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REGULATION OF HEPATIC GLUCOSE HOMEOSTASIS AND CYTOCHROME P450 ENZYMES BY ENERGY-SENSING COACTIVATOR PGC-1α

UNIVERSITY OF OULU GRADUATE SCHOOL;
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MEDICAL RESEARCH CENTER OULU

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REGULATION OF HEPATIC GLUCOSE HOMEOSTASIS AND CYTOCHROME P450 ENZYMES BY ENERGY-SENSING COACTIVATOR PGC-1α

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium A101 of the Department of Anatomy and Cell Biology (Aapistie 7 A), on 22 May 2015, at 12 noon

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Abstract

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a master regulator of energy metabolism and mitochondrial biology in high-energy cell types and tissues. The regulation of PGC-1α is versatile, and both transcriptional and post-transcriptional mechanisms play major roles. External stimuli affect PGC-1α-regulation which in turn adapts cellular signals to meet them. For example, conditions like fasting and diabetes mellitus (DM) are known to activate PGC-1α expression in the liver, resulting in enhanced *de novo* glucose production, gluconeogenesis.

In the present study, the mechanisms of hepatic PGC-1α regulation and PGC-1α-regulated functions were elucidated. We found that PGC-1α was induced by oral type 2 diabetes therapeutic metformin, via AMPK and SIRT1, regulating the mitochondrial gene response, against previous assumptions. Simultaneously, gluconeogenesis was repressed by other means. Furthermore, PGC-1α upregulated the anti-inflammatory interleukin 1 receptor antagonist (IL1Rn). PGC-1α also diminished interleukin 1β-mediated inflammatory response in hepatocytes.

Novel, xenobiotic and endobiotic metabolizing Cytochrome P450 enzymes regulated by PGC-1α were also identified in this thesis. CYP2A5 was induced by PGC-1α through hepatocyte nuclear factor 4α (HNF-4α) coactivation. Also, vitamin D metabolizing CYP2R1 and CYP24A1 were identified as novel genes regulated by PGC-1α, suggesting a role for PGC-1α in the regulation of active vitamin D levels.

The findings presented in this thesis provide insight into the pathology of glucose perturbations such as type 2 diabetes, and stimulate discovery of therapeutic agents to treat this disease. Furthermore, the findings suggest that vitamin D metabolism and energy metabolism are tightly linked, with PGC-1α emerging as a novel mediator.

Keywords: AMPK, CYP24A1, CYP2A5, CYP2R1, diabetes, fasting, IL1Rn, PGC-1α, SIRT1, vitamin D

Aatsinki, Sanna-Mari, Koaktivaattori PGC-1α maksan glukoositasapainon ja sytokromi P450 -entsyymien säätelijänä.
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Tiivistelmä

Peroksisomiproliferaattori-aktivoituvan reseptori γ:n koaktivaattori 1α (PGC-1α) on merkittävä glukoosiaineenvaihdunnan ja mitokondrioiden toiminnan säätelijä korkeaenergisissä soluissa ja kudoksissa. PGC-1α:a säädellään monin tavoin: sekä transkriptionaalisella säätelyllä että transkription jälkeisellä muokkauksella on merkittävä rooli. Monet ulkoiset tekijät säätelevät PGC-1α:n aktiivisuutta, joka puolestaan säätelee solunsisäisiä signaalireittejä vastaamaan tähän signaaliin. Esimerkiksi paasto ja diabetes mellitus (DM) ovat fysiologisia tiloja, jotka lisäävät voimakkaasti PGC-1α:n ilmentymistä maksassa, jolloin glukoosin uudistuotanto eli glukoneogeneesi kiihtyy.

Tässä väitöskirjassa tutkittiin PGC-1α:n säätelyä sekä PGC-1α -säädeltyjä signaalireittejä maksassa. Osoitimme, että tyypin 2 diabeteslääke metformiini indusoi PGC-1α:n ilmentymistä maksassa, vastoin aikaisempia käsityksiä. PGC-1α indusoitui AMPK:n ja SIRT1:n välityksellä, säädelleen edelleen mitokondriaalisten geenien aktiivisuutta. Samalla glukoneogeneesi kuitenkin repressoitui muilla mekanismeilla. Lisäksi osoitimme, että PGC-1α indusoi tulehdusreaktiota vaimentavaa interleukiini 1 reseptorin antagonistia (IL1Rn). PGC-1α esti interleukiini 1β:n aiheuttamaa tulehdusvastetta hepatosyyteissä.

Lisäksi väitöskirjassa tunnistettiin uusia, PGC-1α -säädeltyjä lääkeaineita ja elimistön sisäisiä yhdisteitä metaboloivia sytokromi P450 -entsyymejä (CYP). Hiiren CYP2A5:n ilmentymisen osoitettiin olevan PGC-1α- ja HNF4α-välitteistä. Lisäksi osoitettiin, että D-vitamiinia metaboloivat CYP2R1 ja CYP24A1 ovat uusia PGC-1α -säädeltyjä geenejä. Tämä löydös viittaa siihen, että PGC-1α:lla on rooli aktiivisen D-vitamiinin säätelyssä.

Tämän väitöskirjan löydökset lisäävät tietoa glukoosiaineenvaihdunnan häiriöiden kuten tyypin 2 diabeteksen molekulaarisista mekanismeista, joita voidaan hyödyntää mahdollisten uusien lääkeaineiden kehittämisessä. Lisäksi väitöskirjassa osoitettiin, että D-vitamiinimetabolia on kytköksissä energia-aineenvaihduntaan ja että PGC-1α:lla on tässä rooli, jota ei aiemmin ole tunnettu.

Asiasanat: AMPK, CYP24A1, CYP2A5, CYP2R1, D-vitamiini, diabetes, IL1Rn, paasto, PGC-1α, SIRT1

To Antti and Pinja

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Oulu, April 2015 Sanna-Mari Aatsinki

List of abbreviations

Ad	adenovirus
AHR	aryl hydrocarbon receptor
AICAR	aminoimidazole carboxamide ribofuranoside
ALAS	aminolevulinate synthase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ARNT	AHR nuclear translocator
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
ATP5B	ATP synthase subunit β
BAT	brown adipose tissue
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic AMP
CAR	constitutive androstane receptor
CnA	calcineurin
COX	cytochrome c oxidase
CREB	cAMP response element binding protein
CRTC2	CREB regulated transcription coactivator 2
CYCS	cytochrome c
CYP	Cytochrome P450
DBD	DNA binding domain
ER	estrogen receptor
ERRα	estrogen related receptor α
FOXO	forkhead box O
FXR	farnesoid X receptor
G6Pase	glucose-6-phosphatase
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase 3β
HAT	histone acetyl transferase
HDAC	histone deacetylase
HNF	hepatocyte nuclear factor
IL	interleukin
IL1Rn	interleukin 1 receptor antagonist
KO	knockout
LBD	ligand binding domain

LKB1	liver kinase 1
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
mRNA	messenger ribonucleic acid
NR	nuclear receptor
NRF	nuclear respiratory factor
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	PPAR γ coactivator 1α
PKA	protein kinase A
PKB/Akt	protein kinase B
Pol II	RNA polymerase II
PPAR	peroxisome proliferator-activated receptor
PRC	PGC-related coactivator
PRMT1	protein arginine N-methyltransferase
PXR	pregnane X receptor
qPCR	quantitative PCR
RE	response element
ROS	reactive oxygen species
RXR	retinoid X receptor
SIRT	Sirtuin, silent information regulator T
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TSS	transcription start site
UCP	uncoupling protein
VDR	vitamin D receptor
WAT	white adipose tissue
WT	wild type

List of original articles

This thesis is based on the following articles which are referred to in the text by the Roman numerals I to IV.

I Aatsinki SM, Buler M, Salomäki H, Koulu M, Pavek P and Hakkola J (2014) Metformin induces PGC-1α expression and selectively affects hepatic PGC-1α functions. Br J Pharmacol 171(9) 2351-63.

II Buler M*, Aatsinki SM*, Skoumal R, Komka Z, Tóth M, Kerkelä R, Georgiadi A, Kersten S & Hakkola J (2012) Energy sensing factors coactivator Peroxisome Proliferator-activated Receptor γ Coactivator 1-α (PGC-1α) and AMP-activated protein kinase control expression of inflammatory mediators in liver: induction of Interleukin 1 receptor antagonist. J Biol Chem 287(3):1847-60.

III Arpiainen S, Järvenpää SM, Manninen A, Viitala P, Lang MA, Pelkonen O and Hakkola J (2008) Coactivator PGC-1α regulates the fasting inducible xenobiotic-metabolizing enzyme CYP2A5 in mouse primary hepatocytes. Toxicol Appl Pharmacol 232(1):135-41.

IV Aatsinki SM, Elkhwanky M, Buler M, Viitala P, Mutikainen M, Tavi P, Kersten S, Herzig KH and Hakkola J. Energy-sensing coactivator PGC-1α regulates novel mouse hepatic CYP genes including vitamin D metabolizing CYP2R1 and CYP24A1. Manuscript.

* equal contribution

Contents

1 Introduction

The energy sensing peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a gene expression activator with functions in glucose homeostasis as well as numerous other pathways. It is expressed in high-energy tissues with a central role in mitochondrial function as well as tissue-specific tasks, such as adaptive thermogenesis in brown adipose tissue, adaptation to endurance training in skeletal muscle and *de novo* glucose synthesis from the liver in fasted state (Finck & Kelly 2006, Liu & Lin 2011, Yoon *et al.* 2001). PGC-1α, being a coactivator of the nuclear receptor superfamily, also regulates the Cytochrome P450 (CYP) enzyme family members involved in xenobiotic and endobiotic metabolism (Grasfeder *et al.* 2009, Itoh *et al.* 2006, Martinez-Jimenez *et al.* 2006, Shin & Osborne 2008).

The metabolic syndrome, manifesting as diseases such as type 2 diabetes (T2DM) and obesity, is a complex disease whose pathogenesis is only partly elucidated. Particularly the mechanisms at the very early stages have only recently begun to be understood. Improving patients' quality of life and discovering potential, more specific therapeutics is the ultimate goal, only possible if the cellular and molecular mechanisms are completely unraveled. Although T2DM affects all major tissues in the body, the liver is central due to its ability to both store and produce glucose in fed and fasted state (Israili 2011). Thus, elucidation of the mechanisms behind the pathways regulating glucose balance in the liver is utterly important. The role of PGC-1α in the mechanism of the metabolic syndrome has not been completely characterized and was the main object of this thesis.

Metformin, widely used as an oral T2DM therapeutic (He & Wondisford 2015, Wiernsperger & Bailey 1999), is an old drug, but the mechanism of action has remained obscure, at least until very recently. Several mechanistic reports outline AMPK, another cellular energy sensor, as the key target for metformin action, resulting in repression of glucose output through repression of gluconeogenic genes (Koo *et al.* 2005a, Zhou *et al.* 2001). Interestingly, while it has been known that metformin activates AMPK and PGC-1α in skeletal muscle (Suwa *et al.* 2006), their interactions in the liver have not been completely understood.

On the other hand, the role of PGC-1α can also be extended to the regulation of the drug- as well as endobiotic-metabolizing CYP enzymes. The transcriptional regulation of the CYPs in response to xenobiotics such as clinically used drugs is

well established (Pelkonen *et al.* 2008, Zanger & Schwab 2013), but response to physiological status, such as glucose balance, is not well understood. PGC-1α may serve as a scaffold molecule by sensing the glucose status and regulating the necessary, CYP-regulated pathways.

One of the goals of this thesis was to investigate the role of PGC-1α in mechanisms involved in T2DM. By unraveling mechanisms that regulate PGC-1α and pathways that are under the control of PGC-1α, it is possible to specifically target these pathways. In the future, more specific drugs may also be developed by exploring metformin's hepatic mechanism of action. Also, recognizing PGC-1α:s role as a mediator of anti-inflammatory response provides potential to treat chronic inflammation, typically manifested in T2DM patients.

On the other hand, unraveling the PGC-1α-mediated regulation of the xenobiotic and endobiotic metabolizing CYP enzymes may explain how drug metabolism and energy metabolism interact.

2 Review of the literature

2.1 PGC-1α and PGC-1 coactivators

Transcriptional regulation is a crucial step in constructing a functional protein in the human body. It exploits our heritable DNA, genome, as template material. Transcription factors (TFs) are important mediators of transcriptional activity. Usually, equally important are the following post-transcriptional events that further remodel the messenger RNA (mRNA) produced, the peptide produced and the final protein to become fully functional.

Coactivators are proteins that belong to the group of transcriptional coregulators. They regulate and fine-tune mammalian gene expression but are not transcription factors *per se*. Rather, coregulators are transcription factor interaction partners that alter the DNA structure and either stimulate (coactivator) or repress (corepressor) the transcription of target genes, allowing layers of hierarchy to the event of gene expression. Coregulators' role in human physiology as well as diseases has become increasingly evident during the past few decades (Lonard & O'Malley 2012).

Coactivators, in this context known as nuclear receptor (NR) coactivators, include a wide range of proteins involved in mammalian gene upregulation. The majority of them assemble with transcription factors to target gene promoter regions and interact with basal transcription machinery, thus enhancing gene transcription. Alternatively, coactivators may induce histone protein modifications or cause chromatin remodeling which also allows facilitated gene expression. Some coactivators possess enzymatic activity and perform e.g. phosphorylation, acetylation or methylation events. Roughly, coactivators are divided into four main groups: molecular chaperones that direct and modulate NR trafficking (heat-shock proteins, HSPs), histone modifiers (histone acetyl transferases, HATs), basal transcription machinery recruiters (TRAP/DRIP) and chromatin remodelers (SWI/SNF). Coactivators that act as scaffold proteins to recruit other proteins to form activator protein complexes form an additional class. These coactivators do not possess enzyme activity properties and may be unique in tissue distribution (Wolf *et al.* 2008).

Peroxisome proliferator activated receptor (PPAR) γ coactivator 1 α (PGC-1α) was the first member discovered among the family of PGC-1 coactivators, which also includes PGC-1β and PGC-related coactivator (PRC). All of these

proteins show some similarity in structure and function, being regulators of several metabolic and mitochondrial respiratory pathways. (Liu & Lin 2011). PGC-1β shares high sequence and domain structure homology with PGC-1α, having also partially overlapping functions and tissue distribution (Lin *et al.* 2003) PRC, however, is expressed ubiquitously and has more limited sequence similarity to PGC-1α. It is also somewhat differently regulated but shares similar functional properties with PGC-1α (Andersson & Scarpulla 2001). The main focus of the current review is related to functions of PGC-1α, which is by far the most widely studied protein of the PGC-1 coactivator family.

PGC-1α is a transcriptional coactivator involved in the control of cellular energy homeostasis including fatty acid, carbohydrate and oxidative metabolism in various cell types. PGC-1α was originally discovered from murine brown adipose tissue as a coactivator of PPARγ, regulating thermogenesis when exposed to cold conditions (Puigserver *et al.* 1998). The diversity of the PGC-1α protein became rapidly apparent; it is expressed in various tissues and is related to multiple functions in the cell. The notion that PGC-1α can be regulated at various levels, transcriptionally and post-transcriptionally, by external stimuli is of particular interest compared to other ubiquitous coactivators (Lin *et al.* 2005). The role of PGC-1α in high-energy tissues like brown adipose tissue as well as liver, brain, skeletal muscle, heart and pancreas, is now well established. In addition to tissue-specific functions, PGC-1α is a master regulator of mitochondrial biogenesis and respiration, reactive oxygen species (ROS) compensation mechanisms as well as circadian rhythms by regulating the mammalian clock genes (Liu *et al.* 2007, Liu & Lin 2011).

2.1.1 Structure

PGC-1α gene is located on chromosome 4 in humans and 5 in mice. The gene encodes a protein of 798 and 797 amino acids in humans and mice, respectively, with a predicted size of 91 kDa of the full-length protein. The human protein shows 94-95% amino acid sequence similarity to the mouse ortholog (Esterbauer *et al.* 1999, Larrouy *et al.* 1999, Puigserver *et al.* 1998). The protein is located in the nucleus, being short-lived; the reported half-life is about two hours (Puigserver *et al.* 2001), and it is degraded by the ubiquitin-proteasome pathway (Sano *et al.* 2007). Indeed, phosphothreonine-containing motifs, so-called Cdc4-phosphodegron (CPD) motifs within PGC-1α were shown to be responsible for targeting the protein to ubiquitinylation and proteasomal degradation through

phosphorylation by glycogen synthase 3β (GSK3β) and p38 kinase and subsequent activation of Cdc4 (Olson *et al.* 2008).

Recent studies indicate the existence of multiple PGC-1α isoforms. The full-length (FL), canonical PGC-1α (PGC-1α1) contains 13 exons. N-terminal truncated (NT) PGC-1α was characterized as the second form and unlike the nuclear full-length form, to be predominantly present in cytoplasm. NT-PGC-1α is formed of amino acids 1-270 and was reported to retain and transactivate nuclear receptors by the existing aminoterminal transactivation domain. It was also described as relatively stable (Chang *et al.* 2010). A human liver-specific isoform termed L-PGC-1α has also been described. It possesses deletion of 127 N-terminal amino acids but is otherwise similar to canonical PGC-1α. L-PGC-1α seems functional in terms of inducibility and regulation of target genes and may serve as additional compensation for human hepatic PGC-1α activity (Felder *et al.* 2011). Two promoter areas were found to be regulating the *Pgc-1α* gene, one directly 5' upstream of exon 1 (proximal) and another about 13 kb upstream (distal); this may offer an explanation for the isoform-specific regulation of PGC-1α (Chinsomboon *et al.* 2009, Yoshioka *et al.* 2009). Indeed, three isoforms that are regulated through the distal promoter region have been characterized, named PGC-1α2, PGC-1α3 and PGC-1α4, encoding proteins of 41.9, 41.0 and 29.1 kDa, respectively. These isoforms were shown to be present in relevant amounts in skeletal muscle, heart and BAT whereas the canonical PGC-1α was shown to dominate in the liver. PGC-1α4 resembling the NT-PGC-1α was shown to be induced by resistance training and to specifically upregulate genes involved in muscle hypertrophy (Ruas *et al.* 2012).

PGC-1α itself is not a protein with direct enzymatic activity or capability of binding to DNA. Instead, by recruitment and docking of other coactivators and transcription factors to the promoters of target genes, PGC-1α is indeed a powerful activator of transcription (Finck & Kelly 2006, Lin *et al.* 2005).

The amino-terminal (N-terminal) part of PGC-1α, a region of about 200 amino acids, contains a so-called activation domain (AD) responsible for binding of additional coactivator proteins, e.g. HAT proteins p300, 3'-5'-cyclic adenosine monophosphate (cAMP) response element-binding protein (CBP) and steroid receptor coactivator 1 (SRC-1). They possess the ability to remodel chromatin structure, allowing gene activation by increasing the accessibility of the transcriptional machinery to the specific site. It seems that AD is involved in docking and association of the preinitiation form of transcription machinery (Monsalve *et al.* 2000). Next to the activation domain is a regulatory area also

known as suppression domain spanning a region of about 200 amino acids (aa 170-350). The suppressive effects are relieved by the binding of certain transcription factors such as PPARγ and NRF-1 in this area (Puigserver *et al.* 1999). Also, so-called LXXLL motifs (where L is leucine and X any other amino acid), three in total, which are indispensable for interaction with nuclear receptors, are located at the N-terminal part, L1 and L2 within the AD and L3 within the suppression domain of PGC-1α (Gaillard *et al.* 2006, Huss *et al.* 2002, Liu & Lin 2011).

The carboxy-terminus (C-terminus) of PGC-1α contains sequences for proteins capable of interacting with the general transcription machinery. RNA polymerase II and the thyroid hormone associated protein/vitamin D receptor-interacting protein (TRAP/DRIP, mediator) are such proteins and have been characterized to interact with PGC-1α and to facilitate transcription (Wallberg *et al.* 2003). Along with serine-arginine (SR/RS) rich domains, an RNA recognition motif (RRM) as well as RNA binding and splicing domains are also located at the C-terminal part of PGC-1α This region is capable of RNA processing through interaction with splicing factors, implying a role for PGC-1α also in mRNA variant processing of target genes (Monsalve *et al.* 2000).

