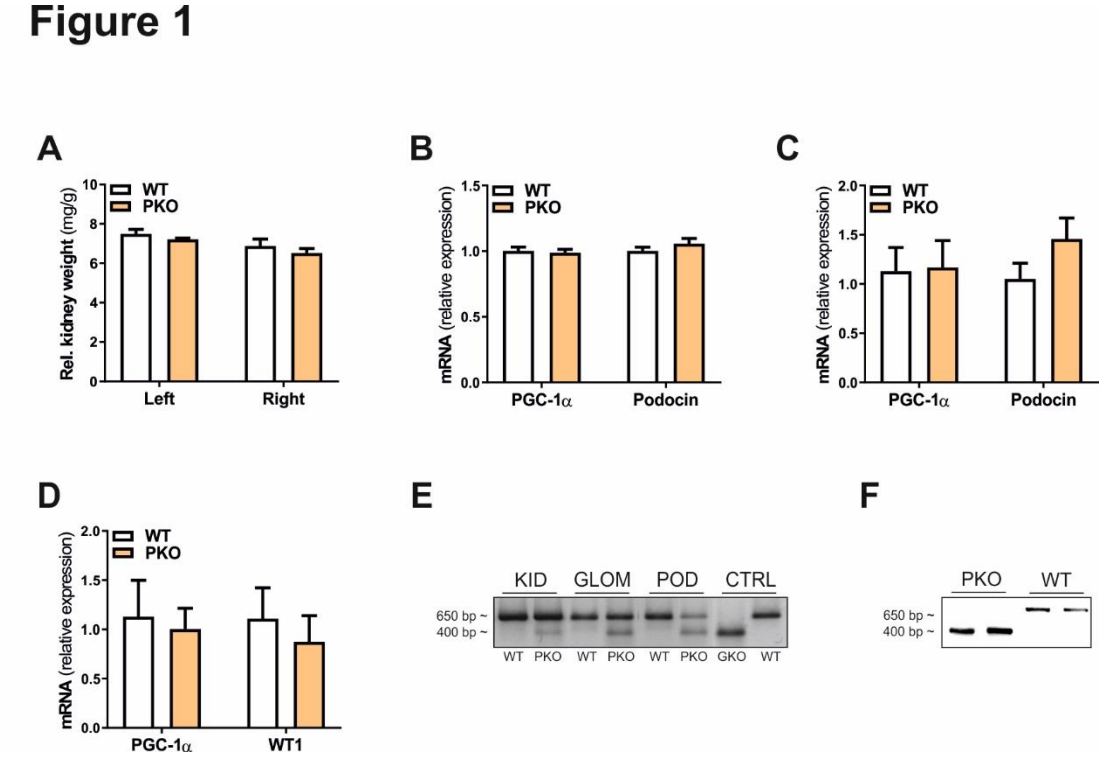

Figure 1. Confirmation of the podocyte-specific PGC-1α knockout

A) Left and right kidney weights of WT and PKO animals relative to their body weight (n=4).

B-D) Gene expression of PGC-1α, podocin and Wilms tumor 1 (WT1) relative to TATA-box binding protein (TBP) in whole kidney (B), primary glomeruli (C) and primary podocytes (D) of WT and PKO mice (n=3-5).

E) Representative PCR gel of whole kidney, primary glomeruli and primary podocytes samples from WT and PKO animals (n=3).

F) Representative PCR gel of FACS sorted primary podocytes from WT and PKO animals (n=4).

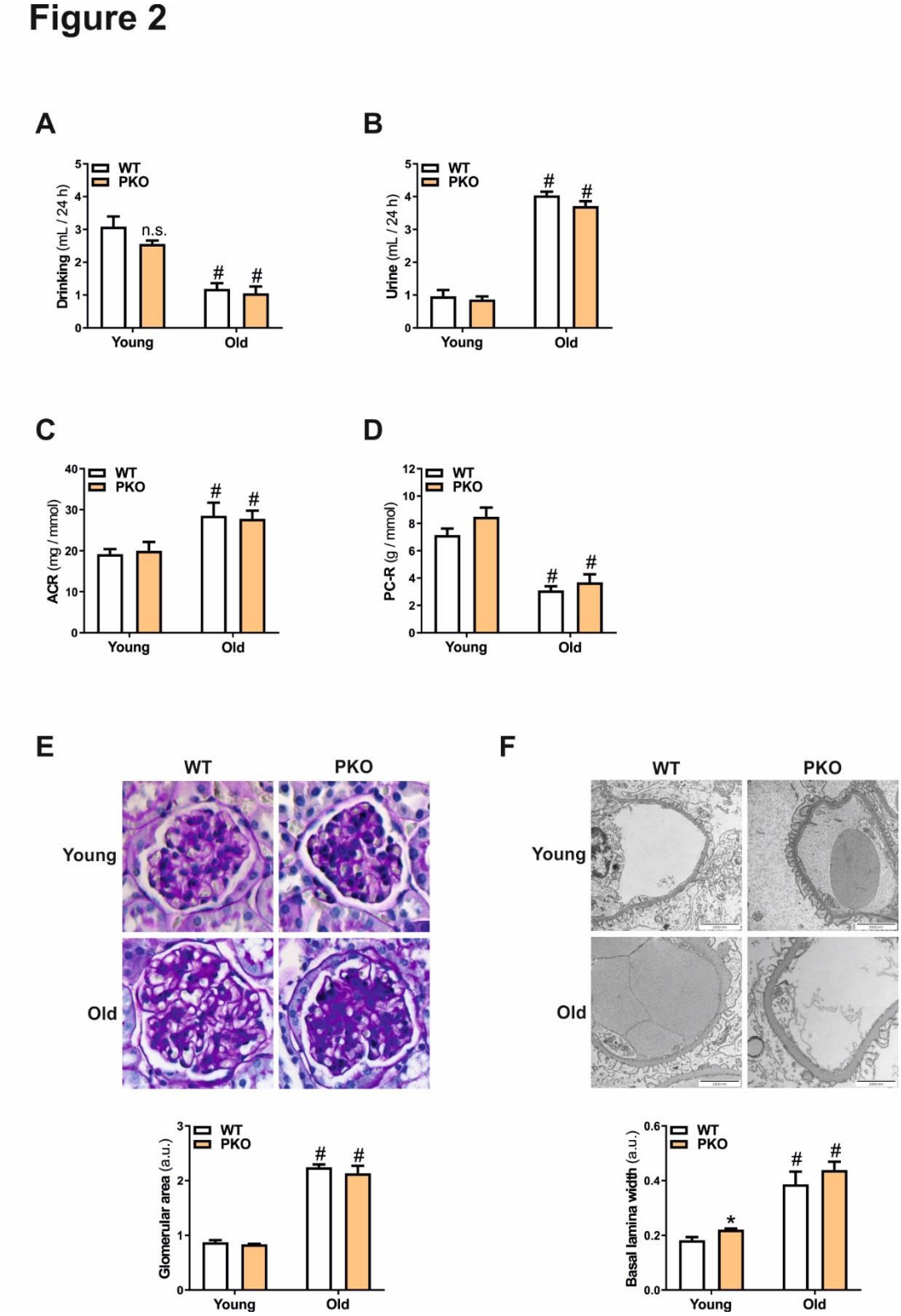

Figure 2. Basal characterization of young and old PKO mice

A-B) Drinking (A) and urine (B) volumes of young and old WT and PKO animals measured over a period of 24 h (n=6-10).

C-D) Albumin to creatinine (ACR) (C) and protein to creatinine ratios (PC-R) (D) in urine of young and old WT and PKO animals (n=6-10).

E) Representative periodic acid-Schiff (PAS) stainings of kidney cross-sections of young and old WT and PKO animals and corresponding quantification (n=4-5).

F) Representative transmission electron microscopy pictures of kidney cortex regions of young and old WT and PKO animals and corresponding quantification (n=4).

* indicating significant differences between WT and PKO mice; # indicating significant differences between young and old mice

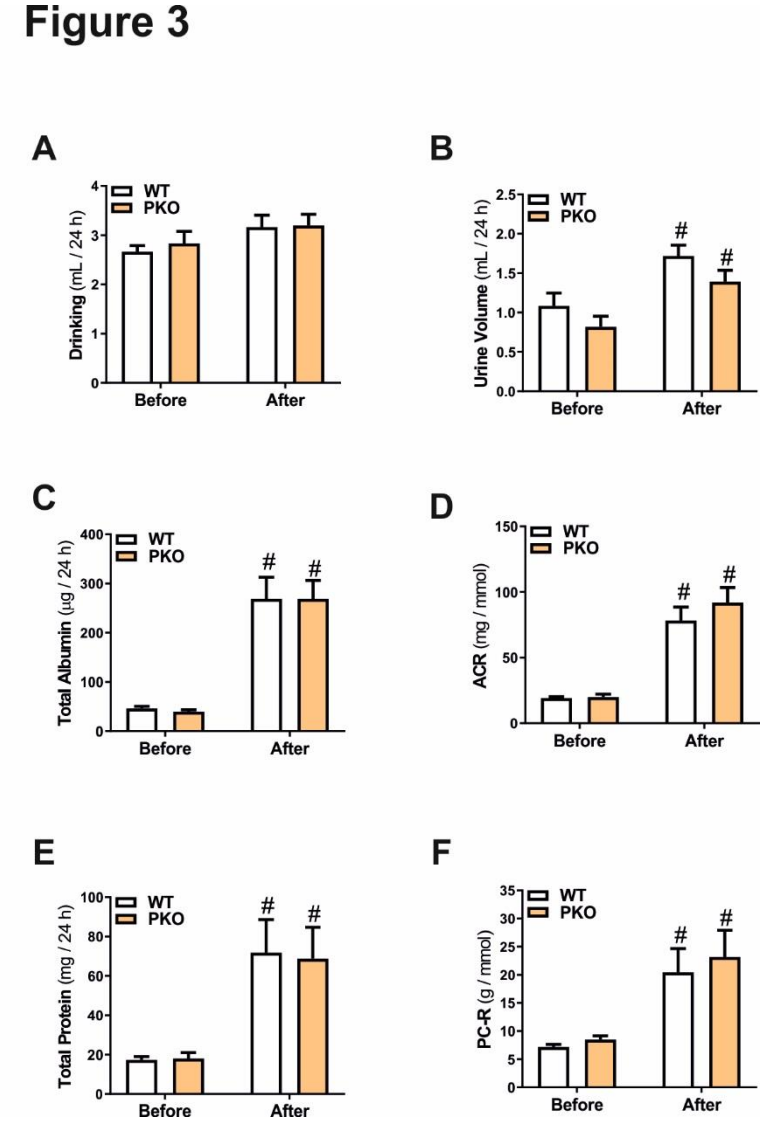

Figure 3. BSA-induced podocyte stress leads to albuminuria and proteinuria independently of PGC-1α

A-B) Drinking (A) and urine (B) volumes of WT and PKO animals before and after intraperitoneal injections of BSA for 7 consecutive days (n=6).

C-F) Total albumin (C), albumin to creatinine (ACR) (D), total protein (E) and protein to creatinine (PC-R) (F) levels in urine of WT and PKO animals before and after intraperitoneal injections of BSA for 7 consecutive days (n=6).

indicating significant differences before and after BSA treatment

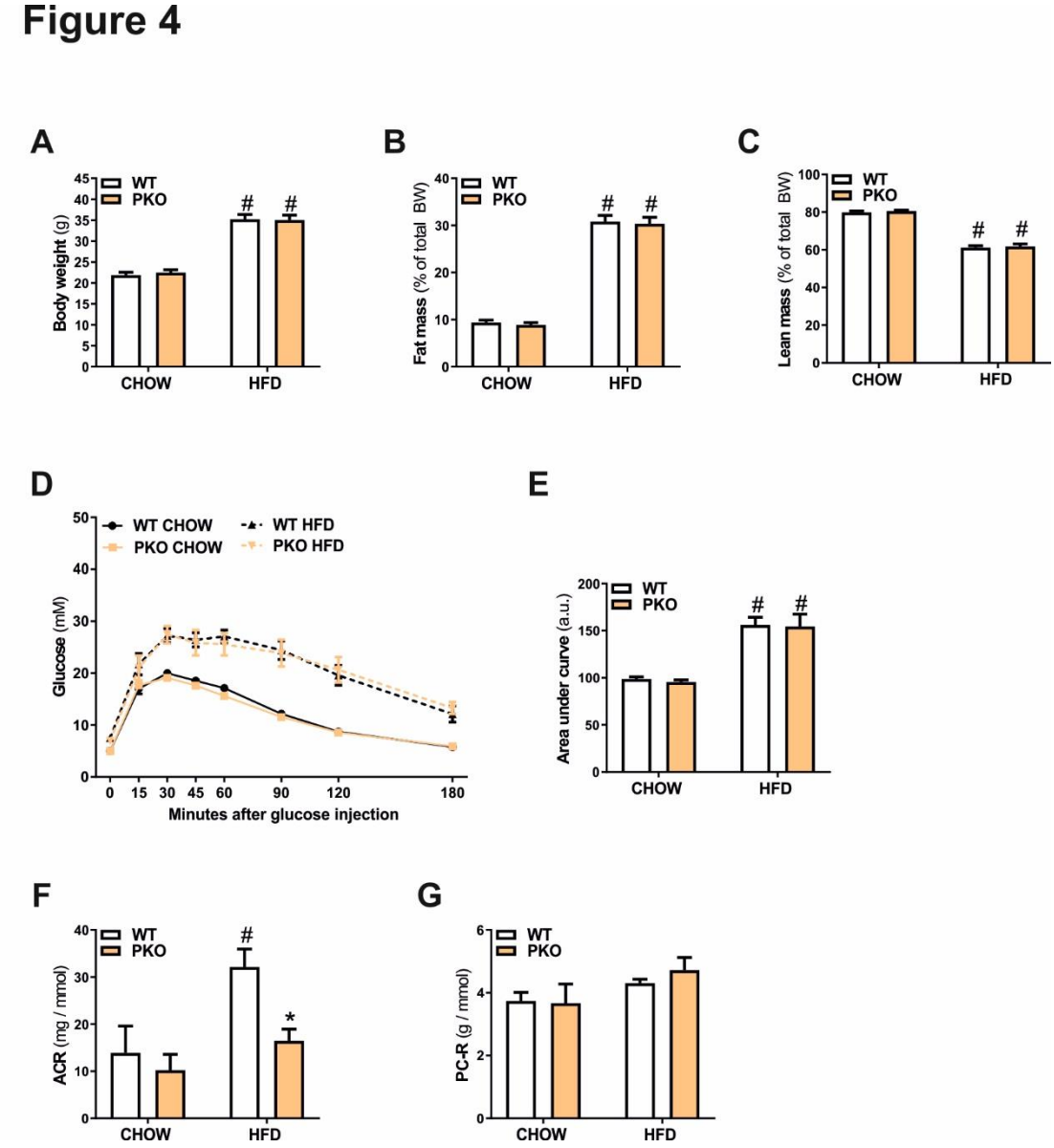

Figure 4. HFD treatment leads to metabolic syndrome and albuminuria in WT mice

A-C) Body weight (A), relative fat mass (B) and relative lean mass (C) of WT and PKO mice before and after 2 months of HFD treatment (n=6).

D-E) Intraperitoneal glucose tolerance test after 16 h of fasting of WT and PKO mice before and after 2 months of HFD treatment. The adjacent bar graph represents the average area under the curve (n=6).

F-G) Albumin to creatinine (ACR) (F) and protein to creatinine ratios (PC-R) (G) of WT and PKO mice before and after 2 months of HFD treatment (n=6).

* indicating significant differences between WT and PKO mice; # indicating significant differences between CHOW and HFD treatments

Discussion

The study of podocyte biology has gained more and more interest in the past view years and emerging data suggest that podocyte injury occurs in many glomerular pathologies that finally progress to chronic kidney diseases (Shankland 2006, Cheng and Harris 2010, Imasawa and Rossignol 2013). Furthermore, mitochondrial metabolism seems to play an important role in the development of podocyte injury and kidney diseases (Hagiwara, Yamagata et al. 2006, Hall and Unwin 2007, Schiffer and Friederich-Persson 2017). Activation and overexpression of PGC-1α, a key protein in the regulation of oxidative metabolism and mitochondrial homeostasis, has been suggested to protect from aldosterone- and adriamycin-induced podocyte injury and mitochondrial dysfunction (Yuan, Huang et al. 2012, Zhu, Xuan et al. 2014, Zhao, Yuan et al. 2016). Thus, we evaluated if PGC-1α is required for basal and stress-induced podocyte function by ablating PGC-1α specifically in podocytes. Our results clearly demonstrate that PGC-1α is not important for normal podocyte metabolism in young and old mice and even dispensable for BSA- and HFD-induced podocyte stress.

Given the important role of PGC-1α in mitochondrial homeostasis (Lin, Handschin et al. 2005, Schnyder, Kupr et al. 2017) and the current literature showing that mitochondrial metabolism is essential for proper podocyte function (Muller-Deile and Schiffer 2014), our data seem to be paradoxical. Mitochondrial respiration in a mouse transformed podocyte cell line has been proposed to account for about 50-75% of cellular respiration (Abe, Sakairi et al. 2010, Ozawa, Ueda et al. 2015), however, these results might not be true for podocytes *in-vivo*. Interestingly, a recent study elucidated that mitochondria are located in the cytosol around the nucleus but not in the cortical area of cultured differentiated podocytes and moreover that mitochondria were not present in foot processes in mouse podocytes (Ozawa, Ueda et al. 2015). Thus, energy metabolism in foot processes seems to be supported by glycolysis rather than mitochondrial respiration. Furthermore, glycolysis was found to contribute to apoptosis regulation, cell motility, lamellipodia formation and local ATP production in the cortical area of podocytes (Ozawa, Ueda et al. 2015). Thus, it is conceivable that PGC-1α is dispensable for energy metabolism in cortical areas of podocytes (e.g. foot processes), which might explain why structural damages of podocytes by BSA or HFD treatments did not lead to any functional differences between WT and PKO animals. Furthermore, ablation of PGC-1α in podocytes protected mice from developing albuminuria upon HFD treatment. In muscle tissue it is well established that PGC-1α knockout mice show a shift from oxidative to more glycolytic muscle fibers (Handschin, Chin et al. 2007) suggesting that the loss of PGC-1α in podocytes could have led to a similar switch in metabolism that seems to be beneficial under HFD conditions. Thus, glycolysis might indeed be more important and crucial for podocyte function in cortical areas and foot processes. Nevertheless, overexpression of PGC-1α seems to be protective against aldosterone- and adriamycin-induced

podocyte injury (Zhu, Xuan et al. 2014, Zhao, Yuan et al. 2016). Both treatments are proposed to increase intracellular levels of reactive oxygen species (ROS) leading to mitochondrial dysfunction and podocyte damage. Ablation of PGC-1α in podocytes does not lead to podocyte damage suggesting that ROS levels and mitochondrial function were normal in PKO mice. Furthermore, this suggests that either PGC-1α is not that important for mitochondrial function in podocytes *in-vivo*, as previously believed, or that the loss of PGC-1α was compensated by other proteins. Indeed, the closely related family member PGC-1β could be a likely candidate compensating for the loss of PGC-1α. For example, dysregulation of PGC-1 coactivators has been associated with the development of skeletal muscle insulin resistance (Mootha, Lindgren et al. 2003, Patti, Butte et al. 2003), however, single knockout models of either PGC-1α or –β did principally not result in insulin resistant or glucose intolerant phenotypes (Lin, Wu et al. 2004, Leone, Lehman et al. 2005, Lelliott, Medina-Gomez et al. 2006, Vianna, Huntgeburth et al. 2006, Handschin, Choi et al. 2007, Sonoda, Mehl et al. 2007, Gali Ramamoorthy, Laverny et al. 2015). Moreover, a large subset of genes seems to be controlled by both PGC-1 coactivators, at least in skeletal muscle (Zechner, Lai et al. 2010). Thus, it may be assumed that the loss of PGC-1α in podocytes could have also resulted in a compensatory increase in PGC-1β expression, which might be another reason why PKO animals did not show any functional abnormalities.

To conclude, our results unraveled that basal PGC-1α expression might not be a key player for normal podocyte function and shed new light on the complex biology of podocyte metabolism. Moreover, glycolysis and mitochondrial respiration might be of equal importance for podocyte energy homeostasis, which possibly opens new avenues for the future development of therapeutic strategies to counteract podocyte injury and chronic kidney diseases.

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7. Discussion

The two PGC-1 family members PGC-1α and PGC-1β are coactivator proteins with pleiotropic functions in tissue-specific and whole body metabolism. During the course of this thesis, we focused on the study of these two coactivators in skeletal muscle physiology as well as of PGC-1α in kidney metabolism. Thereby, we identified new functions and regulatory pathways of PGC-1α and –β in these tissues, which will be discussed in greater detail in the following sections.

