Negative regulation of PGC-1α by NF-κB

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List of Abbreviations

ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AMPK	AMP activated protein kinase
ARB	Angiotensin II receptor blocker
ATP	Adenosine triphosphate
CD36	Cluster of differentiation 36
ChIP	Chromatin immunoprecipitation
CK	Creatine kinase
Cr	Creatine
CPT1	Carnitine palmitoyltransferase I
DMD	Duchenne Muscular Dystrophy
ETC	Electron transport chain
EMSA	Electrophoretic mobility shift assay
FA	Fatty acids
FAO	Fatty acid oxidation
FFA	Free fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GO	Glucose oxidation
HDAC	Histone deacetylase
HF	Heart failure

HIF-2α	Hypoxia inducible factor 2
IκB	Inhibitor of nuclear factor of κ light polypeptide gene enhancer in B-cells
IKK	IκB kinase
IL-6	Interleukin 6
MAPK	Mitogen activated protein kinase
MI	Myocardial infarction
NF-κB	Nuclear factor κ light chain enhancer of activated B cell
PCR	Polymerase chain reaction
PDC	PTCAyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase lipoamide kinase isozyme 4
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PRC	PGC-1-related coactivator
Pr	Phosphocreatine
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
PPAR	Peroxisome proliferator-activated receptor
TCA	Tricarboxylic acid
TOF	Tetraology of Fallot
TSA	Trichostatin A
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

I Abstract

The normal adult heart prefers fatty acids as an energy substrate. In the case of heart failure, the heart switches its preference from fatty acids to glucose, adopting a pattern similar to fetal metabolism profile. Since PGC-1α is heavily involved in fatty acid oxidation, it must be downregulated to shift metabolism towards glucose oxidation. Other than being a regulator of cardiac metabolism, PGC-1α is also involved in mitochondrial development and function. p65, which belongs to the NF-κB transcription factor family is another crucial molecule involved in maintaining cardiac homeostasis. There is a substantial amount of evidence that suggests that PGC-1α and NF-κB directly interact, thereby connecting metabolic and inflammatory processes. Furthermore, dysregulation of either PGC-1α or NF-κB signalling correlates to many diseases including heart disease. In this study, we provide further evidence that the NF-κB family has the ability to repress PGC-1α. We also show that the PGC-1α promoter contains a p65 binding site through which p65 imparts control on the PGC-1α gene. Metabolic homeostasis and inflammation pathways are closely linked and play crucial roles in heart dysfunction.

II Introduction

The average adult human heart pumps 5-6 litres of blood per minute, and requires approximately 6 kg of ATP per day to sustain itself [1]. In a normal situation, metabolic signalling exists in a delicate homeostasis that balances oxygen, fuel intake and fuel use. The overall process is regulated by a vast amount of genes, hormones, proteins and environmental cues [1]. The heart is a metabolic omnivore and has the ability to use either glucose or fatty acids as an energy substrate. Regardless of the substrate, mitochondria within the cardiomyocyte are responsible for maintaining a constant ATP supply via oxidative phosphorylation. Under normal conditions, the adult heart prefers fatty acids as an energy substrate. A commonly observed phenomenon during heart disease is a switch to the fetal metabolic profile. This involves a reduction in fatty acid oxidation genes, which in part depends on the repression of PGC-1α [1, 2]. PGC-1α is a key regulator of cardiac metabolism responsible for regulation of oxidative metabolism, mitochondrial biogenesis and function [1]. PGC-1α is a crucial player in metabolic homeostasis and is implicated in a wide array of physiological and pathological conditions. For this reason it is considered one of the most important metabolic regulators. PGC-1α can be altered in a variety of physiological states such as fasting, exercise, physiological hypertrophy, heart development and mitochondrial biogenesis. Dysregulation of PGC-1α on the other hand is implicated in ischemia, hypoxia, pathological cardiac hypertrophy, heart failure, neurological disorders and diabetes [2-5].

In addition to metabolic dysfunction, another important feature of cardiovascular disease is chronic inflammation [6]. The NF-κB transcription factor family is crucial to the

inflammatory and immune response. p65, one of the five members of the NF-κB family is particularly important due to its role as a transcriptional activator. The NF- κB family is an important player in homeostasis in cardiac physiology, and dysregulation of NF-κB signalling is associated with diseases like cancer, functional deterioration and heart failure. Numerous studies outline the relationship between NF-κB and PGC-1α, highlighting the importance of inflammatory and metabolic pathways in maintaining homeostasis.