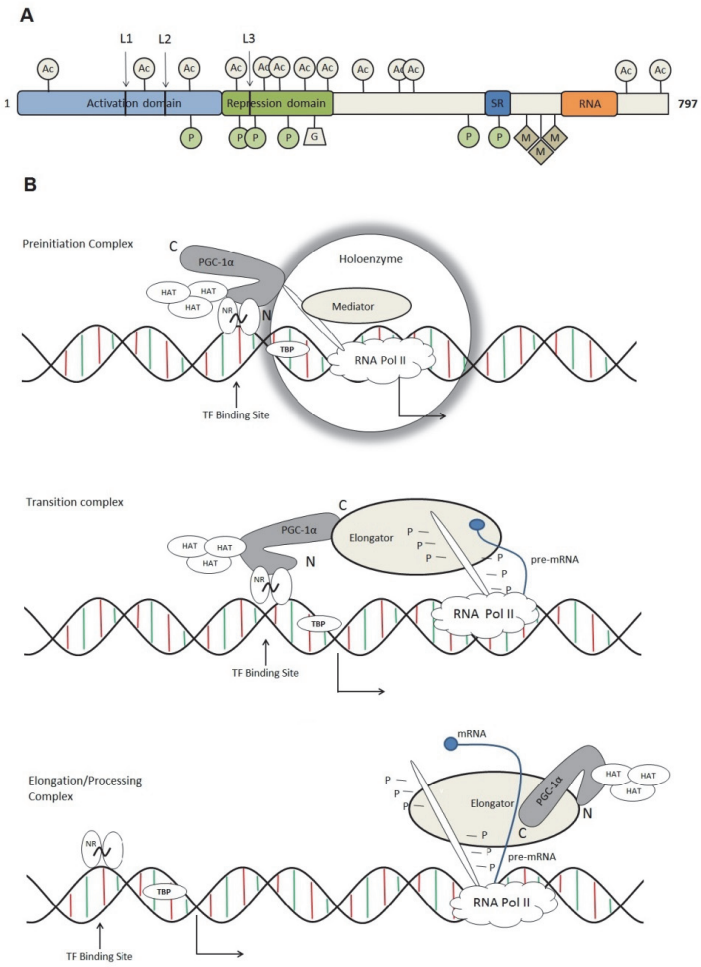

Fig. 1. A. Structure of canonical PGC-1α protein and B. Involvement in initiation of transcription. PGC-1α assembles with transcription factors & coactivators and associates with the preinititation complex. When transcription starts, the C-terminal domain of RNA Pol II becomes phosphorylated. Proteins including PGC-1α associate with it through the C-terminus. The elongation complex that carries PGC-1α coordinately regulates mRNA processing. Ac, acetylation; G, N-acetylglucosamine; L1, L2, L3, LXXLL motifs; M, methylation; P, phosphorylation; SR, serine-arginine-rich domain; RNA, RNA-binding domain; N, N-terminus; C, C-terminus; HAT, histone acetyltransferase; NR, nuclear receptor; RNA Pol II, RNA polymerase II, TBP, TATA binding protein; TF, transcription factor (modified from Canto & Auwerx 2010 & Monsalve 2000).

2.1.2 Regulation of transcription function

The transcriptional regulation of a gene involves multiple steps, such as epigenetic events, along with transcription factor and coregulator docking together with the recruitment of the general transcriptional machinery. As mentioned, there is also need for certain post-transcriptional modifications before the functional protein in that specific cell is produced. These events include various RNA processing methods by so-called RNA-binding proteins, miRNAs affecting the translational efficiency and various post-translational modifications of the protein (Martinez-Salas *et al.* 2013, Schrem *et al.* 2002).

The epigenetic mechanisms are the fundamental basis for gene activation comprising reversible DNA-histone modifications, also known as chromatin modifications, such as acetylations, phosphorylations, ubiquitinations and methylations. The best-known mechanism is acetylation of histone proteins by HATs, allowing the tightly packed chromatin to loosen for the gene activation. The opposite action, deacetylation, is performed by histone deacetylaces (HDACs). The suppression and silencing of gene activity is associated with DNA methylation performed by DNA methyltransferases (DNMTs). DNMTs are able to add methyl groups in cytosine base residues adjacent to guanine bases forming so-called CpG islands at the DNA sequence. The DNMT activity is crucial in the developing embryo but later on it is more restricted (Snykers *et al.* 2009).

A highly regulated step of gene expression is the binding of *trans*-acting transcription factors to the target gene response elements, usually located directly 5' upstream of the transcriptional starting site (TSS). These promoter regions are the spots for sophisticated and fine-tuned regulatory actions involving the binding of transcription factors, additional coregulators such as coactivators and corepressors as well as the complex of transcriptional machinery and accessory proteins. The promoter regions can be subjected to epigenetics as well; methylation of CpG islands usually silences the gene irreversibly and can be inherited to daughter cells (Van den Veyver 2002).

PGC-1α and nuclear receptors

The nuclear receptors (NR) also known as nuclear hormone receptors, are a superfamily of transcription factors involved in various hormone and xenobiotic responses. The NRs regulate several developmental as well as metabolic processes and even novel roles yet to be discovered. Their activation is often

initiated by specific ligands which drive the NR cytoplasmic localization to the nucleus. The classical steroid receptors are classified as class I NRs and typically have ligands such as estrogens, glucocorticoids or androgens. Class II receptors are often retinoid X receptor (RXR) heterodimerization partners and are located either in cytoplasm or nucleus upon ligand activation by nonsteroid molecules such as thyroid hormone, bile acids, vitamins and retinoic acid. However, a large group of 'orphan' NRs are known with no clearly characterized ligand, making them constitutively expressed, and these are classified into group III. These NRs can act as homodimers or heterodimers with RXR. Ligands for orphan NRs are continuously being studied and discovered (Burris *et al.* 2013, Mangelsdorf *et al.* 1995).

The structure of NRs is highly conserved among the family members. The ability of NRs to bind to certain promoter regions lie within their DNA-binding domain (DBD) features. NRs are known to bind to direct (DR), inverted (IR), everted (ER) or non-repeat (NR) consensus sequences spaced by a differing number of nucleotides at the response element of the target gene, and this signature is relatively specific for different NRs (Mangelsdorf *et al.* 1995).

PGC-1α acts as a coactivator for a number of transcription factors, including multiple NR family members. It is well established that coactivators and nuclear receptors form dynamic and multifunctional complexes adding regulatory intricacy to mammalian gene expression. The transcriptional activation domain – the region in the NR that interacts with coactivators - is located at the C-terminal part of the protein, named activation function 2 (AF-2) region. Especially in class I NRs, there is also an N-terminal transcriptional activation domain, AF-1 region. The ligand-binding domain (LBD) is located at the C-terminus, within the AF-2. Thus, ligand binding induces a conformational change at the LBD and subsequently transactivates the AF-2 region to interact with coactivators (Edwards 2000).

Like many other coactivators, also PGC-1α contains conserved leucine-rich domains known as LXXLL (L: leucine, X: any other amino acid) motifs, three in total (L1,L2, L3), of which at least L2 and L3 are indispensable for the interaction with NRs (Gaillard *et al.* 2006, Heery *et al.* 1997, Puigserver *et al.* 1998). Actually, one motif may be bound to NR but produces minimal transactivation power. The other two motifs have synergistic effect on overall transactivation of the PGC-1α complex (Rha *et al.* 2009). Indeed, mutation of the leucines from L2 and L3 results in the inability of PGC-1α to transactivate nuclear receptors (Gaillard *et al.* 2006).

Hepatocyte nuclear factor 4α (HNF-4α) (Yoon *et al.* 2001), pregnane X receptor (PXR) (Bhalla *et al.* 2004), liver X receptor (LXR) (Oberkofler *et al.* 2003) estrogen receptors α and β (ERα,β) (Kressler *et al.* 2002), estrogen-related receptors α, β and γ (ERα,β,γ) (Huss *et al.* 2002, Kamei *et al.* 2003) and PPARα,β/δ,γ (Puigserver *et al.* 1998, Vega *et al.* 2000, Wang *et al.* 2003) are some of the known NR interaction partners of PGC-1α. The complete list of nuclear receptors as well as other transcription factors coactivated by PGC-1α is depicted in Table 1 and discussed in more detail in the context of target tissue.

Table 1. Nuclear receptor and other transcription factor interaction partners of PGC-1α (modified from Lin et al. 2005).

Transcription factor	Nuclear receptor	Function	Tissue	Reference
NRF-1	no	mitochondrial genes	ubiquitous	Wu *et al.* 1999
NRF-2	no	mitochondrial genes	ubiquitous	Mootha *et al.* 2003
PPARα	yes	fatty-acid oxidation		Vega *et al.* 2000
PPARβ/δ	yes	fatty-acid oxidation		Wang *et al.* 2003
PPARγ	yes	UCP1/GyK induction		Guan *et al.* 2005; Puigserver *et al.* 1998
ERRα,β,γ	yes	mitochondrial genes	ubiquitous	Huss *et al.*, 2002; Huss *et al.* 2004, Kamei *et al.* 2003, Mootha *et al.* 2004, Schreiber *et al.* 2004
TRβ	yes	cpt-1 induction		Zhang *et al.* 2004
LXRα,β	yes	lipoprotein secretion		Lin *et al.* 2005, Oberkofler *et al.* 2003
FXR	yes	triglyceride metabolism		Zhang *et al.* 2004a
GR	yes	gluconeogenesis	liver	Yoon *et al.* 2001
ERα,β	yes	estrogen signaling		Tcherepanova *et al.* 2000
PXR	yes	xenobiotic metabolism		Bhalla *et al.* 2004
Sox9	no	chondrogenesis		Kawakami *et al.* 2005
MEF2	no	slow fiber genes	muscle, heart	Lin *et al.* 2002b, Michael *et al.* 2001
FOXO1	no	gluconeogenesis	liver	Puigserver *et al.* 2003
HNF4α	yes	gluconeogenesis	liver	Lin *et al.* 2002a, Yoon *et al.* 2001
VDR	yes	VDR target genes	intestine	Savkur *et al.* 2005
NF-k-B	no	repression of glucose oxidation	heart	Alvarez-Guarrdia *et al.* 2010
YY1	no	mitochondrial function	skeletal muscle	Cunningham *et al.* 2007
ROR	yes	clock gene regulation	liver, skeletal muscle	Liu *et al.* 2007
SF-1	no	steroidogenesis	pituitary	Zhu *et al.* 2010
LRH-1	yes	bile acid biosynthesis	liver	Shin *et al.* 2003, Shin & Osborne 2008

PGC-1α-mediated negative gene regulation

Lately, some reports have emerged introducing the repression of gene regulation or selective targets by PGC-1α. These mechanisms are still mostly unknown, but may involve protein-protein interactions such as competition with transcription factor binding or induction of certain repressor proteins by PGC-1α.

According to a recent study, in skeletal muscle PGC-1α downregulates the NF-kβ mediated transcriptional activity. It was proposed that PGC-1α causes dephosphorylation of the p65 subunit, thus hindering activity of NF-kβ. However, the full mechanism was not established although inhibition of upstream Akt kinase was shown to be involved. Corepressor interactions were postulated to be involved, i.e., induced corepressors may bind to the dephosphorylated p65 and suppress its actions. Alternatively, PPARα was suggested to be induced by PGC-1α and to induce corepressor binding through conjugation by SUMOylation. Repression of target gene activity by this mechanism is called nuclear receptor transrepression (Eisele *et al.* 2013, Ghisletti *et al.* 2007).

There are several studies describing phosphorylation-dependent PGC-1α inhibitory actions; these are discussed in the context of post-transcriptional regulation mechanisms.

2.1.3 Role in mitochondrial biology and ROS

Mitochondrial biogenesis

The mitochondrion can consume huge amounts of fuel (lipids, glucose) and generate robustly ATP, which is the ultimate energy equivalent for the cell in various chemical reactions. The role of functioning mitochondria is crucial; defects in their respiratory capacity leads to diseases such as diabetes, heart failure and neurodegeneration (Scarpulla *et al.* 2012, Wu *et al.* 2014). The PGC-1 coactivators and PGC-1α in particular control almost all aspects of mitochondrial function (Patten & Arany 2012).

The tissue distribution of PGC-1α and tissues with high mitochondrial content are tightly overlapping, implying the dependence on PGC-1α in the control of mitochondrial biology. The importance of PGC-1α in mitochondrial biogenesis is well established; more detailed mechanisms are reviewed (Scarpulla *et al.* 2012) and summarized here.

It was shown already in the first paper describing PGC-1α that this coactivator is capable of increasing the expression of mitochondrially encoded cytochrome C oxidase subunit II (*COXII* or *COX2*) as well as nuclearly encoded cytochrome C oxidase subunit IV (*COXIV* or *COX4*), genes affecting the respiratory function of mitochondria (Puigserver *et al.* 1998). Soon after this it was shown that PGC-1α induces the expression of nuclear respiratory factors 1 (NRF-1) and 2 (NRF-2), acting also as the coactivator of these transcription factors (Wu *et al.* 1999). The NRFs are powerful activators of the mitochondrial genes, including mitochondrial transcription factor A (mtTFA), which is responsible for the transcriptional activation of virtually all gene expression in mitochondria (Virbasius & Scarpulla 1994). The deletion of NRF-1 results in peri-implantation lethality in mice (Huo & Scarpulla 2001). Cells expressing PGC-1α increase the mitochondrial density, i.e., the mitochondrial number compared to the cytoplasmic area, 57% more than cells not expressing PGC-1α. Also mitochondrial DNA content is induced by PGC-1α, through NRF-1 induction (Wu *et al.* 1999). PGC-1α thus acts as a signaling link between signs of stress, e.g. exercise, and the cellular adaptation in the form of mitochondrial biogenesis.

Later it was shown that expression of an orphan NR, estrogen-related receptor α (ERRα), is induced by PGC-1α. Firstly, the transcriptional activity of ERRα is induced and secondly, the activity of ERRα to regulate target genes is activated by PGC-1α, in a "protein" ligand-dependent manner (Schreiber *et al.* 2003). ERRα was then linked to powerful regulation of mitochondrial biogenesis together with PGC-1α, acting in parallel with and perhaps independently of the NRFs in different tissues (Schreiber *et al.* 2004).

The role of PGC-1α also extends to induction of the uncoupling proteins (UCPs), as introduced in chapter 2.1.4, PGC-1α in brown adipose tissue. This phenomenon is, however, closely related to mitochondrial function since UCPs are the uncouplers of ATP synthesis in the mitochondrial membrane.

β-oxidation

Mitochondria are also responsible for the breakdown of fatty acids from the diet, i.e., fatty acid β-oxidation (FAO), which is the main energy producing pathway in the adult heart. PPARα is a key NR in cellular lipid and energy metabolism. It is known to regulate not only the peroxisomal but also the mitochondrial FAO

pathway. Indeed, the majority of the known target genes of PPARα are involved in the FAO (Madrazo & Kelly 2008).

The induction of PGC-1α in cells also induces PPARα activity along with its target genes, muscle carnitine palmitoyltransferase (M-CPT I) and medium-chain acyl-CoA dehydrogenase (MCAD), major enzymes catalyzing the steps of the FAO pathway. Furthermore, oleic acid, a ligand of PPARα, produced additional activation of PPARα response elements at target gene promoters. In summary, it was established that PGC-1α coactivates PPARα and is thus a powerful regulator of the β-oxidation pathway (Lehman *et al.* 2000, Vega *et al.* 2000).

ROS

Reactive oxygen species (ROS) are molecules containing chemically active oxygen radicals, such as superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), produced naturally by the mitochondrial oxidative respiratory chain (OXPHOS). When the respiratory chain is somehow defective, e.g. by drugs inhibiting it, ATP production is diminished and ROS is generated in large amounts due to leaking of electrons and incomplete O_2 reduction. If the excessive ROS is not scavenged it causes cellular damage through the oxidative stress phenomenon. It has been proposed that for example H_2O_2, a known oxygen radical, can activate signaling pathways that regulate mitogenesis, proliferation, cell survival and death responses, by diffusion across mitochondrial membrane. ROS can have direct effects on DNA as well (Balaban *et al.* 2005, Wu *et al.* 2014).

However, there are several mechanisms that can scavenge cellular ROS resulting in minimizing their detrimental effect. These include antioxidant activities by proteins such as superoxide dismutases, thioredoxins, glutaredoxins and glutathione peroxidases, the latter two of which require glutathione as cofactor (Balaban *et al.* 2005). It has been proposed that ROS originate primarily from two sites in the mitochondrial respiratory chain: Complex I (NADH dehydrogenase) and Complex III (Craigen 2012). The defective OXPHOS and ROS induce several signaling pathways that act to maintain cellular homeostasis. H_2O_2, Ca^{2+}, ADP/ATP and NAD^+/NADH ratios act as second messengers to turn on certain pathways.

PGC-1α has been linked to several studies describing that in response to ROS, PGC-1α is activated (Borniquel *et al.* 2006, St Pierre *et al.* 2006, Valle *et al.* 2005). It has been suggested that PGC-1α is a compensation mechanism to scavenge ROS by upregulating ROS-detoxifying proteins like SOD1, SOD2,

GpX and catalase. Also, in PGC-1α null mice, the activation of these genes was diminished in response to H_2O_2. In addition, ROS levels were upregulated in the null mice, suggesting a role of PGC-1α in scavenging (St Pierre *et al.* 2006). Furthermore, it was shown that the Sirtuin 3 (SIRT3) deacetylase was induced by PGC-1α and ERRα in response to ROS, being an additional regulator of ROS scavenging (Kong *et al.* 2010).

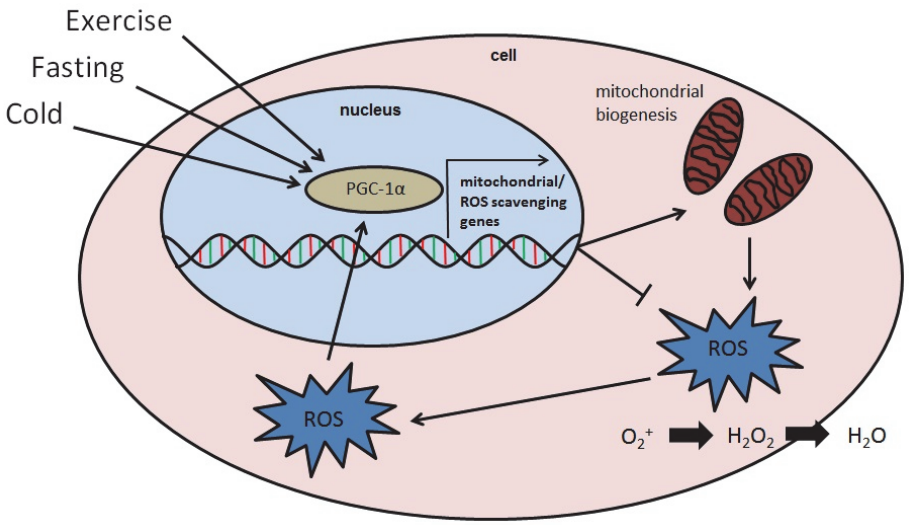

Fig. 2. Regulation of ROS mediated by PGC-1α in the cell (Modified from St-Pierre *et al.* 2006)

2.1.4 Tissue-specific functions

Liver

The liver is a major organ in mammals in terms of size, about 1.5 kg in humans, as well as versatility of functions. It is responsible for e.g. carbohydrate, lipid and bile acid metabolism, xenobiotic catabolism, storing of vitamins and metals as well as synthesis and breakdown of plasma proteins. The central role of the liver in glucose metabolism is apparent from the fact that the liver can both store and release glucose, in addition to which it can endogenously produce glucose, a

unique feature of this organ compared to any other organ in the human body (Baynes & Dominiczak 1999).

PGC-1α is expressed in high-energy tissues, including the liver. In normal healthy conditions, the expression may be low to moderate (Puigserver *et al.* 1998). However, fasting and uncontrolled type 1 and 2 diabetes are conditions known to induce the levels of hepatic PGC-1α (Yoon *et al.* 2001). The fasting response involves numerous molecular events to enable the organism to adapt to nutritional deprivation with rising glucagon levels. *De novo* glucose synthesis (gluconeogenesis), fatty-acid catabolism (β-oxidation), ketone body and bile acid synthesis are major events requiring metabolic changes in the cell (Hardie 2012, Ramnanan *et al.* 2011) and are controlled at the molecular level, in many aspects by PGC-1α.

Indeed, PGC-1α is one of the proteins involved in the regulation of glucose homeostasis in the liver. PGC-1α activates hepatic gluconeogenesis in the fasted state by activating the rate-limiting enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Yoon *et al.* 2001). Fasting causes rapid activation of the cyclic AMP signaling cascade via glucagon and catecholamine stimulus. Furthermore, protein kinase A is activated and subsequently phosphorylates and activates cAMP response element binding (CREB) protein, a transcription factor able to bind to the cAMP responsive elements (CRE) of target genes including *PGC-1α* and *PEPCK*. Although PEPCK is activated in the early stages of fasting through CREB, independent of PGC-1α, at a later stage PGC-1α is needed to maintain gluconeogenesis activated through glucocorticoid receptor (GR) and HNF-4α binding (Herzig *et al.* 2001). Actually, HNF-4α was shown to be absolutely required for the PGC-1α-mediated induction of both PEPCK and G6Pase (Rhee *et al.* 2003). Also transcription factor Forkhead box class-O 1 (FOXO1) has been shown to regulate at least G6Pase coactivated by PGC-1α, negatively regulated by insulin through phosphorylation by Akt kinase (Puigserver *et al.* 2003).

In humans, PGC-1α genetic mutations, polymorphisms, have been shown to cause onset of type 2 diabetes and glucose impairment (Ek *et al.* 2001, Hara *et al.* 2002, Oberkofler *et al.* 2004). Such data suggest the central role of PGC-1α in glucose metabolism in respect to the pathology of the disease. In addition, many PGC-1α target genes involved in oxidative phosphorylation are downregulated in diabetics (Mootha *et al.* 2003). However, the PGC-1α protein was shown to be elevated in the livers of type 2 diabetic mice (Yoon *et al.* 2001) but conversely, downregulated in the livers of obese and insulin-resistant humans (Croce *et al.*

2007). In summary, the role and physiology of PGC-1α in the progress of type 2 diabetes still remains partly unknown, the probable reason being the complex regulation in different tissues (Rowe & Arany 2014).