Skeletal muscle is a highly metabolically active organ with a tremendous capacity to adapt its activity to different stimuli in order to meet the required tissue and whole body energy needs. Physical activity substantially contributes to skeletal muscle metabolism and whole body energy expenditure and is widely accepted as a tool for the treatment of many different metabolic disorders and other diseases (Haskell, Lee et al. 2007, Colberg, Sigal et al. 2010, Egan and Zierath 2013). But also dietary interventions, for example ketogenic diets (KD), have gained more and more popularity in the use as therapeutic strategies (Paoli, Rubini et al. 2013). KDs are known to induce a fasting-like state, also referred to as ketosis, due to the low amount of carbohydrates and the high amount of fatty acids, respectively (Krebs 1966). The concomitant increase in circulating ketone bodies has been proposed to increase fatty acid oxidation, mitochondrial biogenesis and ATP production, however, the exact mode of action is still unclear (Bough and Rho 2007, Tagliabue, Bertoli et al. 2012). Since PGC-1α is an important regulator of all of these processes and furthermore, is involved in ketolysis in skeletal muscle, we explored its role in mediating the local and systemic effects of a long-term KD in the first study of this thesis (Austin and St-Pierre 2012, Svensson, Albert et al. 2016). We demonstrated that PGC-1α affects exercise performance and oxygen consumption and was involved in the induction of genes important for fatty acid oxidation in skeletal muscle upon KD feeding (Schnyder, Svensson et al. 2017). To our surprise, increased circulating ketone bodies did not affect mitochondrial biogenesis and moreover, ATP production was even reduced in skeletal muscle, contrary to what has been shown in brain tissue (Bough and Rho 2007). These discrepancies could be due to different compositions of the administered diet or could indicate that ketone bodies exert different actions depending on the target organ. In fact, ketone bodies have been identified as signaling metabolites that can activate extracellular receptors (Newman and Verdin 2014). β-OHB has been shown to bind two GPRs, hydroxycarboxylic acid receptor 2 (HCAR2) in adipocytes and free fatty acid receptor 3 (FFAR3) in sympathetic ganglions, respectively, to reduce lipolysis and lower the metabolic rate (Taggart, Kero et al. 2005, Kimura, Inoue et al. 2011). Furthermore, β-OHB also binds to and inhibits class I histone deacetylases (HDAC) that protects mice from renal oxidative stress (Shimazu, Hirschey et al. 2013). It

is conceivable that the increase in mitochondrial biogenesis and ATP production in the brain might also be a result of direct β-OHB binding to specific receptors rather than a passive consequence due to the oxidation of ketone bodies. Since GPRs and HDACs are important players in skeletal muscle metabolism, particularly in the regulation of muscle mass, it would be interesting to study the role of β-OHB as signaling metabolite in skeletal muscle (Jean-Baptiste, Yang et al. 2005, Moresi, Williams et al. 2010). First, this could be done in different cell culture settings *in-vitro* using C2C12 or primary myotubes and second, osmotic pumps could be implanted subcutaneously or intraperitoneally to constantly increase circulating β-OHB levels *in-vivo*. These settings would also be of interest in light of PGC-1α, which is known to be regulated by β-adrenergic signaling and HDAC5 (Miura, Kawanaka et al. 2007, Akimoto, Li et al. 2008). Furthermore, direct delivery of β-OHB could also prevent the adverse effects of KD feeding including increased fat mass, cholesterol and liver stress markers (Schnyder, Svensson et al. 2017).

Another point to consider when studying the effects of ketone bodies on whole body metabolism is their acidic nature. Diabetic ketoacidosis is a severe life-threatening complication in type 1 diabetes patients that results from insulin deficiency leading to marked hyperketonemia with plasma ketone body levels exceeding 25 mM (Laffel 1999). Even though KDs only induce a mild hyperketonemia as during caloric starvation (2-6 mM), several studies have shown an association between KDs and metabolic acidosis (Millichap and Jones 1964, Takeoka, Riviello et al. 2002, Yancy, Olsen et al. 2007). This acidosis could induce many adverse effects including gastrointestinal disturbances, nausea, growth retardation in children or cardiometabolic complications (Yuen, Walcutt et al. 2017). However, direct correlations between KDs, increased metabolic acidosis and all of these adverse effects are still missing. Nevertheless, these associations, together with the above mentioned detrimental effects of KD feeding, raise the questions about the usage of KDs as therapeutic strategies and their compatibility with exercise. The latter is still under debate and various studies show different results ranging from positive effects on maximal VO_2 and lactate threshold to no negative effects on aerobic endurance capacity or explosive and strength performance (Phinney, Bistrian et al. 1983, Paoli, Grimaldi et al. 2012, Zajac, Poprzecki et al. 2014). Furthermore, a recent study in healthy non-athlete adults showed that six weeks of KD feeding on one hand led to reduced body weight due to loss in fat and lean mass, decreased VO_2peak and peak power and increased total cholesterol and low density lipoprotein levels (Urbain, Strom et al. 2017). On the other hand, it slightly increased handgrip strength (Urbain, Strom et al. 2017). These data show that KD feeding can result in positive and negative effects on exercise performance and suggest that it would not impair daily activities or aerobic exercise training, however, it might have detrimental effects on training and competition performance in elite athletes (Urbain, Strom et al. 2017). Finally, these inconclusive results strongly emphasize the need of future studies aimed at elucidating the mechanisms and molecular pathways induced by KD feeding.

In the second study, we focused on the acute time-course and chronic exercise responses and the dependence of PGC-1α in these contexts. PGC-1α is an important mediator of exercise-induced molecular responses functioning as metabolic sensor and effector protein (Kupr and Handschin 2015). We now showed that PGC-1α is furthermore required for the timely expression of target genes and defined the PGC-1α-dependent acute core exercise response, which involved stress- and heat-responsive genes. Importantly, PGC-1α muscle-specific knockout mice (PGC-1α MKO) displayed a different time-course expression pattern of exercise-induced gene transcripts and showed a much smaller acute core exercise response. Gene ontology (GO) analysis of the wildtype (WT) acute core exercise response revealed “cellular response to calcium ion” as top category with associated genes of the Fos and Jun gene families. Fos and Jun belong to the immediate-early genes and are known to be induced by exercise and calcium (Curran 1992, Puntschart, Wey et al. 1998, Carrasco, Riveros et al. 2003). Thus, the first acute transcriptional response seems to be initiated to cope with an increase in cytosolic calcium levels (Miledi, Parker et al. 1983). In fact, prolonged increases in cytosolic calcium concentrations pose a major stress factor for any kind of cell and can result in dysfunction, injury and apoptosis (Trump and Berezesky 1992). The importance of cellular calcium homeostasis is also reflected in the severe and life-threatening phenotype of the disease malignant hyperthermia (MH) (MacLennan and Phillips 1992). MH is an inherited syndrome caused by a mutated ryanodine receptor that leads to hypersensitive gating and results in a sustained release of calcium from the sarcoplasmic reticulum (Joffe, Savage et al. 1992). Inhalation anesthetics have been identified as the main triggers of the syndrome and patients experience different symptoms including high fever, skeletal muscle rigidity, lactic acidosis, increased production of CO_2 and organ dysfunction and failure that eventually lead to death (Ohta, Endo et al. 1989, Hopkins 2011). Thus, the severe clinical picture of MH clearly demonstrates the importance of the regulation of calcium homeostasis in skeletal muscle. Furthermore, our results suggest that each bout of exercise poses an enormous stress on skeletal muscle cells that leads to the induction of immediate-early genes such as Fos and Jun. In addition, it is important to note that increases in cytosolic calcium concentrations lead to hyperthermia since the acute core exercise response also included the GO term “response to stress” in which heat shock protein (Hsp) family members clustered. Thus, it might be interesting to study the effect of elevated cytosolic calcium concentrations on the expression of Hsps. In fact, the heat shock transcription factor (HSF) has been shown to be activated by calcium *in-vitro* (Mosser, Kotzbauer et al. 1990). Furthermore, calcium induces Hsp72 and Hsp60 in human cultured keratinocytes and is involved in the induction of Hsp70 upon Hsp90 inhibition in human non-small cell lung cancer H460 cells (Wakita, Tokura et al. 1994, Chang, Lee et al. 2006). Thus, the study of Hsp regulation by increases in cytosolic calcium might also be of interest in skeletal muscle and exercise physiology. However, this would need to be done in a temperature controlled system to avoid confounding effects through mechanical-induced

overheating of the muscle. This could for example be achieved by swimming or treadmill running in a temperature controlled pool or room, respectively (Ishihara, Yamada et al. 2009). Furthermore, it would be interesting to elucidate the role of skeletal muscle PGC-1α in this context, which is known to be regulated by calcium and has been shown to associate with HSF to induce the transcription of Hsps (Wu, Puigserver et al. 1999, Wu, Kanatous et al. 2002, Handschin, Rhee et al. 2003).

To our surprise, the overlap between the acute and the chronic exercise-induced transcriptional responses was almost completely missing. Since chronically trained mice exerted major improvements in exercise performance and oxygen consumption, we suggest that protein rather than transcriptional adaptations in skeletal muscle metabolism occurred. To confirm this, we are currently performing mass spectrometry analysis. Since the chronic exercise samples have been harvested 18 h after the last training session our results indicate that each exercise bout elicited only a transient rather than a continuous increase in mRNA expression levels. Thus, it could be speculated that the acute and the chronic-acute transcriptional responses would show a similar expression pattern of exercise-responsive genes. To evaluate this hypothesis, we plan to repeat the chronic exercise training and harvest samples after the last training session according to the acute time-course. Thereby we will be able to compare the acute and chronic-acute transcriptional responses upon exercise and their dependence on skeletal muscle PGC-1α.

A follow-up study of this second project of the thesis will be the comparison of transcriptional changes elicited by acute exercise and cold exposure in skeletal muscle. Recently, three PGC-1α- and exercise-dependent myokines termed irisin, meteorin-like and β-aminoisobutyric acid have been identified promoting browning of white adipose tissue and thereby increasing energy expenditure (Bostrom, Wu et al. 2012, Rao, Long et al. 2014, Roberts, Bostrom et al. 2014). Even though it seems paradoxical that exercise triggers the release of myokines to induce thermogenesis and energy expenditure, the authors claim that this could just be an evolutionary consequence of muscle shivering, however, direct correlations are missing. This hypothesis suggests that there might be substantial overlap between the responses to exercise and cold exposure in skeletal muscle. Interestingly, PGC-1α expression in skeletal muscle can be induced by physical activity and cold exposure indicating that PGC-1α might also play an important role in shivering thermogenesis (Puigserver, Wu et al. 1998, Wu, Kanatous et al. 2002, Handschin, Rhee et al. 2003). Thus, we started this follow-up study with exposing WT and PGC-1α MKO mice to 4 °C or room temperature for 6 h and harvesting *Quadriceps* muscles immediately after the intervention. We could confirm that PGC-1α together with PPARα and the two uncoupling proteins 2 and 3 were significantly upregulated after 6 h of cold exposure (data not shown). These samples will now be subjected to RNA sequencing. The aim of this follow-up project is to define the PGC-1α-dependent and –independent transcriptional adaptations in response to acute cold exposure and to compare these changes to the ones induced by acute exercise. This study will elucidate how much

overlap these two stress conditions elicit on the transcriptome of skeletal muscle and will give new insights in the regulation of muscle metabolism and the involvement of PGC-1α as a metabolic sensor and effector protein.

The third study of this thesis was aimed at elucidating the role and regulation of PGC-1β in the response to energy deprivation. We were able to demonstrate that PGC-1β expression is regulated and suppressed by increased cAMP levels *in-vitro* and by fasting *in-vivo*. Due to low specificity of commercially available PGC-1β antibodies and protein abundance in mass spectrometry experiments, we did not succeed in having a closer look at the protein levels of PGC-1β under basal and fasted conditions *in-vivo*. Therefore, we claimed that the reduction in PGC-1β mRNA levels is due to a protective mechanism in order to reduce its inhibitory effects on the nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1) to limit muscle mass loss. However, one could also speculate that the observed reduced PGC-1β expression in WT animals could be the result of a negative feedback loop of increased PGC-1β protein stability and content on its own transcription. Interestingly, cAMP has been shown to stabilize the PGC-1β protein most likely through the action of cAMP-dependent protein kinase (PKA) without affecting its expression levels in primary human melanocytes (Shoag, Haq et al. 2013). Thus, it is conceivable that PGC-1β protein stability in skeletal muscle is also increased in response to cAMP and fasting. To test this hypothesis, cell culture models (e.g. C2C12 myotubes) with for example flag-tagged PGC-1β overexpression could be used to circumvent the non-specificity of commercially available antibodies and the low abundance of endogenous PGC-1β protein. These cells could be treated with compounds that are known to induce cAMP such as forskolin or 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate and protein content could be evaluated over time by Western blotting, which would indicate if the half-life of the PGC-1β protein is changed (Bartsch, Zorn-Kruppa et al. 2003). Furthermore, the human PGC-1α protein has been shown to possess several serine PKA binding sites important for its stabilization by cAMP (Shoag, Haq et al. 2013). Mutation of these sites to alanine resulted in abolished protein stability after activation by cAMP (Shoag, Haq et al. 2013). These experiments could also be carried out for PGC-1β and would elucidate if cAMP stabilizes its protein structure through phosphorylation by PKA. If all of these results would be positive, one could conclude that PGC-1β actively participates in the fasting response and most likely contributes to skeletal muscle atrophy. Contrary to this assumption, overexpression of PGC-1β has been shown to protect from muscle wasting through inhibiting FoxO-induced transcription and NF-κB activity during denervation atrophy (Brault, Jespersen et al. 2010). Nevertheless, our results suggest that PGC-1β is required for the induction of muscle atrophy, at least during energy deprivation. This conflicting results could originate from the different stimuli, e.g. fasting versus denervation. Fasting, on one hand, is a continuous progressive response that involves all organs of the body and that triggers

muscle mass loss in the need for amino acids to sustain hepatic gluconeogenesis (Longo and Mattson 2014). Denervation, on the other hand, induces an immediate loss of voluntary contractile activity that results in disuse-atrophy (Batt and Bain 2013). In fact, the regulation of PGC-1β gene expression in these two conditions seems to be distinct. While denervation-induced atrophy leads to a ~80% drop in PGC-1β mRNA levels within the first day, fasting for 24 h only reduces the expression by 30-40% (Sacheck, Hyatt et al. 2007). Conceivably, the regulation of PGC-1β in these two conditions might be different, even though the induction of atrophy markers such as the two E3 ubiquitin ligases MuRF-1 and MAFbx are common during denervation and fasting (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001, Sacheck, Hyatt et al. 2007). Thus, it might be interesting to compare different atrophy models, e.g. denervation, fasting and cancer cachexia, in terms of PGC-1β regulation.

As we propose in our study, PGC-1β could function as an inhibitor and possible corepressor for Nfatc1 transcriptional activity. However, this hypothesis is just based on predictions obtained from the integrated motif activity response analysis (ISMARA) tool as well as of RNA sequencing and qPCR results. Thus, to solidify these data additional experiments should be carried out. For example, the expression of Nfatc1 target genes could be analyzed after overexpression and knockdown of PGC-1β in C2C12 or primary myotubes. If PGC-1β indeed functions as a corepressor for Nfatc1 activity, target genes would be suppressed in the overexpressed and induced in the knockdown condition, respectively. Accordingly, these effects of PGC-1β should be abolished when Nfatc1 is knocked down. Ultimately, this experiment could be repeated *in-vivo* using WT and PGC-1β MKO mice. Knockdown of Nfatc1 should render PGC-1β MKO mice as susceptible to fasting-induced muscle atrophy as WT animals, which would prove that the blunted fasting response in PGC-1β MKO mice resulted from increased Nfatc1 activity. Thus, it might also be interesting to study the role of Nfatc1 in the maintenance of skeletal muscle mass. Nfatc1 has been shown to be important for the determination of skeletal muscle fiber type and muscle-specific Nfatc1 knockout mice display a lower percentage of slow fibers and lack fiber type switching following a seven-day voluntary exercise training in *Soleus* muscle (Ehlers, Celona et al. 2014). Furthermore, another report suggests that Nfatc1 is involved in the signaling of IGF-1-induced skeletal myocyte hypertrophy to activate target gene programs (Musaro, McCullagh et al. 1999). These results indicate that Nfatc1 might indeed be involved in the regulation of muscle mass and increased Nfatc1 activity might protect from fasting-induced muscle atrophy.

In the fourth and last study of this thesis, we focused on the role of PGC-1α in podocyte function and its contribution to renal physiology. In fact, we are the first group that generated podocyte-specific PGC-1α knockout (PGC-1α PKO) mice to elucidate its function in podocyte metabolism *in-vivo*. PGC-1α ablation in podocytes only resulted in a mild increase in the GBM, which had no negative effects on basal or stress-induced podocyte function. These results were quite surprising considering the current

literature on the importance of mitochondrial function in podocyte and kidney metabolism. Oxidative stress and mitochondrial dysfunction have been indicated to be major contributors in the development of podocyte injury and the progression to CKD (Hagiwara, Yamagata et al. 2006, Hall and Unwin 2007, Schiffer and Friederich-Persson 2017). Podocyte injury is often associated with mitochondrial abnormalities and reduced expression of mtDNA and its encoded proteins (Gucer, Talim et al. 2005, Hagiwara, Yamagata et al. 2006, Reidy, Kang et al. 2014). Thus, activation or overexpression of PGC-1α in different experimental settings has been shown to protect from mitochondrial dysfunction and renal disorders (Chung, Lim et al. 2012, Yuan, Huang et al. 2012, Kim, Lim et al. 2013, Hong, Lim et al. 2014, Tran, Zsengeller et al. 2016). Conversely, global- or kidney-specific PGC-1α knockout mice display difficulties in the response to different stressors such as sepsis, HFD feeding or ischemia (Tran, Tam et al. 2011, Svensson, Schnyder et al. 2016, Tran, Zsengeller et al. 2016). Contrary to these results, our data revealed that PGC-1α is not required for basal or stress-induced podocyte function *in-vivo*. This could be due to two different reasons: either podocytes are not that dependent on oxidative metabolism and mitochondrial function as previously believed or the applied stresses were too mild to trigger a possible phenotype in PGC-1α PKO mice. Indeed, it has recently been shown that foot processes, the actual barrier-forming units of podocytes, mainly rely on glycolysis to produce ATP rather than on mitochondrial respiration (Ozawa, Ueda et al. 2015). This suggests that the loss of PGC-1α in podocytes did not lead to any structural defects in podocyte architecture and foot processes, which might explain why bovine serum albumin- and HFD-induced albuminuria was not different between WT and PGC-1α PKO mice. Conceivably, other stressors such as sepsis, ischemia or aldosterone could have led to different results. In fact, aldosterone seems to be a potent mediator of podocyte injury and its inhibition has recently been implicated to lower proteinuria and to slow the progression of CKD (Fourkiotis, Hanslik et al. 2012, Buglioni, Cannone et al. 2015, Buglioni, Cannone et al. 2015). Thus, future experiments could include the use of subcutaneously implanted mini-pumps to chronically administrate a constant dose of aldosterone followed by the assessment of albuminuria and the study of glomerular and podocyte function in WT and PGC-1α PKO mice. These experiments could clarify if PGC-1α is indeed dispensable for basal and stress-induced podocyte function or if its required to protect from aldosterone-induced podocyte injury and might thus be involved in the development and progression of CKD.

8. Outlook

To conclude, PGC-1α and PGC-1β are coactivator proteins with pleiotropic functions in tissue-specific and whole body metabolism.

Our results in the first study of this thesis revealed that PGC-1α in skeletal muscle is involved in the response to chronic ketogenic diet feeding being essential for the maintenance of adequate energy levels during exhaustive endurance exercise. In the second project, we identified PGC-1α to be important for the timely expression of target genes upon acute exercise and defined the PGC-1α-dependent acute core exercise response. The third study was aimed at elucidating the role of skeletal muscle PGC-1β in the response to fasting. We demonstrated that PGC-1β is substantially involved in fasting-induced muscle atrophy, most likely by inhibiting Nfatc1 transcriptional activity. Finally, we showed that PGC-1α is dispensable for normal and stress-induced podocyte and kidney function *in-vivo* in the fourth and last study of this thesis.

Taken all together, we contributed important new aspects to the current knowledge of the function and role of the two PGC-1 coactivators in skeletal muscle and of PGC-1α in kidney metabolism. Nevertheless, many new questions regarding the regulation and role of the two PGC-1s arose that have been discussed in the previous section. In addition, there is still limited knowledge about PGC-1 protein regulation in different physiological and pathophysiological settings, in particular in the case of PGC-1β. Thus, future studies should focus on the control of PGC-1 protein modifications and their contributions to cellular homeostasis and whole body metabolism. Furthermore, PGC-1 coactivators have been implicated to possess therapeutic potential for the treatment of different pathological states. Therefore, intervention strategies should be developed to target and activate PGC-1s in disease situations to exert their beneficial effects on tissue-specific and whole body metabolism.

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Appendices

Appendix A

PLOS | ONE

RESEARCH ARTICLE

Loss of Renal Tubular PGC-1α Exacerbates Diet-Induced Renal Steatosis and Age-Related Urinary Sodium Excretion in Mice

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OPEN ACCESS

Citation: Svensson K, Schnyder S, Cardel B, Handschin C (2016) Loss of Renal Tubular PGC-1α Exacerbates Diet-Induced Renal Steatosis and Age-Related Urinary Sodium Excretion in Mice. PLoS ONE 11(7): e0158716. doi:10.1371/journal.pone.0158716

Editor: Franziska Theilig, Anatomy, SWITZERLAND

Received: February 9, 2016

Accepted: June 21, 2016

Published: July 27, 2016

Data Availability Statement: The expression array data have been deposited in the Gene Expression Omnibus (GEO) repository under the accession number GSE80618.

Funding: This project was funded by the ERC Consolidator grant 616830-MUSCLE_NET, the Swiss National Science Foundation, SystemsX.ch, the Swiss Society for Research on Muscle Diseases (SSEM), the "Novartis Stiftung für medizinisch-biologische Forschung", the University of Basel and the Biozentrum. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

The kidney has a high energy demand and is dependent on oxidative metabolism for ATP production. Accordingly, the kidney is rich in mitochondria, and mitochondrial dysfunction is a common denominator for several renal diseases. While the mitochondrial master regulator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is highly expressed in kidney, its role in renal physiology is so far unclear. Here we show that PGC-1α is a transcriptional regulator of mitochondrial metabolic pathways in the kidney. Moreover, we demonstrate that mice with an inducible nephron-specific inactivation of PGC-1α in the kidney display elevated urinary sodium excretion, exacerbated renal steatosis during metabolic stress but normal blood pressure regulation. Overall, PGC-1α seems largely dispensable for basal renal physiology. However, the role of PGC-1α in renal mitochondrial biogenesis indicates that activation of PGC-1α in the context of renal disorders could be a valid therapeutic strategy to ameliorate renal mitochondrial dysfunction.