The relationship between PGC-1α and NF-κB in cardiac tissue has already been established, but the exact mechanism of their interaction is unclear. We hypothesize that NF-κB is involved in the down-regulation of the PGC-1α gene expression in hypoxic neonatal cardiomyocytes. The first portion of the study is an *in vitro* examination of the physical interaction between the PGC-1α promoter and p65. This is the focus of this thesis. The second portion of our study focuses on *how* PGC-1α is repressed in a hypoxic rat cardiomyocyte model. This data is not included in this thesis.

The first part of the study began with an *in silico* analysis of the PGC-1α promoter which found two putative p65 binding sites: site 1: GGGACTTT (1564-1571 bp) and site 2: GGGACTGT (2959-2966 bp). Both sites are highly homologous to the mouse p65 consensus sequence GGGACTTTCC. We examined the mechanism by which the NF-κB p65 subunit represses PGC-1α promoter via mutagenesis and sequential deletion studies. Our data shows that either mutation or ablation of both p65 binding sites on the PGC-1α

promoter rescues PGC-1α promoter activity when transfected together with p65 to various degrees.

Another group reports an NF-κB site on the human PGC-1α proximal promoter in skeletal muscle. The NF-κB site was found at position -1575 and reads: GGGAATTTAC [7]. Additionally, they also found a binding site at p53 at position −1237. An NCBI Blast sequence alignment between the mouse and human PGC-1α promoter showed moderate similarity, though the mouse and human PGC-1α promoters are of different lengths. The p65 binding site found on the human PGC-1α promoter does not align with any sequence on the mouse PGC-1α promoter. However, when we searched the human PGC-1α promoter for our putative p65 binding sites #1 and #2, we found very similar regions on the human promoter. Our p65 site #1 is a near perfect fit with the human PGC-1α promoter (from 3878 to 3886 bp) matching 6 out of 8 base pairs while p65 site #2 is an exact match (from 5159 to 5166 bp). Interestingly, the area that we identify as p65 site #2 appears to be a part of a human p65 consensus sequence on the human PGC-1α promoter. It reads as: GGGGACTGTAGTAA and is underlined in Figure 1. Figure 1 shows the alignment between the mouse and human PGC-1α promoters at putative p65 binding sites #1 and #2 found via our analysis. It is not clear why the paper did not identify either region outlined in Figure 1 as a potential for NF-κB binding (likely because they did not focus on the NF-κB binding site in their study). It is encouraging to see similar NF-κB binding sites present on the human PGC-1α promoter, especially that of putative p65 binding site #2. It supports our theory that PGC-1α promoter repression is mediated by NF-κB binding at the promoter level in both mice and humans.

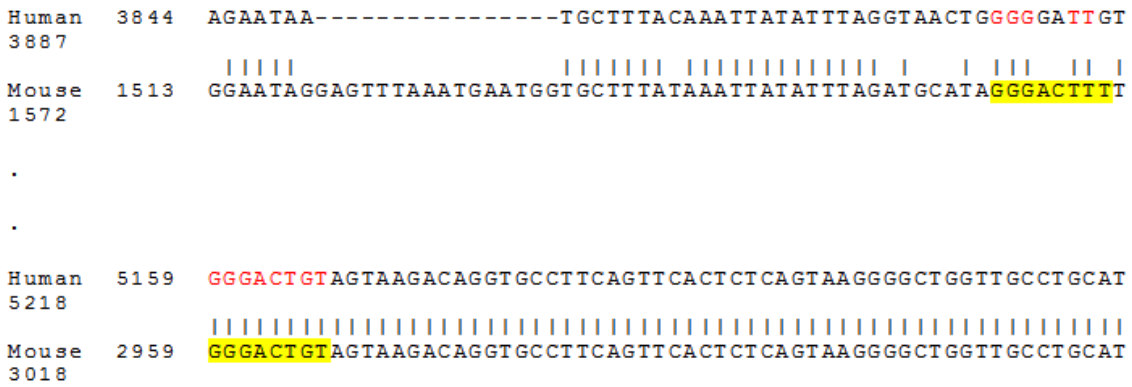

Figure 1: Comparison between human and mouse PGC-1α promoters at putative p65 binding site #1 and #2

The top line represents the human PGC-1α promoter sequence which is aligned with the bottom mouse PGC-1α promoter sequence. Highlighted in yellow are the two putative p65 binding sites found on the mouse PGC-1α promoter. Red letters represent the nucleotides on the human PGC-1α promoter which match the mouse PGC-1α promoter. Lastly, the underlined region on the human promoter from 5159-5171bp represents the human p65 binding site consensus sequence.