The true importance of PGC-1α in the liver has been studied in PGC-1α$^{-/-}$ deleted (null) animals. The first two whole-body null mouse studies were published at about the same time (Leone *et al.* 2005, Lin *et al.* 2004). However, there were also unexpected consequences in the animals, presumably due to systematic PGC-1α-independent compensatory mechanisms. To avoid the systemic effects, tissue-specific PGC-1α$^{-/-}$ studies have also been performed (Handschin *et al.* 2005, Handschin *et al.* 2007).

In the PGC-1α whole-body null study by Lin *et al.* isolated hepatocytes from knockout mice were resistant to hormone-stimulated activation of gluconeogenesis, as expected. Namely, the PEPCK and G6Pase inductions seen in PGC-1α$^{+/+}$ was greatly diminished in PGC-1α$^{-/-}$ hepatocytes treated with dexamethasone and forskolin. This illustrates the central role of PGC-1α in the regulation of gluconeogenic genes. However, in these mice the basal gluconeogenic gene expressions were distorted. The PEPCK, and especially G6Pase expression, were constitutively active in the null mice in normal, fed state compared to the wild type mice. In the fasted state their expression was similarly elevated in both the wild type and null mice. The constitutive expression was shown to be due to PGC-1α independent factor by the induction of C/EBPβ, which is also able to upregulate the gluconeogenic genes (Lin *et al.* 2004).

The other study by Leone *et al.* did not address the question of gluconeogenic gene response *per se*. However, they discovered that fasting caused severe hepatic steatosis manifested as inability to maintain cellular lipid balance in the fasted state. The phenotype of PGC-1α$^{-/-}$ mice was mildly obese but surprisingly, their glucose tolerance was similar to wild types with a normal diet. On a high-fat diet the null mice were even more glucose tolerant and insulin sensitive than the control mice, indicating again some systemic compensation mechanism (Leone *et al.* 2005).

In the liver-specific knockout model of PGC-1α, the PEPCK and G6Pase expression behavior was as expected. Their induction in the fasted state was significantly lower in PGC-1α$^{liver-/-}$ compared to the wild type mice. No constitutive activity of the gluconeogenic genes was observed, either. The role of PGC-1α in the control of hepatic response to gluconeogenesis in fasting was thus clearly established (Handschin *et al.* 2005).

The role of PGC-1α in hepatic mitochondrial β-oxidation is also well known, as already described.

Brown adipose tissue (BAT)

Originally discovered as the interaction protein of PPARγ in cold-exposed, β-adrenergic stimulated murine BAT, PGC-1α possesses important function in this tissue (Puigserver *et al.* 1998). Namely, the role of PGC-1α is the general regulation of adaptive thermogenesis, which is characteristic for BAT compared to white adipose tissue (WAT) where PGC-1α expression is negligible (Rosen & Spiegelman 2000). Adaptive thermogenesis is achieved through the action of uncoupling proteins (UCP) which are located at the mitochondrial inner membrane. They are able to uncouple ATP synthesis through proton 'leak', dissipating the energy into producing heat required by the organism in cold conditions. The first member discovered was UCP-1 which is expressed solely in the BAT; of its closest homologs, UCP-2 is ubiquitous and UCP-3 is present also in skeletal muscle in addition to BAT (Dulloo & Samec 2001). UCP-1 was shown to be regulated by the nuclear receptors PPARγ and thyroid receptor TR coactivated by PGC-1α in BAT (Puigserver *et al.* 1998).

The importance of PGC-1α in the regulation of adaptive thermogenesis was shown with PGC-1α null mice. Namely, these animals were unable to maintain their core body temperature compared to wild types (Leone *et al.* 2005, Lin *et al.* 2004). According to Leone *et al.* there was no difference in the UCP-1 expression between the two groups, indicating that UCP-1 upregulation is not the main mechanism regulating adaptive thermogenesis by PGC-1α. A role of general reduced capacity of mitochondrial respiration was suggested (Leone *et al.* 2005). However, Lin *et al.* reported that the expression of UCP-1 and UCP-2 in the null mice was markedly reduced, which did in indeed suggest a UCP-mediated mechanism (Lin *et al.* 2004).

It was also shown that the levels of UCP-1 through PGC-1α are negatively regulated by the orphan receptor SHP, a general repressor of gene expression. In $SHP^{-/-}$ BAT the levels of PGC-1α as well as UCP-1 were markedly higher, implying a role for SHP in the control of the thermogenic response. It was postulated that SHP targets the PGC-1α transcriptional activator ERRγ inhibiting its transactivation properties, but this phenomenon is limited to the BAT. SHP may also have a direct repressive effect on the *UCP-1* promoter (Wang *et al.* 2005).

Skeletal muscle

PGC-1α was shown to promote the formation of slow-twitch, type I muscle fibers. This was evidenced through overexpression of PGC-1α in type II muscle fibers which resulted in upregulation of slow-twitch type fiber proteins such as myoglobin, troponin I and the mitochondrial enzymes. The mechanism was shown to be calcium-induced, promoted by exercise, through calcium/calmodulin-dependent protein phosphatase calcineurin A (CnA) signaling, which finally docks PGC-1α and a transcription factor myocyte enhancer factor 2 (MEF2) to the regulatory areas of target genes to drive their gene expression (Lin *et al.* 2002). MEF2 was previously shown to regulate the slow-twitch muscle-specific gene expression through calcineurin (Wu *et al.* 2000).

The plasticity of mammalian skeletal muscle allows fiber-type switching in response to contractile demands, i.e., higher loads or endurance training leads to a higher relative amount of slow-twitch muscle fibers rich in mitochondria (Booth & Thomason 1991) Not surprisingly, both short-term and long-term *in vivo* exercise induces PGC-1α in murine skeletal muscle (Akimoto *et al.* 2005, Pilegaard *et al.* 2003) in line with the role in promoting type I fiber formation.

In the soleus muscle of PGC-1α null mice the amount and size of mitochondria were smaller; the levels of genes responsible for mitochondrial metabolism were also lower, implying reduced mitochondrial biogenesis. The exercise behavior between the PGC-1$\alpha^{-/-}$ and PGC-1$\alpha^{+/+}$ groups was also different. The null mice were unable to maintain as high a running capacity as the control mice; they were less resistant to muscle fatigue (Leone *et al.* 2005). Also in the study by Lin *et al.* the genes involved in mitochondrial function in quadriceps muscle were expressed at lower levels in PGC-1$\alpha^{-/-}$ mice. Interestingly, the expression of ERRα, regulated by PGC-1α and regulating many mitochondrial functions, was also reduced in the PGC-1α null mice (Lin *et al.* 2004). Recently, in contrast to the previous assumption, it was shown using PGC-1α muscle-specific knockout mice that PGC-1α is dispensable for the exercise-induced mitochondrial biogenesis. It was speculated that the p38γmitogen-activated protein kinase (MAPK) pathway may be relevant in this setup. The role of PGC-1β as compensatory mechanism was ruled out, as was AMP activated kinase (AMPK) action (Rowe *et al.* 2012).

Skeletal muscle functions as glucose storing, oxidizing and releasing tissue in response to differing conditions. The glucose transporter 4 (GLUT4) is regulated

in skeletal muscle upon insulin or exercise stimulus, promoting the uptake of excess glucose from blood stream to muscle (Benton *et al.* 2008). The expression of GLUT4 was reported to be increased by PGC-1α overexpression *in vitro* and through MEF2-mediated transcriptional activation of GLUT4, coactivated by PGC-1α. The GLUT4 translocation to the cell membrane was higher even in the absence of insulin, making PGC-1α an important regulator of endogenous GLUT4 expression and function in muscle cells (Michael *et al.* 2001). In muscle-specific knockout mice GLUT4 expression was clearly lower than in control mice, but there was no apparent abnormality in peripheral insulin sensitivity, probably due to the compensatory mechanisms already mentioned (Handschin *et al.* 2007).

Heart and vasculature

The post-natal, normally functioning heart favors fatty acid oxidation as the main energy source over glucose, which is utilized in the fetal heart. This is performed via activation of the mitochondrial fatty acid β-oxidation pathway (FAO) producing reduced equivalents for the electron transport chain, finally yielding high amounts of ATP. The heart contains a huge number of mitochondria capable of reacting to needs of energy, and more can be generated by means of mitochondrial biogenesis. The mitochondrial mass is roughly one third of an adult's total heart mass (Lehman & Kelly 2002).

PGC-1α was shown to activate the genes related to FAO and mitochondrial biogenesis in cardiac myocytes (Lehman *et al.* 2000). However, studies from PGC-1α$^{-/-}$ mice revealed that mitochondrial biogenesis is not solely dependent on PGC-1α since the overall volume and content appeared unchanged in these mice compared to controls. Only slight phenotypic differences were found by electron microscopy (Arany *et al.* 2005, Lin *et al.* 2004) Later it was shown that ERRα is an essential TF interaction partner for PGC-1α in the control of mitochondrial regulation, also in cardiac tissue (Rangwala *et al.* 2007).

It appears that PGC-1α together with ERRα induces the vascular endothelial growth factor (VEGF) and some other vasculature factors leading to the production of new blood vessels. In summary, PGC-1α promotes angiogenesis, thus increasing the fuel uptake from blood circulation into the cells and mitochondria. This phenomenon is important, for example in cardiac adaptation to pregnancy, and imbalances in PGC-1α expression result in peripartum

cardiomyopathy, a fatal disease in late gestation (Arany *et al.* 2008, Patten *et al.* 2012).

In general, PGC-1α is needed for the proper function of the heart, but many other factors are also involved. During pathological hypertrophy, a switch to the fetal phenotype occurs as glucose becomes the main substrate for ATP production. PGC-1α and FAO genes have been suggested to be downregulated in the pathologic pressure overload hypertrophy in rodents (Arany *et al.* 2006, Lehman & Kelly 2002), but results showing no effect on PGC-1α have also been reported (Hu *et al.* 2011).

An overall assumption suggests that PGC-1α is the mechanistic link between substrate switch from FAO to glucose utilization. Although it remains to be poorly understood whether PGC-1α is the factor mediating the downregulation of genes in hypertrophy, a positive outcome is seen in patients with a failing heart by exercise-stimulated, probably PGC-1α activation-mediated effects (Riehle & Abel 2012).

Brain

PGC-1α expression is abundant also in the brain (Puigserver *et al.* 1998, Ruas *et al.* 2012). The importance of PGC-1α in the brain becomes evident in the PGC-1α knockout mice which display signs of neurodegenerative disorders such as disordered movements, frequent limb clasping, dystonic posturing and abnormal startle responding. Indeed, the brain area striatum suffered from spongiform lesions in the PGC-1α null mice, apparently as a result from loss of axons in this area. Many mitochondrial genes but also other neuronal genes were downregulated in the brains of the null mice, suggesting a role for PGC-1α in regulation of proper neuron and brain function (Lin *et al.* 2004).

Moreover, it was shown that there was increased sensitivity to ROS in the brains of PGC-1α null animals. Following injections of the neurotoxin MPTP and the oxidative stressor kainic acid, more severe damage was observed in the null animals compared to wild type littermates. It was shown that PGC-1α induces ROS-detoxifying enzymes, mainly the SOD enzymes, GpX and UCP2, thus protecting neuronal cells from oxidative stress (St Pierre *et al.* 2006).

2.1.5 Involvement in cancer

Lately, the role of perturbed cellular metabolism in promoting and maintaining cancer has garnered considerable attention. It has been known for a long time that cancer cells prefer glycolytic metabolism over oxidative phosphorylation, also called the Warburg effect (Warburg 1956). However, more recent studies suggest a significant role for mitochondria in tumor metabolism, suggesting that oxidative metabolism is of importance after all (Fogal *et al.* 2010, Funes *et al.* 2007).

Since PGC-1α plays a major role in the regulation of mitochondrial function, it is not surprising that the link between OXPHOS and cancer has been suggested to be PGC-1α-mediated. Namely, some years ago it was shown that PGC-1α is induced in a cell model of tumorigenesis (Ramanathan *et al.* 2005). Moreover, studies with PGC-1α null mice demonstrated that loss of PGC-1α protects against induced colon cancer (Bhalla *et al.* 2011). The mechanisms by which PGC-1α may promote cancer have been extensively reviewed and suggested: tumor cell glucose uptake, induction of anabolic catabolism to extend biomass and promoting cell survival in signs of apoptotic circumstances or stress situations (Girnun 2012).

2.1.6 Involvement in circadian oscillation and longevity

The role of PGC-1α in the regulation of the clock genes was described some time ago. In myotubes and hepatocytes, overexpression of PGC-1α induces BmalI and Clock, the essential transcription factors regulating circadian responsive genes. This mechanism involves the action of an NR called RAR-related orphan receptor (ROR) and Rev-erbα, the latter of which acting as repressor (Liu *et al.* 2007). Both of these factors are known clock gene regulators (Sato *et al.* 2004, Yin *et al.* 2006). RORα activation is regulated by the natural ligand melatonin (Carlberg 2000). It was shown that the expression of clock genes was disturbed in the PGC-1α null mice, especially in skeletal muscle but also in the liver. In conclusion, PGC-1α acts as a central component in the mammalian clock gene regulation (Liu *et al.* 2007).

Caloric restriction (CR) has for quite some time been known to enhance the life span of many invertebrate and vertebrate animals, with numerous reports describing the phenomenon. CR can be defined as a reduced (from 10 up to 50% reduction) caloric intake compared to *ad libitum* conditions, without any signs of malnutrition. The positive effects on longevity are higher in rodents that follow

the most reduced caloric intake, until they reach malnutrition which prevents the beneficial effects (Weindruch 1996).

The molecular mechanisms behind the life spanning process of CR are under extensive study. As PGC-1α is induced by fasting, it has become a key molecule in the longevity studies. The evolutionarily conserved insulin/insulin growth factor I (IGF-I) pathway was shown to be a key mediator of the life spanning process. Indeed, mice having disturbances or loss of the insulin receptor live longer and have higher resistance to oxidative stress, also a marker of longevity (Bluher *et al.* 2003, Holzenberger *et al.* 2003). As it is known that fasting and CR inhibit the insulin/insulin growth factor I (IGF-I) pathway, the FOXO proteins have emerged as key molecules. Namely, it was shown that Daf-16 action is needed for the increased longevity in the nematode *C.elegans* where the insulin/IGF-I pathway was inactivated. Daf-16 target genes included stress-response genes and antimicrobial genes, which were speculated to play a key role in the longevity (Murphy *et al.* 2003). The FOXO proteins are mammalian homologs for Daf-16.

The sirtuin family members have also been suggested as the FOXO down-stream effectors. Like PGC-1α, SIRT1 is activated by CR and negatively affected by insulin/IGF-I (Cohen *et al.* 2004). SIRT1 is known to be a post-transcriptional activator of the FOXO proteins, thus promoting their action towards cell-cycle arrest and stress genes (Brunet *et al.* 2004, Daitoku *et al.* 2004).

As described in the next chapters, both FOXO1 and SIRT1 are modulators of PGC-1α activity in numerous tissues, andPGC-1α is thus a key player in the CR-mediated longevity process (Corton & Brown-Borg 2005). Interesting findings have been collected from so-called dwarf mice, which are genetic models of longevity. These mice have increased β-oxidation and xenobiotic metabolism, processes that are under the control of PGC-1α. Also, many target genes of PPARα are constitutively active in these mice, and the PGC-1α–PPARα axis was suggested to be a key element mediating the transcriptional responses to longevity (Corton & Brown-Borg 2005, Liang *et al.* 2003).

2.1.7 Regulation of heme biosynthesis

Heme is a key component of the respiratory cytochromes and present in e.g. hemoglobin and the cytochrome P450 enzymes. The heme biosynthesis pathway contains eight enzymatic steps and is a highly regulated process due to the potential toxicity of heme. Namely, it can generate oxidative stress when reacting

with O_2. The rate-limiting enzyme in the heme biosynthesis pathway, aminolevulinate synthase (ALAS-1), was shown to be positively regulated by PGC-1α. This is in accordance with the fact that many PGC-1α target genes are heme-containing proteins such as cytochromes (Handschin *et al.* 2005).

Later on it was shown that heme is a physiological ligand for Rev-erbα, a clock gene and NR acting as repressor of gene transcription (Raghuram *et al.* 2007). In addition, it was shown that heme and Rev-erbα repress PGC-1α transcription. Thus heme regulates its own levels through Rev-erbα by a negative feedback loop (Wu *et al.* 2009).

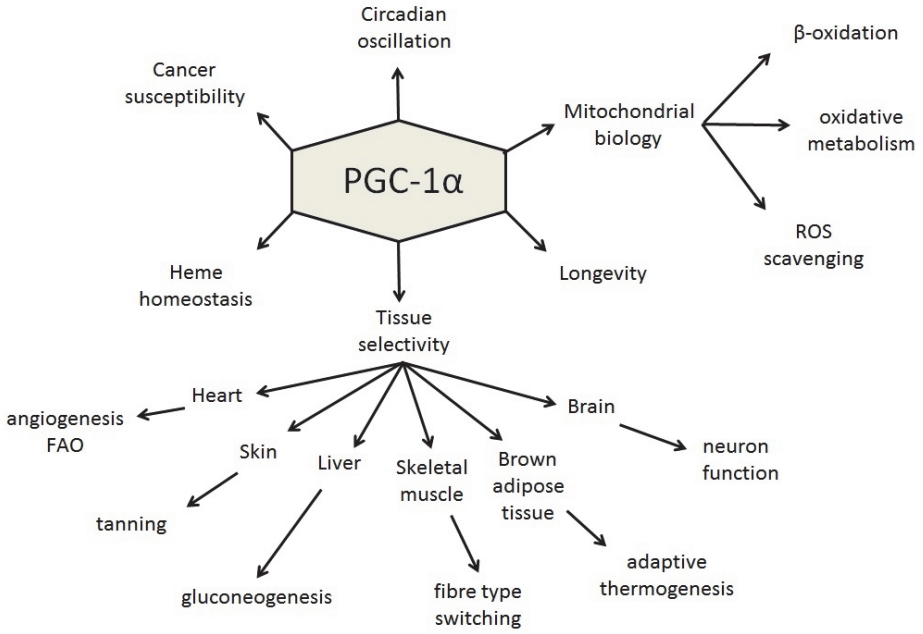

Fig. 3. Processes regulated by PGC-1α in the human body. FAO, fatty acid oxidation; ROS, reactive oxygen species.

2.2 Regulation of PGC-1α expression

The regulation of PGC-1α coactivator is exceptionally versatile, including transcriptional as well as numerous post-transcriptional events which are summarized in the following chapters.

2.2.1 Transcriptional regulation

The transcriptional regulation of PGC-1α is partly tissue- and condition-specific although some general pattern of regulation exists by the response elements found at the *PGC-1α* proximal promoter. However, considering the wide range of functions, more mechanisms of *PGC-1α* gene regulation will presumably be discovered in the future.

A well-known transcriptional activation mechanism of PGC-1α is mediated through cAMP response element binding protein CREB. During food deprivation, glucagon and catecholamines activate the well-known protein kinase A (PKA) signaling cascade in hepatocytes, which activates CREB by phosphorylation of serine 133 (Gonzalez & Montminy 1989). The human *PGC-1α* CREB response element (CRE) is located -133 to -116 bp from the transcription starting site in the promoter region (-146 to -129 bp in mouse). A mutation of the PGC-1α CRE site by two nucleotides results in the complete loss of induction by agents such as forskolin (Handschin *et al.* 2003, Herzig *et al.* 2001).

Another factor that activates PGC-1α through CREB and CRE is calcium/calmodulin-dependent protein kinase IV (CaMKIV) (Handschin *et al.* 2003, Wu *et al.* 2002), activated by exercise and calcium (Ca^{2+}) as a signaling molecule in skeletal muscle (Berchtold *et al.* 2000). However, the activation by calcineurin A (CnA), a protein phosphatase also activated by Ca^{2+}, induces PGC-1α transcription through myocyte enhancer factor 2 (MEF2) response element which is located at -1464 to -1447 bp (-1308 to -1293 in mouse). Interestingly, PGC-1α enhances its own transcription by the subsequent coactivation of MEF2 in its own promoter creating an autoregulatory mechanism (Handschin *et al.* 2003).

p38 mitogen-activated kinase (p38 MAPK) is a kinase also activated by calcium and exercise in skeletal muscle (Widegren *et al.* 1998). p38 was shown to activate the downstream target activating transcription factor 2 (ATF2) by phosphorylation, required for induction of *PGC-1α* through the previously identified CRE site (Akimoto *et al.* 2005). p38 MAPK is a well-known, direct activator of the MEF2 as well (Zhao *et al.* 1999).

AMP-activated protein kinase (AMPK), a cellular energy sensor, is activated in energy-deprived conditions when cellular AMP/ATP rises and energy-producing pathways are needed to stimulate ATP production. AMPK has been suggested to regulate gene expression, but these mechanisms are not well understood. Direct phosphorylation of transcription factors has been suggested as

one possible mechanism (Fernandez-Marcos & Auwerx 2011). Exercise is known to activate AMPK in skeletal muscle and to increase PGC-1α transcription (Pilegaard *et al.* 2003). However, the main mechanism of induction is postulated to be direct phosphorylation of the PGC-1α protein at threonine 177 and serine 538, which by unknown mechanism also improves the transcription of PGC-1α, possibly through autoregulation, i.e., coactivation by PGC-1α (Canto & Auwerx 2009, Jager *et al.* 2007). A negative regulation of PGC-1α by AMPK also exists. Phosphorylation of CREB-regulated transcription coactivator 2 (CRTC2) by AMPK at Ser171 prevents nuclear entry of CRTC2 and subsequent transcriptional activation of the CREB-activated gluconeogeneic genes, including PGC-1α, PEPCK and G6Pase (Koo *et al.* 2005a).