Introduction

The kidney is an important organ for the clearance of metabolic waste products from the blood, for maintaining body salt and fluid balance and for blood pressure homeostasis. This is achieved through passive filtration of plasma in the glomerulus, which is coupled to a system of transporters along the nephron, responsible for maintaining systemic nutrient- and salt homeostasis [1]. Tubular reabsorption is an energy-demanding process and the majority of ATP (~95%) in the kidney is produced through oxidative metabolism [2,3]. Consequently, mitochondrial density is highest in tubule segments associated with high basal transcellular transport rates, such as the proximal tubules and the thick loop of Henle [3]. The integral role of mitochondrial metabolism in renal function is underscored by the prevalence of renal dysfunction in patients suffering from mitochondrial cytopathies [2,4,5]. The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is an important regulator of mitochondrial function [6]. While PGC-1α is highly expressed in the kidneys [7], the role of PGC-1α in renal physiology is so far unclear. To address this gap in knowledge, we have generated and characterized mice with a nephron-specific inducible PGC-1α knockout (NiPKO). Inactivation of PGC-1α in the kidney resulted in reduced

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: DOX, doxycycline; HFD, high fat diet; ISMARA, Integrated Motif Activity Response Analysis; LSD, low salt diet; mtDNA, mitochondrial DNA; NiPKO, nephron-specific inducible PGC-1α knockout; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; PPAR, peroxisome proliferator-activated receptor; rtTA, reverse tetracycline-controlled transactivator; TF, transcription factor.

expression of mitochondrial enzymes. NiPKO mice displayed a mild sodium-losing phenotype, but otherwise showed normal regulation of salt and water balance and blood pressure. Importantly, we found that PGC-1α is necessary for the transcriptional induction of lipid metabolic processes in the kidney upon high fat diet feeding. Consequently, NiPKO mice develop renal hypertriglyceridemia in this dietary context. Collectively, our results indicate a minor role for PGC-1α in basal renal physiology, mainly affecting age-related sodium excretion. Moreover, we observed a central role for PGC-1α in the transcriptional regulation of mitochondrial and metabolic processes in the kidney, most notably during high-fat diet feeding, with consequences on lipid accumulation and inflammation.

Materials and Methods

Animals and diets

Animals were housed in a conventional facility with a 12-h light/12-h dark cycle with free access to food and water. Mice were sacrificed by CO_2 inhalation or terminal bleeding of anaesthetized animals. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt under the consideration of 3R and to ensure minimal pain and stress in the animals. To generate nephron-specific inducible PGC-1α knockout (NiPKO) mice, we crossed mice with transgenic expression of the reverse tetracycline-dependent transactivator (rtTA) under control of the Pax8-promoter (Pax8-rtTA, a kind gift from Dr. Robert Koesters) [8] with transgenic (tetO-cre)-LC1 mice (obtained from the European Mouse Mutant Archive) [9]. These double-transgenic mice were subsequently crossed with mice having two floxed PGC-1α alleles (PGC-1αfl/fl, from internal breeding) [10]. While the PGC-1αfl/fl mice are in a C57BL/6 strain background, the LC1 and the Pax8-rtTA were in a mixed background. To account for that, littermate controls were used in all experiments. All experiments were performed in male mice. To induce the knockout of PGC-1α, doxycycline (DOX) (Sigma) (0.2 mg/mL) was administered *ad libitum* to the drinking water of 12 week old mice, with the addition of 2% sucrose (Sigma) to enhance palatability. After two weeks, mice were switched back to regular drinking water and were allowed at least one week of rest before experiments started. Recombination PCR was performed using primers binding to a region surrounding exons 3–5 of PGC-1α; forward 5'-TCCAGTAGGCAGAGA TTTATGAC-3', reverse 5'- CCAACTGTCTATAATTCCAGTTC-3'. This primer pair yields a product when exons 3–5 of PGC-1α are excised [10]. A control PCR reaction was performed using the following primers; forward 5'- ACCTGTCTTTGCCTATGATTC-3', reverse 5'-CCA GTTTCTTCATTGGTGTG-3'. The experimental diets used for this study were either obtained from Harlan Teklad (low sodium diet (LSD, <0.02% Na) (TD.90228)) or from Research Diets Inc (high fat diet (HFD, 60 kcal% fat, D12492)). Blood pressure was measured in restrained conscious mice using a non-invasive tail-cuff blood pressure analyzer (BP-2000, Visitech system, Bioseb). The mice were acclimatized to this method for 5 consecutive days, measurements were taken for 5 days and values averaged similar to previous studies [11]. Body composition was measured using an EchoMRI-100™ analyzer (EchoMRI Medical Systems).

Blood and urine analysis

For urine collection, mice were housed in single mouse metabolic cages (3600M021, Tecniplast) overnight (16 hours) or for 24 hours. Amount of food and water consumed was recorded, and urine was collected for further analysis. For plasma analysis, whole tail-vein blood was collected in Microvette tubes (Sarstedt). Levels of sodium, chloride, potassium, calcium, protein, urea and creatinine (enzymatic method) levels in urine and plasma were determined using an automated biochemical analyzer (Cobas c111 analyzer; Roche).

RNA extraction and RT-PCR

Frozen tissue was homogenized using TRIzol reagent (Invitrogen) before addition of chlorophorm an centrifugation at 12'000 g for 15 min. Clear supernatant was removed and incubated with isopropanol before centrifugation at 12'000 g for 10 min. Then, pellet was washed twice with 75% ethanol and final pellet dissolved in water. RNA concentration was adjusted and cDNA synthesis was performed using 1 μg of total RNA. Semi-quantitative real-time PCR analysis was performed using Fast SYBR Green master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels for each gene of interest were calculated with the ΔΔCt method, normalizing against mRNA levels of eukaryotic elongation factor 2 (*eEF2*) or TATA-binding protein (*Tbp*). A list of primer sequences used in this study can be found in S1 Table.

Gene expression array

Gene expression analysis of control and NiPKO kidneys was performed using 4 samples per group, with the Affymetrix GeneChip Mouse Gene 2.0 ST microarray. Transcripts were considered significantly altered if having a p-values <0.05 and a fold-change of >1.2. Pathway enrichment analysis was performed using Babelomics [12] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [13]. KEGG terms were considered significantly enriched with a cut-off of p-values<0.05. Integrated System for Motif Activity Response Analysis (ISMARA) [14] was used to predict the core transcription factors driving the transcriptional changes observed in our gene expression array.

Mitochondrial DNA (mtDNA) analysis

Analysis of mtDNA was performed similar to previous descriptions [15]. Frozen tissue was homogenized and DNA was extracted via a standard phenol/chloroform extraction protocol. DNA concentration was adjusted and the relative amount of mtDNA (primers against mtDNA D-loop region) to nuclear DNA (*Ndufv1* primers) was analyzed using Fast SYBR Green master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems).

Protein isolation and immunoblotting

Frozen tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer and after centrifugation at 13'000 g protein concentration of the supernatant was measured using the Bradford method (Biorad). Equal amounts of proteins were separated on SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Whatman). Proteins of interest were detected using the following primary antibodies: Mitoprofile (MS604, MitoSciences), NKCC2 (sc-133823, Santa Cruz Biotechnology), NCCT (sc-21554, Santa Cruz Biotechnology) and eEF2 (2332; Cell signaling). Densitometric analysis of immunoblots was performed on 6 individual samples using Image-J software and a representative selection from this group is presented in each figure.

Histology

After extraction, kidneys were fixed in 4% paraformaldehyde/PBS at 4°C overnight. Kidneys were subsequently dehydrated, embedded in paraffin and 5 μm thick sections were cut using a microtome. For general histology, a Periodic Acid-Schiff (PAS) staining was performed according to the manufacturer's instructions (PAS Kit, Sigma).

Isolation of triglycerides from kidney

Triglycerides were isolated from snap-frozen kidney as previously described (Svensson et al., 2015). Briefly, frozen kidney tissue was homogenized in a 2:1 chloroform/methanol mixture. The organic phase was subsequently dried under N2, re-suspended and cleaned on a solid phase extraction column. Triglycerides were measured using a commercial enzymatic kit (TG PAP 150, BioMérieux) and normalized to the initial weight of the tissue used for extraction.

Statistical analysis

All data are presented as means ± SEM. Unpaired student two-tailed t-test was used to determine differences between groups.

Results

Knockout of PGC-1α in renal tubular cells results in a mild salt-losing phenotype

To inactivate PGC-1α in the kidney, we generated nephron-specific inducible PGC-1α knockout (NiPKO) mice by crossing transgenic Pax8rtTA-(tetO-cre)-LC1 animals [8,9] with mice harboring floxed PGC-1α alleles (PGC-1αfl/fl) [10] (Fig 1A). Through administration of doxycycline (DOX), this system allows specific inactivation of PGC-1α in the complete renal tubule

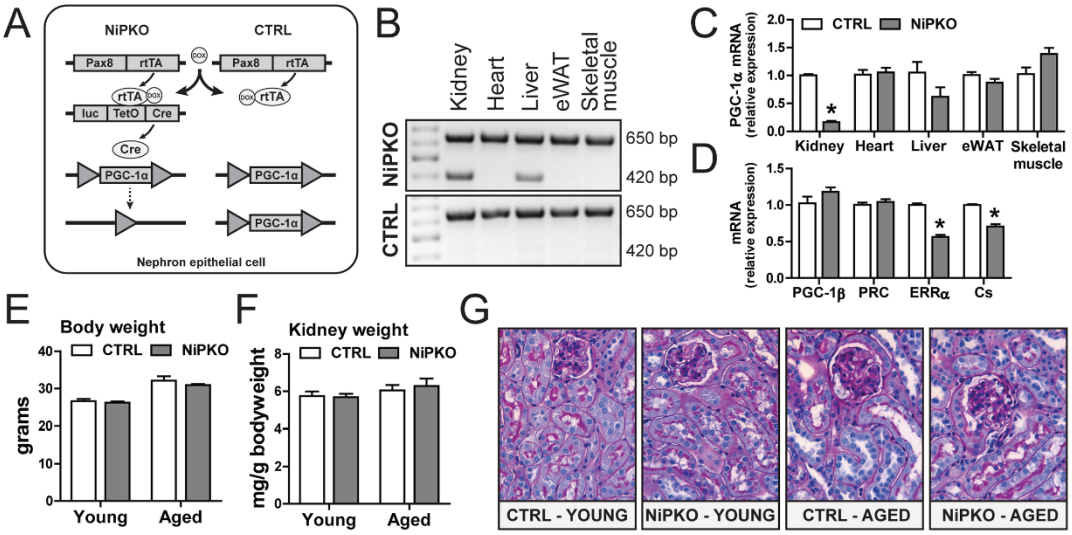

Fig 1. Kidney-specific inactivation of PGC-1α in NiPKO mice. (A) Nephron-specific inducible PGC-1α knockout (NiPKO) mice were generated by crossing transgenic Pax8rtTA-(tetO-cre)-LC1 mice with mice having two floxed PGC-1α alleles (PGC-1αfl/fl). Knockout was induced through doxycycline administration for 14 days. (B) Representative recombination PCR for PGC- 1α on DNA extracted from kidney, heart, liver, epididymal white adipose tissue (eWAT) and skeletal muscle. The amplified products for the wild-type and knockout alleles are approximately 650 bp and 420 bp, respectively. (C) mRNA levels of PGC-1α in kidney, heart, liver, eWAT and skeletal muscle, normalized to eukaryotic elongation factor 2 (eEF2) mRNA levels (n = 5–7). (D) mRNA levels of indicated genes in kidney normalized to eEF2 mRNA levels (n = 7–8). (E) Body weight and (F) average kidney weight normalized to body weight of mice at 1 and 12 months after doxycycline administration (n = 6–16). (G) Representative pictures of kidney histology at 1 and 12 months after doxycycline administration (n = 3). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*).

doi:10.1371/journal.pone.0158716.g001

system while sparing the glomerulus [8]. Successful recombination of exon 3–5 of PGC-1α was confirmed in kidney from NiPKO mice after DOX-administration (Fig 1B). In line with a low *Pax8* expression in liver [8], we also detected recombination of PGC-1α in liver of NiPKO mice (Fig 1B). However, while PGC-1α mRNA expression was strongly diminished in the kidney, there was no significant reduction in PGC-1α transcript levels in the liver (Fig 1C). All other tissues tested showed unchanged PGC-1α mRNA levels between control and NiPKO mice (Fig 1C). The inactivation of PGC-1α in kidney did not affect expression of the related family members PGC-1β and PGC1-related coactivator (PRC) (Fig 1D), but resulted in significantly reduced transcript levels of known PGC-1α target genes such as estrogen related receptor α (ERRα) and citrate synthase (Cs) (Fig 1D). Next, the phenotype of the NiPKO mice was investigated in young mice (1 month after DOX administration) and aged mice (12 months after DOX administration). Inactivation of PGC-1α in kidney led to no significant changes in either body weight (Fig 1E) or relative kidney weight (Fig 1F) in either age category. Moreover, kidneys from NiPKO mice displayed normal histological features compared to control mice (Fig 1G, S1A and S1B Fig). Subsequently, we analyzed salt and water homeostasis and blood pressure in control and NiPKO mice. Inactivation of PGC- 1α in the kidney did not affect water intake or urine output in either young (Fig 2A) or aged (Fig 2B) NiPKO mice compared to control mice. Similarly, systolic and diastolic blood pressure was indistinguishable between the groups at these time points (Fig 2C and 2D). Interestingly, NiPKO mice displayed a significant increase in urinary sodium excretion when normalized to urinary creatinine excretion in both young (Fig 2E) and aged animals (Fig 2F). However, there was no significant difference for the urinary excretion of chloride, potassium and calcium or for proteins and urea (Fig 2E and 2F), or in plasma levels of sodium or any of the above-mentioned parameters (S2A and S2B Fig). The increased urinary sodium excretion was not connected to alterations in transcript levels of the epithelial Na+ channel α (ENaCα), ENaCβ or ENaCγ subunits in either young or aged mice (Fig 2G and 2H). Likewise, no differences in transcript (Fig 2G and 2H) or protein (Fig 2I and 2J) levels of the Na+-Cl− cotransporter (NCCT) or Na+-K+-Cl− cotransporter (NKCC2) were detected. These data indicate that NiPKO mice display a largely normal regulation of water intake, urine output and blood pressure, but display a mild sodium-losing phenotype in the basal state.

Aged NiPKO mice cannot adapt their sodium excretion during low salt diet feeding

The small elevation in urinary sodium excretion in NiPKO mice indicates a role for PGC-1α in the maintenance of renal salt homeostasis. To test this notion, we exposed control and NiPKO animals to a dietary salt stress and assessed salt- and water homeostasis. Control and NiPKO mice were administered a standardized diet containing <0.02% NaCl (low salt diet, LSD) for 5 consecutive weeks, starting at either 1 or 12 months after DOX-administration. LSD-feeding did not affect body weight in either group (Fig 3A) and there was no significant difference in food intake (Fig 3B) or water intake (Fig 3C) between LSD-fed control and NiPKO mice. Interestingly, despite the mild salt-losing phenotype in the basal state (Fig 2E), young NiPKO mice could adapt their urinary sodium excretion to the same extent as control mice during LSD feeding (Fig 3D). Consequently, there were no differences in either urine output (Fig 3E) or urine-to-water ratio (Fig 3F) between young control and NiPKO mice with LSD feeding. In contrast to the young mice however, aged NiPKO mice displayed elevated urinary sodium levels (Fig 3D) with a concomitant elevated urine output (Fig 3E) and urine-to-water ratio (Fig 3F) in aged NiPKO mice compared to control mice with LSD feeding. Collectively, these data indicate that while young NiPKO mice can adapt their reabsorption of urinary sodium during

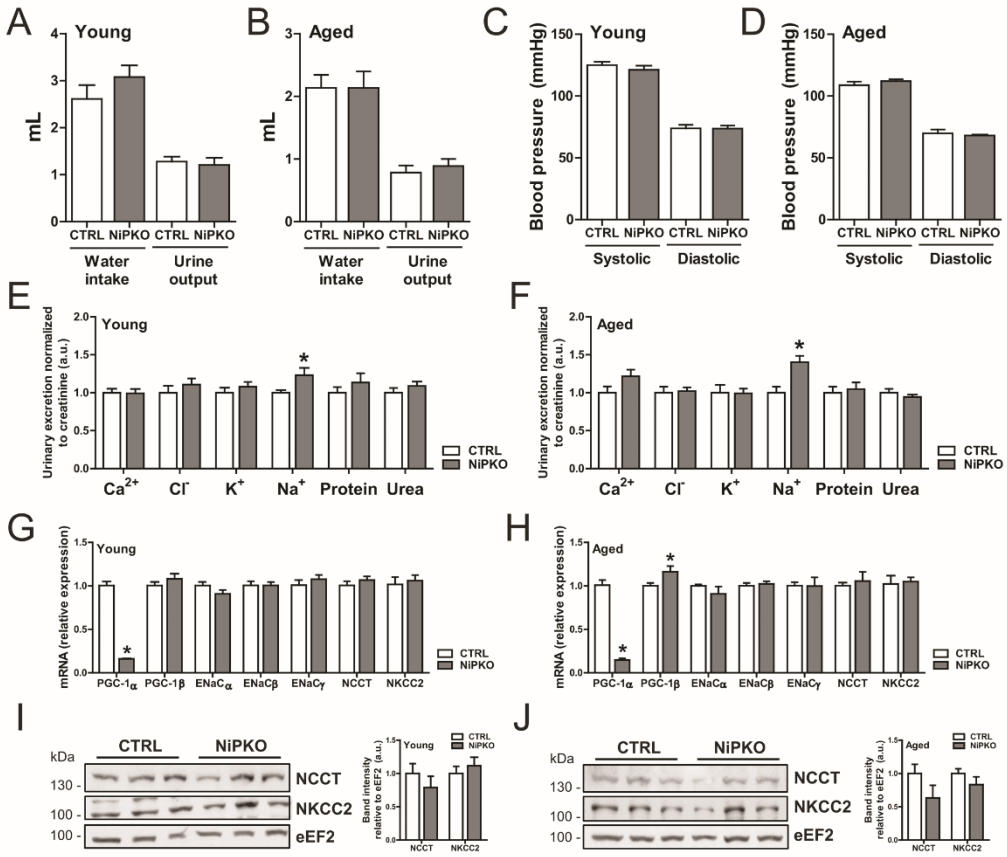

Fig 2. NiPKO mice display elevated sodium-excretion in urine. (A-B) Water intake and urine output in mice over 24 hours, at (A) 1 month and (B) 12 months after doxycycline administration (n = 8–13). (C-D) Systolic and diastolic blood pressure measured via tail-cuff photoplethysmography at (C) 1 month and (D) 12 months after doxycycline administration (n = 6–8). (E-F) Urinary levels of calcium (Ca2+), chloride (Cl-), potassium (K+), sodium (Na+), protein and urea over 24 hours, normalized to urinary excretion of creatinine, at (E) 1 month and (F) 12 months after doxycycline administration (n = 8–13). (G-H) mRNA levels of indicated genes in kidney normalized to *Tbp* mRNA levels, at (G) 1 month and (H) 12 months after doxycycline administration (n = 6). (I-J) Representative immunoblots of NCCT, NKCC2 and eEF2 in kidney. Bar graph shows quantification of band intensities of NCCT and NKCC2 relative to eEF2 (n = 6). Error bars represent mean ±SEM. Significant differences (p-value<0.05) between genotypes are indicated by an asterisk (*).

doi:10.1371/journal.pone.0158716.g002

a reduced dietary salt intake to the same extent as control mice, this process is impaired in aged NiPKO mice. Similar to chow-fed mice (Fig 2G and 2H), there were no differences in transcript levels of the sodium transporters ENaC (α/β/γ subunits), NCCT or NKCC2 between young or aged control and NiPKO mice (Fig 3G and 3H). Interestingly, while protein levels of NCCT and NKCC2 were comparable between young LSD-fed control and NiPKO mice (Fig 3I), aged NiPKO mice displayed significantly reduced renal protein levels of both NCCT and NKCC2 compared to control mice while the phosphorylated forms of NCCT and NKCC2 were not

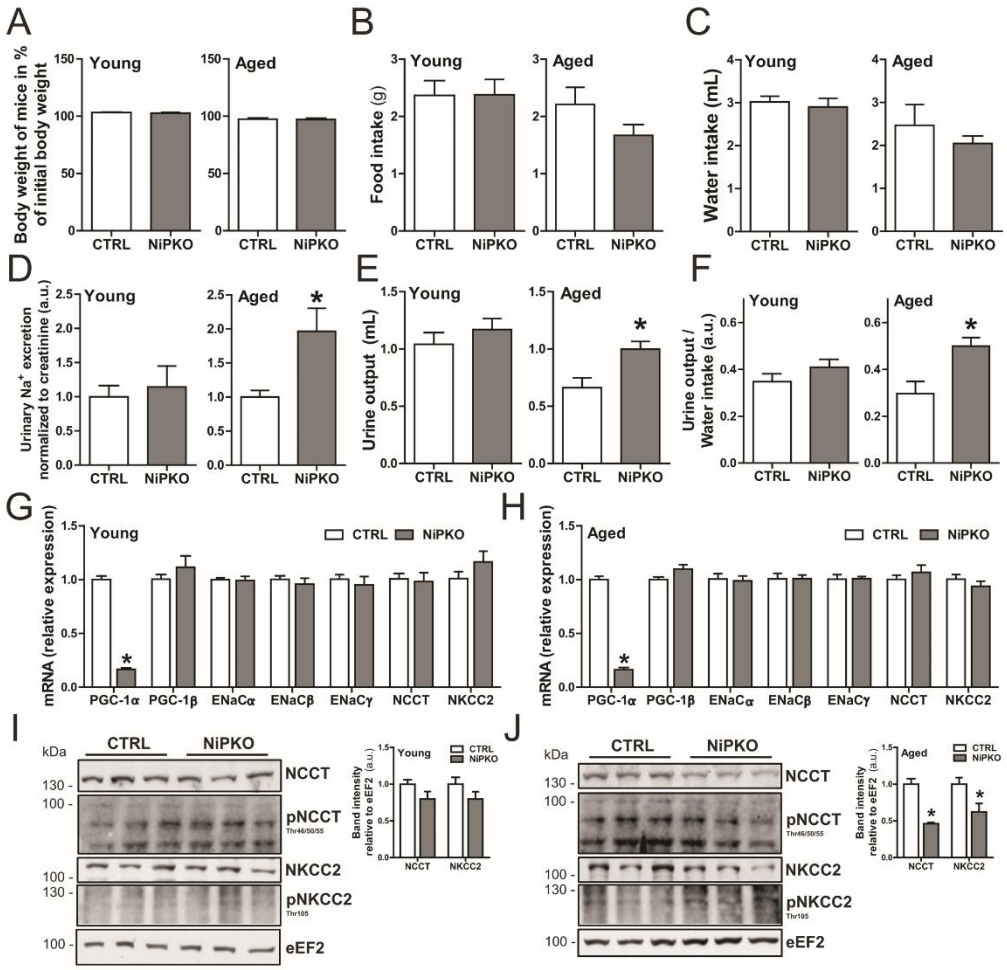

Fig 3. Aged NiPKO mice cannot adapt their salt- and water homeostasis to a reduced dietary salt intake. Control (CTRL) and NiPKO mice were fed a standardized diet containing <0.02% NaCl (low salt diet, LSD) for 5 weeks, starting at either 1 month or 12 months after doxycycline administration. (A) Body weight at end of LSD feeding period expressed as percentage of initial body weight (n = 6–8). (B) Food intake over 24 hours (n = 6–8). (C) Water intake over 24 hours (n = 6–8). (D) Urinary sodium (Na+) excretion normalized to urinary creatinine excretion (n = 6–8). (E) Urine output over 24 hours (n = 6–8). (F) Urine-to-water ratio over 24 hours (n = 6–8). (G-H) mRNA levels of indicated genes in kidney normalized to *Tbp* mRNA levels, at (G) 1 month and (H) 12 months after DOX administration (n = 6). (I-J) Representative immunoblots of NCCT, p-NCCT (Thr45/50/55), NKCC2, p-NKCC2 (Thr105) and eEF2 in kidney. Bar graph shows quantification of band intensities of total NCCT and NKCC2 relative to eEF2 (n = 6). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*).

doi:10.1371/journal.pone.0158716.g003

different between the groups at either age (Fig 3I and 3J). Importantly, the reduced levels of the renal sodium-transporters NCCT and NKCC2 in aged NiPKO mice could explain the salt

losing phenotype specifically in this group. However, since transcription of the genes encoding these channels was unaltered (Fig 3G and 3H), the reduced protein levels of NCCT and NKCC2 is most likely an indirect effect of PGC-1α inactivation in aged mice.