As already mentioned, the second part of this study is in progress and aims to determine how PGC-1α is repressed in a hypoxic rat cardiomyocyte model. Previous studies have reported that HDACs have angiogenic properties and are up-regulated during severe hypoxia. HDAC inhibition prevents the switch to the fetal metabolic program, and can supress hypoxia-induced cardiopulmonary remodelling and hypertension [8-14]. Previous studies from our lab have shown that over-expression of HDACs inhibits PGC-1α expression, while hypoxia reduces histone acetylation within the PGC-1α promoter [4, 15]. Furthermore, treatment with Trichostatin A (TSA), a pan-HDAC inhibitor prevented the deacetylation of histones within the PGC-1α promoter and therefore prevented PGC-1α down-regulation. As a result of these findings we hypothesize that in oxygen-limiting conditions, PGC-1α expression is reduced via an HDAC mechanism [4]. This research is still ongoing so it will not be discussed in this thesis.

Overall, our work links the PGC-1α and NF-κB pathways and provides a better understanding of the role that metabolic homeostasis and inflammation play in heart dysfunction. Understanding the interaction between PGC-1α and NF-κB pathways is important for future therapeutic strategies targeting heart failure.

III Statement of hypothesis

We hypothesize that the PGC-1α promoter contains p65 binding site(s) and is directly repressed by p65, an NF-κB subunit. Under hypoxic conditions, we expect the interaction between PGC-1α promoter and p65 to become increased. The figure below outlines the proposed working model, which involves HDAC mediated PGC-1α repression. As already mentioned, the focus of this thesis will be on the relationship between p65 and the PGC-1α promoter and not the involvement of HDACs.