Insulin is a hormone sensing the glucose balance, eventually leading to the uptake and storage of glucose from the bloodstream. Insulin is also a powerful suppressor of gluconeogenesis and the signaling involves the activation of protein kinase B PKB/Akt kinase. The PGC-1α promoter contains three insulin response elements (IRS1-3) which are sites for Forkhead box class-O 1 (FOXO1) transcription factors. In muscle, insulin phosphorylates both Akt and FOXO1, which results in the nuclear exclusion of FOXO1 and the transcriptional inhibition of PGC-1α (Daitoku *et al.* 2003, Southgate *et al.* 2005). Daitoku *et al.* suggest a mutual role of CREB and FOXO1 in regulating the *PGC-1α* during times of feeding and fasting to maintain an appropriate blood glucose level (Daitoku *et al.* 2003). However, Puigserver *et al.* reported that in forskolin-treated, PKA-activated hepatocytes, insulin does not repress the transcriptional activity of PGC-1α, but the effect of gluconeogenesis inhibition is preferably mediated at the functional level by reducing the interaction capability of FOXO1 and PGC-1α (Puigserver *et al.* 2003). Interestingly, it was shown later that PGC-1α is directly phosphorylated by Akt, rendering the coactivator inactive (Li *et al.* 2007).

Mammalian target of rapamycin (mTOR) kinase, another nutrient sensor, regulates transcription of many genes involved in growth, size and mitochondrial function. mTOR has been linked to PGC-1α and PGC-1α-driven mitochondrial gene regulation by a transcription factor called yin-yang 1 (YY1). YY1 binds to PGC-1α and other target gene promoters and is also coactivated by PGC-1α protein. Inhibition of mTOR results in the disruption of PGC-1α coactivation although YY1 retains its binding ability. Less is known about the transcriptional activation of YY1 at the *PGC-1α* promoter (Cunningham *et al.* 2007).

Very recently also ERRα was postulated to regulate the transcription of PGC-1α at least in cardiomyocytes. An evolutionary conserved ERRα binding site was found in the PGC-1α promoter located in reverse orientation between -1978 and -2038 bp in humans before TSS (Ramjiawan *et al.* 2013). It has been known for some time that PGC-1α controls the levels of ERRα, the master regulator of mitochondrial biology (Schreiber *et al.* 2003).

Less is known about the repression of PGC-1α transcription. Bile acids were shown to induce SHP and via that mechanism repress HNF-4α and FOXO1 (Yamagata *et al.* 2004). Later, the same group showed that also PGC-1α was repressed through the same mechanism by bile acids (Yamagata *et al.* 2007). Also Rev-Erbα, a repressive clock gene, was shown to repress the transcription of PGC-1α. Two putative Rev-Erbα binding (RORE) elements within the first intron of *PGC-1α* were established as the targets of the repressive effects by Rev-Erbα (Wu *et al.* 2009).

Interestingly, epigenetic regulation of PGC-1α has been reported. The PGC-1α promoter is a target for methylation and silencing of transcription, since CpG sites have been found within the *PGC-1α* promoter. In skeletal muscle, hypermethylation of *PGC-1α* was observed, associated with increased methyl transferase DNMT3B expression in type 2 diabetic patients (Barres *et al.* 2009). Also, in the liver *PGC-1α* methylation status was associated with the insulin resistance phenotype and overall reduction in mitochondrial biogenesis in these patients (Sookoian *et al.* 2010).

The activity of a corepressor, namely RIP140, in inhibiting PGC-1α-mediated gene expression has been suggested. Interestingly, RIP140 can directly bind to PGC-1α and inhibit its coactivation properties. RIP140 and PGC-1α may thus act as parallel factors determining the activity of NRs in the control of metabolic genes (Hallberg *et al.* 2008, Parker *et al.* 2006).

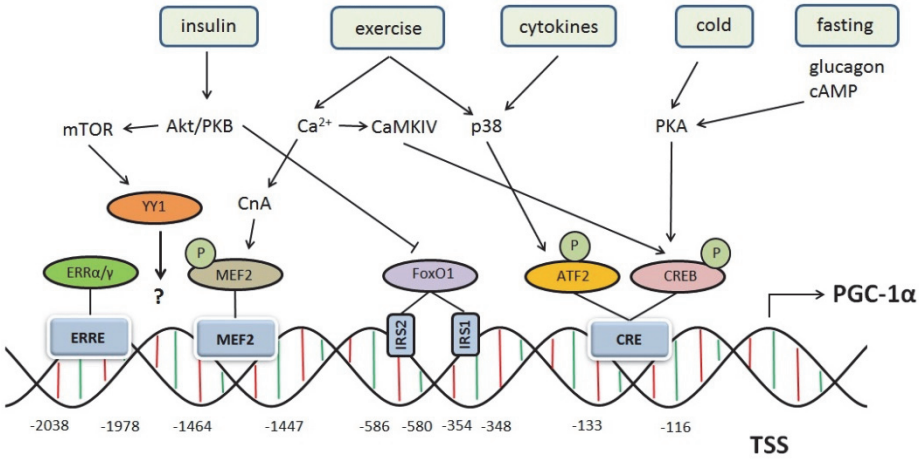

Fig. 4. Illustration of PGC-1α transcriptional regulation. At the PGC-1α promoter, there are binding sites (response elements) for transcription factors indicated in the circles. These factors are modulated by different signaling pathways triggered by certain stimulus like insulin, exercise, cytokines, cold or fasting. Akt/PKB, protein kinase B; ATF2, activating transcription factor 2; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CnA, calcineurin, CRE/CREB, cAMP response element/binding protein; ERRE, estrogen-related receptor response element; ERRα/γ, estrogen-related receptor; FoxO1, forkhead box O1 protein; MEF2, myocyte enhancer factor 2; P, phosphorylation; PKA, protein kinase A; p38, p38 MAPK kinase (modified from Fernandez-Marcos & Auwerx 2011).

2.2.2 Post-transcriptional regulation

The post-transcriptional regulation of PGC-1α has emerged as a major means of determining the activity of PGC-1α. It seems that the mechanisms of PGC-1α regulation are different in different tissues, in principle due to the nature of PGC-1α functions. A summary of the modifications is depicted in Table 2.

Phosphorylation

It seems that PGC-1α regulation is under the control of several protein kinases, either directly or indirectly, which exert their effects under different physiological and pathological conditions (Canto & Auwerx 2009, Fernandez-Marcos & Auwerx 2011).

AMPK, as mentioned, directly phosphorylates the PGC-1α protein at Thr177 and Ser538 in skeletal muscle and increases autoregulation as well as the activity of PGC-1α to further regulate targets, and this is dependent on the phosphorylation by AMPK. Namely, two chemical AMPK activators, 5-aminoimidazole-4-carboxamide 1-beta-D-ribonucleoside (AICAR) and the diabetes drug metformin, are able to upregulate PGC-1α, GLUT4, CytC, UCP-2 and -3 in wild-type primary muscle cells whereas none of these genes were affected in PGC-1α-deficient cells. GLUT4 is responsible for the glucose uptake in muscle and AMPK activation seems to promote this phenomenon through PGC-1α (Jager *et al.* 2007).

p38 MAPK is able to regulate PGC-1α also post-transcriptionally in addition to transcriptional regulation, by phosphorylation of the sites in the negative regulatory domain. In addition to exercise, also inflammatory stimuli activate the p38 MAPK pathway in muscle cells. Cytokines such as IL-1α, IL-1β, TNFα and lipopolysaccharide (LPS) induce the activity of PGC-1α by p38-mediated direct phosphorylation of Thr262, Ser265 and Thr298 residues which are in the conserved negative regulatory domain of the PGC-1α promoter. This phosphorylation renders PGC-1α more stable with augmented transcriptional activity compared to the unphosphorylated form (Puigserver *et al.* 2001). Later it was established that this area contains a PGC-1α repressor called p160 myb-binding protein which reduces the transcriptional and biological activity of PGC-1α. p38 MAPK phosphorylation is able to prevent the p160-PGC-1α interaction, mechanistically explaining at least partly the role of p38 MAPK in the activation of PGC-1α (Fan *et al.* 2004). Adding more complexity, Cao *et al.* demonstrated that in the fasted state, p38 MAPK is activated by glucagon in hepatocytes and through CREB phosphorylation, it regulates the gluconeogenic program, including PGC-1α (Cao *et al.* 2005).

As discussed earlier, the PKB/Akt kinase phosphorylates many targets in response to the postprandial hormone insulin. It was shown that Akt phosphorylates also hepatic PGC-1α directly at Ser570, located at the SR domain, producing an inhibitory effect on the coactivator properties. This was established by showing that independently of FOXO1 regulation, PGC-1α target genes were downregulated by Akt activation while genes in Ser570 mutant-treated livers were not (Li *et al.* 2007). The additional finding that insulin and Akt phosphorylates FOXO1, preventing the nuclear translocation and thus both reducing *PGC-1α* transcription as well as PGC-1α coactivation, results in multiple regulation mechanisms by the Akt kinase on PGC-1α activity (Daitoku *et al.* 2003,

Puigserver *et al.* 2003). Also, Cdc2-like kinase (Clk2) was shown to phosphorylate the SR domain of PGC-1α also containing the Akt phosphorylation site. Phosphorylation by Clk2 renders PGC-1α inactive and was thus suggested to be another responsive kinase to insulin. It was hypothesized that while Akt mediates the early refeeding response to suppress glugoneogenesis, Clk2 is a late-response mediator of the insulin action. In addition, the Clk2 action does not affect the coactivation properties of PGC-1α in regulating mitochondrial biogenesis (Canto & Auwerx 2010, Rodgers *et al.* 2010).

Recently, it was reported that a serine/threonine kinase, S6K1 (S6), activated by insulin and feeding, also directly phosphorylates PGC-1α in hepatocytes at two residues, Ser568 and Ser572. S6 is the downstream target of mTOR, another kinase affecting PGC-1α transcription as discussed earlier. Interestingly, the S6-phosphorylated protein is unable to promote gluconeogenic genes and coactivate HNF-4α but its ability to activate mitochondrial genes and β-oxidation is intact. Taken together, S6 is able to selectively affect the coactivation functions of PGC-1α through phosphorylation events (Lustig *et al.* 2011).

PGC-1α protein has also phosphothreonine containing motifs, so-called Cdc4-phosphodegron (CPD) motifs. Glycogen synthase kinase 3β (GSK-3β) was shown to phosphorylate Thr295 located at the CPD2 motif and to finally target PGC-1α for ubiquitin-mediated proteasomal degradation, primed by p38 MAPK phosphorylation. CPDs are reported to be even crucial for the coactivation ability of PGC-1α because mutations in them result in reduced transcriptional activation of PGC-1α. Controversially, the CPDs are also recognized by the protein degradation machinery (Olson *et al.* 2008).

Acetylation

In addition to phosphorylation, reversible acetylation of PGC-1α has emerged as a key means of determining PGC-1α activity. Tandem mass spectrometry has revealed 13 acetylation prone lysine residues on the PGC-1α protein (Rodgers *et al.* 2005). Sirtuin 1 (SIRT1) is the well-established deacetylase of PGC-1α (Nemoto *et al.* 2005). SIRT1 is the first member identified in the family of Sirtuins 1-7. It requires the coenzyme nicotineamide adenine dinucleotide (NAD^+) as a cofactor, linking the cellular NAD^+/NADH as a regulator for Sirt1 activity which is activated upon cellular stress caused e.g. by exercise or nutritional deprivation (Canto & Auwerx 2009, Rodgers *et al.* 2005). The deacetylation results in enhanced PGC-1α activity on target gene expression.

Namely, the over-expression of Sirt1 in mouse liver leads to activation and increase in the PGC-1α-dependent gluconeogenic program. The knockdown of Sirt1 diminishes this effect also *in vivo,* making SIRT1 a relevant, although not indispensable, determinant of PGC-1α activity in fasted state (Rodgers & Puigserver 2007). Interestingly, also AMPK seems to activate SIRT1 and subsequently PGC-1α by upregulating NAD^+/NADH. Namely, the deacetylation of PGC-1α by SIRT1 in skeletal muscle requires a priming phosphorylation by AMPK through Thr177 and Ser538. (Canto *et al.* 2009). The activation of PGC-1α in muscle after cellular stress such as exercise, thus involves both phosphorylation by AMPK independently as well as together with SIRT1 by deacetylation (Canto *et al.* 2009, Jager *et al.* 2007).

Under high nutrient conditions and low NAD^+ PGC-1α is hyperacetylated by acetyltransferase GCN5 inhibiting the PGC-1α-dependent hepatic transcriptional activation of gluconeogenic genes. GCN5 seems to affect the intranuclear localization of PGC-1α, possibly by interaction with a nuclear corepressor of hormone nuclear receptors, RIP140, thus explaining the repressive effect of PGC-1α on target genes. The inhibition by GCN5 is not limited to gluconeogenic genes; the mitochondrial PGC-1α target genes (βATPs) and Cytochrome C (Cycs) are also repressed by GCN5 (Lerin *et al.* 2006).

The finding that a coactivator, SRC-3, is able to promote GCN5 expression, leading to the subsequent hyperacetylation and inactivation of PGC-1α, adds more complexity to the acetylation of PGC-1α. In SRC-3 null mice, fasting plasma glucose and insulin levels were lower, as were the lipid levels. The expression of PGC-1α and mitochondrial genes was notably higher in the muscles of these mice but interestingly, the ratio of acetylated over total PGC-1α was lower, due to GCN5 inhibition. This finding indicates that the coactivator SRC-3 is able to affect the acetylation status of PGC-1α through GCN5 (Coste *et al.* 2008).

Methylation

The major protein arginine methyltransferase 1 (PRMT1) controls the methylation status of PGC-1α. PRMT1 can regulate protein methylation, which may determine the cellular localization, protein-protein interactions or signal transduction properties of the target protein (Lee *et al.* 2005). PRMT1 can act as coactivator by binding to target gene promoters, especially RNA-binding proteins' promoters, performing chromatin remodeling to facilitate transcription

(Koh *et al.* 2001). PRMT1 methylates PGC-1α within the C-terminal RNA-binding region through two or three arginine residues (Arg665, 667 and 669), enhancing the coactivator properties of PGC-1α (Teyssier *et al.* 2005).

Sumoylation

The small ubiquitin-like modifier (SUMO) proteins are able to connect with lysine residues of target proteins and covalently add the SUMO peptide affecting e.g. the subcellular localization, interaction properties and transcriptional activity of targets. The Lys183 at the PGC-1α N-terminal activation domain was found to be a SUMOylation target site. The SUMOylation repressed the PGC-1α coactivator activity, adding another layer of fine-tuning to the regulation of PGC-1α activity by post-translational modifications (Rytinki & Palvimo 2009).

Table 2. Summary of PGC-1α post-translational modifications (modified from Canto & Auwerx 2010, Fernandez-Marcos & Auwerx 2011).

Modification	Protein/enzyme	Residue	Effect
Phosphorylation	AMPK	T177, S538	Transcriptional activation, Facilitated deacetylation
	Clk2	SR region	Repression of hepatic glucose output
	GSK-3β	T295	
	p38MAPK	T262, S265, T298	Stabilization and transcriptional activation
	PKA (NT-PGC-1α)	S194, S241, T256	Inhibition of nuclear transport, Transcriptional activation
	Akt/PKB	S570	Repression of hepatic glucose output
Acetylation	GCN5 (+)	K77, K144, K183, K200, K223, K253, K270, K277, K320, K346, K412, K441, K450, K757, K778	Transcriptional repression
	SIRT1 (-)		Transcriptional activation, Mitochondrial biogenesis, Hepatic glucose production
Methylation	PRMT1	R665, R667, R669	Transcriptional activation
Sumoylation	PIAS 1, 3 (+)	K183	Transcriptional attenuation
	SNEP-1,2 (-)		
Ubiquitination			Inactivation / degradation

2.3 Glucose metabolism and perturbations in the liver

2.3.1 Insulin and glucagon

Blood glucose homeostasis is controlled by the anabolic hormone insulin and in opposing conditions by the so-called anti-insulin hormones, mainly glucagon, catecholamines, cortisol and growth hormone. Insulin is the postprandial hormone secreted by pancreatic βcells; this results in normalizing the blood glucose levels after a burst of carbohydrates after a meal. Glucagon, secreted by the pancreatic αcells acts to stimulate glucose production and release of glucose between meals, also in conditions of prolonged food deprivation (Baynes & Dominiczak 1999).

2.3.2 AMPK

The AMP-activated kinase (AMPK) is a versatile protein kinase capable of sensing cellular energy status and acting to balance deprivation conditions. It acts as a stimulator of catabolic processes which produce energy, subsequently suppressing biosynthetic pathways. The increased ADP/ATP or AMP/ATP ratio, caused by metabolic stress or xenobiotics, rapidly activates AMPK through the upstream liver kinase B1 (LKB1). After a conformational change, LKB1 phosphorylates and activates AMPK at Thr172, which is thought to be the main activation site. AMPK is highly conserved in eukaryotes, including in mammals, and forms a heterotrimeric complex comprising α, β and γ subunits. The α subunit contains the catalytic properties while β and γ are regulatory subunits. All the subunits have multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3) (Hardie *et al.* 2012).

Since the elucidation of AMPK as the target for the glucose-lowering antidiabetic drug metformin, the AMPK pathway has been under extensive study for potential therapy and treatment of type 2 diabetes (Zhang *et al.* 2009, Zhou *et al.* 2001).

As already described, the AMPK activation in skeletal muscle leads to the increased expression and phosphorylation of PGC-1α (Jager *et al.* 2007). Furthermore, treatment with AMPK activators such as AICAR, metformin, resveratrol, A-769662 and dinitrophenol (DNP) resulted in decreased acetylation status of PGC-1α in myotubes and skeletal muscle, mediated through SIRT1 activation. This activation was mediated through increasing the NAD^+/NADH by AMPK, as already discussed (Canto *et al.* 2009).

However, it is surprising that not very much is known about the hepatic actions of AMPK in respect to SIRT1 and PGC-1α. One recent report by Caton and co-workers finally showed that AMPK, activated by metformin, activated SIRT1 in the liver. The SIRT1 activity leads to the deacetylation and activation of PGC-1α by the AMPK action. However, the acetyltransferase GCN5 was additionally shown to be induced by metformin independently of AMPK, causing the inhibition of PGC-1α activity. In summary, the implication was that AMPK activation *per se* activates PGC-1α in the liver but gluconeogenic genes are inhibited by other means (Caton *et al.* 2010). This issue is further introduced in chapter 2.3.3.

2.3.3 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a heterogeneous disease resulting in defects of both insulin secretion and action. The latter is often called insulin resistance, manifested by a decrease in glucose uptake upon insulin stimulus. Hepatic *de novo* glucose synthesis, gluconeogenesis, attempts to compensate for the poor uptake, leading to a vicious net result, hyperglycemia. The excess of glucose persists in the patients even in fasted state (DeFronzo 1999). The elevated glucose in the circulation eventually leads to severe complications that mostly affect the vascular system and are classified as micro- and macrovascular. The microvascular complications cause damage to the small blood vessels and include diseases such as retinopathy, nephropathy and neuropathy. Macrovascular complications include myocardial infarction and cerebrovascular disease manifesting as strokes (Forbes & Cooper 2013).

The molecular mechanisms preceding the major complications in T2DM patients have been under extensive study. Many factors are partly overlapping, one affecting the other. Low-grade, chronic inflammation in the patients is a well-established factor participating in and leading to the phenomenon of insulin resistance (Donath & Shoelson 2011, Tilg & Moschen 2008). Hyperglycemia generates ROS in the mitochondria of pancreatic βcells and is particularly devastating because of a diminished amount of antioxidant enzymes. The ROS, which is not properly scavenged, releases pro-inflammatory molecules such as cytokines to the circulation causing both insulin resistance and inflammation in several sites of the body. These severe consequences are typically seen in T2DM patients (Akash *et al.* 2013).

Obesity in T2DM patients causes problems by the caloric over-load in the diet, causing an imbalance in the fat and lipid profile of vasculature and other non-adipose tissues. Hepatic steatosis and steatohepatitis are both major players in the non-alcoholic fatty liver diease (NAFLD) for which there is strong evidence as being a risk factor for T2DM. NAFLD also promotes the future development of the metabolic syndrome (Lonardo *et al.* 2015).

Cardiovascular diseases such as hypertension also promote diabetic vascular events. Additional molecular factors like ROS and inflammation are major players affecting the complications seen in the T2DM patients (Forbes & Cooper 2013).

Insulin resistance

Insulin resistance manifests as defects in cellular insulin action as well as compensatory, increased insulin secretion from pancreatic βcells resulting in hyperinsulinemia. Insulin resistance effects are seen in major target tissues, most importantly in skeletal muscle, but also in the liver and adipose tissue (Kashyap & Defronzo 2007).

Skeletal muscle is responsible for about 80% uptake of postprandial glucose from the circulation in response to insulin. Upon stimulus, insulin binds to insulin receptor (IR), also known as insulin tyrosine kinase receptor, at the cell membrane and induces autophosphorylation of the IR with subsequent activation of the insulin receptor substrate (IRS) 1 migration to cell membrane. IRS-1 becomes phosphorylated at tyrosine residues and activates a phosphorylation-dephosphorylation cascade, eventually leading to activation of protein kinase B (Akt) and translocation of GLUT4 to the cell membrane. GLUT4 allows glucose uptake to the cell. The activity of the IRS-1/Akt signaling pathway is essential for normal insulin-stimulated glucose uptake in skeletal muscle (Kashyap & Defronzo 2007).