PGC-1α regulates mitochondrial gene transcription in the kidney

PGC-1α is an important transcriptional regulator of mitochondrial and metabolic processes [6]. Consequently, we were interested in how inactivation of PGC-1α affects the renal transcriptome in mice fed a chow diet and in the context of high fat diet (HFD)-induced metabolic stress. Accordingly, starting one week after the end of the DOX administration, control and NiPKO mice were fed either a chow diet or a HFD (60 kcal% fat) for five months. At the end of this period, HFD-fed control and NiPKO mice were significantly heavier than the chow fed cohorts (Fig 4A). HFD-fed control and NiPKO mice gained a similar amount of weight and displayed no difference in body composition (Fig 4A and 4B). Moreover, systolic and diastolic blood pressure was unaltered between genotypes during HFD-feeding (Fig 4C). Similar to chow fed mice (Fig 2E and 2F), we could confirm the salt-losing phenotype in HFD-fed

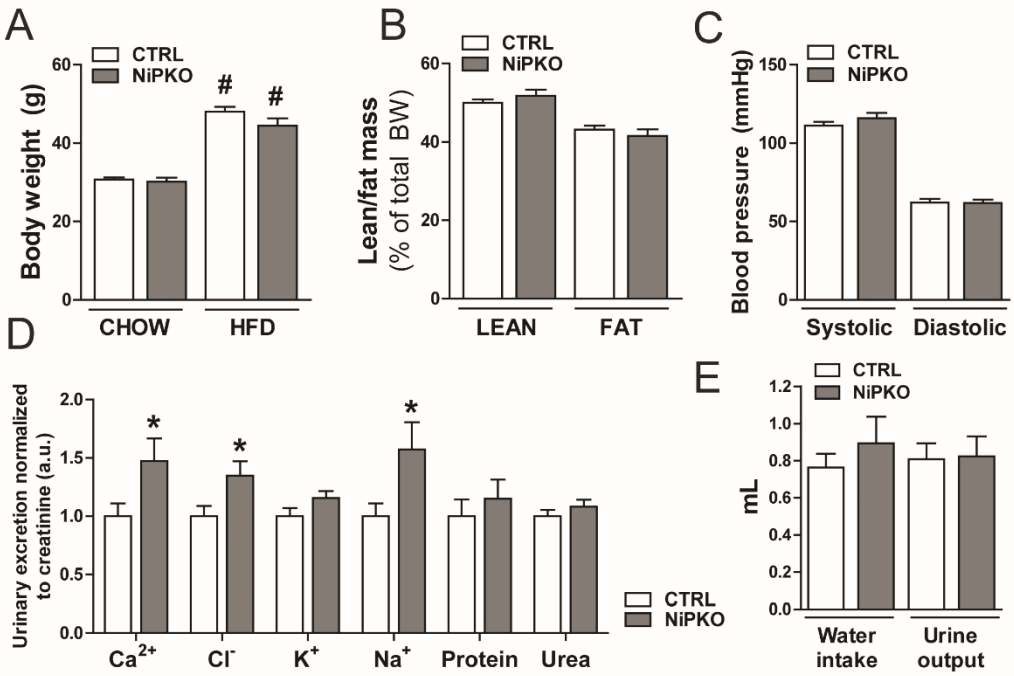

Fig 4. High fat diet-induced changes in control and NiPKO mice. Control (CTRL) and NiPKO mice were fed either a chow-diet (CHOW, 10 kcal % fat) or a high fat diet (HFD, 60 kcal% fat) for 5 months, starting at 2 weeks after DOX-administration. (A) Body weight at the end of chow or HFD feeding period (n = 10–15). (B) Body composition expressed as percentage lean and fat mass of total body weight of HFD-fed mice (n = 16). (C) Systolic and diastolic blood pressure measured via tail- cuff photoplethysmography of HFD-fed mice (n = 10). (D) Urinary levels of calcium (Ca2+), chloride (Cl-), potassium (K+), sodium (Na+), protein and urea over 16 hours, normalized to urinary excretion of creatinine, at the end of HFD feeding period (n = 9). (E) Water intake and urine output over 16 hours of HFD-fed mice (n = 15–16). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*) and between chow- and HFD-fed groups by a number sign (#).

doi:10.1371/journal.pone.0158716.g004

NiPKO mice (Fig 4D). Interestingly, HFD- fed NiPKO mice displayed enhanced urinary excretion not only of sodium, but also of chloride and calcium (Fig 4D). Despite the elevated levels of electrolytes in the urine, HFD-fed NiPKO mice displayed unaltered water intake and urine output compared to control mice (Fig 4E). Moreover, differences in food intake cannot account for the modulation of chloride and calcium (S3 Fig). Overall, HFD-feeding exacerbated the loss of ions in the urine, but did not significantly impact the overall ability of NiPKO mice to adjust their salt and water homeostasis and blood pressure regulation. Accordingly, the transcriptional regulation of several transporters by genotype or diet remains relatively mild (S4 Fig).

Next, we focused on changes in the renal transcriptome by performing a gene expression array on kidney from chow- and HFD-fed control and NiPKO mice. A total of 1013 unique genes (502 down, 511 up) were significantly changed in kidneys from chow-fed NiPKO mice compared to chow-fed control mice. For HFD- fed NiPKO mice compared to HFD-fed control mice, 825 genes (409 down, 416 up) were significantly changed (Fig 5A, S2 and S3 Tables). In control mice, 1065 unique genes (545 down, 520 up) were significantly altered with HFD-feeding while in NiPKO mice, the same comparison yielded 1041 significantly changed genes (505 down, 536 up) (Fig 5A, S2 and S3 Tables). To elucidate the functional networks regulated by PGC-1α in kidney, we performed pathway enrichment analysis on differentially regulated transcripts in NiPKO compared to control mice, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [13]. Terms associated with metabolic and mitochondrial processes, including *oxidative phosphorylation (mmu00190)*, *the citrate cycle (mmu00020)* and *glycolysis (mmu00010)* were significantly enriched amongst the downregulated genes in NiPKO mice both in the chow-fed and HFD-fed cohorts (Fig 5B and 5C, S4 Table). Additionally, the KEGG pathway enrichment analysis revealed terms such as *Parkinson's disease (mmu05012)*, *Huntington disease (mmu05016)* and *Alzheimer's disease (mmu05010)*, which contain many genes encoding mitochondrial proteins (e.g. cytochrome oxidase 7a1/*Cox7a1*, *ATP-synthase subunit 5h/Atp5h* and cytochrome c/*Cycs*) to be downregulated in NiPKOs (Fig 5B and 5C, S4 Table). Hence, inactivation of PGC-1α in kidney leads to a robust reduction in the transcription of genes associated with mitochondrial function and metabolism. In particular, in the KEGG-category *oxidative phosphorylation (mmu00190)*, many genes encoding proteins of the mitochondrial electron transport chain were down- regulated in both chow- and HFD-fed NiPKO mice compared to control mice (Fig 5D). We could furthermore validate the down-regulation of some selected mitochondrial oxidative phosphorylation components by RT-PCR (Fig 6A). Thus, in analogy to other tissues (Villena, 2015), PGC-1α is an important transcriptional regulator of mitochondrial genes also in the kidney. To investigate whether inactivation of PGC-1α affects mitochondrial biogenesis in the kidney, we then assessed mitochondrial DNA (mtDNA) content and mitochondrial protein expression in chow- and HFD-fed NiPKO and control mice, respectively. HFD-feeding reduced relative mtDNA content in the kidney, but there was no difference between genotypes (Fig 6B). On the other hand, there was a robust reduction in protein levels of ATP5A and UQCRC2 in the kidney of chow-fed (Fig 6C), HFD-fed (Fig 6D) and aged chow-fed (Fig 6E) NiPKO mice compared to control mice. In chow-fed and aged chow-fed mice, COX1 levels were also significantly reduced in NiPKO compared to control mice (Fig 6C and 6E), while NADH:ubiquinone oxidoreductase subunit B8 (NDUFB8) protein levels were specifically reduced in kidneys from HFD-fed NiPKO mice (Fig 6D). Other mitochondrial proteins tested were not significantly altered between control and NiPKO mice (Fig 6C–6E). Taken together, PGC-1α is important for the transcriptional regulation of metabolic and mitochondrial gene programs in the kidney. This leads to a reduction in protein levels of some, but not all mitochondrial oxidative phosphorylation components, and does not affect mitochondrial content in kidney, at least based on mtDNA levels.

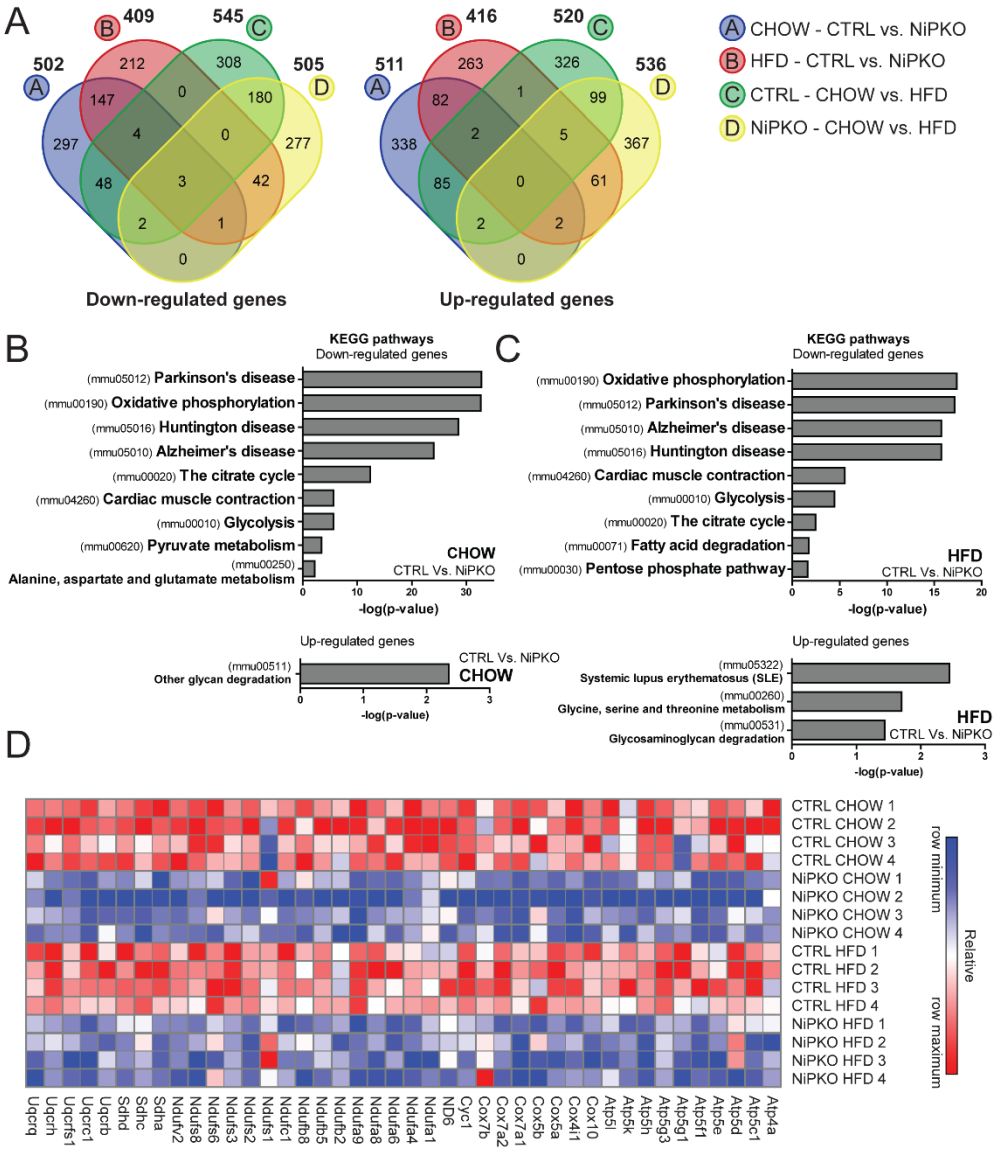

Fig 5. PGC-1α regulates transcription of mitochondrial and metabolic genes in kidney. (A) VENN diagrams displaying the number of unique or overlapping down- or up-regulated genes ($p<0.05$ and fold change >1.2 cut-off) in either chow- or HFD-fed control (CTRL) or NiPKO mice. (B-C) Top significant terms for KEGG pathway enrichment analysis of down- or up-regulated genes in kidneys from (B) chow- fed NiPKO mice compared to CTRL mice or in (C) HFD-fed NiPKO mice compared to CTRL mice. (D) Heat map generated using probe set intensities for transcripts associated with the KEGG-category "*Oxidative phosphorylation*" in Fig B and C, for CHOW and HFD-fed CTRL and NiPKO mice. Row minimum = -3, row maximum = 3 fold change.

doi:10.1371/journal.pone.0158716.g005

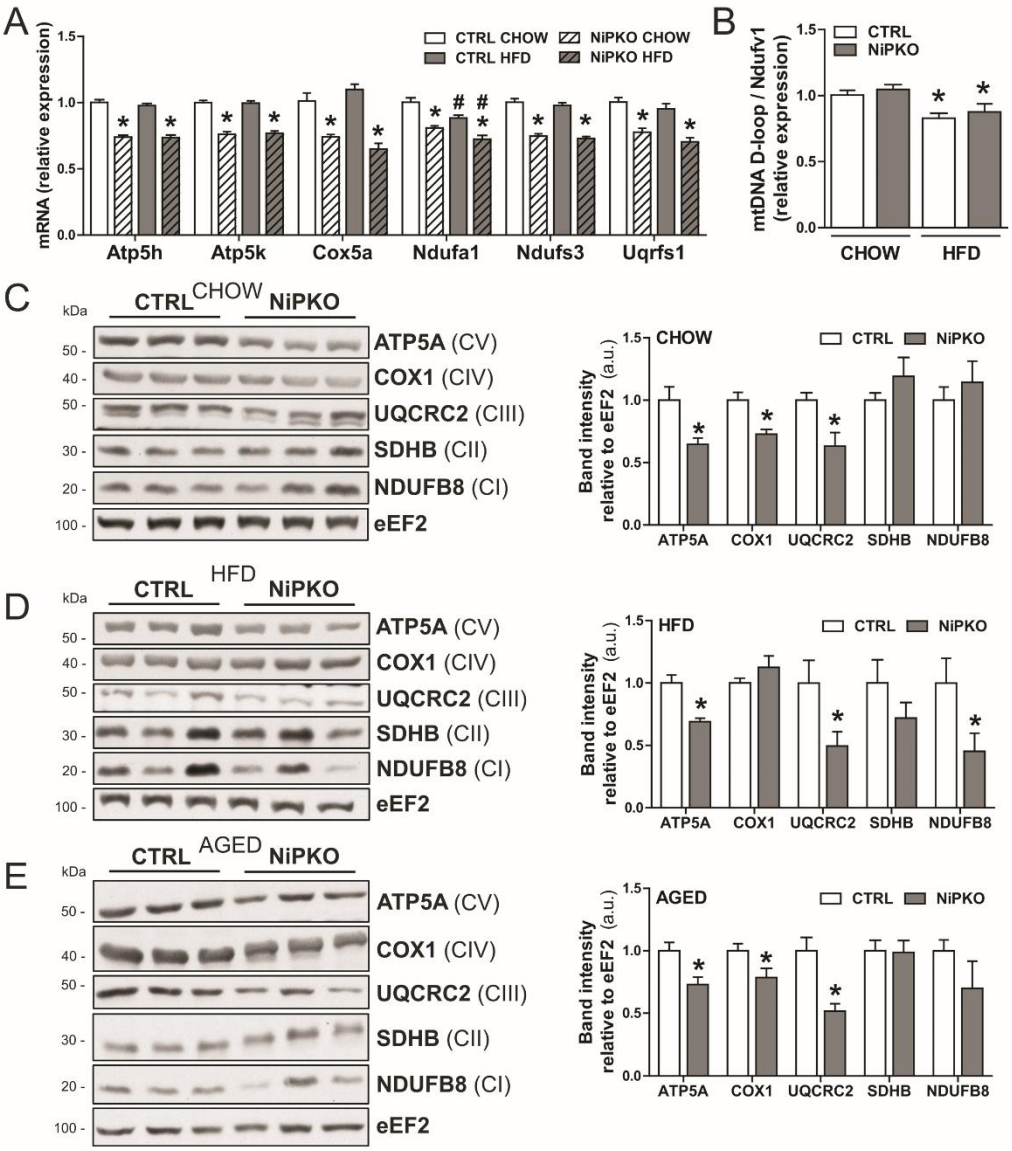

Fig 6. NiPKO mice display reduced levels of mitochondrial enzymes in kidney. (A) mRNA levels of indicated genes in kidney normalized to *eEF2* mRNA levels, in either CHOW- or HFD- fed control (CTRL) or NiPKO mice (n = 7–8). (B) Mitochondrial DNA (mtDNA) content expressed as the relative content of mtDNA D-loop compared to the nuclear *Ndufv1* gene (n = 7). (C-E) Representative immunoblots of ATP5A, COX1, UQCRC2, SDHB, NDUFB8 and eEF2 in kidney. Bar graph shows quantification of band intensities relative to eEF2 for either (C) chow-fed, (D) HFD-fed or (E) aged chow- fed CTRL and NiPKO mice (n = 6). CI, CII, CIII, CIV and CV represents subunits of electron transport chain complex I, II, III, IV and V,

respectively. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*) and between chow- and HFD-fed groups by a number sign (#).

doi:10.1371/journal.pone.0158716.g006

PGC-1α is important for adaptation of renal lipid metabolism with HFD feeding

To further elucidate the PGC-1α-regulated transcriptional network in kidney, we used Integrated Motif Activity Response Analysis (ISMARA) [14] to assess the core set of transcription factors (TF) that display altered activity with inactivation of PGC-1α. Interestingly, ERRα (ESRRA.p2) was amongst the top-scoring TF motifs with reduced activity in both chow-fed (S5A Fig) and HFD-fed (S5B Fig) NiPKO mice. ERRα is a well- known partner of PGC-1α in the regulation of mitochondrial gene transcription [16], and impaired ERRα activity in both chow-fed and HFD-fed NiPKO animals (S5C Fig) likely contributes to the reduction in mitochondrial gene transcription in the kidney of these mice. Additionally, in chow-fed NiPKO mice, the recently described transcriptional partner of PGC-1α/ERRα, Krüppel-like factor 4 (KLF4) [17], was predicted to have reduced activity (S5A Fig). Inversely, nuclear factor κB (NF-κB) activity (NFKB1_REL_RELA.p2) was predicted to be specifically increased in HFD-fed (S5E Fig) but not in the chow-fed (S5D Fig) NiPKO mice compared to control animals. NF-κB is a central transcriptional regulator of the inflammatory gene program, which is upregulated in kidney during high fat diet feeding (Stemmer et al., 2012). Furthermore, in skeletal muscle cells, the transcriptional activity of NF-κB and pro- inflammatory gene expression is reduced by PGC-1α [18]. Accordingly, when comparing chow-fed to HFD-fed groups, NF-κB activity was predicted to be increased with HFD feeding in NiPKO mice, but not in control mice (S3C and S3D Fig). Thus, NF-κB activity seems to be induced in NiPKO mice upon HFD-feeding to a higher extent than in control mice (S3F Fig), which could indicate that there is a difference in the inflammatory response to HFD feeding between control and NiPKO mice.