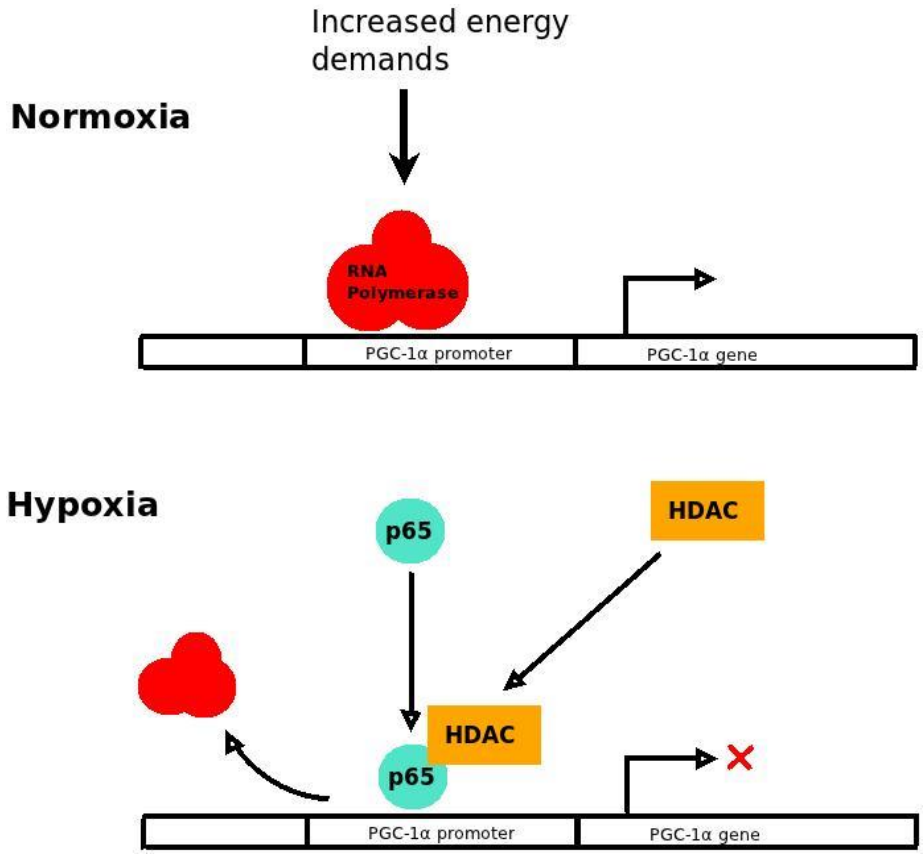

Figure 2: Schematic representation of our working hypothesis

Under hypoxic conditions, p65, a subunit of NF-κB may displace RNA polymerase and recruit the HDAC(s) that prevents expression of PGC-1α, thus promoting the switch to glucose. This diagram focusses on p65within the NF-κB complex, so p65 is drawn and as a separate unit (blue circle). In reality however, the NF-κB complex exists as a hetero/homo dimer.

IV Objectives

A) Observe and quantify the repression of the PGC-1α promoter by p65 (NF-κB subunit) in 3T3 cells.

B) Determine the location of the p65 binding site(s) on the PGC-1α promoter using either point mutations or sequential deletions.

C) Observe whether NF-κB/PGC-1α promoter complexes increase during hypoxia in neonatal cardiomyocytes.

V Literature Review

1.0 Oxygen and the mammalian heart

1.1 Evolution of oxygen and the mammalian heart

Oxygen sustains most life on Earth, and is thought to have appeared approximately 2.3 billion years ago. Our ability to intake sugars and fats to produce energy largely depends on oxygen availability and the mitochondria within our cells. It is estimated that eukaryotic cells evolved at least 1.45 billion years ago, mitochondria arising as a result of endosymbiosis between single cell organisms [16]. Mitochondria eventually evolved to become energetic factories for cells, which utilize oxygen as well as products from sugar and fat burning pathways to harness the energy required to maintain life [2]. The presence of multiple mitochondria per cell resulted in a dramatic increase in energetic capacity and potential, allowing for evolution of larger and more complex systems [17].

The heart is an organ designed to shuttle blood enriched with nutrients and oxygen throughout the body. The first heart appeared 600-700 million years ago and likely resembled a pulsating tube shaped structure lacking a septum, chambers, valves and uni-directional blood flow. The vertebrate heart evolved approximately 500 million years ago [17]. Vertebrates which exist today can be organized based on whether they have two, three or four chambered hearts, the latter being the most efficient. All mammals have a four chambered heart. The average adult human heart beats around 72 beats and pumps 5-6 litres of blood per minute at rest, pushing blood into blood vessels that spread throughout the body. The right atrium receives deoxygenated blood, which moves to the

right ventricle and then into the lungs for reoxygenation. Once the blood is oxygenated, it returns via the left atrium. From there it moves through the left ventricle, the aorta and eventually to the rest of the body. Oxygen is required for of the majority of energy (or ATP) produced within the body. The human heart uses more ATP than any other organ, adding up to a daily weight of 6 kg of ATP. To meet the demands of the heart and other organs, metabolic signalling has evolved to exist in a stringently regulated balance between glucose, fatty acid and oxygen utilization [1].

1.2 Failure to accommodate energy requirements

Many diseases compromise our ability to get adequate access to oxygen and/or energy substrates, affecting the lungs, hearts or metabolic pathways. If the heart cannot pump with sufficient force or speed to accommodate the needs of the body, disease will ensue. Disease that affects oxygen use and energy metabolism typically involves the lungs, vascular system, mitochondria, specific metabolic pathways or numerous types of cancer. Examples are peripheral vascular disease caused by atherosclerosis or stenosis, stroke, mitochondrial myopathies, diabetes, cardiomyopathies and many varieties of congenital diseases which involve defects in metabolism or heart structure. Additionally, chronic oxygen deprivation can lead to many secondary diseases such as kidney disease and cirrhosis of the liver [18-21].