The glucose taken to cells is rapidly phosphorylated to glucose-6-phosphate and is either stored as glycogen (about two thirds) or subjected to metabolic pathways such as glycolysis (about one third). Impaired glycogen synthesis along with reduced glycogen synthase activity is the very first metabolic defect preceding T2DM and causing insulin resistance. However, in addition to insulin resistance, also reduced pancreatic βcell insulin secretion is required for the evolution of T2DM (DeFronzo & Tripathy 2009).

The molecular mechanisms behind insulin resistance have been under extensive study. Mechanisms linked to the impaired IRS-1/Akt phosphorylation cascade are of major importance. For example, serine phosphorylation of IRS-1 by MAP kinases, caused by hyperinsulinemia, has been associated with inability of tyrosine phosphorylation and thus resulting in defects in the insulin signaling cascade (De Fea & Roth 1997). Also, hyperinsulinemia may cause the expression of IR number to be downregulated (DeFronzo & Tripathy 2009). Additionally, caloric overload and obesity cause elevated free fatty acid (FFA) levels, which are a hallmark of insulin resistance. FFAs result in the induction of ROS, inflammatory molecules as well as cellular cascades that interfere with the insulin-IRS1-Akt pathway, impairing its action. Insulin is a potent inhibitor of

lipolysis, thus exacerbating the detrimental effects of fats (Abdul-Ghani & DeFronzo 2010, DeFronzo & Tripathy 2009, Kashyap & Defronzo 2007).

Inflammation

Population studies have been conducted in order to link some specific inflammatory mediators and T2DM. The pro-inflammatory cytokines found in patients are shown to be versatile and vary between subjects and may not reflect the severity of inflammation in individual tissues. Adipose tissue and liver are big organs which contribute heavily to the levels of cytokines in the blood. However, inflammation in the small pancreatic islets does not result in remarkable elevation in the circulating cytokines, but the inflammation may still be devastating for the onset and progress of the disease (Donath & Shoelson 2011).

Nevertheless, the acute phase proteins C-reactive protein (CRP), interleukin 6 (IL-6) (Pradhan *et al.* 2001) and interleukin 1β (IL-1β) (Herder *et al.* 2009, Spranger *et al.* 2003) have been shown to be predictive indicators of developing T2DM. Cytokines such as tumor necrosis factor α (TNFα) also play an important role, especially in promoting insulin resistance (Tilg & Moschen 2008).

Adipose tissue macrophages are the main source of many pro-inflammatory mediators (Zhao *et al.* 2006). Also pancreatic β cells are a source of glucose-induced IL-1β secretion, leading to the apoptosis and destruction of these cells (Maedler *et al.* 2002). A naturally occurring antagonist for IL-1 in the body is interleukin 1 receptor antagonist (IL-1Ra or IL-Rn). IL-1β acts as an agonist for the membrane-bound IL-1 receptor, causing the intracellular signaling activation leading to the inflammatory response. The antagonist IL-1Rn binds to the receptor and efficiently prevents the ligands IL-1β, also IL-1α, from binding and exerting their effects. A recombinant form of IL-1Rn, also known as Anakinra, is available for the treatment of rheumatoid arthritis (Dinarello 2009).

Interestingly, the levels of IL-1Rn have also been shown to be elevated in the circulation of T2DM patients about 5 years before the onset of the disease, being one predictive marker as well. This antagonism is the initial protective mechanism for IL-1β effects but fails at a later stage as the disease progresses (Carstensen *et al.* 2010).

Pharmacological treatment of type 2 diabetes consists of a wide variety of drugs possessing somewhat different mechanisms of action. Their usefulness depends highly on the type and severity state of the disease. Traditionally, the therapeutics are classified as insulin sensitizers (metformin & thiazolidinediones; TZDs) that inhibit gluconeogenesis, or secretagogues (sulofonylureas, glinides, incretin mimetics) that improve insulin secretion. In addition, insulin as rapid- short-intermediate- or long-acting is routinely used in combination with the therapeutics mentioned, depending on the severity of the disease.

TZDs such as rosiglitazone or pioglitazone are PPAR agonists that are excellent insulin sensitizers in adipose and muscle tissue. They also improve pancreatic βcell function. However, the use of TZDs in treatment of T2DM is declining due to their certain detrimental effects and the development of more beneficial drugs. The incretin-based therapy represents one of these newest classes of drugs. Incretins such as GLP-1 (glucagon-like peptide 1), GIP (glucose-dependent insulinotropic polypeptide) or DPP-4 (dipeptidyl peptidase 4) are hormones secreted from the gastrointestinal tract after a meal rich in carbohydrates, lipids and proteins. GLP-1 and GIP promote insulin secretion but are short-lived and degraded by DPP-4. GLP-1 analogs and DPP-4 inhibitors are one of the most recent drug candidates in the T2DM field (DeFronzo 2010). Sodium glucose co-transporter 2 (SGLT2) inhibitors represent an additional novel therapy to treat T2DM. SGLT2 inhibitors suppress renal glucose reabsorption and increase urinary glucose excretion (Moses *et al.* 2014). However, continuous development is ongoing and necessary to discover more efficient and specific drugs as treatment for this disease.

Metformin, a biguanide class drug, is widely used as the first line therapeutic option for the treatment of T2DM in humans. The drug, introduced in 1957, has been on the market for decades, but surprisingly, its mechanism of action has been under extensive study ever since and has been poorly elucidated. Metformin is not bound to plasma proteins and is not a substrate of drug-metabolizing enzymes, being mainly eliminated unchanged through the kidneys. (Bailey 1992).

The therapeutic effects of metformin are thought to be most importantly transmitted by the hepatic action of the drug. Suppression of glucose output, increased peripheral glucose utilization, reduced fatty acid utilization and increased glucose turnover are clinical effects of metformin. It also has beneficial effects on the lipid balance by reducing hypertriglyceridemia and FAO

(Wiernsperger & Bailey 1999). The more detailed hepatic mechanisms of action suggested during the past years are introduced here.

The well-known hypothesis is that metformin exerts its effects mainly through the activation of AMPK (Zhou *et al.* 2001) and upstream kinase LKB1 (Shaw *et al.* 2005). The deletion of LKB1 in the liver resulted in decrease in AMPK Thr172 phosphorylation and subsequent loss of activation. Also severe hyperglycemia was manifested in the LKB1$^{-/-}$ mice. The administration of metformin did not lower blood glucose levels in these mice, suggesting that the LKB1-AMPK axis plays a critical role in the control of glucose balance in metformin therapy (Shaw *et al.* 2005). More specific details were subsequently reported describing the role of CREB coactivator 2 (CRTC2, also known as TORC2) in AMPK; however, not in metformin action. CRTC2 was shown to be phosphorylated by AMPK activators promoting the cytoplasmic retention and disruption of the CREB-mediated gluconeogenic gene expression (Koo *et al.* 2005a). He *et al.* reported that metformin suppresses gluconeogenesis by phosphorylating the CREB binding protein (CBP) via atypical protein kinase C (PKCι/λ). The activity of PKCι/λ was stimulated by the action of AMPK. CBP acts as a coactivator for CREB along with CRTC2 and is critical for the gene regulation of CREB targets. The phosphorylated CBP resulted in disruption from the CREB-CRTC2 complex, nuclear exclusion and transcriptional suppression of gluconeogenic genes (He *et al.* 2009).

Meanwhile, Kim and co-workers claimed that metformin induces SHP, the repressional NR, and by that mechanism, inhibits the gluconeogenic genes, i.e., by interfering with HNF-4α, FOXO1 and FOXA2 activity. The phenomenon was reversed in hepatocytes with siSHP which knocked down the SHP expression. The SHP induction was also shown to be present in metformin-treated, B6*Lep*$^{-/-}$ mice representing a T2D model (Kim *et al.* 2008).

In contrast to the traditional AMPK-mediated mechanism, more recent publications suggest also other mechanisms of action for metformin. It has previously been reported that metformin targets and inhibits the mitochondrial respiratory chain complex I, thus inhibiting ATP production and activating AMPK (El-Mir *et al.* 2000, Owen *et al.* 2000). However, Foretz *et al.* showed that the LKB1-AMPK is dispensable for the metformin action in liver and that the decrease in ATP correlates with the reduced, ATP-dependent glucose production in metformin-treated hepatocytes. In summary, the low cellular ATP caused by metformin explains the inhibition of gluconeogenesis (Foretz *et al.* 2010).

Interestingly, Ota *et al.* suggested ATP inhibition-independent as well as AMPK activation-independent effect of the suppression of G6Pase by metformin. However, no mechanistic details for their observations were reported (Ota *et al.* 2009). Another suggested mechanism involved the regulation of glucokinase by metformin. Namely, the glucose transporter 2 (GLUT2) is responsible for glucose release in the liver and is regulated by glucokinase activity. Metformin and AICAR were shown to inhibit the glucokinase translocation from nucleus to cytosol, also in AMPK-deficient cells, implying an AMPK-independent mechanism. The phenomenon was also suggested to be result of a fall in ATP concentration (Guigas *et al.* 2006).

A recent study suggested that metformin induces the activity of the counteracting SIRT1 and GCN5 to regulate the gluconeogenic gene response (Caton *et al.* 2010). Earlier it was shown that SIRT1 deacetylates CRTC2 and promotes its uibiquitin-mediated degradation (Liu *et al.* 2008). In the study by Caton *et al.* SIRT1 activation by metformin, dependent of AMPK activation, led to the inhibition of gluconeogenesis due to the suppression of the CRTC2-CREB axis. However, SIRT1 deacetylates also PGC-1α, leading to the activation of gluconeogenesis (Nemoto *et al.* 2005, Rodgers & Puigserver 2007). Interestingly, PGC-1α mRNA and protein was induced by metformin in db/db mice, but the inactivity of PGC-1α was explained by GCN5-mediated acetylation leading to inactivation of the protein (Caton *et al.* 2010). Furthermore, metformin reduces the expression of SIRT3, a mitochondrial sirtuin member responsible for the deacetylation of mitochondrial proteins. This mechanism was hypothesized to play a role in reducing ATP production by metformin, not involving AMPK, however. (Buler *et al.* 2012).

Very recently, the mechanism of metformin action was shown to be directly the antagonizing effect of glucagon. Namely, metformin promoted the AMP accumulation which then inhibited the adenylate cyclase directly and reduced the cellular cAMP levels. Thus, the PKA-mediated CREB-regulated gene expression would be diminished as a result of metformin-mediated inhibition of cAMP signaling (Miller *et al.* 2013).

Another more recent study showed that the mechanism by which metformin acts is by altering the cytosolic redox state. Namely, it was shown that metformin inhibits mitochondrial glycerophosphate dehydrogenase (mGPD). This mechanism was concluded to be a primary therapeutic target for early metformin action (Madiraju *et al.* 2014).

2.4 Drug and xenobiotic CYP metabolism in the liver

2.4.1 Cytochrome P450 enzymes

Cytochrome P450 (CYP) enzymes belong to a superfamily of heme-thiolate containing proteins that can metabolize a wide variety of substrates. The P450 superfamily comprises 18 CYP gene families in mammals, formed by sequence homology (more than 40% homology) and moreover, several subfamilies (more than 55% homology). The substrates of CYP families 1-3 usually cover xenobiotics such as most clinically used drugs, environmental pollutants and foodstuffs. The endobiotics, e.g. hormones, eicosanoids, cholesterol and vitamins, are usually substrates of the CYP families 4-51. This classification is not, however, strictly defined (Nebert & Dalton 2006, Nelson *et al.* 1996).

The expression pattern of CYPs is very broad in terms of tissue selectivity. However, the liver is the main organ comprising the highest abundance and largest amount of individual CYPs. Substantial amounts of CYPs can also be found in the intestine, lung, kidney, brain, adrenal gland, gonads, heart, nasal and tracheal mucosa, and the skin, which may contain the highest expression of certain CYP isoforms (Pelkonen *et al.* 2008).

The cellular localization of the CYPs is generally thought to be in the endoplasmic reticulum (ER) membranes, routinely referred to as microsomes. However, expression in other cellular compartments such as mitochondria and plasma membrane may be substantial and is regulated through so-called targeting signals within the CYP amino acid sequence. There are CYP enzymes that are expressed in either the ER or in the mitochondria, but interestingly, also so-called bimodal regulation exists. Namely, CYP1A1, 2B1, 2E1 and 2D6 have chimeric targeting signals to both ER and mitochondria which are dynamically regulated. The physiological consequences of the bimodal regulation are poorly understood but may suggest toxicity-related mechanisms (Avadhani *et al.* 2011).

Drug metabolizing CYPs and drug-drug interactions

The research of CYP enzymes and CYP genes has been extensive since the discovery of cytochrome P450s in the 1950s and 60s (Omura & Sato 1962). CYP enzymes catalyze a wide variety of reactions. A common feature is that CYPs can through the iron in their heme group react with molecular oxygen (O2) using NADPH as cofactor, resulting in the formation of an oxidized substrate and water

as a by-product. The most common reactions that the CYPs catalyze include carbon hydroxylation, heteroatom oxygenation, heteroatom release (dealkylation), epoxidation and aromatic hydroxylation, but some more complex reactions also exist (Guengerich 2001).

As mentioned, CYP enzymes have a wide variety of substrates including commonly prescribed drugs that are usually lipophilic in nature and which require metabolism to facilitate their clearance from the body. The estimation is that roughly half of the drugs are metabolized through the P450 system (of the top 200 drugs prescribed in the US) (Zanger *et al.* 2008). This shows the importance of studying both the enzyme actions and regulation mechanisms of the CYPs. CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are the most important ones in the metabolism of clinical drugs. Drug-drug interactions (DDI) are formed in the case of inhibition or induction of the CYPs by other drugs or xenobiotics. Mechanisms leading to these phenomena have been studied extensively with continuous novel factors found affecting the process especially at the gene transcription level (Michalets 1998, Pelkonen *et al.* 2008, Zanger *et al.* 2014).

2.4.2 Transcriptional regulation of CYPs

Cytochrome P450 genes are regulated by the NR superfamily but also by liver-enriched transcription factors such as HNF1α, HNF3, C/EBP and DBP. The Cytochrome P450 family transcriptional regulation has been extensively studied; see reviews by Akiyama & Gonzalez and Honkakoski & Negishi (Akiyama & Gonzalez 2003, Honkakoski & Negishi 2000).

PGC-1α and CYPs

PGC-1α, as the coactivator of the NR superfamily, is naturally involved in the regulation of the CYP family members. The first study describing the PGC-1α regulation on CYPs was the finding that CYP7A1 is induced by PGC-1α in fasting conditions and in streptozotocin-induced diabetic mice. The activation by PGC-1α was mediated by several nuclear receptors: HNF-4α, COUP TFII and LRH-1. CYP7A1 catalyzes the first enzymatic step in the bile acid biosynthesis pathway, which is the route by which excess cholesterol is eliminated from the body. CYP7A1 catalyzes the conversion of cholesterol into 7αhydroxycholesterol.

It was speculated that PGC-1α could be the link in the altered bile acid metabolism seen in diabetic patients (Shin *et al.* 2003).

PGC-1α was shown to induce the CYP2A6 in hepatocytes, which metabolizes substrates like nicotine and coumarin and to a lesser extent some clinically used drugs in humans. The induction was shown to be mediated through PXR and synergistic effect by HNF-4α (Itoh *et al.* 2006). Also, the activation of CYP1A1, CYP1A2, CYP2C9, CYP3A4, CYP3A5, and CYP2D6 by PGC-1α over-expression in hepatocytes has been suggested, and to be mediated by HNF-4α (Martinez-Jimenez *et al.* 2006).

Interestingly, a more specific study came out underlining the importance of PGC-1α in the regulation of CYP17A1 and CYP11A1, two enzymes involved in the initial steps of steroidogenesis in the liver. These enzymes catalyze the production of dehydroepiandrosterone (DHEA), a precursor for more specific steroid synthesis. Additionally, ERRα and HNF-4α were established as novel transcription factors regulating the induction of these genes dependent on PGC-1α. It was speculated that PGC-1α-induced DHEA production could be the mechanism behind the beneficial effects in liver metabolism since DHEA has been shown to reduce hyperglycemia and hyperinsulinemia in diabetic and obese mice (Grasfeder *et al.* 2009).

CYP2A5

The mammalian CYP2A subfamily consists of more than twenty (twenty-three so far) genes and non-functional pseudogenes (p). These include CYP2A1, 2A2 and 2A3 in rats; Cyp2a4, 2a5, 2a12, 2a20p, 2a21p, 2a22 and 2a23p in mice; and CYP2A6, 2A7, 2A13, 2A18P in humans (Nelson *et al.* 1996, Wang *et al.* 2003).

Mouse CYP2A5, also known as coumarin 7-hydroxylase, catalyzes the metabolism of the well-known alkaloid coumarin. In addition, it metabolizes toxins such as nicotine and its metabolite cotinine, (Zhou *et al.* 2010), aflatoxins and nitrosamines (Camus *et al.* 1993, Kirby *et al.* 1994). Recently also the by-product of heme metabolism, bilirubin, has been suggested as an endogenous substrate for CYP2A5 (Abu-Bakar *et al.* 2011). CYP2A5 is the major CYP in the mouse olfactory mucosa but expression is also detected in the liver, kidney, lung, brain and small intestine (Koskela *et al.* 1999, Su *et al.* 1996, Su *et al.* 1998)

The human ortholog CYP2A6 is called nicotine hydroxylase and has 82% amino acid similarity to CYP2A5 and also overlapping substrate specificity (Poso

et al. 2001, Raunio *et al.* 1988). CYP2A6 catalyzes 70-80% of nicotine to cotinine and is solely responsible for the hydroxylation of this metabolite.

Several chemicals including phenobarbital, pyrazole, cobalt, griseofulvin, PCN, TCPOBOP, thioacetamide and aminotriazole induce the expression of CYP2A5 (Cai *et al.* 2002, Donato *et al.* 2000, Hahnemann *et al.* 1992, Salonpaa *et al.* 1995). Many of these inducers also induce the human CYP2A6 *in vitro,* suggesting common mechanisms of regulation of transcription in both *Cyp2a5* and *CYP2A6* genes (Donato *et al.* 2000). However, regulation of *Cyp2a5* has been studied in more detail and seems to display complex and diverse characteristics compared to other classical *CYP* genes. Especially in situations of liver insults such as injury or toxicity, the expression of CYP2A6/CYP2A5 is upregulated while most other CYPs are suppressed. This mechanism suggests a role for this enzyme as both a marker of hepatic stress and a putative protective enzyme in these situations; see extensive review by Abu-Bakar *et al.* (Abu-Bakar *et al.* 2013).

Transcription factors known to regulate *Cyp2a5* include the aryl hydrocarbon receptor (AhR) together with the aryl hydrocarbon receptor nuclear translocator (ARNT). AhR was previously known as the typical inducer of the CYP1 family members upon exposure to environmental chemicals such as dioxins (Abel & Haarmann-Stemmann 2010). The *Cyp2a5* promoter was shown to contain a xenobiotic response element (XRE) at distal position, established as being responsible for the binding of Ahr/ARNT heterodimer (Arpiainen *et al.* 2005). In addition, ARNT was shown to regulate *Cyp2a5* as homodimer through a common E-box site also located at distal promoter region. Upstream stimulatory factors (USF) were shown to utilize the same site HNF-4α-dependently to induce CYP2A5 (Arpiainen *et al.* 2007).

Moreover, nuclear receptors HNF-4α and nuclear factor I (NF-I) have been shown as regulators of CYP2A5 expression together with CAR and PXR (Cai *et al.* 2002, Ulvila *et al.* 2004).

Interestingly, CYP2A5 was also shown to be induced by physiological stimuli such as glucagon through cAMP-mediated mechanism. In addition, fasting induces its expression in the liver (Bauer *et al.* 2004, Viitala *et al.* 2001). CYP2A5 is also regulated by circadian oscillation, the highest peak in expression being in the evening (Lavery *et al.* 1999, Viitala *et al.* 2001). The induction is mediated mainly through the albumin-D-site binding protein (DBP) and was demonstrated with DBP null (-/-) mice where the circadian rhythm of CYP2A5 expression was severely impaired (Lavery *et al.* 1999).

Recent studies suggest that CYP2A5 has a significant role in cellular heme homeostasis. Heme is the central cofactor in hemoproteins, such as the CYP family members. The heme catabolism pathway is tightly regulated due to the cytotoxicity of bilirubin, a metabolite of heme, at high amounts. At moderate levels, bilirubin putatively acts as an antioxidant in cellular stress situations (Jansen *et al.* 2010). Interestingly, CYP2A5 was established as a novel bilirubin oxidase. In addition, bilirubin seems to stabilize the protein, thus acting as an endogenous substrate for CYP2A5 and inducer of its own metabolism in circumstances of oxidative stress (Abu-Bakar *et al.* 2011).