Next, we analyzed the transcriptional networks affected by HFD feeding in the kidney of both genotypes via KEGG pathway enrichment analysis to investigate whether NiPKO mice display any alterations in their adaptation to a HFD compared to control mice. In regard to transcripts significantly down-regulated by HFD feeding, the term *steroid biosynthesis (mmu00100)* was significantly enriched in both NiPKO and control mice (Fig 7A and 7B, S5 Table). This indicates that this process is downregulated by HFD feeding in the kidney, but not further affected by the absence of PGC-1α. However, in line with the predicted increase in NF-κB activity, HFD-fed NiPKO mice displayed several KEGG terms enriched amongst their upregulated genes that were associated with inflammation and immune response, such as *primary immunodeficiencies (mmu05340)*, *the JAK/STAT pathway (mmu04630)* and *Toll-like receptor signaling pathway (mmu04620)* (Fig 7B, S5 Table). Importantly, none of these terms were found associated with the upregulated transcripts in HFD-fed control mice (Fig 7A, S5 Table). Contrariwise, the two most significant KEGG terms for the upregulated genes in HFD-fed control mice, *PPAR signaling pathway (mmu03320)* and *fatty acid degradation (mmu00071)* (Fig 7A), were not enriched amongst the up- regulated processes in NiPKO mice (Fig 7B). Within the KEGG-categories *PPAR signaling pathway* and *fatty acid degradation*, we found several genes associated with lipid metabolism that were induced by HFD feeding in control mice, but not in NiPKO mice (e.g. acetyl-CoA acyltransferase 2/*Acaa2*, apolipoprotein A2/*Apoa2*, fatty acid binding protein 1/*Fabp1*) (Fig 7C). Collectively, this indicates that induction of lipid metabolic processes with HFD-feeding in kidney is dependent on PGC-1α. Since one of the main upregulated terms in HFD-fed control mice was *PPAR signaling pathway*, we measured transcript levels of the peroxisome proliferator-activated receptors (PPAR) in kidney of control

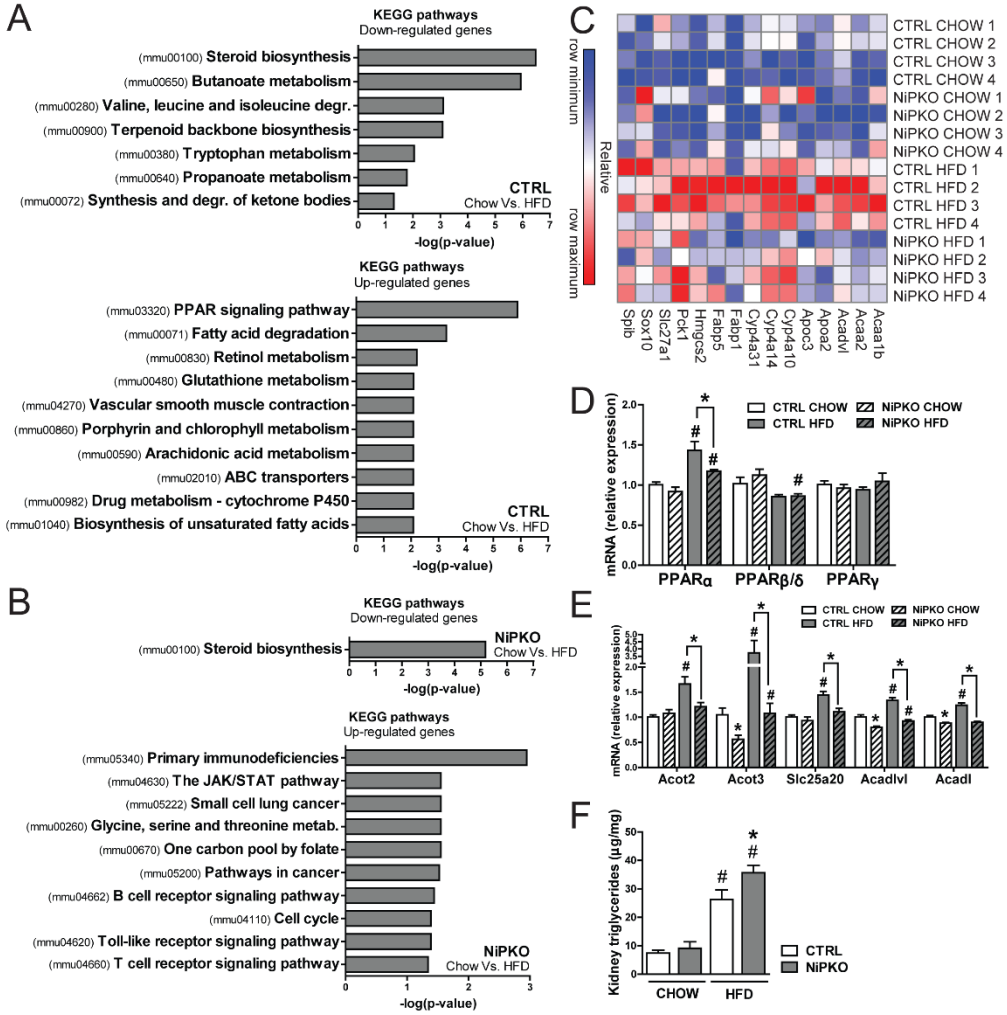

Fig 7. NiPKO mice develop exacerbated renal steatosis with high fat diet-feeding. KEGG pathway enrichment analysis performed on gene expression array data from kidney from either CHOW or HFD-fed control (CTRL) or NiPKO mice. (A-B) Top significant terms for KEGG pathway enrichment analysis of transcripts either down- or up-regulated with HFD-feeding in (A) CTRL or (B) NiPKO mice. (C) Heat map generated using probe set intensities for transcripts associated with the KEGG-categories "*PPAR signaling pathway*" and "*Fatty acid degradation*" in figure A, for CHOW- and HFD-fed CTRL or NiPKO mice. Row minimum = -10, row maximum = 10 fold change. (D-E) mRNA levels of indicated genes in kidney normalized to eEF2 mRNA levels, in CHOW or HFD-fed CTRL or NiPKO mice (n = 7–8). (F) Triglycerides in kidney from CHOW- and HFD-fed CTRL or NiPKO mice (n = 6–8). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*) and between chow- and HFD-fed groups by a number sign (#).

doi:10.1371/journal.pone.0158716.g007

and NiPKO mice. Interestingly, while neither PPARβ/δ nor PPARγ were induced, PPARα transcript levels were increased in the kidney of HFD-fed mice (Fig 7D) and this induction of PPARα was blunted in HFD- fed NiPKO mice (Fig 7D). Accordingly, a similar transcriptional pattern could be observed for several PPARα target genes involved in fatty acid metabolism (Fig 7E), since HFD feeding led to an increased transcription of acyl-CoA thioesterase 2 (*Acot2*), *Acot3*, solute carrier family 25 (carnitine/acylcarnitine translocase) member 20 (*Slc25a20*), acyl-CoA dehydrogenase very long chain (*Acadvl*) and acyl-CoA dehydrogenase long chain (*Acadl*) in kidneys of control mice, and this induction was again blunted in NiPKO mice (Fig 7E). Importantly, triglyceride levels were significantly elevated in kidney of HFD-fed NiPKO mice compared to control mice (Fig 7F) indicating that the reduced transcription of fatty acid metabolism in kidney of NiPKO mice was indeed associated with alterations in renal lipid handling. Thus, PGC-1α is important for the adaptation of renal lipid metabolism in response to HFD feeding, and inactivation of PGC-1α in the kidney exacerbates renal hypertriglyceridemia and subsequent inflammation during HFD feeding.

Discussion

The kidney has a high energy demand and relies almost exclusively on oxidative phosphorylation for ATP production [2,3]. The importance of mitochondria for renal function is underscored by the prevalence of renal dysfunction in mitochondrial cytopathies [2,4,5]. Additionally, mitochondrial dysfunction is a hallmark sign of several renal disorders [19,20,21,22]. PGC-1α is a key regulator of mitochondrial oxidative phosphorylation and oxidative metabolism [6]. Surprisingly, despite the importance of mitochondrial energy metabolism for renal function, relatively little is known about how PGC-1α influences renal function and physiology. We now demonstrate that PGC-1α is an important transcriptional regulator of mitochondrial gene programs in kidney, such as oxidative phosphorylation, TCA cycle and fatty acid metabolism. However, despite the role of PGC-1α in transcriptional regulation of mitochondrial genes, inactivation of PGC-1α in the kidney had only minor effects on the regulation of blood pressure and salt- and water homeostasis. Notably, NiPKO mice displayed a mild loss of sodium in the urine, and this phenotype was exacerbated in aged NiPKO mice upon dietary sodium restriction. Aging is associated with a reduced ability to retain sodium during salt depletion in humans [23], and with reduced renal PGC-1α levels in mice [24]. Thus, our study provides a potential link between reduced PGC-1α levels and the reduced capacity to reabsorb sodium with age. While the underlying mechanism(s) needs to be elucidated, we can demonstrate that inactivation of PGC-1α in kidney leads to decreased protein levels of the sodium transporters NKCC2 and NCCT in kidney during low-salt diet feeding. Even though the phosphorylation status of these two proteins remains unchanged, reduced levels of the two important sodium channels during LSD-feeding could explain the urinary loss of sodium with ablation of renal PGC-1α. However, without a more detailed segmental study, it is unclear how PGC-1α is involved in the function of different parts of the nephron. A rudimentary expression analysis using microdissected nephron segments indicates a relatively high expression of PGC-1α in different parts of the tubulus, at least compared to transcript levels in the glomerulus (S6 Fig). Intriguingly, a gradient towards increasing expression of PGC-1α in the distal direction of the tubulus is observed, which could be linked to the salt-losing phenotype in the knockout mice.

The importance of renal PGC-1α for the transcriptional regulation of mitochondrial and metabolic processes in kidney is in agreement with the effect of PGC-1α in other organs, such as brown adipose tissue, skeletal muscle and heart [25]. Importantly, the similarities in transcriptional profiles between these tissues could result not only from high basal expression of

PGC-1α, but could also be due to the expression of the same TF partners of PGC-1α relevant for mitochondrial transcription, such as nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM) and ERRα. Indeed, renal ERRα levels equal those of brown adipose tissue and heart and exceed the levels in skeletal muscle [26,27]. Based on motif activity response analysis of our gene expression data, we predicted a significant blunting of ERRα transcriptional activity in our NiPKO mice. ERRα is an established partner of PGC-1α in the regulation of mitochondrial gene transcription [16], and reduced activity ERRα correlates with the impaired transcription of mitochondrial genes when PGC-1α is ablated in kidney. We also predicted a significant reduction of KLF4 transcriptional activity in kidney of NiPKO mice. KLF4 was recently shown to be an essential component of the transcription of mitochondrial genes via PGC-1α and ERRα in heart [17]. Thus, KLF4 could likewise be a novel regulator of mitochondrial gene transcription working together with PGC-1α in the kidney. Furthermore, in our analysis, nuclear receptor 5A1 (NR5A1) and zinc finger protein 143 (ZNF143) were predicted to have a reduced transcriptional activity in NiPKO mice, and these TFs have been either shown or at least predicted to be transcriptional partners of PGC-1α [28,29]. Interestingly, ISMARA analysis implies both NR5A1 and ZNF143 to regulate mitochondrial gene transcription in our dataset (data not shown). However, further studies are needed to elucidate the potential role of these TFs as transcriptional partners of PGC-1α-mediated mitochondrial gene transcription in kidney.

Mitochondrial dysfunction and decreased ATP production are associated with reduced renal function and impaired transtubular transport [4,5]. Despite the inactivation of PGC-1α in kidney and the reduction in mitochondrial transcription, NiPKO mice displayed a largely normal renal phenotype. It is important to note that the effect of PGC-1α inactivation on mitochondrial protein content in our study was moderate, which in turn could explain the mild phenotype. One likely explanation for this could be that the related family member PGC-1β could compensate for the loss of PGC-1α in NiPKO mice, especially since redundancy between PGC-1α and PGC-1β on mitochondrial gene transcription has been previously shown in skeletal muscle [30]. Another explanation for this observation would be that the role of PGC-1α in kidney is more prominent during states of increased metabolic stress, as we demonstrated in the context of HFD feeding, but also in other renal disease states. Indeed, several etiologically distinct renal disorders, such as diabetic nephropathy, ischemia/reperfusion injury and sepsis are associated with reduced mitochondrial function [19,20,21,22] and reduced levels or activity of PGC-1α in the kidney [22,31,32]. The link between PGC-1α and renal disease was recently explored in two studies by Tran et al. [22,33], where the authors show that deletion of PGC-1α specifically in the renal proximal tubules leads to a worsened renal phenotype in mice during acute ischemic kidney injury [22] while overexpression of PGC-1α helps to protect against damage [33]. Recent studies have also shown that treatment with the SIRT1-activator SRT1720 [20] or calorie restriction [31], which are linked to increased PGC-1α activation, improves the renal mitochondrial phenotype and reduces ischemia/reperfusion injury in mice. Moreover, overexpression of PGC-1α in cultured proximal tubule cells protects against both TNFα- [22] and aldosterone-induced [32] mitochondrial dysfunction. Hence, while our current study demonstrates a minor role for PGC-1α in basal salt- and water handling and blood pressure regulation in kidney, increased PGC-1α activity could be a valid therapeutic strategy to ameliorate renal mitochondrial dysfunction in a broad spectrum of renal disorders. In particular, our data highlight the importance of PGC-1α in the kidney during metabolic stress, since we found that absence of PGC-1α leads to enhanced deposition of triglycerides in kidney of HFD-fed mice. Transcriptional analysis showed that upon HFD feeding, induction of PPARα and several PPARα-targets involved in fatty acid metabolism were significantly blunted in the kidney of NiPKO mice. Indeed, PPARα is a well-known transcriptional partner of PGC-1α for the

regulation of lipid metabolism [34]. Importantly, ablation of PPARα in mice leads to an increased susceptibility to diabetic nephropathy [35] and exacerbated free fatty acid-induced injury in the kidney [36]. Moreover, increased PPARα-activity in obese rodents protects against renal lipotoxicity [37,38] and has been associated with increased levels of PGC-1α in kidney [39]. Obesity and HFD feeding lead to ectopic deposition of triglyceride in the kidney [38,40]. Both PGC-1α and PPARα have been described as inhibitors of NF-κB activity [18,41]. Thus, both PGC-1α and PPARα could be instrumental in attenuating an inflammatory reaction triggered by pathological accumulation of lipids in the kidney as in other tissues [42]. Our findings highlight an important link between reduced renal PGC-1α levels and deregulated lipid metabolism in kidney. Further studies are now needed to address the potential protective effect of increased PGC-1α activity in diseases associated with defective renal lipid metabolism and lipotoxicity.

In summary, we found that PGC-1α plays a marked role in the transcriptional regulation of several interconnected mitochondrial gene programs in the kidney, such as oxidative phosphorylation, TCA cycle and fatty acid metabolism. While inactivation of tubular PGC-1α resulted in a minor increase in urinary sodium excretion that was exacerbated by age and high fat diet feeding, there was no further impairment in salt- and water homeostasis or blood pressure regulation in NiPKO mice. Importantly, our data demonstrate a crucial role for PGC-1α in the regulation of renal lipid metabolism during metabolic stress, leading to elevated renal steatosis in the absence of functional PGC-1α. While our findings point towards a minor role of PGC-1α in basal renal physiology, we thus hypothesize that activation of PGC-1α in the context of renal disorders could be a valid therapeutic strategy to ameliorate renal mitochondrial dysfunction in a broad spectrum of renal disorders.

Supporting Information

S1 Fig. Histological kidney sections. A-B) Representative PAS staining of kidney regions or whole kidney sections at 1 and 12 months after doxycycline administration (n = 3).
(PDF)

S2 Fig. Plasma ion levels and ISMARA prediction. (A-B) Plasma levels of calcium (Ca^{2+}), chloride (Cl^-), potassium (K^+), sodium (Na^+), protein, urea at (A) 1 month and (B) 12 months after doxycycline administration (n = 5–12). (C-D) Transcription factor motifs predicted to have altered activity in ISMARA analysis of CHOW-fed and HFD-fed (C) control and (D) NiPKO mice. Error bars represent mean ±SEM. Significant differences (p-value<0.05) between genotypes are indicated by an asterisk (*).
(PDF)

S3 Fig. Food intake. Food intake in Control and NiPKO mice. Food intake was measured for individually-housed mice in metabolic cages on 5 consecutive days and normalized for intake over 24 hours.
(PDF)

S4 Fig. Transporter gene expression analysis. mRNA levels of indicated genes in kidney normalized to *eEF2* mRNA levels, in either CHOW- or HFD- fed control (CTRL) or NiPKO mice (n = 7–8). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between genotypes are indicated by an asterisk (*) and between chow- and HFD-fed groups by a number sign (#).
(PDF)

S5 Fig. Motif activity prediction. A-B) Transcription factor (TF) motifs predicted to have decreased activity in ISMARA analysis between (A) CHOW-fed control and NiPKO mice or (B) HFD-fed control and NiPKO mice. (C) Changes in ERRα (ESRRA.p2) activity in kidney from either chow or HFD-fed CTRL or NiPKO mice, as predicted by ISMARA analysis. (D-E) Transcription factor (TF) motifs showing increased activity in ISMARA analysis between (D) CHOW-fed control and NiPKO or (E) HFD-fed control and NiPKO mice. (F) Changes in NF-κB (NFKB1_REL_RELA.p2) activity in kidney from either chow or HFD-fed CTRL or NiPKO mice, as predicted by ISMARA analysis.
(PDF)

S6 Fig. Expression of PGC-1α in different kidney segments. Expression of PGC-1α was measured from RNA extracted from microdissected nephron segments and glomeruli. PGC-1α transcript levels are normalized to TATA-binding protein gene expression and depicted according to the ΔCt method.
(PDF)

S1 Table. Primer sequences. qPCR primer sequences.
(PDF)

S2 Table. List of down-regulated genes. List of genes significantly down-regulated ($p<0.05$, $FC<1.2$) between chow-fed control (CWT), chow-fed NiPKO (CKO), HFD-fed control (HWT) and HFD-fed NiPKO (HKO) groups. The heading "Groups" lists in which comparison the relevant gene(s) are found to be down-regulated.
(PDF)

S3 Table. List of up-regulated genes. List of genes significantly up-regulated ($p<0.05$, $FC<1.2$) between chow-fed control (CWT), chow-fed NiPKO (CKO), HFD-fed control (HWT) and HFD-fed NiPKO (HKO) groups. The heading "Groups" lists in which comparison the relevant gene(s) are found to be up-regulated.
(PDF)

S4 Table. Functional analysis of gene expression in chow-fed mice. Genes associated with KEGG pathways in CTRL vs. NiPKO animals.
(PDF)

S5 Table. Functional analysis of gene expression in HFD-fed mice. Genes associated with KEGG pathways CHOW vs. HFD treated animals.
(PDF)

Author Contributions

Conceived and designed the experiments: KS CH. Performed the experiments: KS BC SS. Analyzed the data: KS CH SS. Contributed reagents/materials/analysis tools: KS BC SS. Wrote the paper: KS CH.

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Appendix B

Biochimie 136 (2017) 49–54

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Mini-review

Coregulator-mediated control of skeletal muscle plasticity – A mini-review

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ARTICLE INFO

Article history:
Received 3 October 2016
Received in revised form
13 December 2016
Accepted 19 December 2016
Available online 3 January 2017

Keywords:
Coregulators
Metabolism
Skeletal muscle
PGC-1

ABSTRACT

Skeletal muscle plasticity is a complex process entailing massive transcriptional programs. These changes are mediated by the action of nuclear receptors and other transcription factors. In addition, coregulator proteins have emerged as important players in this process by linking transcription factors to the RNA polymerase II complex and inducing changes in the chromatic structure. An accumulating body of work highlights the pleiotropic functions of coregulator proteins in the control of tissue-specific and whole body metabolism. In skeletal muscle, several coregulators have been identified as potent modulators of metabolic and myofibrillar plasticity. In this mini-review, we will discuss the control, function and physiological significance of these coregulators in skeletal muscle biology.

1. Introduction

Maintenance of cellular metabolic homeostasis requires the precise integration of internal and external cues in a highly regulated manner. Transcriptional programs are central to many plastic changes, and often involve the action of numerous nuclear receptors (NRs) and other transcription factors (TF). Importantly, coregulator proteins provide an additional layer of control, and vastly expand the repertoire of specificity and fine-tuning of transcription [1,2]. In many cases, coregulators are metabolic sensor and effector proteins and thereby directly link the cellular environment to the transcriptional output [3]. Coregulator proteins lack an intrinsic DNA binding domain and therefore require a direct or indirect interaction with TFs to be recruited to target gene enhancer and promoter elements. Direct binding partners of TFs are referred to as coregulators, while other members of the coregulatory complex are called secondary coregulators or co-coregulators [4]. Importantly, such complexes often encompass 10 or even more individual proteins, the composition of which can change in a highly dynamic manner. Moreover, coregulator complexes exist in three hierarchical layers with the stable core modules at the center, escalating to a more dynamic core complex formation between such modules and ultimately network-forming complex-complex interactions that are highly context-dependent [5]. Coregulators can be broadly categorized into coactivators and corepressors that induce or inhibit gene expression, respectively, even though some members of this protein family can act as both, depending on the local state of chromatin, TF activation or conformation [1]. While some coregulators primarily serve as scaffold for the assembly of complexes, others harbor intrinsic enzymatic activity, mainly targeted at the modification of histones and thereby affecting chromatin structure. Importantly, alternative splicing of the transcripts and post-translational modifications of coregulator proteins collectively allow a fine-tuned and specific assessment of homeostatic conditions, followed by a rapid, efficient and reversible transcriptional control to initiate the appropriate physiological response [1,2]. Since the discovery of the first coregulator, SRC-1, two decades ago [6], more than 450 coregulator proteins, and hundreds of co-coregulators, have been identified and implicated in the general control of transcription or the regulation of tissue-specific programs [5]. Skeletal muscle tissue is one of the best examples of an organ that exhibits enormous plasticity upon various challenges, including specific and well-controlled short- and long-term adaptations. Even though the integration, regulation and coordination of numerous signaling pathways and the ensuing transcriptional programs are still poorly understood, several TFs and coregulators have been implicated in the regulation of this complex system. Skeletal muscle accounts for around 40–50% of total body mass and is one of the most active metabolic organs. This tissue is one of the main energy storage sites, generates force, maintains

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http://dx.doi.org/10.1016/j.biochi.2016.12.011
0300-9084/

Abbreviations

AMPK	AMP-activated protein kinase
BAT	Brown adipose tissue
CACT	Carnitine/acyl-carnitine translocase
ERRα	Estrogen-related receptor α
GLUT4	Glucose transporter 4
Gys1	Glycogen synthase 1
HDAC	Histone deacetylase
HFD	High fat diet
KO	Knockout
MEF	Myocyte enhancer factor
MyHC	Myosin heavy chain
NCOR1	Nuclear corepressor 1
NR	Nuclear receptor
Pgam2	Muscle phosphoglycerate mutase 2
PGC-1	Peroxisome proliferator-activated receptor γ coactivator-1
PKB	Protein kinase B/Akt
PPARγ	Peroxisome proliferator-activated receptor γ
PRC	PGC-related coactivator
PRMT4	Protein arginine methyltransferase 4
Pygm	Muscle glycogen phosphorylase
RAR	Retinoic acid receptor
RER	Respiratory exchange ratio
RID1	Receptor interacting domain 1
RIP140	Receptor interacting protein 140
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SRC	Steroid receptor coactivator
TF	Transcription factor
TR	Thyroid hormone receptor
UCP-1	Uncoupling protein 1
WAT	White adipose tissue

posture, is essential for shivering thermogenesis, acts as an endocrine organ and can detoxify excessive endogenous metabolites. However, the adaptation of skeletal muscle to exercise and inactivity are of particular physiological and pathological importance. Training can substantially improve metabolic and contractile functions of the muscle while inactivity has the opposite effect. This review will summarize the advances in understanding the role of coregulators in skeletal muscle plasticity.