1.3 Heart failure and treatment

In particular, heart failure (HF) is a multifactorial disease characterized by the inability of the heart ventricle to fill and eject blood. Many HF patients also suffer from diabetes, kidney disease, sleep apnea, loss of metabolic flexibility, ventricular remodelling, cardiac

hypertrophy and hypertension [22-26]. Typical HF treatments use a combinational approach, targeting blood pressure, water retention and strength of contraction. Both angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) act on the renin-angiotensin system (RAS). RAS is a hormonal response system that regulates blood pressure and fluid balance. It is activated during fluid loss or drops in blood pressure. The end product of RAS is angiotensin II, which increases water absorption, sodium absorption and vasoconstriction. ACE inhibitors inhibit angiotensin converting enzyme (ACE) and prevent formation of angiotensin II, while ARB's block angiotensin receptor 1, preventing angiotensin II from binding to it [27, 28]. Diuretics are often taken in combination with ACE inhibitors [29]. Aldosterone antagonists target aldosterone which is produced by the adrenal glands when stimulated with angiotensin II. Aldoesterone induces sodium and water re-absorbption as well as stimulates the sympathetic nervous system. Hyperaldosteronism induces oxidant stress, cardiac fibrosis, cardiac apoptosis and vascular inflammation [30]. Beta blockers target the beta receptor cells involved in sympathetic activity. By blocking beta receptors, adrengergic activity, blood pressure, heart rate, myocardial workload and oxygen demand are all reduced. There is also some evidence to suggest that beta blockers shift metabolism from fatty acid to glucose [31, 32]. Dixogin is a cardiac glycoside and is the oldest cardiovascular therapy that has been used for more than two hundred years. Cardiac glycosides reduce sinoatrial (SA) and atrioventricular (AV) node firing and increase the refractory period. Digitalis in combination with beta blockers is especially effective in HF patients experiencing atrial fibrillation. Digitalis also increases intracellular calcium and therefore acts as a positive inotrope [29, 33]. If pharmacological intervention is not sufficient,

surgical options include heart valve repair or replacement, implantable defibrillators, pacemakers and heart transplantation.

2.0 Key regulators of metabolism, PPAR and PGC-1α

2.1 PPAR function

The peroxisome proliferator-activated receptor (PPAR) transcription factor family are nuclear receptors involved in fatty acid (FA) metabolism. There are 3 isoforms: PPARα, PPARβ/δ and PPARγ [5]. All 3 isoforms of the PPAR family heterodimerize with the retinoid X receptor (RXR) and initiate target gene transcription by binding to a conserved DNA binding sequence called peroxisome proliferator response element (PPRE). All three isoforms are found in the heart, but PPARα and PPARβ/δ predominate [34-36].

The expression of PPAR isoforms is different depending on the tissue, and can fluctuate in various pathologies. PPARα is involved in FA oxidation and can be found in the heart, muscle and liver. Numerous studies report a downregulation of PPARα during hypoxia, cardiac hypertrophy and heart failure models [4, 36-40]. PPARα knock out studies show decreased FA oxidation and cardiac hypertrophy while PPARα overexpression models result in hearts that rely almost entirely on fatty acids [41, 42]. Additionally, Jamshidi et al. report a polymorphism in PPARα that significantly increases LV growth as a result of either hypertension or exercise [43]. PPARβ/δ is widely expressed, with high levels in cardiac and skeletal muscle. PPARβ/δ is also involved in the transcriptional regulation of FA oxidation enzymes, as well as glucose utilization [44, 45]. *In vivo* gene deletion

studies have shown that a lack of PPARβ/δ leads to a decrease in fatty acid and glucose oxidation as well as cardiomyopathy [46, 47]. Finally, PPARγ is involved in adipocyte differentiation [48, 49]. PPARγ overexpression causes lipid accumulation, mitochondrial abnormalities and cardiac dysfunction [50]. PPARs are well documented to have beneficial effects in inflammatory diseases, and are considered important therapeutic targets. PPARδ has been found to have protective post-stroke effects and PPARγ can reduce post-ischemic injury and ventricular remodelling after MI [51-54]. In contrast, Heinrich Taegtmeyer's group reported that artificial activation of PPARα genes further debilitated the hypertrophied heart as well as prevented substrate switching. This suggests that PPARα, down-regulation could be crucial for possible adaptive responses that may occur during hypertrophy [55].