Moreover, CYP2A5 is induced by Nuclear factor erythroid-derived 2 -like 2 (Nrf2) pathway activation (Abu-Bakar *et al.* 2007). Nrf2 is a well-known mediator of the oxidative stress response (Copple *et al.* 2008). Several stress-inducing chemicals, such as cadmium or heavy metals, induce both the Nrf2 pathway and CYP2A5 expression, suggesting CYP2A5 to be a strong candidate for acting as a biomarker of Nrf2 pathway activation (Abu-Bakar *et al.* 2004, Lamsa *et al.* 2010).

2.5 Endobiotic CYP metabolism in the liver

The function of Cytochrome P450s is not solely limited to xenobiotic metabolism. Namely, just under half of the human CYPs can have roles in endobiotic functions, mainly in sterol, fatty acid, eicosanoid and lipid-soluble vitamin (A&D) metabolism. In addition, there are several CYPs whose substrates are yet to be identified and which may be potential endobiotic metabolizing enzymes (Guengerich & Cheng 2011).

2.5.1 Vitamin D biosynthesis

Vitamin D is an essential fat-soluble vitamin in the body. Vitamin D refers to two highly lipophilic secosteroids: cholecalciferol, also known as vitamin D_3, and ergocalciferol, also known as vitamin D_2. D_2 is produced in fungi whereas D_3 is produced in the skin of animals by solar ultraviolet B radiation from precursor 7-dehydrocholesterol (7-DHC). Active vitamin D can be synthetized as such in the human body or taken as part of a diet rich in vitamin D or a supplement. The supplement is especially needed when access to sun is diminished, for example in the northern latitudes in the winter. Low vitamin D levels cause well-established problems in bone mineralization processes, i.e., osteoporosis and in most severe

cases, vitamin D-dependent rickets.(Kulie *et al.* 2009, Schuster 2011, Stocklin & Eggersdorfer 2013). It is increasingly evident that vitamin D deficiency may contribute to pathogenesis of cardiovascular diseases, metabolic syndrome, multiple sclerosis and several cancers (Kulie *et al.* 2009). Vitamin D has been established as a modulator of the immune system as well (Baeke *et al.* 2010).

Active vitamin D is formed via the vitamin D biosynthesis pathway which consists of both non-enzymatic and CYP enzyme catalyzed hydroxylation steps. First, 7-DHC, which is the "pro-vitamin D" synthesized in the skin as part of cholesterol biosynthesis (Glossmann 2010), undergoes non-enzymatic isomerization reaction upon ultraviolet B radiation to form previtamin D_3. Additionally, depending on adequate temperature, this form is spontaneously isomerized to vitamin D_3 (cholecalciferol). This molecule is then transported to the liver bound to vitamin D binding protein (DBP, also known as GC-globulin) and through enzymatic steps converted to active vitamin D, transported to target sites and finally inactivated in the body (Lehmann & Meurer 2010, Schuster 2011). The vitamin D biosynthesis pathway is summarized in Figure 5. These enzymatic steps are introduced in the next few chapters.

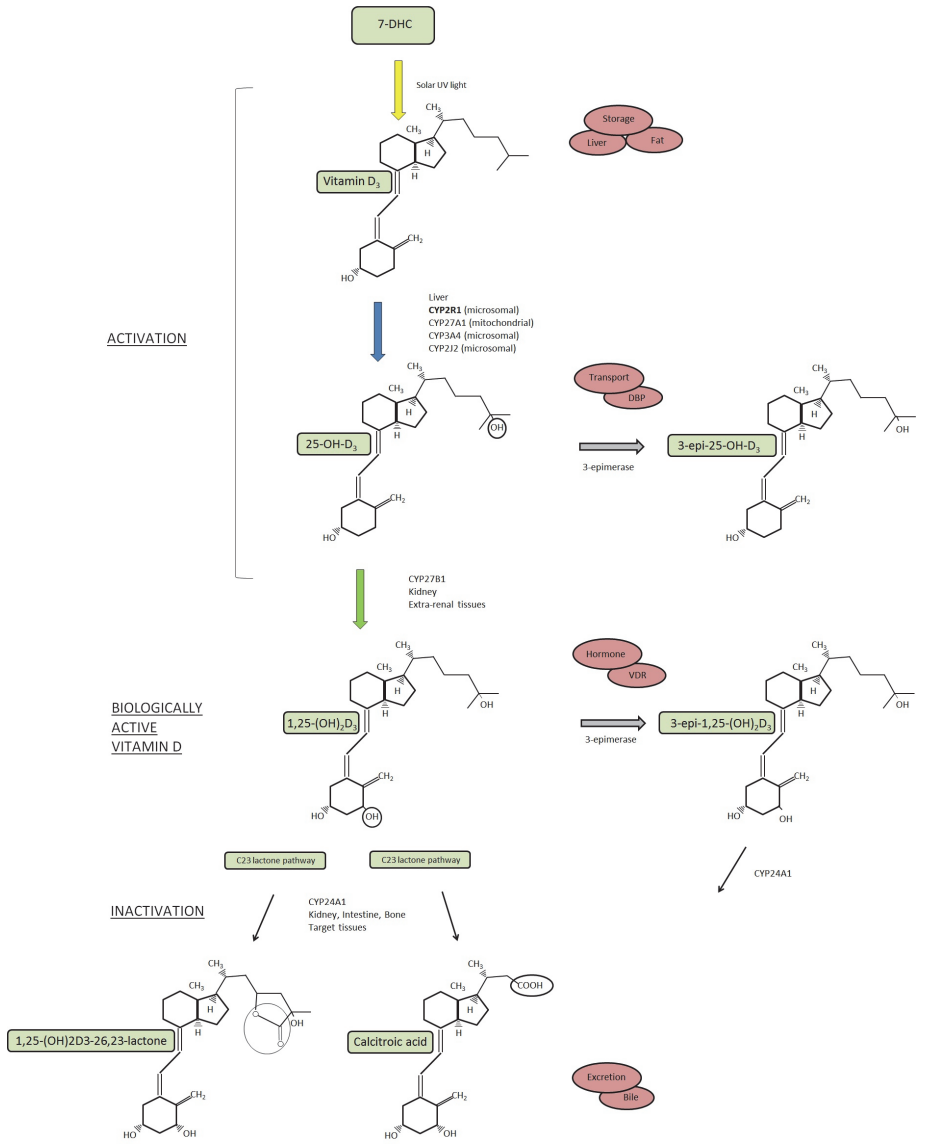

Fig. 5. The vitamin D biosynthesis pathway. A scheme illustrating the generation of active vitamin D3 from 7-dehydrocholesterol (7-DHC) upon sunlight exposure (yellow arrow) and the two subsequent activation steps (blue & green arrows) to the active hormone $1,25(OH)_2D_3$. The catabolism is performed by CYP24A1. In addition, both $25\text{-}OHD_3$ and $1,25(OH)_2D_3$ can also be converted to metabolites through C3-epimerization (modified from Schuster 2011 & Jones *et al.* 2012).

2.5.2 Formation of biologically active vitamin D

25-hydroxylation

The step of vitamin D 25-hydroxylation (carbon-25 or C-25 hydroxylation) occurs mainly in the liver and is the first of the two activating enzymatic reactions producing the "storage" form of vitamin D: 25-dihydroxyvitamin D_3, also known as calcifediol or simply $25(OH)D_3$. There are four CYP enzymes known to have substantial 25-hydroxylation activity: CYP2J2, CYP3A4, CYP27A1 and CYP2R1. Of these, CYP27A1 is mitochondrial while all others are microsomal enzymes (Schuster 2011).

Even though 25-hydroxylation is not the only activation step it seems that genetic defects in 25-hydroxylase activity can cause vitamin D-dependent rickets (VDDR type IB), a condition resulting from impaired vitamin D metabolism in children (Casella *et al.* 1994). Lately, several reports have suggested that of the four enzymes mentioned, CYP2R1 is the most important 25-hydroxylase *in vivo* since defects in its expression and activity cause vitamin D insufficiency (Al Mutair *et al.* 2012, Cheng *et al.* 2004). Also, CYP2R1 has been reported to have highest affinity and specificity towards vitamin D (Shinkyo *et al.* 2004). According to a very recent report describing the *Cyp2r1* knockout mice, it was confirmed that CYP2R1 is indeed the major 25-hydroxylase *in vivo*. However, the ablation of CYP2R1 did not result in complete loss of the circulating $25(OH)D_3$ in the blood. It was postulated that an additional, as yet unknown 25-hydroxylase enzyme exists (Zhu *et al.* 2013).

It has been thought that the step of 25-hydroxylation is loosely regulated, if at all. More tight regulation is involved in the next step of activation. In fact, the transcriptional regulation of CYP2R1 is poorly characterized. One report describes that in dermal fibroblast cells treatment with phenobarbital and efavirenz as well as active vitamin D suppresses the expression of CYP2R1 (Ellfolk *et al.* 2009). Another study shows that in testis Leydig cells, osteocalcin can modulate the CYP2R1 protein expression, thus affecting the vitamin D 25-hydroxylation and bone mineralization function of testis (De Toni *et al.* 2014). An increasing amount of evidence suggests that the promoter regions of the vitamin D biosynthesis genes, including CYP2R1, are epigenetically, reversibly suppressed by e.g. vitamin D status in the circulation (Fetahu *et al.* 2014).

1α-hydroxylation

It is well established that mitochondrial CYP27B1 is the single enzyme responsible for the 1α-hydroxylation (carbon 1α or C-1α hydroxylation) step, which results in the biologically active 1α,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$. This step occurs primarily in the kidney. Low 1α-hydroxylation activity has been reported for the enzyme CYP27A1 *in vitro* but it is not a probable player *in vivo* (Sawada *et al.* 2007). VDDR (type I) caused by missense mutations in *CYP27B1* gene results in disturbance of the enzyme activity (Kitanaka *et al.* 1999, Sawada *et al.* 2001).

The regulation of CYP27B1 is controlled positively by parathyroid hormone (PTH) which is released at low calcium levels from parathyroid glands. As a result, CYP27B1 is induced and active vitamin D is formed from the circulating storage 25(OH)D_3. CYP27B1 expression is suppressed by 1,25$(OH)_2D_3$; this is performed by methylation silencing through the promoter region, and PTH is able to reverse the methylation and result in activation (Schuster 2011). Interestingly, the mechanism of activation–suppression of CYP27B1 activity seems to be limited to the kidneys. In the respiratory track and in the skin it has been reported and suggested that CYP27B1 expression is constitutively active and 1,25$(OH)_2D_3$ plays a role in the protection against external agents by inducing host defense genes. In these cases, the active vitamin D hormone levels are downregulated, probably by induced catabolic CYP24A1 and not by the suppression of CYP27B1 action through 1,25$(OH)_2D_3$ (Hansdottir *et al.* 2008, Schuster *et al.* 2001).

In addition, fibroblast growth factor 23 (FGF23) is an important negative regulator of 1,25$(OH)_2D_3$. FGF23 is activated and secreted by high serum phosphate and 1,25$(OH)_2D_3$ concentrations from bone osteocytes. In the kidney proximal tubules FGF23 binds to the FGF receptor and results in transcriptional suppression of CYP27B1 (Henry *et al.* 2011, Shimada *et al.* 2004).

2.5.3 Vitamin D action and inactivation

Once active, vitamin D has genomic and non-genomic actions. All the genomic actions involve the activation of vitamin D receptor (VDR), a member of the NR superfamily, activated by ligand 1,25$(OH)_2D_3$ and subsequent heterodimerization with RXR to regulate target gene promoter regions (Noda *et al.* 1990). One such target gene is the catabolic enzyme CYP24A1 which is induced by the VDR

activation and acts as a negative regulatory loop for the action of vitamin D (Schuster 2011).

The non-genomic action of vitamin D refers to the effect on several signal transduction pathways. These mechanisms are clearly more rapid than the genomic ones requiring transcriptional activation. For example, the Ca^{2+} influx [Ca^{2+}] is rapidly increased by vitamin D; also at very high levels the intracellular stores of Ca^{2+} are being released. In addition, adenylate cyclase, phospholipase c, protein kinases C and D, mitogen-activated protein kinase (MAPK) and rapidly growing fibrosarcoma kinase (Raf) pathways are modulated by active vitamin D (Civitelli *et al.* 1990, Lehmann & Meurer 2010).

Active vitamin D is short-lived due to the promotion of its own catabolism via induction of 24-hydroxylase (carbon-24 or C-24) CYP24A1. This enzyme is solely responsible for the inactivation of the active form by hydroxylation to water-soluble, biliary metabolite calcitroic acid. The CYP24A1 can recognize not only the $1,25(OH)_2D_3$ but also the $25(OH)D_3$ as substrate. CYP24A1 is expressed at highest levels in oocytes and kidneys, but also in many other tissues at lower levels. The expression pattern suggests a role for CYP24A1 and vitamin D inactivation in extrarenal tissues as well. Interestingly, in addition to regulation by vitamin D and VDR, also PXR and its ligands have been reported to induce CYP24A1 (Pascussi *et al.* 2005). In addition, CYP24A1 overexpression has been detected in several malignancies and associated with poor prognosis and has thus been considered a potential oncogene (Schuster 2011).

3 Aims of the present study

The major objective of the study was to unravel molecular mechanisms behind PGC-1α-regulated functions in the liver. It is utterly important to establish how PGC-1α itself is regulated as it is such an important protein in high-energy tissues, regulating endogenous glucose metabolism as well as drug metabolism. Additionally, also investigated in this thesis were PGC-1α-regulated pathways in physiological and pathological conditions which induce its expression, models of starvation and T2DM.

Specifically, the aims of the study were:

1. To characterize the role of PGC-1α in metformin's mechanism of action, in particularly, its relation to AMPK.
2. To study how PGC-1α regulates inflammatory mechanisms in response to conditions inducing PGC-1α expression.
3. To identify novel, mouse PGC-1α-regulated hepatic CYP genes.
4. To study mechanisms of CYP2A5 induction by fasting and glucagon.
5. To establish the role of PGC-1α in the regulation of vitamin D metabolism.
6. To show that vitamin D 25-hydroxylase CYP2R1 is regulated by previously unknown metabolic factors in the liver.

4 Materials and methods

An overview of the materials and methods used throughout the thesis is presented here. More detailed information is found in the original publications.

4.1 Reagents and antibodies

All chemicals, the oligos used in qPCR and gene silencing siRNA oligos were purchased from Sigma-Aldrich (St. Louis, MO, USA) with the exception of the following: A769662 was from Tocris Bioscience (Bristol, UK). TaqMan gene expression assays were from Applied Biosystems, Life Technologies (Carlsbad, CA, USA). Recombinant human IL1β protein (ab9617) was from Abcam (Cambridge, UK). siIL1Rn (siGENOME SMARTpool) and appropriate controls were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Real-time qPCR mastermixes, FastStart Universal Probe Master and FastStart SYBR Green Master came from Roche Diagnostics (Mannheim, Germany).

Reagents used in cell culturing experiments such as fetal bovine serum (FBS), antibiotics (Penicillin-streptomycin), Dulbecco's modified eagle medium (DMEM), trypsin, phosphate buffered saline (PBS), all cell culture grade, were purchased from Gibco, Life Technologies. Collagenase type II was purchased from Biochemical Corporation (Lakewood, NJ, USA). William's E medium used in primary cell cultures came from Sigma-Aldrich.

Rabbit polyclonal anti-CYP2R1 IgG (#T2849) was purchased from Epitomics (Burlingame, CA, USA), mouse monoclonal anti-β-actin IgG (A1978) from Sigma-Aldrich. Goat polyclonal anti-IL1Rn IgG (sc-8482), goat polyclonal anti-IL-15Rα IgG (sc-1524), rabbit polyclonal anti-CYP24A1 IgG (H-87, sc-66851), rabbit anti-PGC-1α IgG (sc-13067), rabbit anti-HNF-4α IgG (sc-8987) were from Santa-Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-acetylated lysine (Ac-Lys) IgG (#9681) and rabbit anti-phospho-acetyl-CoA carboxylase (pACC) IgG (#3661) were from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-PGC-1α IgG (ST1202), used in immunodetecting of immunoprecipitated PGC-1α, was from Calbiochem (Darmstadt, Germany). Goat anti-rabbit IgG-HRP (sc-2004) and anti-mouse IgG-HRP used as secondary antibodies were purchased from Santa Cruz Biotechnology and GE healthcare (Little Chalfont, UK), respectively.

4.2 Hepatocyte cell cultures and animals

4.2.1 Primary hepatocytes (I-IV)

Mouse primary hepatocytes were prepared by two-step collagenase method, as described previously (Salonpaa *et al.* 1994). Briefly, after isolation, centrifugation and filtration the hepatocytes were dispersed into William's E medium containing 2.0 g/L D-glucose and ITS (insulin 5 mg/L, transferrin 5 mg/L, sodium selenate 5 μg/L). To conduct experiments, cells were seeded into appropriate cell culture-compatible multiwell plates or dishes, usually 12-well or 6-well plates or 10 cm (in diameter) dishes.

Primary hepatocytes were usually incubated overnight before additional treatments with chemicals or viral particles. These experiments were performed using serum-deprived Williams E medium (Sigma-Aldrich).

In original publication I one batch of human primary hepatocytes was purchased from Biopredic International (Rennes, France) and cultivated in the medium according to protocol from Biopredic. RNA samples from the second batch of human hepatocytes were obtained from Dr. Petr Pavek's laboratory (Charles University in Prague, Czech Republic).

4.2.2 Continuous cell lines

All cell lines expect the adenoviral packaging cells were obtained from American Type Culture Collection (ATCC, Manassas, VI, USA). The cells were cultured following aseptic techniques, according to media and protocols recommended by the ATCC.

Recombinant adenoviral particles were produced in the cell line QBI-293A (HEK-293A) obtained from Qbiogene Inc. (Carlsbad, CA, USA).

4.2.3 In vivo animal experiments (I-IV)

All animal experiments were approved by the local animal welfare agencies. In original publication I *in vivo* work with oral metformin (300 mg/kg/day) treatment was conducted using either pregnant female (Salomaki *et al.* 2013) or male C57BL/6 mice aged 16-17 weeks. In original publications II and IV male Sprague-Dawley rats were used in the fasting experiment. In publication IV, female C57BL/6 ob/ob and wild type mice aged 5-6 months were used. In

addition, male DBA/2 mice aged 8-12 weeks were used in the fasting experiment (IV).

4.3 Transient transfections into primary hepatocytes (I-III)

4.3.1 Expression plasmid transfections

The 2,000 bp PGC-1α-LUC and the mutation promoter constructs (I) have been described previously (Handschin *et al.* 2003) and were purchased from Addgene (Cambridge, MA, USA) (plasmids #8887 and #8888). The mouse 1,900 bp IlRn-LUC promoter construct (II) has been described previously (Stienstra *et al.* 2007). The Cyp2a5 5'-LUC promoter constructs (III) and the site-directed mutation of them have been described previously (Ulvila *et al.* 2004).

Renilla luciferase reporter vector (pRL-TK) (Promega) was used as control in all transfection experiments. Plasmids were transfected into primary hepatocytes or HepG2 cells using Tfx-20 reagent (I, III) from Promega, Fugene HD reagent (I, III) from Roche Diagnostics with optimized DNA–lipid composition, according to manufacturer's protocol. Luciferase activities were assayed with Dual-Luciferase Reporter Assay System (Promega).

4.3.2 Small interfering RNA (siRNA) transfections

For gene silencing, small interfering RNA (siRNA) oligos were used, purchased from Sigma-Aldrich or Thermo Scientific; specific sequences or product numbers can be found in the original publications. Scramble and target gene siRNAs were transfected into hepatocytes using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol followed by chemical or adenoviral treatment 5-24 hours post-transfection.

Cells were incubated for an additional 24-72 hours to achieve sufficient downregulation of the target gene, which was confirmed by qPCR analysis.

4.4 Adenoviral transductions (I-IV)

All recombinant adenoviruses used in original publications I-IV were constructed according to the protocol modified from the AdEasy Adenoviral vector system (Stratagene, La Jolla, CA, USA). Briefly, the target gene of interest was cloned

into a shuttle vector pShuttle-CMV (Qbiogene Inc.) with appropriate restriction enzymes. This vector contains CMV promoter along with SV40 polyadenylation signal. The gene of interest was confirmed by sequence analysis. The shuttle vector was then linearized with *Pme*I restriction enzyme and complete digestion was confirmed by agarose gel electrophoresis. The linearized vector was electroporated by Gene Pulser Transfection apparatus (Bio-Rad, Hercules, CA, USA) into BJ5183-AD-1 bacterial competent cells (Stratagene) transformed with adenoviral vector backbone, allowing recombination with the shuttle. Positive, recombinant clones were selected for kanamycin resistance and confirmed by *Pac*I restriction digest. Resulting recombinant viral plasmids were amplified in *E.coli* XL-1 bacteria and purified with maxiprep kit (Sigma-Aldrich), with ethanol-sodium acetate cleanup to produce a sufficient amount of plasmid for transfection.

The recombinant adenoviral construct was linearized with *Pac*I restriction enzyme after which it was transfected with Lipofectamine 2000 (Invitrogen) into adenoviral packaging cell line QBI293, modified from HEK293 (Q-Biogene Inc.). The transfected, detached cells were collected by centrifugation. Infectious particles were released from the cells by freeze-thaw cycles allowing lysis. Lysates were centrifuged and fresh QBI293 cells were infected with the supernatant. Three to four such amplification cycles were performed, and the final supernatant was purified by density gradient ultracentrifugation using iodixanol (Optiprep, Axis-Shield PoC AS, Oslo, Norway).