1.1. Steroid receptor coactivators (SRCs)

SRCs have been the first coactivator proteins identified [6] consisting of 3 homologous family members termed SRC-1 (also known as NCoA1), −2 (also known as NCoA2, GRIP1 and TIF1) and −3 (also known as NCoA3, ACTR, AIB1, p/CIP, RAC3 and TRAM-1). SRCs mediate steroid hormone actions and are involved in transcriptional initiation, cofactor recruitment, elongation, RNA splicing and translation [4]. In different tissues, SRC-1 and SRC-2 are inversely regulated and these two coactivator proteins compete for binding to the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and transcription factors [4,7]. Muscle- specific ablation of SRC-2 leads to SRC-1-dependent enhanced skeletal muscle uncoupling via regulation of the uncoupling protein 3 (UCP-3), resulting in increased whole body energy expenditure, which protects mice from type 2 diabetes and high fat diet (HFD)-induced obesity. Moreover, SRC-2 muscle knockout (KO) mice show enhanced oxidative capacity in comparison to age-matched control littermates due to increased transcription levels of PGC-1α and its target genes involved in fatty acid and β-oxidation. Thus, skeletal muscle SRC-2 is important to maintain low levels of SRC-1 in order for optimal mitochondrial function and skeletal muscle as well as whole body homeostasis [8]. Intriguingly, SRC-1/SRC-2 double KO animals exhibit no difference in UCP-3 expression, body temperature, energy expenditure or mitochondrial function, indicating that the induction of SRC-1 is at least in part responsible for the phenotype of muscle-specific SRC-2 KO mice [4]. In myogenesis, SRC-1 and SRC-2 show likewise opposing roles in the regulation of myogenic differentiation (MyoD)-mediated transcription: domain-specific binding of SRC-1 results in coactivation and of SRC-2 in corepression of MyoD, respectively [9]. Furthermore, SRC-2 coactivates the myocyte enhancer factor-2C (MEF-2C) and myogenin, and thereby potentiates the differentiation of cultured C2C12 myoblasts into myotubes [10].

SRC-3 has been implicated in the regulation of long- and very long-chain fatty acid metabolism in skeletal muscle. SRC-3 $^{/}$ mice show significantly reduced expression levels of the carnitine/acyl-carnitine translocase (CACT) in skeletal muscle, which leads to the accumulation of long-chain fatty acids. The loss of SRC-3 resembles the genetic deficiency of CACT in humans and is accompanied by hypoketonemia, hypoglycemia, cardiac abnormality, hyperammonemia, abnormal electrical discharge in the brain and severe muscle weakness [11]. The modulation of muscle endurance and hypoglycemia in the SRC-3 KO can be alleviated by a short-chain fatty acid diet. In summary, similar to other tissues, SRC-1 and SRC-2 promote a program of energy expenditure and energy storage in muscle, respectively, while SRC-3 controls energy substrate usage and thereby affects endurance.

1.2. Peroxisome proliferator-activated receptor γ coactivators-1 (PGC-1)

The first member of the peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1) family, PGC-1α, has been discovered as a coregulator protein interacting with PPARγ in brown adipose tissue (BAT) almost twenty years ago. Since then, two other family members have been identified termed PGC-1β and PGC-related coactivator (PRC). The PGC-1s are highly regulated, potent coactivators and are able to interact with a large variety of different TFs to induced distinct biological processes in a tissue-specific manner [12,13]. Expression of PGC-1α in skeletal muscle is strongly controlled by contractile activity in humans and rodents. Accordingly, muscle-specific PGC-1α transgenic animals show increased oxidative metabolism, a higher proportion of slow-twitch muscle fibers and improved endurance capacity [12,14,15]. In contrast, muscle-specific PGC-1α KO mice display impaired exercise performance, myopathy, decreased oxidative metabolism and abnormal glucose tolerance [16,17]. Curiously, the high-endurance phenotype is predominantly evoked by the specific PGC-1α isoforms PGC-1α1, PGC-1α2 and PGC-1α3 [18]. Recently, the so-called PGC-1α4 isoform has been described to be implicated in the regulation of muscle hypertrophy by inducing IGF-1 and repressing myostatin gene expression [19].

In contrast to the well-established role of PGC-1α in exercise-adaptation, the regulation and function of PGC-1β is less clear.

Muscle-specific overexpression of PGC-1β drives the formation of oxidative type IIx fibers [20] and different PGC-1β global KO models show impaired muscle mitochondrial function [21]. Moreover, muscle-specific ablation of PGC-1β reduces exercise performance, oxidative capacity and increases oxidative stress [21,22]. Thus, PGC-1α and PGC-1β are crucial for oxidative metabolism and mitochondrial function and loss of both family members in skeletal muscle leads to a dramatic reduction in exercise performance due to mitochondrial structural and functional abnormalities [23]. These results indicate that the two PGC-1 coactivators have overlapping, but also distinct functions in skeletal muscle.

1.3. Nuclear corepressor 1 (NCoR1) and silencing mediator for retinoid and thyroid hormone receptor (SMRT)

NCoR1 and SMRT (also known as NCoR2) have been found to interact with thyroid hormone receptor (TR), retinoic acid receptor (RAR) and other TFs to suppress target gene transcription. The two corepressors show a high degree of amino acid sequence homology and display similar functional domains [24]. Like the SRCs and the PGC-1s, NCoR1 and SMRT are scaffold coregulators and thus recruit heterogeneous corepressor complexes in a context-specific manner.

Loss of NCoR1 in skeletal muscle enhances exercise performance due to an increase in muscle mass, mitochondrial number and activity [25,26]. Interestingly, NCoR1 muscle KO mice show a similar phenotype as PGC-1α muscle transgenic mice and in fact, NCoR1 and PGC-1α inversely control the transcription of a common subset of genes by competitive binding to the estrogen-related receptor α (ERRα) [26] and/or PPARβ/δ [25].

The corepressor SMRT may be involved in aging-associated metabolic processes, since mRNA and protein levels are induced in older mice in BAT and muscle. Furthermore, in mice with a disrupted SMRT receptor interacting domain 1 (RID1), muscles show reduced insulin-stimulated protein kinase B (PKB/Akt) phosphorylation, glucose transporter 4 (GLUT4) protein levels and a blunted insulin-induced glucose uptake [27]. These changes in glucose handling lead to premature aging and other metabolic diseases by shifting the SMRT repression to RID2- associated NRs such as the PPARs. Thus, these results suggest that SMRT might be involved in the development of insulin resistance induced by dietary conditions or aging. Importantly, both NCoR1 and SMRT modulate mitochondrial biogenesis and function in different tissues, thereby reducing oxidative metabolism and energy expenditure [24]. Thus, both corepressors are major factors in the regulation of energy homeostasis. Intriguingly, the repressive action of NCoR1 on clock gene expression could imply that this corepressor, and potentially SMRT, links metabolism to the circadian rhythm [24].

1.4. Thyroid hormone receptor-associated protein complex component/mediator complex subunit 1 (TRAP220/MED1)

The Mediator complex forms a bridge from transcription factors to the RNA polymerase II transcriptional machinery. In fat cells, PGC-1α is a crucial interaction partner of the Mediator complex for the dynamic changes from chromatin remodeling to transcriptional initiation. Direct recruitment of PGC-1α to activated transcription factors results in the co-binding of CBP/p300 and other histone acetyltransferases to modify nucleosomes and thereby open the chromatin conformation. Subsequently, interaction between PGC-1α and the Mediator subunit Med1 engages the basal transcription machinery for transcriptional initiation [28]. Med1 binds to the C-terminus of PGC-1α, displaces this coactivator from the transcription factor, and thereby promotes the formation of an enhanced complex for transcriptional activity [29]. Somewhat unexpectedly, chow-fed muscle-specific MED1 KO mice display increased glucose tolerance and insulin sensitivity suggesting a role of muscle MED1 in whole body glucose homeostasis. Furthermore, these MED1 KO mice are protected against HFD-induced obesity. Gene expression analyses of white muscles revealed elevated levels of the mitochondrial genes, but also the BAT-typical uncoupling protein-1 (UCP-1) and Cidea, in MED1 KO mice. As a result, MED1 ablation in white muscles results in higher mitochondrial numbers and causes a switch toward slow oxidative fibers [30]. Interestingly, the increase in mitochondrial density and oxidative metabolism does not require an elevation in either PGC-1α or PGC-1β transcription suggesting that basal PGC-1 levels are sufficient to induce the respective metabolic and myofibrillar genes in the absence of MED1. It is thus conceivable that MED1 actively suppresses PGC-1 target genes, which can only get activated when PGC-1 expression itself is induced and overcomes the suppressive effect of MED1. This mechanism of action would ensure the fine-tuning of specific gene sets upon different external stimuli.

1.5. Receptor interacting protein 140 (RIP140)

RIP140 was first discovered in breast cancer cells as an estrogen receptor modulator and has now been identified as a corepressor for a large number of ligand-bound NRs involved in metabolism [31]. RIP140 transcript levels are most elevated in white adipose tissue (WAT) followed by skeletal muscle, where its mRNA levels are higher in glycolytic compared to oxidative muscle fibers [32,33]. Ablation of RIP140 in skeletal muscle results in induced oxidative metabolism through enhanced fatty acid oxidation in addition to increased oxygen consumption of chow and HFD-fed mice while lowering the respiratory exchange ratio (RER). Furthermore, RIP140 regulates glucose trafficking in oxidative muscles. Deletion of muscle RIP140 increases the expression of UCP-1, leading to energy dissipation through mitochondrial uncoupling. Concomitantly, AMP-activated protein kinase (AMPK) gets activated, which in turn stimulates the translocation of GLUT4 vesicles to the plasma membrane enabling the entrance of glucose from the blood [34]. Thus, RIP140 functions as a transcriptional corepressor that reduces oxidative metabolism and inhibits glucose uptake into oxidative muscles. It is conceivable that these effects of RIP140 are achieved by competing with PGC-1α for binding to ERRs and PPARs in muscle [35].

1.6. Protein arginine methyltransferases (PRMT)

PRMT are coactivators that possess intrinsic enzymatic activity, which catalyze the formation of monomethylarginine as well as symmetrical and asymmetrical dimethylarginine. These post-translational modifications affect the function of target proteins and regulate their ability to interact with DNA, RNA or other proteins [36].

PRMT4 (also known as CARM1) is the most abundantly expressed PRMT in skeletal muscle and controls glycogen metabolism. PRMT4 siRNA suppresses the expression levels of glycogen synthase 1 (GYS1), muscle phosphoglycerate mutase 2 (PGAM2) and muscle glycogen phosphorylase (PYGM) and decreases mRNA levels of myosin heavy chains (MyHC) type IIb and IIx. Moreover, expression of a mutant PRMT4 with attenuated methyltransferase and coactivator activities decreases glycogen levels in skeletal muscle cells [36]. These data indicate that expression of PRMT4 and the associated enzymatic activity are important for muscle glycogen metabolism and might play a role in human glycogen storage diseases. In myogenesis and differentiation, PRMT4 interacts with SRC-2 to recruit and activate MEF2 to the creatine

kinase promoter. Ablation of PRMT4 gene expression reduces the expression of MEF2 and myogenin and thereby leads to impaired myogenesis [37].

1.7. Histone deacetylases (HDACs) and histone acetyltransferases (HATs)

HDACs are categorized into class I, IIa, IIb and IV HDACs, which require zinc as a cofactor for enzymatic activity and class III HDACs, better known as sirtuins, which require NAD^+ as a cofactor [38]. Some of these proteins deacetylate histones as well as other proteins. For example, in skeletal muscle, HDAC3, a class I HDAC, is often found in a complex with NCoR1/SMRT, and directly contributes to target gene promoter recruitment and the transcriptional repression activity via histone deacetylation. In addition, HDAC3 also deacetylates MEF2 and could thereby affect myogenesis and the expression of oxidative fiber genes [3,38].

HDAC5, a member of the class IIa HDACs, also represses MEF2, which then inhibits transcription of MEF2 target genes such as PGC-1α [39]. The positive autoregulatory loop that controls PGC-1α expression in skeletal muscle thus hinges on the competition of HDACs and PGC-1α to bind to and repress and activate MEF2s, respectively, on the PGC-1α promoter [40]. Since class IIa HDACs exhibit only limited deacetylase activity, recruitment of HDAC3 most likely is responsible for the enzymatic effect [3]. HDAC5 activity is inhibited by phosphorylation through AMPK, which enables the MEF2-dependent induction of GLUT4 in myotubes in the right metabolic context [41]. HDAC4 and HDAC5 are furthermore involved in the induction of E3 ubiquitin ligases in neurogenic fiber atrophy [42]. In both of these scenarios, PGC-1α mediates opposing effects, e.g. by being activated by AMPK [43] or by reducing the denervation-induced expression of pro-atrophic E3 ubiquitin ligases [44]. Thus, collectively, several members of the class I and class II HDACs have been implicated in the metabolic reprogramming of skeletal muscle tissue [38].

Sirtuin 1 (SIRT1), a class III HDAC, exerts the most studied effects in skeletal muscle by deacetylation and thereby activation of PGC-1α [45]. An interdependent AMPK-SIRT1-PGC-1 axis ensures a tightly controlled sensing of the metabolic state of the cell based on AMP/ATP and NAD^+/NADH ratios [46]. There are several open questions in regard to SIRT1 action in skeletal muscle. For example, SRT1720, a proposed, though disputed synthetic activator of SIRT1, improves muscle endurance and promotes an oxidative phenotype [47], even though the engagement of muscle PGC-1α seems non-obligatory for the systemic effects of SRT1720 and resveratrol to occur [48]. Directly opposed to SIRT1, GCN5 not only acetylates histones but also other targets, including PGC-1α [49]. The debate about the relative contributions of SIRT1 and the counteracting protein acetyltransferase GCN5 (also called KAT2A) to control oxidative metabolism in skeletal muscle remains currently unresolved [50].

In addition to GCN5, other HATs have also been implicated in the regulation of myogenesis and muscle function. For example, CBP/p300 and pCAF (also known as KAT2B) interact to control MyoD activity in myogenic differentiation [51]. However, even though a strong association of CBP/p300 and PGC-1α has been demonstrated [7], the relevance of this interaction in endurance exercise-induced muscle plasticity has not been studied so far.

1.8. cAMP-regulated transcriptional coactivators (CRTC)

CRTCs (also called transducers of regulated CREB-binding proteins, TORCs), have been identified as coactivators of the cAMP-responsive element binding protein (CREB) [3]. In most tissues, the CRTCs are strongly regulated by the hormonal and metabolic environment. CRTC1 emerged as the most potent activator of PGC-1α gene expression in a screen of 10′000 human full-length cDNAs in muscle cells [52]. In addition to CRTC1, the other two CRTC family members CRTC2 and CRCT3 likewise strongly induce PGC-1α transcription and downstream targets, including genes encoding mitochondrial oxidative phosphorylation enzymes, thereby linking external cues that activate calcium- and cAMP-dependent signaling pathways to an oxidative muscle cell phenotype in a PGC-1α- and

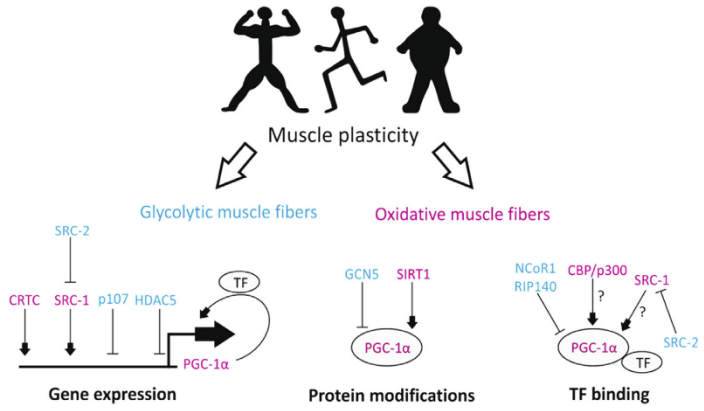

Fig. 1. The complex regulatory network of the coactivator PGC-1α. Gene expression: PGC-1α is induced by itself via the interaction with different transcription factors (e.g. MEF2, possibly PPARδ), CRTC family members and SRC-1, which in turn is repressed by SRC-2. Furthermore, PGC-1α is repressed by HDAC5 and p107; Protein modifications: PGC-1α activity is controlled by GCN5 and SIRT1, which acetylate or deacetylate it, respectively; TF binding: NCoR1 and RIP140 compete with PGC-1α in the binding to TFs while CBP/p300 and SRC-1 enhance its binding to the transcriptional machinery, even though this has only been shown in fat and not in muscle tissue, which is why the interaction is shown with a question mark. Also, the direct correlation between the glycolytic and oxidative metabolism and the corresponding color-coded coregulators (blue: glycolytic; pink: oxidative) is often missing, however, KO studies strongly suggest this association.

Table 1
Coregulator proteins involved in skeletal muscle plasticity.

Family	Short name	Members	In skeletal muscle involved in …
Steroid receptor coactivators	SRC	SRC-1, SRC-2, SRC-3	Steroid hormone metabolism, uncoupling, myogenesis, fatty acid metabolism
Peroxisome proliferator-activated receptor γ coactivators-1	PGC-1; PRC	PGC-1α, PGC-1β, PRC	Oxidative metabolism, mitochondrial homeostasis, exercise performance
Nuclear corepressors	NCoR	NCor1, SMRT	Energy homeostasis, hypertrophy, aging, glucose homeostasis
Thyroid hormone receptor-associated protein complex component/Mediator Complex Subunit 1	Mediator complex	TRAP220, MED1	Initiation of transcription, glucose homeostasis, mitochondrial homeostasis
Receptor interacting protein 140	RIP140	RIP140	Oxidative metabolism, uncoupling, glucose homeostasis
Protein arginine methyltransferases	PRMT	PRMT4	Glycogen metabolism, myogenesis
Histone deacetylases	HDAC	HDAC3, HDAC4, HDAC5, sirtuins	Myogenesis, oxidative metabolism, atrophy, energy homeostasis
Histone acetyltransferases	HAT	GCN5, CBP/p300, pCAF	Oxidative metabolism, myogenesis
cAMP-regulated transcriptional coactivators	CRTC	CRCT1, CRCT2, CRCT3	Oxidative metabolism
Retinoblastoma proteins	pRB	pRB, p107	Oxidative metabolism

CREB-dependent manner [52], most likely via the cAMP-response element in the PGC-1α promoter [40,52].

1.9. Retinoblastoma proteins (pRbs)

pRb was initially identified as a tumor suppressor gene in the regulation of the cell cycle [3]. E2F1, the target of the repressive action of pRb in this context, however also exerts potent functions in cellular metabolism. Transcriptional derepression of E2F1 by deletion of pRb thus results in the promotion of an oxidative phenotype in skeletal muscle, thereby potentially linking cell proliferation, differentiation and metabolism in this tissue [53]. Similarly, p107, another member of the pRb protein family, inhibits PGC-1α gene expression and hence an oxidative muscle phenotype by repressing the activity of E2F4 on the PGC-1α promoter [54].

2. Conclusion

In this review, we tried to summarize the still very rudimentary insights into the role of coregulator proteins in muscle plasticity (Fig. 1 and Table 1). Even though the complex transcriptional networks that are controlled by these proteins are still very poorly understood [13], small steps have recently been made to understand the recruitment and activity of some of these proteins in muscle cells [55,56]. The synergistic and antagonizing effects of coregulators, and the potential to modulate the activity of many different TFs not only increases the complexity of transcriptional regulation, but could also help to identify potential therapeutic targets in the treatment of metabolic and muscle diseases. For example, transgenic elevation of PGC-1α in skeletal muscle confers therapeutic effects on many different muscle pathologies, even though diet-induced insulin resistance is not ameliorated without additional exercise interventions [57]. Furthermore, chronic administration of a synthetic class IIa HDAC inhibitor enhanced muscle endurance, and ameliorated systemic lipid and glucose handling [58]. Thus, to design safe and specific drugs, future studies will hopefully unravel how coregulators orchestrate complex metabolic networks and how their transcriptional fine-tuning abilities are regulated in muscle tissue.

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Appendix C

Bone 80 (2015) 115–125

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Review

Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise

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ARTICLE INFO

Article history:
Received 15 August 2014
Revised 27 January 2015
Accepted 8 February 2015

Keywords:
Skeletal muscle
Exercise
Myokines
Inflammation
PGC-1α

ABSTRACT

An active lifestyle is crucial to maintain health into old age; inversely, sedentariness has been linked to an elevated risk for many chronic diseases. The discovery of myokines, hormones produced by skeletal muscle tissue, suggests the possibility that these might be molecular mediators of the whole body effects of exercise originating from contracting muscle fibers. Even though less is known about the sedentary state, the lack of contraction-induced myokines or the production of a distinct set of hormones in the inactive muscle could likewise contribute to pathological consequences in this context. In this review, we try to summarize the most recent developments in the study of muscle as an endocrine organ and speculate about the potential impact on our understanding of exercise and sedentary physiology, respectively.

This article is part of a Special Issue entitled "Muscle Bone Interactions".