The viral particle infectivity was measured according to the AdEasy Viral Titer kit (Stratagene).

4.5 mRNA analyses (I-IV)

Total RNA was isolated using either TRI reagent (Molecular research center, Cincinnati, OH, USA), RNAzol RT (Sigma-Aldrich) or NucleoSpin RNA II kit (Düren, Germany) according to manufacturer's protocol. Five µg of total RNA was treated with DNAse (Promega) to avoid genomic DNA contamination, after which RNA, usually 1 µg, was converted into cDNA using M-MuLV enzyme (Promega) and random hexamers (Roche Diagnostics) before real-time qPCR analysis.

Gene expressions were quantified either with SYBR green or TaqMan probe chemistries, by specific primers and/or probe sets for each target gene. All oligonucleotide sequences used can be found in the original publications. The

qPCR reactions were performed on ABI 7300 qPCR system (I, II, IV) (Applied Biosystems) or Mx3000P qPCR system (III) (Stratagene). The resulting fluorescence values were corrected by the passive reference dye ROX after which gene expression fold-values were calculated by the comparative C_t method (Livak method), also known as the ΔΔCt method using 18S as normalizer gene.

4.6 Protein analyses (I-IV)

Protein fractions were prepared from cultured primary hepatocytes or tissue samples according to two main approaches. Total protein fractions were prepared by sonication of harvested cell pellet suspended in cold PBS, and centrifugation of the lysate at 13,000 g, after which the supernatant was subjected to SDS-PAGE. Alternatively, the nuclear and cytosolic proteins were fractionated according to protocol a modified from Schreiber *et al.* (Lamsa *et al.* 2010, Schreiber *et al.* 1989).

The protein fractions (usually 10-50 μg) obtained were subjected to SDS-PAGE and transferred to nitrocellulose or PVDF membrane with semi-dry blotting apparatus (Bio-Rad). After blocking the unspecific background binding the membrane was treated with primary antibody, usually overnight, in 5% skimmed milk in TBS-Tween buffer after with washing was performed. Membrane was then treated with secondary antibody with conjugated horseradish peroxidase and after washing, the immunoreactive bands were visualized using chemiluminescent peroxidase substrate CPS-1 reagent (Sigma-Aldrich).

The resulting band intensities were measured with Quantity One software (Bio-Rad). The proteins of interest were normalized against β-actin protein intensity.

Chromatin immunoprecipitation (ChIP) experiments were conducted according to a previously described method (Abu-Bakar *et al.* 2007) with the addition of re-ChIP experiment.

4.7 Statistical analyses

Statistics were calculated either with Graphpad Prism v.5.04 (I, II, IV) or SPSS (III). Comparison of two groups was performed using Student's t-test. For the comparison of multiple groups, one-way ANOVA was used, followed by Dunnett, Tukey (I, II, IV) or LSD (III) *post hoc* test. Two-way ANOVA was used when

comparing two variables in two groups, followed by Dunnett *post hoc* test. Differences were considered significant when $p<0.05$.

5 Results and discussion

Only the most relevant findings from the original publications are presented as well as discussed here. The original publications are referred to with the Roman numerals I-IV and figures with corresponding numbers as in the original publications.

5.1 Type 2 diabetes drug metformin induces coactivator PGC-1α in the liver (I)

In original publication I, metformin's hepatic mechanism of action in relation to PGC-1α was investigated. Metformin, a widely used oral treatment for T2DM, is known to act on diminishing hepatic glucose output (DeFronzo 1999). However, the specific action still remained obscure and surprisingly, the regulation of PGC-1α by metformin in the liver had not been reported before.

The role of LKB1 as an upstream kinase for AMPK and as a target for metformin action in the liver has been established (Shaw *et al.* 2005). In addition, it has been shown that AMPK activation causes phosphorylation of the CREB coactivator CRTC2, resulting in its cytoplasmic retention and inability to induce gluconeogenic genes (Koo *et al.* 2005b). We aimed specifically to study the role of the coactivator PGC-1α in the hepatic metformin action. Surprisingly, we found that metformin was able to induce PGC-1α mRNA and protein in *ex vivo* mouse hepatocyte culture (I, Fig. 1A-C). The results were reproducible in human hepatocyte culture as well (I, Fig. 1D) but not in rat (data not shown). Concomitantly, the gluconeogenic genes were clearly downregulated, suggesting another, PGC-1α-independent mechanism for the suppression.

We also studied the phenomenon *in vivo*. C57BL/6 mice were administered 300 mg/kg/day metformin for seven and sixteen days in two studies with slightly different set-ups (I, Fig. 1E-F). In mice treated with metformin for seven days, receiving the last dose 4 hours prior to killing, PGC-1α mRNA level was clearly induced in the livers compared to controls. However, there was no clear difference in PEPCK expression levels in mice treated with metformin compared to control mice. In the second study, in mice receiving metformin for 16 days, the last dose 24 hours prior to killing, there was no change in PGC-1α level, but PEPCK was clearly downregulated by metformin. Although the results were not as striking as in *in vitro* hepatocytes, we were able to show that hepatic PGC-1α is most certainly not repressed but slightly induced by metformin *in vivo,* in contrast

to previous assumptions. Possibly, there could be circadian fluctuation in the expression of PGC-1α in response to metformin. Alternatively, PGC-1α expression may be dependent on the time of sample collection after last metformin dosing, shown by the two study set-ups.

5.2 PGC-1α is induced by AMPK action and retains regulation on mitochondrial genes (I)

The induction of PGC-1α seems to be AMPK-dependent. Over-expression by constitutively active AMPKα1 resulted in the induction of PGC-1α in the hepatocytes (I, Fig. 2E). Also, knocking down the AMPK isoforms α1 and α2 resulted in abolishment of the PGC-1α induction by metformin (I, Fig. 2F). Phosphorylation of PGC-1α by AMPK, which has been reported in skeletal muscle (Jager *et al.* 2007), could be a potential mechanism of activation. We studied this effect by over-expressing mouse hepatocytes with PGC-1α-Ad with mutated phosphorylation sites (T177A, S538A) responsible for the phosphorylation in skeletal muscle. Hepatocytes were additionally treated with metformin. When comparing the results with wild-type PGC-1α over-expression there was no clear difference in the expression of any target genes, suggesting that the phosphorylation status of PGC-1α at these sites does not play a role in metformin action in the liver (data not shown). Since SIRT1 was shown to be activated by AMPK (Canto *et al.* 2009), we also addressed the question whether PGC-1α acetylation status is changed by metformin. We were able to detect mild deacetylation at the protein level by metformin (I, Fig. 5A). In addition, knocking down SIRT1 in hepatocytes resulted in diminished induction of PGC-1α by metformin treatment (I, Fig. 2G).

Clearly, PGC-1α is not able to activate the gluconeogenic program upon metformin treatment. Interestingly, we found that metformin was still activating the genes responsible for mitochondrial biogenesis, such as cytochrome c, ATP synthase and the cytochrome c subunits (I, Fig. 4B). In summary, we show that metformin-induced PGC-1α does not act as an activator of gluconeogenesis, which is dramatically downregulated by metformin. However, PGC-1α still retains the ability to activate mitochondrial genes by metformin.

5.3 PGC-1α regulates a set of inflammatory response genes including *IL-1Rn* (II)

In original publication II, inflammatory cytokines regulated by PGC-1α expression were characterized. During recent years it has become evident that the chronic inflammatory response in metabolic perturbations such as diabetes and obesity is one important factor contributing to their pathogenesis. All major metabolic organs including the liver as well as brain, adipose tissue and skeletal muscle play a role in the inflammatory response (Lumeng & Saltiel 2011). However, the role of PGC-1α in regulating inflammatory genes or cytokines in the liver has not been clearly elucidated.

PGC-1α was over-expressed in mouse primary hepatocytes by adenoviral transduction. On the treated cells' mRNA, a microarray was performed resulting in mouse genes up- or downregulated by PGC-1α-Ad compared to control virus treatment. A set of inflammatory pathway genes was identified using Ingenuity Pathway Analysis software. The results were compared to a previously performed microarray using fasted mice (GSE17865) and genes regulated in the same direction by PGC-1α and fasting (-/+1.5-fold compared to controls) were selected for further studies (II, Fig. 1A).

From the analysis performed, it became evident that PGC-1α regulates a set of inflammatory responsive genes in the liver. Namely, PGC-1α downregulated several inflammatory cytokines, one being TNF-related weak inducer of apoptosis (TWEAK). In contrast, PGC-1α upregulated anti-inflammatory proteins including IL-1Rn and IL15-Rα (II, Fig. 1B). Glucagon and cAMP treatments showed that these genes were responsive to the cAMP signaling pathway while siPGC-1α experiment showed that PGC-1α is indeed the mediator of IL-1Rn and IL-15-Rα induction in hepatocytes (II, Fig. 2). The mechanism was also investigated *in vivo*. In rat livers exposed to fasting and exercising conditions, both known to induce PGC-1α, IL-Rn and IL-15Rα mRNAs were significantly increased (II, Fig. 3).

Next, the mechanism behind the induction of the anti-inflammatory cytokines was investigated. PPARα has been shown to regulate *IL-1Rn* (Stienstra *et al.* 2007) and is a known interaction partner to PGC-1α (Vega *et al.* 2000). Thus, the role of PPARα involvement was studied, and indeed, knock-down of PPARα in hepatocytes treated with PGC-1α-Ad did abolish the induction of IL-1Rn and IL-15-Rα mRNAs (II, Fig. 4).

Furthermore, the role of IL-1Rn induction by PGC-1α in inflammatory conditions was assessed. IL-1Rn was knocked down by siRNA in mouse primary hepatocytes. To achieve inflammatory response, recombinant IL-1β, which is the target for IL-1Rn, was dosed into the cell medium. In addition, cells were treated with PGC-1α-Ad. Firstly, IL-1β alone caused inflammatory response, seen by induction of inflammatory genes CRP and haptoglobin (II, Fig. 5). Secondly, in the presence of PGC-1α-Ad the expression of these genes was low. However, when IL-1Rn was knocked down, in PGC-1α-Ad and IL-1β treated cells the expressions of CRP and haptoglobin were significantly higher. This suggests that IL-1Rn mediates largely the anti-inflammatory effect in response to PGC-1α over-expression.

5.4 Induction of *IL-1Rn* is modulated by AMPK (II)

Since PGC-1α action is activated by AMPK we also investigated the role of AMPK towards IL-1Rn induction by PGC-1α. When overexpressing hepatocytes by constitutively active AMPKα1 also IL-1Rn was significantly induced (II, Fig. 6C-D). Treatment with metformin, a known AMPK activator, combined with either cAMP or glucagon also induced IL-1Rn (II, Fig. 6A-B).

Furthermore, *IL-1Rn* promoter was studied by transfecting a 1,900-bp 5' flanking region promoter construct together with a PGC-1α phosphorylation mutant (T177A, S538A) plasmid and AMPK-Ad into mouse hepatocytes. The results showed that there was clear induction of the *IL-1Rn* promoter activity in response to AMPK-Ad and wild type PGC-1α. However, the activity was significantly diminished by the PGC-1α phosphorylation mutant, suggesting that AMPK may affect *IL-1Rn* transcription through phosphorylation of PGC-1α at these sites (II, Fig. 6E).

5.5 PGC-1α and AMPK; mutual regulators of glucose homeostasis

AMPK is induced in energy deprivation, i.e., when cellular ATP/ADP or ATP/AMP rises, basically through two mechanisms. When energy fuels are low, AMPK can directly interact with AMP by docking it to the γ subunit, changing the conformation of the kinase to promote activation. Secondly, AMPK is activated by phosphorylation at threonine 172 by upstream kinases, mainly LKB1 or Ca^{2+}-dependent CaMKK. Also the conformational change by AMP binding promotes the phosphorylation at Thr172 and inhibits dephosphorylation by

phosphatases (Viollet *et al.* 2009b). LKB1, as already mentioned, was reported to activate AMPK in metformin action (Shaw *et al.* 2005). The ultimate role of AMPK is to promote cellular energy-producing pathways and to arrest energy consumption (Viollet *et al.* 2009a).

Activation of AMPK phosphorylates and activates the PGC-1α protein by compounds like metformin or AICAR in skeletal muscle. Interestingly, also gene expression is induced by the AMPK modulators (Jager *et al.* 2007, Suwa *et al.* 2006). Furthermore, AMPK activation decreases the acetylation status of PGC-1α in myotubes and skeletal muscle, mediated through SIRT1 activation. This activation is mediated through increased NAD^+/NADH ratio by AMPK (Canto *et al.* 2009).

However, in the liver the role of PGC-1α as well as AMPK is profoundly different from that in skeletal muscle, with differences in the mechanisms of interactions of these two. Namely, activation of AMPK leads to decreased and the activation of PGC-1α to increased glucose output from the liver. Decrease in glucose production by AMPK activation results from phosphorylation of CREB coactivators, with subsequent inactivation and downregulation of CREB-mediated gene expression (Koo *et al.* 2005a). According to a previous hypothesis, through AMPK, metformin causes the phosphorylation of CRTC2 and sequestration to the cytosol thus preventing the transcription of PGC-1α and other CREB target genes (Viollet *et al.* 2009a). We clearly show in original publication I that this is not the case for PGC-1α. We do not rule out the possible effect on PEPCK and G6Pase expression by this mechanism. In fact, it seems that regulation of gluconeogenesis upon metformin stimulus is more versatile than was previously assumed. We found that several transcription factors responsible for gluconeogenesis–HNF-4α, FOXO1 and KLF15–are downregulated by metformin, suggesting additional mechanism for the suppression. In contrast, we and others (Krausova *et al.* 2011) did not detect induction, but rather dramatic downregulation of the repressor protein SHP by metformin. The induction of SHP by metformin was suggested to explain the downregulation of the gluconeogenic program by acting as repressor for the HNF-4α mediated regulation (Kim *et al.* 2008).

PGC-1α activity is also regulated through acetylation status and interestingly, AMPK activates also the deacetylase SIRT1, although indirectly by altering the NAD^+/NADH ratio in skeletal muscle (Canto *et al.* 2009). In the study by Caton and co-workers, metformin treatment in the diabetic liver activated AMPK and subsequently SIRT1, leading to the deacetylation of PGC-1α (Caton *et al.* 2010) rendering the coactivator active in terms of activation of gluconeogenesis

(Rodgers *et al.* 2005). However, intriguingly, the acetyltransferase GCN5 was additionally shown to be AMPK-independently activated by metformin, rendering PGC-1α inactive (Caton *et al.* 2010). We detected deacetylation of PGC-1α protein by metformin; additionally, SIRT1 knock-down diminished the induction of PGC-1α by metformin.

In summary, according to our hypothesis metformin activates AMPK in hepatocytes and subsequently activates SIRT1, which induces PGC-1α at mRNA as well as protein level. However, gluconeogenesis is inhibited by other, PGC-1α-independent means. We additionally show that PGC-1α, induced by metformin, retains the ability to activate mitochondrial gene response.

These findings may offer explanation for the "beneficial" effects of metformin treatment. Metformin acts to suppress ATP production and one of the primary targets is claimed to be mitochondrial respiratory chain complex I, resulting in activating AMPK (El-Mir *et al.* 2000, Owen *et al.* 2000). The induction of PGC-1α may have a protective role in retaining the mitochondrial gene expression in response to metformin.

5.6 Role of PGC-1α in mechanisms of the metabolic syndrome

Unraveling the mechanisms and involvement of PGC-1α in the pathogenesis of the metabolic syndrome, e.g. inflammation or insulin resistance, is utterly important to be able to treat patients as well as develop potential therapeutics for the disease. It is well established that the signaling pathways and molecules involved, such as kinases and receptors, are key components and possible target molecules. Also PGC-1α has emerged as a crucial protein since it regulates as well as is regulated in such a versatile manner in metabolic disturbances. In addition, PGC-1α is affected through external stimuli such as fasting or exercise. Thus, PGC-1α can be considered a key scaffold protein, adapting cellular signaling to extracellular needs.

Skeletal muscle insulin resistance has emerged as one of the early signs of T2DM. It has detrimental effects on the whole body glucose homeostasis due to the key role of storing excess glucose from the body (DeFronzo & Tripathy 2009). In skeletal muscle, the reduction of PGC-1α expression has been observed in obesity and T2DM with insulin resistance (Crunkhorn *et al.* 2007, Patti *et al.* 2003). It was hypothesized that due to the reduced action of PGC-1α as well as key transcription factors, the expression of metabolic genes in the mitochondria is reduced. As a consequence, lipids accumulate in skeletal muscle causing insulin

resistance (Patti *et al.* 2003). Also, long chain fatty acids were found to decrease the expression of PGC-1α, creating a vicious cycle (Crunkhorn *et al.* 2007). The role of these free fatty acids (FFAs) in the pathogenesis of insulin resistance is well known (Abdul-Ghani & DeFronzo 2010). In the light of this, the upregulation of PGC-1α in skeletal muscle is advantageous with improved mitochondrial function. Indeed, e.g. rosiglitazone or metformin improve PPAR and PGC-1α action along with oxidative enzyme activity in skeletal muscle (Mensink *et al.* 2007, Suwa *et al.* 2006).

It is generally known that T2DM and obese patients have low-grade inflammation manifesting as circulating inflammatory cytokines, e.g. CRP, IL-6 (Pradhan *et al.* 2001), IL-1β (Herder *et al.* 2009, Spranger *et al.* 2003) and TNFα (Tilg & Moschen 2008) originating mainly from the WAT. It is currently not fully known what the trigger for inflammation is in the patients. ROS, generated from excessive exposure to fat from diet and incomplete oxidation in adipocytes, is a highly potent factor inducing inflammatory response by activating the NF-kβ pathway and activating expression of inflammatory cytokines. Thus, these factors leak and are secreted throughout the body (Furukawa *et al.* 2004). According to our findings, PGC-1α is able to attenuate the inflammatory response by inducing anti-inflammatory cytokines such as IL-1Rn through PPARα in hepatocytes.

However, as has already become clear, the tissue-specificity of PGC-1α action is different in the liver than in skeletal muscle. In T2DM patients' liver, PGC-1α expression status is not so clearly established. PGC-1α polymorphisms in humans cause onset of type 2 diabetes and glucose impairment (Ek *et al.* 2001, Hara *et al.* 2002, Oberkofler *et al.* 2004), which would suggest a major role for PGC-1α in normal liver function. In addition, many PGC-1α target genes involved in oxidative phosphorylation are downregulated in diabetics (Mootha *et al.* 2003). However, PGC-1α protein is elevated in the livers of type 2 diabetic mice (Yoon *et al.* 2001). Downregulation in the livers of obese and insulin-resistant humans has been reported (Croce *et al.* 2007), as has, controversially, upregulation in T2DM patients' livers (Misu *et al.* 2007). The findings could in general suggest that PGC-1α action regulating the mitochondrial response is most importantly an advantage while the action on gluconeogenesis is a disadvantage in patients with the metabolic syndrome.

5.7 PGC-1α regulates the mouse coumarin-7-hydroxylase CYP2A5 (III)

In original publication III, the regulation of CYP2A5 by the energy sensor PGC-1α was studied. Previously, CYP2A5 had been shown to be induced by starvation (Bauer *et al.* 2004). Additionally, the cAMP pathway activator fasting-inducible hormone glucagon was shown to upregulate CYP2A5 in mouse hepatocytes (Salonpaa *et al.* 1994, Viitala *et al.* 2001).

In the present study we show that in addition to PEPCK, over-expression of PGC-1α by adenoviral transduction in mouse primary hepatocytes also induces CYP2A5 mRNA. CYP2A5 was induced both time- and dose-dependently in the PGC-1α-Ad-treated hepatocytes (III, Fig. 2). CYP2A5 was significantly induced at a later time point (12 h) than PEPCK, induced already after 2 hours, probably suggesting that CYP2A5 is a true target for PGC-1α action. PEPCK is regulated also directly by CREB, in addition to PGC-1α-dependent regulation, which has been suggested as a mechanism for prolonged fasting response while CREB serves as acute response (Herzig *et al.* 2001).

5.8 Induction is mediated through nuclear receptor HNF-4α (III)

Mouse primary hepatocytes were transiently transfected with *Cyp2a5* proximal promoter luciferase constructs: wild-type promoter region (-3033 bp from transcription start site) and promoter region with mutated HNF-4α response element (description (Ulvila *et al.* 2004)). PGC-1α expression vector was co-transfected with the *Cyp2a5*-LUC constructs into mouse primary hepatocytes and measured for luciferase activity. As expected, PGC-1α cotransfected with the wild-type promoter showed about 3- to 4-fold induction in the LUC-activity compared to control expression vector (III Fig. 3). However, when using the mutated HNF-4α site, where HNF-4α is unable to bind, there was no induction compared to the control vector. This clearly suggests that HNF-4α mediates the CYP2A5 induction response by PGC-1α. In addition, chromatin immunoprecipitation (ChIP) and re-ChIP using primary hepatocytes showed that endogenous *Cyp2a5* promoter region was bound by PGC-1α and HNF-4α either directly or indirectly, suggesting that PGC-1α and HNF-4α are in the same protein complex regulating the *Cyp2a5* gene (III, Fig. 4).