Contents

Abbreviations: AMPK, AMP-dependent kinase; AP-1, Activator protein 1; aP2, Adipocyte protein 2; BAIBA, β-Aminoisobutyric acid; BAT, Brown adipose tissue; BDNF, Brain-derived neurotrophic factor; CaMK, Calcium/calmodulin-dependent protein kinase; C/EBPα, CCAAT-enhancer-binding protein α; CnA, Calcineurin A; CNTF, Ciliary neurotrophic factor; CREB, Cyclic-AMP-responsive-element-binding protein; CXCR2, CXC receptor 2; ERRα, Estrogen-related receptor α; FGF21, Fibroblast growth factor 21; GLP-1, Glucagon-like peptide-1; GLUT4, Glucose transporter 4; HIF-1α, Hypoxia inducible factor 1α; IGF-1, Insulin-like growth factor 1; IL, Interleukin; JAK, Janus kinase; MEF, Myocyte-enhancer factor; Metrnl, Meteorin-like; MSTN, Myostatin; NFAT, Nuclear factor of activated T cells; NFκB, Nuclear factor κB; NRF-1, Nuclear respiratory factor 1; OSM, Oncostatin-M; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, Peroxisome-proliferator activated receptor γ coactivator 1α; PI3K, Phosphatidylinositol 3-kinase; PPAR, Peroxisome-proliferator activated receptor; REE, Resting energy expenditure; SERCA, Sarcoplasmic/endoplasmic reticulum calcium ATPase; SPARC, Secreted protein acidic rich in cysteine; SPP1, Secreted phosphoprotein 1; STAT, Signal transducer and activator of transcription; TFAM, Mitochondrial transcription factor A; TGF-β, Transforming growth factor β; TNFα, Tumor necrosis factor α; UCP, Uncoupling protein; VEGF, Vascular endothelial growth factor; VLDL, Very low density lipoproteins

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http://dx.doi.org/10.1016/j.bone.2015.02.008
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1. Introduction

1.1. Skeletal muscle morphology and function

The human body consists of around 600 muscles that contribute to approximately 40–50% of the total body weight. Similar to cardiac muscle, skeletal muscle is a striated muscle, is attached to the skeleton and thereby facilitates the movement of the body by applying force to bones and joints. Skeletal muscle is composed of myofibers that are formed by the fusion of individual myoblasts during a process called myogenesis. Muscle plasticity, for example the adaptation to exercise, is facilitated by a switch between oxidative, slow-twitch and glycolytic, fast-twitch muscle fibers [1]. The former are characterized by a high mitochondrial number, rich capillary supply, slow twitch frequency and high resistance against fatigue. The ample vascularization and the abundance of heme-containing proteins confer a red color to muscle beds with a high proportion of oxidative fibers. On the other hand, low mitochondrial number, fast twitch contraction kinetics, high peak force and low endurance are the functional hallmarks of glycolytic muscle fibers. Muscle beds consisting of glycolytic fibers appear more whitish in color. In rodents, type I and type IIa fibers are considered oxidative while type IIx and type IIb are more glycolytic. In humans, the spectrum of muscle fiber types is restricted to types I, IIa and IIx as well as hybrid fibers [2].

Myofibrils are composed of actin and myosin filaments arranged in sequentially repeated units called sarcomeres, the basic functional units of a muscle fiber that enables the muscle to contract. Skeletal muscle cells are the only voluntary type of muscle cells in contrast to cardiac muscle, smooth muscle and myoepithelial cells. Skeletal muscle cells are innervated by motor neurons and action potentials are exclusively initiated by the neurotransmitter acetylcholine in this context. Once a muscle cell is sufficiently depolarized, the sarcoplasmic reticulum releases calcium in a ryanodine receptor-dependent manner. Calcium subsequently binds to the troponin C subunit of the troponin complex. Troponin and tropomyosin are two important regulatory proteins, which are associated with actin to prevent interaction with myosin in the rested state. Calcium-bound troponin C then undergoes a conformational change that leads to an allosteric modulation of tropomyosin, which subsequently allows myosin to bind to actin. ATP-dependent cross bridging between myosin and actin then leads to a shortening of the muscle. Finally, calcium is pumped back into the sarcoplasmic reticulum by sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) ultimately resulting in muscle fiber relaxation.

It is important to note that muscle function is not limited to the generation of power, locomotion, posture and breathing. In fact, shivering skeletal muscle is the most important organ for the maintenance of body temperature besides non-shivering thermogenesis by brown adipose tissue. Furthermore, skeletal muscle is one of the largest energy stores with substantial amounts of triglycerides and glycogen, in particular in trained subjects. In addition, anaerobic glycolysis and breakdown of skeletal muscle tissue during starvation releases lactate and amino acids, respectively, some of which subsequently are utilized to fuel hepatic (mainly alanine and lactate) and renal (mainly glutamine) gluconeogenesis. Thus, skeletal muscle has long been known to be capable of secreting factors in order to communicate with non-muscle tissues. However, more recently, such auto-, para- and endocrine mediators produced and released by skeletal muscle have been termed "myokines", in analogy to the adipokines produced by adipose tissue. These secreted factors potentially have far-reaching effects on non-muscle tissue and thereby could provide a molecular link between muscle function and whole body physiology. This review will mainly focus on the biology of a selection of such myokines.

1.2. Skeletal muscle plasticity

Repeated contractions, for example in training, result in a pleiotropic adaptation of muscle cells with a dramatic remodeling of contractile and metabolic properties. Importantly, the diametrically opposite activation pattern in endurance and resistance training elicit distinct cellular consequences. For example, endurance training results in a switch to oxidative, slow-twitch muscle fibers while resistance exercise increases the proportion as well as the cross-sectional area of glycolytic muscle fibers. Surprisingly, the molecular mediators of these adaptations are only poorly understood [3]. It is clear that slow-type activation results in a more continuous elevation of intracellular calcium with low amplitude whereas these transients are characterized by more intermittent spikes with a high amplitude in glycolytic fibers in resistance training [4]. Nevertheless, it is unclear how the distinct calcium levels are interpreted to result in either a slow- or a fast-type gene program. For example, in both cases, the intracellular increase in calcium activates the catalytic subunit of the phosphatase calcineurin A (CnA) and members of the calcium/calmodulin-dependent protein kinase family (CaMK) leading to a change in the phosphorylation status of different transcription factors and coactivators. These transcription factors include the cyclic-AMP-responsive-element-binding protein (CREB), myocyte-enhancer factor 2C (MEF2C) and MEF2D, and members of the nuclear factor of activated T cells (NFAT) family. In resistance-trained muscle, growth factor signaling, in particular that initiated by the insulin-like growth factor 1 (IGF-1), seems to be an important modifier of the ensuing muscle cell remodeling. In endurance-trained muscle, MEF2C/2D and NFAT regulate the gene expression of slow-type genes. In addition, CREB, MEF2C/2D and NFAT control the transcriptional rate of the peroxisome-proliferator activated receptor γ coactivator 1α (PGC-1α) [5]. This transcriptional coactivator constitutes a regulatory nexus in the adaptation of skeletal muscle to endurance training [6,7]. Accordingly, in addition to the regulation by calcium signaling, PGC-1α gene expression and posttranslational modifications are affected by every major signaling pathway that is activated in a contracting muscle fiber (Fig. 1). In turn, PGC-1α coordinates the entire program of endurance training adaptation in skeletal muscle. For example, PGC-1α is recruited to more than 7500 distinct sites in the mouse genome and induces and inhibits the transcription of around 984 and 727 genes, respectively, in muscle cells [8]. Such a complex transcriptional response is enabled by a specific interaction with a high number of transcription factor binding partners and furthermore, selective modulation of the PGC-1α activity by additional co-regulators, e.g. competition with the corepressor NCoR1 for binding to the estrogen-related receptor α (ERRα) [9] in the regulation of mitochondrial genes [10]. Thus, when overexpressed in muscle, transgenic mice exhibit a trained phenotype [11] while skeletal-muscle-specific knockout animals for PGC-1α suffer from a reduced endurance capacity as well as other signs of pathological inactivity [12,13]. In individual exercise bouts, stabilization of existing PGC-1α protein by the p38 mitogen-activated protein kinase (p38 MAPK) [14], phosphorylation by the AMP-dependent kinase (AMPK) [15], other posttranslational modifications and subsequent transcriptional induction ensure a rapid and robust elevation of PGC-1α activity [16,17]. Upon cessation of muscle contraction, the short half-life of the PGC-1α protein and a reduction in the gene transcription help to revert PGC-1α levels back to baseline expression within hours. Chronic exercise,

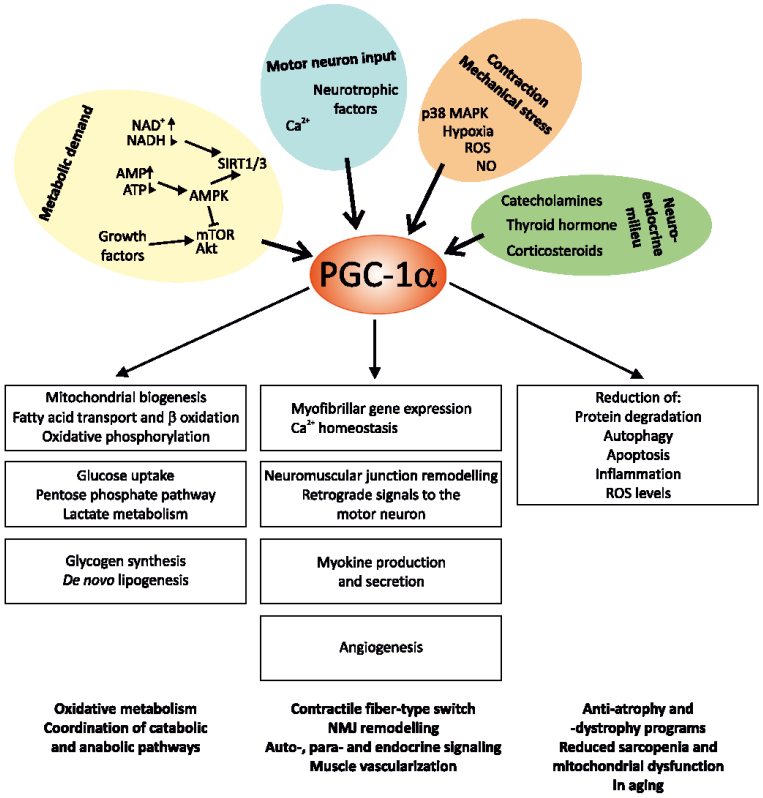

Fig. 1. Central role of PGC-1α in the regulation of skeletal muscle cell plasticity. Every major signaling pathway that is activated in a contracting muscle fiber during and after endurance training converges on PGC-1α by modulating PGC-1α gene expression and/or post-translational modifications of the PGC-1α protein. As a consequence, PGC-1α in turn coordinates the transcriptional network that controls the biological program of exercise-induced muscle remodeling.

e.g. in endurance training, promotes a shift towards a higher proportion of slow-type muscle fibers. Due to the fiber-type preference in PGC-1α gene expression, endurance-trained muscle exhibits higher basal transcript levels of PGC-1α while retaining the acute induction in each exercise bout [11,18]. Due to the potent effect of PGC-1α on muscle function, modulation of the levels and/or activity of this coactivator might provide an interesting therapeutic avenue for metabolic and other diseases that are linked to an inactive muscle [19,20]. At least experimentally, elevation of PGC-1α and its homolog PGC-1β indeed ameliorated several different muscle wasting pathologies in various mouse models of Duchenne muscular dystrophy [21,22] and a mitochondrial myopathy [23]. In the context of metabolic diseases, the results are less clear and it seems that *bona fide* physical activity is required to synergize the effect of overexpressed PGC-1α to improve diet-induced insulin resistance [24,25].

1.3. The broad effect of physical inactivity on health

A sedentary lifestyle is defined by a lack of or irregular physical activity and is one of the leading causes of preventable deaths worldwide [26,27]. For example, sedentariness can contribute to the development of a number of chronic diseases, such as type 2 diabetes [28], cardiovascular pathologies [29], obesity [30,31], postmenopausal breast cancer and other tumors [32]. Furthermore, physical inactivity may also play a role in the development of dementia [33], depression [34] and neurodegenerative events [35].

A persistent, sterile, inflammation is one of the most obvious features of physical inactivity [36]. Chronic, systemic inflammation most likely promotes the development of insulin resistance, atherosclerosis, neurodegeneration and tumor growth [36]. Historically, an excess of adipose tissue, as in the context of obesity, has been demonstrated to secrete increased amounts of the pro-inflammatory cytokines tumor necrosis factor-α (TNFα), interleukin 1-β (IL-1β) and IL-6 in both rodents and humans [37,38], thus, contributing substantially to the chronic systemic inflammation. More recently, other organs, including liver and skeletal muscle, have been discovered as additional sources of pro-inflammatory cytokines in pathological settings [39,40]. For example, the reduced expression of PGC-1α in skeletal muscle resulting from a sedentary lifestyle or gene ablation results in a low-level local as well as systemic inflammatory response, which then elicits negative impacts on other tissues such as pancreatic β cells [41]. Inversely, PGC-1α reduces the activity of the nuclear factor κB (NFκB), the master

regulator of pro-inflammatory gene expression [42]. Thus, by contributing to the production of pro-inflammatory cytokines, inactive muscles in a sedentary individual might negatively affect other organs and ultimately, whole body homeostasis [36].

1.4. Exercise and its beneficial effects on skeletal muscle and whole body metabolism

The beneficial effects of exercise transcend improved skeletal muscle functionality. For example, moderate exercise has been shown to increase life span in rats [43], to improve neuromuscular and neurological performance in mice [44] and to lower hyperglycemia [45,46], hypercholesterolemia [47] and hypertension [48]. Similarly, systemic responses to exercise have been observed in distal organs like heart, kidney, brain and liver in various animal models [44,49–51]. Epidemiological studies have likewise linked an active life-style to systemic adaptations and an elongated health-span, thus time of life in good health, in different human cohorts [52]. Intriguingly, many of these effects even occur in elderly individuals that only initiate exercise at advanced age highlighting the high efficacy of exercise as an intervention for the prevention and/or treatment for various ailments. To date, the mechanisms that link physical activity to health remain unknown. Nevertheless, several hypotheses have been proposed to contribute to the beneficial effects of exercise. For example, the sought-after whole body adaptations to exercise could be induced by a general reduction in systemic inflammation since inversely, a persistent, sterile inflammatory state has been linked to the etiology of many chronic diseases [36,53]. Thus, regular physical activity acutely increases the release of adrenaline, cortisol, growth hormone, prolactin and other factors with immunomodulatory effects [54]. Importantly however, long-term training is associated with decreased circulating levels of the classical stress hormones. Moreover, exercise reduces the expression of Toll-like receptors at the surface of monocytes that have been implicated as mediators of systemic inflammation [55]. Importantly, very high intensity exercise bouts themselves trigger systemic inflammation, a subsequent immunodepression and thus a higher risk for infections [56]. Muscle function, inflammation and exercise are hence intrinsically linked in a complex manner [36,40]. Therefore, not surprisingly, many myokines, for example interleukin 6, have also been described as prototypical pro-inflammatory cytokines. Induction of beneficial versus detrimental effects therefore seems highly context-specific and might depend on the amplitude, frequency and other variables in secretion.

2. Myokines

It is now firmly established that skeletal muscle tissue produces and secretes cytokines and other proteins, which have been named "myokines" [57]. Myokines subsequently exert auto-, para- and/or endocrine effects (Fig. 2). Thus, skeletal muscle can be classified as an endocrine organ. Since the description of the first members, the list of myokines has constantly been growing demonstrating that skeletal muscle has the capacity to express several myokines, some simultaneously, others in a temporally- or context-controlled manner. For most currently described myokines, contractile activity is the key regulatory element for expression and secretion. In this review we will focus on some of the classical as well as some of the more exotic myokines, describe their regulation in skeletal muscle and their possible systemic effects on distal non-muscle tissues. Furthermore, we will highlight the newest members of the myokine family and their potential application in combating metabolic diseases.

3. Myostatin

Myostatin (MSTN) is a member of the transforming growth factor β (TGF-β) superfamily that is expressed in the developing and adult skeletal muscle. The main function of myostatin is to negatively regulate muscle mass [58]. Evolutionarily, actively limiting muscle growth might have helped to prevent the build-up of energy-consuming muscle mass beyond the needs of the current situation. Accordingly, myostatin null mice exhibit a massive muscle hypertrophy that is characterized by an increased fiber cross-sectional area as well as an elevated number of fibers. The hyperplasia in this animal model most likely originates from accelerated primary and secondary myogenesis. Importantly, the myostatin gene is highly conserved among different vertebrate species. For example, myostatin mutations in some domestic breeds of cattle including the Piedmontese, Belgian Blue and Marchigiana result in a so called double-muscling phenotype and hence pronounced muscle hypertrophy [59–61]. Similar double muscling phenotypes have been observed in sheep and the whipped dog breed that leads to increased muscle mass and racing performance in the latter [62]. Finally, a mutation in the myostatin gene has also been associated with muscle hypertrophy in a male child with extraordinary muscularity and several relatives with self-reported unusual strength [63].

In addition to the hypertrophic muscle phenotype, myostatin null mice show reduced total and intramuscular body fat compared to wild-type animals [58,64]. An increase in muscle mass leads to increased resting energy expenditure (REE), which in turn could account for the reduction in fat mass in myostatin knockout animals [58]. Accordingly, circulating levels of leptin, the "satiety hormone", are reduced in these mice [64,65] hence suggesting that despite decreased leptin secretion, myostatin null mice are protected from the counter-regulatory consequence of leptin signaling on energy expenditure. Moreover, myostatin might directly influence the cellular physiology of adipocytes even though the results are conflicting. For example, myostatin inhibits the differentiation of 3T3-L1 preadipocytes and reduces the expression of several adipogenic markers and transcription factors such as the peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer-binding protein α (C/EBPα), adipocyte protein 2 (aP2) and leptin [66,67]. In contrast, in a multipotent mesenchymal cell line (C3H10T1/2), myostatin promotes adipogenesis, which would be more in line with the reduced adiposity in myostatin knockout animals [68,69]. Inversely however, pharmacological administration of myostatin *in vitro* and *in vivo* is not able to increase lipolysis and to reduce fat mass [67] suggesting that the reduction in body fat in myostatin null animals is indeed due to the increased muscle mass and only to a lesser extent to direct effects on adipose tissue.

Even though it was not called a myokine at the time of its discovery, myostatin is one of the first members of this class of proteins. However, since both aerobic exercise [70–73] and strength training [74–78] in animals and humans significantly reduce expression in skeletal muscle, myostatin is more like an "inverse" myokine compared to most other family members that are elevated by exercise. In addition to transcript levels, serum myostatin also decreases with resistance training in young men [79].

4. Decorin

In contrast to myostatin, decorin is a more recently discovered myokine that is elevated in mice and humans after acute and chronic resistance exercise [80]. Overexpression of decorin in transgenic models resulted in a pro-hypertrophic gene program, for example elevated Mighty, MyoD and follistatin gene expression. Decorin seems to act in a paracrine manner by directly binding to and thereby inhibiting the action of myostatin [80]. Therefore, decorin can act as a myokine that antagonizes the effects of another myokine, in this case myostatin, and in addition also neutralize myostatin of non-muscle origin, for example myostatin released from tumor cells in cancer cachexia [81].

5. Interleukin-6

Interleukin-6 (IL-6) has originally been classified as a prototypical pro-inflammatory cytokine while later, anti-inflammatory properties

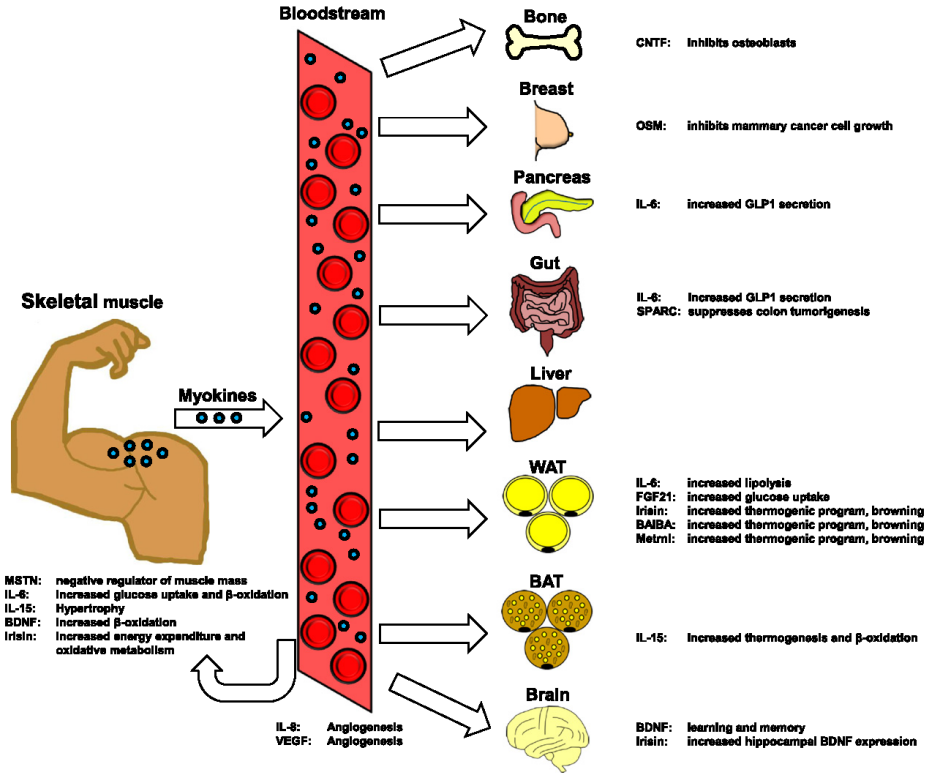

Fig. 2. Auto-, para- and endocrine effects of myokines. Selected examples of the physiological consequences of the production and release of myokines on skeletal muscle and other organs are depicted.

have also been described [82]. Besides the production of IL-6 in activated immune cells, the systemic elevation of IL-6 in patients with metabolic diseases has contributed to the link of IL-6 and inflammation. Moreover, overexpression of IL-6 in transgenic mice results in reduced body mass and impaired insulin-stimulated glucose uptake by skeletal muscle [83]. Thus, IL-6 has been proposed as one of the pro-inflammatory factors that promote the development of peripheral insulin resistance. In stark contrast however, exercise-induced elevation of IL-6 plasma levels lead to increased circulating levels of several potent anti-inflammatory cytokines such as IL-1ra and IL-10, suggesting that IL-6 may also have anti-inflammatory properties [84,85].

IL-6 is produced by a number of cells *in vivo* including stimulated monocytes/macrophages, fibroblasts and vascular endothelial cells [86]. Skeletal muscle fibers also express and release IL-6 during and after exercise [86–89]. IL-6 production is likewise boosted in connective tissue, the brain and adipose tissue post-exercise [90]. Exercise-induced plasma IL-6 concentrations peak at the end or shortly after cessation of an acute exercise bout and quickly return to pre-exercise levels [91]. IL-6 mRNA levels are generally induced in contracting skeletal muscle [87,92]: however, exercise further enhances the transcriptional rate of IL-6 if muscle glycogen stores are low [93]. As IL-6 is a classical inflammatory cytokine, it was initially thought that exercise-induced IL-6 is released in response to muscle injury. However, several lines of evidence indicate that substantial amounts of IL-6 are produced independent of muscle injury [85,87,94].