5.9 Regulation of novel CYP genes by PGC-1α, including vitamin D metabolizing CYP2R1 and CYP24A1 (IV)

In original publication IV, novel, PGC-1α-regulated CYP genes in mouse hepatocytes were investigated in the same microarray data as in original publication II, PGC-1α-Ad overexpression study. Six CYP genes were identified as regulated by PGC-1α: *Cyp24a1, Cyp2s1, Cyp17a1, Cyp4a12, Cyp2r1* and *Cyp39a1* (IV, Fig. 1A-B). Two of these, *Cyp2r1 and Cyp24a1,* are involved in the vitamin D biosynthesis pathway. Interestingly, a novel cluster of RXR/VDR regulated gene set by the metabolic coactivator PGC-1α was found (data not shown) suggesting a novel role for PGC-1α in vitamin D receptor signaling pathway in the liver.

CYP2R1, which is the vitamin D 25-hydroxylase, an enzyme responsible for the first of two activating steps, is expressed and functional in the liver. We aimed to characterize the mechanisms behind the downregulation of the enzyme by PGC-1α. In addition, we aimed to study the phenomenon of CYP24A1 induction by PGC-1α, although this catabolic enzyme is primarily active and expressed in the kidneys.

5.10 CYP2R1 expression is significantly affected *in vivo* by models of metabolically challenged/disturbed mice (IV)

We studied the effect of fasting, which is known to activate PGC-1α and gluconeogenic genes in mouse liver (Yoon *et al.* 2001), on CYP2R1 expression. Mice were fasted for 12 or 24 hours, sacrificed and the livers collected, after which PGC-1α, PEPCK, and CYP2R1 mRNA levels were measured (IV, Fig. 2A). Surprisingly, the mRNA level of PGC-1α was not induced in the fasted mice; if anything, it was downregulated after 12 h fasting. However, PEPCK expression was significantly upregulated compared to control animals at both time points: 1.9-fold at 12 h and 4.1-fold at 24 h, indicating the apparent fasting response of the model. The expression of CYP2R1, on the other hand, was significantly downregulated in the fasted animals; a more pronounced effect was seen over time as after 24 h fasting the expression was only 0.2-fold compared to control animals. In addition, the protein level of CYP2R1 was dramatically downregulated at both time points, seen by western blotting of the microsomal fractions prepared from the same livers as the mRNA measured (IV, Fig. 2B).

Similar results were obtained with rats fasted for 24 hours after which liver samples were collected and mRNA levels of PGC-1α and CYP2R1 determined (IV, Fig. 2C). The average level of PGC-1α in the fasted animals was 1.7-fold compared to control animals, but high individual variation was observed. However, as also seen in mice, CYP2R1 expression was significantly downregulated, 0.3-fold compared to controls.

Next we studied the livers of *leptin*-deficient ob/ob mice, i.e., the obese, type 2 diabetic rodent model. From the livers we measured PGC-1α and CYP2R1 mRNA (IV, Fig. 2D). As expected, expression of PGC-1α was significantly induced, 1.6-fold, whereas CYP2R1 expression was clearly not affected in this model.

We wanted to assess CYP2R1 expression also in type 1 diabetes rodent model: the streptozotocin (STZ) treated mice which develop insulin deficiency after destruction of pancreatic β-cells by STZ. Franco and co-workers performed a microarray experiment (GSE39752) (Franko *et al.* 2014) by administration of STZ into mice for five consecutive days (i.p. 60 mg/kg) and analyzing liver tissues 2 months later. In these livers, the PGC-1α expression was significantly upregulated, about 1.9-fold compared to control animals (IV, Fig 2E). Moreover, the expression of CYP2R1 was significantly downregulated, to 0.7-fold.

5.11 CYP2R1 downregulation by fasting *in vivo* is reversed in PGC-1 α null mice (IV)

We examined the dependence of PGC-1α in repressing CYP2R1 in the fasted state by using PGC-1α knock-out animals with the model described by Lin *et al.* (Lin *et al.* 2004). Mice were fasted for 12 hours after which PGC-1α, PEPCK, and CYP2R1 mRNA levels were measured. The expression of PGC-1α was not significantly induced by fasting in wild type PGC-1$\alpha^{+/+}$ animals, although modest upregulation was observed, on average about 1.3-fold compared to controls (IV, Fig. 3). In the PGC-1$\alpha^{-/-}$ animals the expression of PGC-1α was at undetectable levels, as expected. PEPCK expression was significantly induced by fasting in wild type mice, 2.3-fold, whereas in knock-out animals there was no difference in the expression between control and fasted animals, suggesting PGC-1α involvement in the fasting response.

In CYP2R1 expression, there was significant downregulation in the wild type animals by fasting as expected, being 0.4-fold compared to controls. However, the downregulation was abolished in the PGC-1$\alpha^{-/-}$ mice, being 0.7-fold compared to

control mice, yielding no statistical significance, implying a PGC-1α-dependent mechanism of CYP2R1downregulation in the fasted state.

5.12 The effect of ERRα in CYP2R1 downregulation and CYP24A1 induction mechanism by PGC-1α (IV)

We next studied the effect of PGC-1α NR-binding domain mutations on the regulation of PGC-1α, PEPCK and genes related to mitochondrial function: ATP-synthase β subunit (ATPB5) and Cytochrome c (CYCS), in addition to CYP2R1, to explore the mechanism behind the downregulation in more detail. We used either wild type PGC-1α (WT), selectively HNF-4α or ERRα binding PGC-1α (PGC-1α-2x9) or PGC-1α unable to bind any nuclear receptors (PGC-1α-L2L3M) adenoviruses and infected mouse primary hepatocyte culture.

As expected, the expression of gluconeogenic gene PEPCK was significantly induced by PGC-1α WT and 2x9-Ad but not affected by the L2L3M mutant (IV, Fig. 4). Similar results were obtained for ATB5B and CYCS expression, being in line with previous data (Gaillard *et al.* 2006). In the case of CYP2R1 the PGC-1α-Ad WT downregulated the expression significantly as expected, to 0.3-fold compared to control. Moreover, 2x9-Ad mutant also downregulated CYP2R1 expression significantly to 0.5-fold. Interestingly, the L2L3M-Ad reversed the CYP2R1 suppression and even induced the mRNA to about 1.4-fold. The difference between WT and L2L3M-Ad as well as between 2x9-Ad and L2L3M-Ad on CYP2R1 expression was statistically significant, suggesting the downregulation to be nuclear receptor-dependent.

The role of ERRα was assessed in more detail since the 2x9 mutant is specific for ERRα and HNF-4α interaction with PGC-1α and the ERRα coactivation by PGC-1α in multiple metabolic regulatory mechanisms is well-established. Knock-down of ERRα by shERRα-Ad was performed in primary hepatocytes, in combination with PGC-1α-Ad treatment, after which the expression of PGC-1α and CYP2R1 was assessed.

ERRα mRNA was measured from the samples to confirm the knock-down which was clearly effective: only 10% ERRα expression was present when using shERRα-Ad (IV, Fig. 5). PGC-1α induces the expression of ERRα (Schreiber *et al.* 2003). This was also apparent in PGC-1α-Ad-treated cells with scrambled shRNA: ERRα was significantly induced to about 7-fold, as expected. PGC-1α expression was induced to 530-fold by the over-expression when combined with scrambled shRNA. When using shERRα, the expression was about 630-fold and

still extremely significant compared to LacZ-Ad. The effect of shERRα combined with PGC-1α-Ad on the expression of CYP2R1 was opposite compared to the PGC-1α-Ad combined with scramble shRNA; the expression was significantly induced to about 1.6-fold with PGC-1Ad compared to LacZ-Ad (IV, Fig. 5) strongly suggesting an ERRα-dependent mechanism of the downregulation.

We also explored the induction mechanism of CYP24A1 in mouse hepatocytes, although the physiological relevance of the phenomenon is obscure. However, it may be that similar regulatory mechanisms are found in other target tissues of CYP24A1. We studied the induction of CYP24A1 by treating hepatocytes with both the classical inducer calcitriol and by PGC-1α over-expression (IV, Fig. 6.A-B). CYP24A1 mRNA was dramatically induced by PGC-1α, 9,000-fold, and significantly also by calcitriol, 60-fold. CYP24A1 expression is induced vitamin D through VDRE (Jones *et al.* 1998, Zierold *et al.* 1995). VDR mRNA was significantly induced by calcitriol treatment but not by PGC-1α over-expression, which in turn significantly induced ERRα expression.

Based on these results we investigated the expression of CYP24A1 by treatment with the PGC-1α 2x9 and L2L3M mutant adenoviruses and by knocking down ERRα (IV, Fig. 6.C-D). CYP24A1 was significantly induced by both WT and 2x9 PGC-1α. Moreover, knock-down of ERRα abolished the dramatic induction caused by the WT PGC-1α. These results suggest that CYP24A1 expression is regulated through the ERRα-PGC-1α axis.

5.13 Cytochrome P450 genes regulated by PGC-1α

PGC-1α is an abundant interaction partner to the NR superfamily and thus regulates, as we and others have shown, the expression of multiple CYP enzymes in the liver. CYP7A1 is induced by PGC-1α in fasting conditions and in type I diabetic mice (Shin *et al.* 2003). Also, the activation of CYP1A1, CYP1A2, CYP2C9, CYP3A4, CYP3A5, and CYP2D6 is induced by PGC-1α and mediated via HNF-4α (Martinez-Jimenez *et al.* 2006). Interestingly, PGC-1α regulates CYP17A1 and CYP11A1, two enzymes involved in the initial steps of steroidogenesis in the liver. Additionally, ERRα and HNF-4α were established as novel transcription factors regulating the induction of these genes dependent on PGC-1α (Grasfeder *et al.* 2009). It seems that PGC-1α is emerging as a novel regulator of endobiotic CYP metabolism in addition to involvement in xenobiotic metabolism. Accordingly, we establish PGC-1α as novel regulator of *Cyp24a1, Cyp2s1, Cyp4a12, Cyp2r1* and *Cyp39a1* genes in mouse hepatocytes. It remains

to be discovered what the role of PGC-1α is in the induction of CYP2S1, CYP4A12 and CYP39A1 metabolizing retinoids, fatty acids and cholesterol, respectively.

In addition, PGC-1α induces CYP2A6 in hepatocytes, mediated through PXR and synergistically by HNF-4α (Itoh *et al.* 2006). We report that PGC-1α also induces the CYP2A6 mouse ortholog CYP2A5 in the liver. It was previously known that both CYP2A5 and PGC-1α are fasting-inducible through cAMP pathway activation (Bauer *et al.* 2004, Salonpaa *et al.* 1994, Yoon *et al.* 2001). Regulation of CYP2A5 is particularly interesting compared to many other CYP2 family members, and especially regulation in perturbed cellular redox state is of relevance considering interaction with PGC-1α.

Namely, CYP2A5 is regulated through the ROS-activated Nuclear factor erythroid-derived 2-like 2 (Nrf2) pathway (Abu-Bakar *et al.* 2007). Nrf2 is a cellular redox-sensing transcription factor regulating cytoprotective genes such as heme-oxygenase (HMOX1, HO-1), γ glutamyl cysteine ligase (GCL), peroxiredoxin 1 (Prx1), glutathione S-transferase (GST), glutathione synthetase (GS), superoxide dismutases (SOD) and UDP-glucuronosyl transferases (UGT) (Copple *et al.* 2008). Interestingly, PGC-1α also regulates many of the same genes involved in ROS-scavenging (St Pierre *et al.* 2006). In addition, Nrf2, CYP2A5 and PGC-1α have roles in the heme biosynthesis pathway. HO-1 is the enzyme responsible for catabolism of heme, which Nrf2 induces. And bilirubin, the metabolite of heme, has been suggested as a novel substrate for CYP2A5 enzyme (Abu-Bakar *et al.* 2011). The rate-limiting enzyme in the heme biosynthesis pathway, aminolevulinate synthase (ALAS-1), is induced by PGC-1α (Handschin *et al.* 2005). On the other hand, heme directly represses PGC-1α transcription through Rev-erbα, possibly acting as a regulatory feed-back loop (Wu *et al.* 2009). Perhaps PGC-1α induces CYP2A5 due to having a role in bilirubin clearance in the liver since PGC-1α and fasting both activate heme biosynthesis (Handschin *et al.* 2005).

Intriguingly, in Nrf2 null mice, the expression of CYP2A5 is dramatically induced by PGC-1α over-expression, although CYP2A5 is also induced in wild type animals by PGC-1α (Lamsa *et al.* 2012) suggesting a yet unknown crosstalk for Nrf2 and PGC-1α in the regulation of heme and ROS homeostasis. It is possible that the two major regulators compensate the effect of each other in cellular ROS disturbances. Nevertheless, detailed mechanisms remain to be determined.

5.14 Role of PGC-1α and metabolic syndrome in the regulation of vitamin D pathway

Growing evidence supports the role of vitamin D in multiple aspects of health and disease in the human body. It is well established that vitamin D plays a major role in the bone mineralization process as well as calcium and phosphorus absorption and homeostasis (Holick 2007). Furthermore, more recent data describe the effect of impaired vitamin D status on cardiovascular disease (Norman & Powell 2014), certain cancers (Spina *et al.* 2006), multiple sclerosis (Pakpoor & Ramagopalan 2014), inflammation (Wobke *et al.* 2014) and most interestingly, the metabolic syndrome including type 1 and 2 diabetes (Awad *et al.* 2012). However, the molecular mechanisms of how the metabolic syndrome and vitamin D status interact are poorly understood, along with a general consensus on consequence and cause.

Examples on the connection between vitamin D insufficiency and metabolic syndrome have been described in the literature. In obese and T2DM patients, low circulating 25-hydroxyvitamin D has been reported in several studies (Bajaj *et al.* 2014, Bell *et al.* 1985, Compston *et al.* 1981, Husemoen *et al.* 2012, Liel *et al.* 1988). Interestingly, the association between T2DM and hypovitaminosis D has been shown to be independent on other risk factors such as body mass index, fasting insulin levels or insulin resistance (Lim *et al.* 2013). Furthermore, diet supplementation of type 2 diabetics with vitamin D has resulted in beneficial effects on hypertension (Breslavsky *et al.* 2013, Nasri *et al.* 2014), although no clear positive outcome on glycemic factors has been detected. In a Finnish population study, dietary vitamin D supplementation was associated with reduced risk for type 1 diabetes (Hypponen *et al.* 2001). In rodents with T2DM, the blood glucose lowering effects of vitamin D has been confirmed (Meerza *et al.* 2012).

We show that in mice, fasting and type I diabetes *in vivo* modulate the expression of the major vitamin D 25-hydroxylase, CYP2R1, the activating enzyme in the vitamin D biosynthesis pathway. Using rodents we show that CYP2R1 expression is significantly downregulated in the fasted state. In addition, in type 1 diabetic rodents CYP2R1 expression is also perturbed. These results clearly suggest that CYP2R1 is under the control of hepatic glucose homeostasis. Even though CYP2R1 is considered the main 25-hydroxylase *in vivo*, the regulation of this gene is surprisingly poorly understood with only a few mechanistic reports (De Toni *et al.* 2014, Ellfolk *et al.* 2009). A recent review suggests that epigenetics may play a significant role in the regulation of vitamin

D metabolizing genes including VDR, CYP27B1, CYP2R1 and CYP24A1. Many of them have cytosine and guanine (CpG)-rich promoter regions, making them prone to methylation events, generally associated with silencing and repressing gene expression. Interestingly, CYP2R1 has been suggested to be methylated in response to low vitamin D and reversed with high vitamin D in the serum (Fetahu *et al.* 2014). Perhaps CYP2R1 repression mechanism in fasted or diabetic state could involve silencing through such promoter methylation.

Our results additionally suggest that the downregulation mechanism of CYP2R1 in the fasted state is PGC-1α-dependent, shown by PGC-1α knock-out model. Furthermore, we show that in mouse primary hepatocytes, PGC-1α overexpression results in dramatic downregulation of CYP2R1. The phenomenon was reversed when knocking down ERRα, a key metabolic regulator and interaction partner of PGC-1α, suggesting a central role for ERRα in the downregulation mechanism.

The role of ERRα as activator of target gene expression is well known. However, the repressive effects of ERRα on gene regulation are not well documented. One such report describes that human hydroxysteroid sulfotransferase (SULT2A1) is repressed by ERRα-mediated mechanism in HepG2 cells. Namely, the repression was shown to demonstrate competition between other nuclear receptors within the crucial promoter region of SULT2A1 (Huang *et al.* 2011). A putative mechanism may involve SUMOylation, an event often connected with repression of gene expression. The induction of corepressor binding can occur within target gene promoters through conjugation by SUMOylation, a mechanism often called SUMOylation-dependent transrepression, reported for many NR family members (Ghisletti *et al.* 2007). ERRα has been shown to be SUMOylated by phosphorylation at the N-terminus rendering the receptor transcriptionally inactive (Tremblay *et al.* 2008). Interestingly, a repression domain has been found in the N-terminus of ERRα which may render its activity cell and promoter-dependently. The circumstances in which ERRα exerts repressive effect are not well-established (Zirngibl *et al.* 2008).

Recently, PGC-1α was linked to the regulation of tanning process in the skin. In keratinocytes tanning is produced by melanin which is formed in melanocytes. The process starts by UV light exposure which activates the secretion of the peptide α melanocyte stimulating hormone (α-MSH). α-MSH binds to melanocortin receptor and activates the cAMP pathway leading to induction of the microphthalmia-associated transcription factor (MITF), a master regulator of

melanocyte differentiation and pigment formation. PGC-1α was shown to be induced and stabilized by α-MSH and to induce MITF, thus being a critical player in the tanning process (Shoag *et al.* 2013). Interestingly, melanin is known to reduce vitamin D levels by blocking UV radiation at the synthesis wavelength: people with dark pigmentation need more sunlight compared to light-skinned people to obtain sufficient vitamin D synthesis (Macdonald 2013). These results support evidence for the role of PGC-1α in diminishing active vitamin D levels.

It is tempting to speculate that PGC-1α could play a major role in regulating vitamin D levels in the liver in response to metabolic stress. Such a role suggests inhibition of active vitamin D by PGC-1α since the coactivator significantly represses the activating enzyme CYP2R1 while dramatically inducing the inactivating enzyme CYP24A1. Perhaps PGC-1α has a role in other tissues such as skin or kidney as well. Still, the complete physiological meaning of PGC-1α in vitamin D metabolism remains to some extent unknown.

5.15 Clinical implications and future studies

The mechanisms of glucose imbalance and type 2 diabetes are ambiguous, with many factors affecting each other. Investigations by us and others suggest a central role for PGC-1α in the pathogenesis of T2DM serving as a possible therapeutic target. However, our studies revealed that repression of PGC-1α expression is not involved in mechanism of action of metformin, although metformin may affect PGC-1α target genes. Discovering the mechanism of action of metformin, an oral T2DM therapeutic, is of high importance due to its wide use in the clinic. Altough, there are several novel types of T2DM drugs, continuous development on safer and more specific drugs is needed to enable treatment of T2DM at various disease stages. Intriguingly, metformin is a good example of how its action is not restricted to only one target molecule. More studies are still needed to fully understand the mechanisms behind the benficial effects of metformin.

Low, chronic inflammation is associated with different stages of T2DM. Interestingly, we showed that PGC-1α induces and activates the anti-inflammatory IL-1Rn. Thus, PGC-1α activation could be beneficial in the livers of these patients resulting in lowering of the inflammatory response. Yet, there is a wide variety signalling pathways and target molecules to be investigated in the crosstalk of T2DM and inflammation.

We established PGC-1α as a regulator of active vitamin D in the body. Vitamin D levels seem to be regulated by a previously unknown mechanism, through repression of the vitamin D 25-hydroxylase, CYP2R1 which is responsible for the first of two activation steps in vitamin D biosynthesis pathway. This novel finding suggests that vitamin D status is affected by caloric and glucose status of the body and that the association of diabetes with low vitamin D levels may be PGC-1α-dependent, again stressing role of PGC-1α in metabolic disturbancies. This also suggests that diabetes would be the cause of low vitamin D level and not vice versa.

PGC-1α's post-transcriptional modifications have emerged as a key mechanism on how this coactivator is regulated under metabolic stress. The proper characterization of PGC-1α activation by various external stimuli affecting glucose balance, mitochondrial biology as well as novel functions is still needed in all target tissues.

6 Conclusions

In the present study, the pathways involving PGC-1α regulation-dependent signaling as well as novel genes regulated by PGC-1α were investigated using primary hepatocytes *ex vivo* and livers from *in vivo* experiments. The following main findings were established based on this work:

1. Metformin, a type 2 diabetes drug, induces PGC-1α in hepatocytes, contradictory to previous assumptions. The regulation of mitochondrial biogenesis by PGC-1α remains intact while regulation towards gluconeogenesis is perturbed and blocked by other mechanisms including transcription factor suppression.
2. PGC-1α is induced by metformin-AMPK activation in the liver, involving also SIRT1 action.
3. PGC-1α overexpression and conditions inducing PGC-1α induce several anti-inflammatory cytokines, most strikingly IL-1Rn in the liver. PGC-1α diminishes the inflammatory response caused by pro-inflammatory IL-1β.
4. PGC-1α induces murine CYP2A5 through HNF-4α coactivation and acts as putative mediator of CYP2A5 induction upon fasting.
5. PGC-1α regulates a set of novel CYP genes in mouse liver including vitamin D biosynthesis regulating enzymes CYP2R1 and CYP24A1 in hepatocytes.
6. CYP2R1 emerges as novel gene downregulated by metabolic factors and PGC-1α. ERRα mediates the repressive effect by PGC-1α.

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Original articles

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