When IL-6 was discovered and classified as one of the founding members of the myokine class of proteins, Pedersen et al. suggested IL-6 to possess some of the characteristics of a true "exercise factor", which exerts its effects both locally within the muscle bed and peripherally on distal organs in an endocrine-like fashion [95]. In skeletal muscle, IL-6 signals through a gp130Rβ/IL-6Rα homodimer leading to the activation of AMPK and/or phosphatidylinositol 3-kinase (PI3K) and, subsequently, to an increase in glucose uptake and fatty acid oxidation [96,97]. Similarly, enhanced AMPK activity upon IL-6 signaling has also been reported in adipose tissue [98]. Furthermore, IL-6 has been suggested to stimulate hepatic glycogenolysis, gluconeogenesis and glucose release [99]. Furthermore, IL-6 stimulates the secretion of glucagon-like peptide-1 (GLP-1), which results in an enhanced secretion of insulin from intestinal L-cells and pancreatic α-cells [100].

Thus, IL-6 release in response to exercise seems to have pleiotropic effects by increasing glucose uptake and fatty acid oxidation locally in skeletal muscle and enhancing insulin secretion, which further increases glucose uptake into muscle cells. At the same time hepatic glucose output [99] and fatty acid release from adipose tissue [101] are stimulated thereby providing energy substrates for the exercising muscle.

6. Interleukin-8

Interleukin-8 (IL-8) is a chemokine that attracts primary neutrophils [102]. In addition, IL-8 associates with the CXC receptor 2 (CXCR2) and

thereby promotes angiogenesis [103–105]. IL-8 mRNA levels in skeletal muscle are elevated after exercise [106–108] and IL-8 plasma levels were found to be increased after eccentric muscle contractions [106, 109–112]. Curiously, systemic IL-8 plasma concentrations are unchanged following concentric exercise [106–108,113], suggesting that the main part of the systemic increase in IL-8 after eccentric exercise, which is associated with a higher degree of fiber damage compared to concentric contractions, may account for a general inflammatory response [95]. Nevertheless, a small and transient net release of IL-8 has been reported across a concentrically exercising limb [107], indicating that muscle-derived IL-8 still acts locally in an auto- and/or paracrine manner while not substantially contributing to the systemic IL-8 plasma concentration in this context.

Even though the physiological function of IL-8 on skeletal muscle is still unknown, the association with CXCR2 suggests at least a role in promoting exercise-induced neovascularization of muscle tissue. This assumption is further supported by the fact that CXCR2 mRNA and protein levels are induced upon concentric exercise in the vascular endothelial cells in muscle tissue [114].

7. Interleukin-15

Interleukin-15 (IL-15) is a pro-inflammatory cytokine with structural similarities to IL-2 and regulates T and natural killer cell activation and proliferation [115,116]. IL-15 binds and signals through the IL15Rαβγ complex, which is upstream of the Janus kinases 1 and 3 (JAK1, 3) and signal transducer and activator of transcription-3 and 5 (STAT3, 5) [117, 118]. Expression of IL-15 mRNA has been detected in several human tissues including heart, lung, liver and kidney, but most abundantly in placenta and skeletal muscle. Similarly, peripheral blood mononuclear, epithelial and fibroblast cells seem to express significant levels of IL-15 [116].

Originally, IL-15 has been described as an anabolic factor in skeletal muscle. For example, even one bout of resistance training accordingly elevated IL-15 mRNA expression in human skeletal muscle [119]. Furthermore, IL-15 stimulates the production of contractile proteins [120] and overexpression of IL-15 *in vitro* results in muscle cell hypertrophy [121]. Moreover, IL-15 treatment of tumor-bearing rats antagonizes cancer cachexia [122] demonstrating the therapeutic potential in muscle wasting diseases. More detailed analyses of these *in vivo* studies however provided a different picture [123]: for example, administration of IL-15 did not affect muscle mass in the healthy control animals [122]. Transgenic overexpression of IL-15 [121] and muscle-specific ablation of the IL-15 receptor α [124] strongly implicated IL-15 in promoting an oxidative, high-endurance muscle phenotype. Accordingly, IL-15 promotes skeletal muscle glucose uptake and fatty acid oxidation [125,126]. Moreover, 12 weeks of endurance training resulted in an elevation of IL-15 protein in skeletal muscle of human volunteers, even though mRNA expression and IL-15 plasma levels remained unchanged [127]. Thus, future experiments will have to elucidate whether the assignment of IL-15 as an anabolic factor has been spurious and based on *in vitro*-specific effects or if a dual role for IL-15 in both resistance as well as endurance training adaptation could be possible in a context-specific manner.

Interestingly, diametrically opposite to the postulated anabolic effects on skeletal muscle, IL-15 inversely shrinks adipose tissue mass. In humans, a negative correlation between IL-15 plasma levels and trunk fat mass has been demonstrated and likewise, IL-15 overexpression in mouse skeletal muscle decreases visceral fat mass [128]. Besides the strong IL-15-dependent remodeling of skeletal muscle, this reduction in fat mass might also be associated with the effects of IL-15 on liver metabolism. For example, chronic administration of IL-15 decreases hepatic lipogenesis [129] and, at the same time, boosts hepatic fatty acid oxidation [126]. Together, these two effects potentially reduce the export of very low density lipoproteins (VLDL). Accordingly, IL-15 treatment of animals diminishes circulating VLDL levels in comparison to non-treated control animals [130].

Finally, IL-15 administration decreases brown adipose tissue (BAT) mass in rats. At the same time however, the transcript levels of the uncoupling proteins 1 and 3 (UCP1, 3), lipid-related transcription factors and other proteins involved in membrane and mitochondrial transport and fatty acid oxidation are induced [131] and thereby, BAT thermogenesis and fatty acid oxidation are boosted.

8. Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, is strongly expressed in the brain [132] and to a lesser extent in skeletal muscle [133]. In the CNS, BDNF regulates neuronal development and modulates synaptic plasticity, playing a role in the regulation of survival, growth and maintenance of neurons [134,135]. Furthermore, hypothalamic BDNF has been identified as a key factor in the control of body mass and energy homeostasis [136]. BDNF also influences learning and memory [137] and brain samples of patients with Alzheimer's disease exhibit reduced expression of BDNF [138]. Similarly, BDNF serum levels of patients with depression, obesity and type 2 diabetes are decreased [139,140]. Inversely, exercise increases circulating BDNF levels in humans [141] and recent studies suggest that the brain contributes 70–80% of the circulating BDNF in this context [142]. BDNF mRNA and protein levels are also increased in skeletal muscle in response to exercise and contribute to enhanced fat oxidation by activating AMPK [133]. However, muscle-derived BDNF seems not to be released into the circulation in significant amounts indicating that BDNF primarily acts in an auto- and/or paracrine manner. Accordingly, besides the effects on metabolic properties, the main consequences of modulation of muscle BDNF extend to myogenesis, satellite cell activation and skeletal muscle regeneration [143,144]. Other members of the neurotrophin factor family, in particular neurotrophin-3 (NT-3) and NT-4/5, have also been found in skeletal muscle tissue [145,146]. However, even though a potential role of NT-3 and NT-4/5 in the repair of peripheral nerve injury has been suggested [145], the regulation and function of NT-3 and NT-4/5 in muscle remains unclear.

9. Ciliary neurotrophic factor

Like BDNF, ciliary neurotrophic factor (CNTF) is also a member of the neurotrophin family with important functions in the regulation of neural survival, development, function and plasticity [132]. Surprisingly, CNTF is also involved in the regulation of osteoblasts. For example, female CNTF-deficient mice exhibit increased bone mass and osteoblast activity compared to control animals. Moreover, CNTF decreases the mineralization and production of the osteoblast differentiation factor osterix in osteoblasts *in vitro* [147].

Recently, CNTF has been identified as a myokine that negatively regulates osteoblast gene expression together with the soluble CNTF receptor [148]. Therefore, muscle-derived CNTF could be a molecular link that underlies reduced bone formation in inactive individuals and therefore might contribute to osteoporosis boosted by a sedentary life-style. Inversely however, CNTF production in inactive or resting muscle fibers may prevent heterotopic ossification of the muscle or regulate periosteal expansion during bone growth.

10. Vascular endothelial growth factor and secreted phosphoprotein 1

Vascular endothelial growth factor (VEGF) is a mitogen with specificity for vascular endothelial cells [149] and is a crucial regulator of embryonic vascular development (vasculogenesis) as well as blood vessel formation (angiogenesis). In fact, VEGF is most likely the most important pro-angiogenic growth factor in most tissues including skeletal muscle [150]. VEGF mRNA and protein levels are upregulated in skeletal muscle following an acute bout of exercise [151–153]. Furthermore,

interstitial VEGF levels likewise increase markedly after exercise [152,154] suggesting that VEGF is indeed secreted from contracting skeletal muscle fibers. This is further supported by a study showing that electrostimulation of cultured muscle cells leads to the secretion of VEGF into the culture medium [150]. Thus, skeletal muscle controls its own capillary supply be secreting VEGF into the extracellular space where VEGF acts on the vascular endothelial cells to increase blood vessel formation, and thus ultimately improve oxygen and energy substrate transport to the exercising muscle. In many different cell types, VEGF activity is determined by the hypoxia inducible factor 1α (HIF-1α), one of the main regulators of VEGF gene transcription, in addition to a strong regulation at the post-transcriptional level [155]. In contracting skeletal muscle tissue, an alternative, HIF-1α-independent regulation of VEGF transcription has been discovered centered on PGC-1α, in functional interaction with ERRα [156] and the activator protein-1 (AP-1) [8]. Interestingly, VEGF-induction by PGC-1α is further coordinated with a PGC-1α-dependent elevation of the secreted phosphoprotein 1 (SPP1). SPP1 is a new myokine that helps to orchestrate physiological angiogenesis by stimulating macrophages and in turn, activating endothelial cells, pericytes and smooth muscle cells [157].

11. Fibroblast growth factor 21

Fibroblast growth factor 21 (FGF21) belongs to the FGF super family with major functions in modulating cell proliferation, growth and differentiation as well as metabolism [158,159]. FGF21 is predominantly expressed and secreted by the liver [160] but also by adipose tissue, pancreas and skeletal muscle [161]. The liver mainly secretes FGF21 in response to fasting [162] while BAT secretes FGF21 upon noradrenergic stimulation [163]. In the liver, FGF21 induces the expression of PGC-1α that in turn increases fatty acid oxidation, TCA cycle flux and gluconeogenesis [164].

In adipose tissue, FGF21 stimulates glucose uptake and transgenic overexpression of FGF21 protects mice from diet-induced obesity. Furthermore, FGF21 administration to diabetic rodents lowers blood glucose and triglyceride levels demonstrating its potential therapeutic application [165].

A recent study suggests that FGF21 is an insulin-regulated myokine that is expressed in skeletal muscle in response to insulin stimulation [166]. Moreover, cold-induced FGF-21 in muscle could contribute to an activation of thermogenesis [167]. FGF21 expression in skeletal muscle is also increased in a mouse model for a mitochondrial myopathy and the ensuing FGF21 elevation in the circulation promotes a starvation-like response in this disease context [168]. These results suggest that FGF21 might be more of a stress-induced than a "classical" exercise-induced myokine.

12. Irisin, meterorin-like and β-aminoisobutyric acid

Since PGC-1α is a crucial regulator of skeletal muscle plasticity after exercise [16] and accordingly, PGC-1α levels often correlate with those of myokines [169], PGC-1α gain- and loss-of-function models have been used to identify novel myokines. For example, screening of muscle cells that overexpress PGC-1α in three recent studies led to the identification of three new PGC-1α-dependent myokines, irisin [170], BAIBA [171] and meteorin-like [172]. Intriguingly, all three myokines seem to be involved in promoting beige fat thermogenesis.

12.1. Irisin

Irisin is cleaved off from FNDC5, a membrane-bound protein in skeletal muscle that is induced by exercise and muscle shivering [167]. Irisin exerts its action on white adipose tissue cells to stimulate UCP-1 expression and other brown fat-like genes thereby inducing browning and thermogenesis of white adipose tissue. Collectively, these effects lead to an increase in energy expenditure and result in an improvement of adiposity and glucose homeostasis [170]. Besides skeletal muscle, FNDC5 is also expressed in the brain [173,174]. By elevating systemic irisin levels, endurance exercise induces FNDC5 expression in the hippocampus in a PGC-1α-dependent manner, which then leads to increased hippocampal BDNF expression and ultimately neurogenesis in this brain region. Accordingly, peripheral delivery of FNDC5 via adenoviral vectors is sufficient to induce BDNF expression in the brain [175]. Therefore, FNDC5/irisin might be the molecular mediator of exercise-induced neurogenesis in a direct skeletal muscle-brain cross-talk.

In addition to these endocrine effects, irisin also positively regulates muscle metabolism. Myocytes treated with irisin *in vitro* express higher levels of PGC-1α, nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), glucose transporter 4 (GLUT4), UCP3, and irisin implying a positive autoregulatory loop between PGC-1α and FNDC5 [176]. Thereby, energy expenditure and oxidative metabolism in muscle cells is elevated by irisin. Importantly, while data in mice are robust, the regulation and function of irisin in humans is currently under debate. Future studies will have to show why irisin is only induced in some, but not all exercise cohorts. As with other factors, irisin regulation could depend on the specific training protocol (e.g. intensity, endurance vs. resistance, acute vs. chronic, time of blood sampling after exercise), age, gender, ethnicity, fitness and other variables. Second, the presence of circulating irisin in humans will have to be further investigated since the human FNDC5 gene lacks a functional start codon and detection of the protein has been questioned due to supposed suboptimal antibody specificity [177]. Notably however, 2–4% of eukaryotic genes contain an atypical start codon and nevertheless can be transcribed [178,179]. Moreover, circulating human irisin has been described in several studies using different antibodies and assays such as highly sensitive mass spectroscopy [167].

12.2. Meteorin-like

Meteorin-like (Metrnl) is a hormone that has been identified as a myokine, which is induced in skeletal muscle upon exercise and in white adipose tissue upon cold exposure [172]. Interestingly, in contrast to FNDC5/irisin, Metrnl primarily is dependent on the PGC-1α isoform 4 (PGC-1α4). PGC-1α4 is a splice variant of PGC-1α with a very distinct gene regulation pattern [74]. In contrast to the other known PGC-1α variants that primarily promote an oxidative, high endurance muscle phenotype, PGC-1α4 is induced by resistant training and enhances muscle hypertrophy and strength [74]. Nevertheless, even though the regulation of irisin and Metrnl is specific, Metrnl also increases whole body energy expenditure and improves glucose tolerance in obese/diabetic mice by indirectly activating the browning gene program in white adipose tissue via stimulation of an eosinophil-macrophage signaling cascade to ultimately activate a pro-thermogenic program [172].

12.3. BAIBA

BAIBA (β-aminoisobutyric acid) is an atypical myokine inasmuch BAIBA is not a cytokine-like molecule or even a protein. Nevertheless, BAIBA behaves clearly in a myokine-specific manner by being secreted from PGC-1α-expressing myocytes and subsequently activating the thermogenic program and beigeing of white adipose tissue [171]. In a similar endocrine fashion, BAIBA increases β-oxidation in hepatocytes both *in vitro* and *in vivo* by activating the transcription factor PPARα. In mice and in humans *in vivo*, circulating BAIBA levels increase with exercise and are inversely correlated with cardiometabolic risk factors in humans. Thus, exercise-induced circulating BAIBA has been suggested to protect from metabolic diseases [171].

Strikingly, all of these three newly identified PGC-1α-dependent myokines exert a strong endocrine effect on white adipose tissue by promoting a browning/thermogenic response and thereby increasing energy expenditure. The subsequent improvement of adiposity and whole body metabolism is of obvious interest for the treatment of

obesity and obesity-associated diseases like type 2 diabetes. Moreover, increased adrenergic signaling during exercise can at least mechanistically be linked to a browning effect [180]. Nevertheless however, browning of white adipose tissue and, as a consequence, elevation of energy expenditure in the context of exercise raises interesting conceptual questions. With a degree of efficiency of around 15–25%, exercising muscle already produces significant heat that has to be dissipated by vasodilation and sweating in order to sustain prolonged contractions. It thus seems counterintuitive for skeletal muscle to directly initiate a myokine-dependent program that results in even further heat production in beige adipose tissue and as a consequence, usage of energy substrates that should optimally be available for muscle, and not adipose tissue, both for contractions as well as post-exercise refueling of energy stores. In the original manuscript, the authors speculated that such a program could be an evolutionary consequence of shivering thermogenesis that became increasingly important in higher organisms [170]. Indeed, recent work identified irisin and FGF-21 as cold-induced endocrine activators of thermogenesis in humans [167]. In fact, irisin secretion closely correlated with shivering intensity further supporting the hypothesis of a specification of skeletal muscle to sustain and coordinate both shivering in muscle as well as non-shivering thermogenesis in beige and brown adipocytes [181].

13. Anti-tumorigenic myokines: SPARC and OSM

An active life-style is not only associated with a decreased risk for the development of metabolic diseases such as cardiovascular pathologies and type 2 diabetes, different studies also imply a possible link between physical activity and certain types of cancer [27,182]. For example, the World Cancer Research Fund proposed that exercise reduces the risk for developing breast and colon cancer by 25–30% [183]. There is a growing list of potential mechanisms on how exercise may exhibit anti-tumorigenic effects, even though the molecular pathways are still largely unknown. Recently, two myokines have been identified, secreted protein acidic and rich in cysteine (SPARC) [184] and oncostatin-M (OSM) [185], which suppress tumor formation in the colon and inhibit mammary cancer cell growth, respectively. Both myokines inhibit proliferation and induce apoptosis of cancer cells. While such effects are of obvious therapeutic importance, the physiological roles of these two myokines remain unclear. Future studies might elucidate whether SPARC and OSM likewise regulate cell proliferation and apoptosis after being produced and secreted in contracting muscle fibers or if these two myokines have completely different effects in non-tumor cells.

14. Training, myokines and "exercise factors"

As described in the individual sections above, a clear regulatory and functional role linked to exercise has been found for some myokines. For example, IL-6 expression closely correlates with muscle contraction [91]. In turn, e.g. by promoting hepatic gluconeogenesis [99] and lipolysis in adipose tissue [98], IL-6 subsequently contributes to an adequate supply of energy substrates for the contracting muscle by affecting distal organs. Accordingly, IL-6 has been declared to constitute a *bona fide* "exercise factor" [95]. For other myokines, such a direct link to exercise is lacking; in some cases, the physiological relevance of the consequences of myokine action in the context of exercise remains enigmatic (e.g. the increase in brown fat-associated thermogenesis). Accordingly, the definition of "exercise factors" as a subgroup of myokines has been proposed based on the following criteria [186]: "exercise factors" are regulated by exercise and subsequently released into the circulation in order to exert distal effects. Non-exercise factor myokines are not necessarily controlled by muscle contraction and might not have a systemic function. Importantly, myokines do not have to be exclusively produced by skeletal muscle—indeed, the vast majority of the currently described myokines are also found in other tissues [186]. Finally, "exercise factors" can furthermore be stratified as acute or chronic based on their secretion pattern since skeletal muscle will elicit dramatically different phenotypic consequences in distal tissues following an acute bout or long-term training [187]. Based on these criteria and the presence of a complete chain of evidence, Catoire and Kersten proposed IL-6, SPARC, Angptl4, CX3CL1 and CCL2 as myokines with the highest potential to constitute "exercise factors" in humans [186]. Obviously, more work is needed to refine and expand this list in the future.

15. Conclusion

The discovery of myokines has opened a vast new and exciting field in the study of muscle, and, in particular, exercise physiology. Even now, members of the myokine group of signaling molecules cover a whole range of auto-, para- and endocrine effects. Importantly, different *in vitro* and *in vivo* approaches identify novel potential myokines at a breath-taking pace. For example, recent secretome analyses of cultured human muscle cells, with or without electrical stimulation, revealed over 50 novel candidate proteins [188–190]. Obviously, a myokine function of these proteins remains to be tested in murine and human *in vivo* models. Likewise however, a complete chain-of-evidence of the skeletal muscle cell origin of many myokines described *in vivo* is still missing. Therefore, future studies aimed at the identification and characterization of novel myokines will optimally combine *in vitro* and *in vivo* experiments. Next, the current studies aimed at the identification of exercise-induced myokines might be combined with a "myokineome" analysis of the inactive, sedentary muscle. So far, only myostatin or CNTF might fit that description—however, it would be surprising if myostatin and CNTF were the only "sedentary" myokines. Finally, non-protein myokines such as BAIBA might gain further relevance in the future to explain systemic plasticity initiated by skeletal muscle. Intriguingly, muscle-mediated conversion of kynurenine to kynurenic acid, a metabolite unable to cross the blood–brain barrier, could underlie the beneficial effect of physical activity on stress-induced depression and thereby provide another example for a metabolite link between muscle and brain [191]. In any case however, myokines not only provide a molecular explanation for the extensive cross-talk between muscle and other tissues in our body, but also reveal novel therapeutic avenues for the treatment of a myriad of chronic diseases that are associated with a sedentary life-style. Thus, the study of myokines will remain an extremely interesting research topic for our understanding of basic as well as translational aspects of skeletal muscle physiology in the years to come.

Acknowledgments

Work in our laboratory is supported by the ERC Consolidator grant 616830-MUSCLE_NET, the Swiss National Science Foundation, SystemsX.ch, the Swiss Society for Research on Muscle Diseases (SSEM), the Neuromuscular Research Association Basel (NeRAB), the Gebert-Rüf Foundation "Rare Diseases" Program, the "Novartis Stiftung für medizinisch-biologische Forschung", the University of Basel, and the Biozentrum.

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Acknowledgments

First of all, I would like to thank Christoph Handschin, my supervisor, for giving me the opportunity of doing my PhD in his lab. He allowed me to work very independently and to bring in and follow up my own ideas and hypothesis but nevertheless, was always there to support me whenever I had difficulties or needed advice.

Furthermore, I want to thank all the members of the Handschin and Rüegg labs for creating such a nice working atmosphere and for helping me in different kinds of techniques and experiments.

Special thanks goes to Barbara Kupr, Bettina Karrer and Regula Furrer, who were always there for me to discuss any kind of stupid problem and who became friends over the last years.

Last but not least, the biggest thank you goes to my family and especially my boyfriend, Marc Schmid, who experienced all the ups and downs and always encouraged and supported me with great patience and understanding.