2.2 PGC-1α function

PPARγ coactivator-1α (PGC-1α) was first discovered in a two hybrid yeast system by Bruce Spiegelman's group as a PPARγ interacting protein. As mentioned in the previous section, PPARγ is responsible for adipocyte differentiation. PGC-1α mRNA was found to be increased in brown adipose and skeletal muscle tissues of mice exposed to cold. [48]. Though PGC-1α was first known as a co-activator of PPARs, it has an astounding number of other interaction partners and functions [5, 56, 57].

The PGC-1α protein is a roughly 800 amino acid protein encoded on human chromosome 4 [58]. PGC-1α is the best studied member of the PGC-1 family. The other two members are PGC-1β and PGC-1-related coactivator (PRC). All three have a transcription

activation domain and the major nuclear hormone receptor-interacting motif (LXXLL) at the N terminus. The C terminus has an RNA binding motif and a serine/arginine-rich domain.

Co-activators exist as complexes and mediate a broad number of pathways PGC-1α is a potent coactivator that responds to cues from the environment and regulates entire biological networks accordingly [56]. PGC-1α functions as a scaffold in order to recruit other proteins which possess enzymatic activity such as kinases, methyltransferases and histone acetyltransferases [5]. It also directly interacts with transcription factors, nuclear hormone receptors, chromatin remodelling proteins and splicing machinery in order to coordinate biochemical networks on both a transcriptional and post-translational level. PGC-1α also undergoes a high level of post-translational modifications itself, which includes phosphorylation, acetylation/deacetylation, methylation and sumoylation [2, 40, 59-63]. PGC-1α is a crucial player in metabolic homeostasis, and it is especially regulated at points where signalling pathways converge on one another. It is highly regulated by numerous molecular pathways. Furthermore, it has the capacity to induce its own expression and may be capable of splicing its own mRNA [35, 64].

PGC-1 protein orthologs are conserved in mammals, birds and fish suggesting that PGC-1 evolved millions of years ago to contribute a crucial metabolic function [56, 65, 66]. PGC-1α is involved in regulating the switch from glucose to FA, oxidative phosphorylation, respiration, gluconeogenesis, mitochondrial biogenesis, angiogenesis, thermogenesis, antioxidant production and fiber type switching in skeletal muscle PGC-

1α localizes to the nucleus and is highly expressed in mitochondria-rich oxidative tissues, such as kidney, brain, brown fat, skeletal muscle and the heart [58, 67-72].

While the details of PGC-1α's role in heart dysfunction are not fully understood, it is generally agreed that the pathologic changes seen in the failing/hypertrophied heart is in part due to dysregulation of the PGC-1α circuit [40].

2.3 PGC-1α up-regulation

PGC-1α expression is increased as a response to increased energy demands [5]. PGC-1α is involved in cardiomyocyte differentiation and is up-regulated in the developing mouse heart [56, 73], as well as under physiological hypertrophy [5]. Chronic/acute exercise, fasting and exposure to cold all increase PGC-1α expression in respective tissues: skeletal muscle, liver and brown fat [2, 4]. Increasing PGC-1α expression increases FA oxidation, respiration and mitochondrial synthesis. PGC-1α overexpression also leads to an increased resistance against fiber damage, reduced muscle soreness and overall better endurance [3, 4]. PGC-1α overexpression has also been found to improve the outcome of Duchenne Muscular Dystrophy (DMD), a disease caused by dystrophin mutation. PGC-1α is able to induce expression of a dystrophin homologue, which partly compensates for its absence [71, 72, 74-77]. Some studies also report that PGC-1α is increased in patients with idiopathic pulmonary arterial hypertension (IPAH) [73]. However, despite the promising effects of PGC-1α overexpression, there are numerous associated problems. Studies have found that heart specific overexpression of PGC-1α causes uncontrolled mitochondrial biogenesis, which causes myofibrillar displacement and loss of sarcomeric structure. These structural changes impair the heart's ability to effectively pump blood

leading to hypertrophy, dilated cardiomyopathy and eventually heart failure [40, 66, 68, 78].

2.4 PGC-1α down-regulation

PGC-1α levels tend to decrease with age [66]. PGC-1α down-regulation is also linked to local and systemic inflammation markers, dysregulation of metabolism, chronic obstructive pulmonary disease, heart failure, ischemia and decompensated cardiac hypertrophy [3, 5, 69, 70, 79, 80]. Of note, it has been observed that PGC-1α dysregulation occurs early on in pathological hypertrophy, meaning it is not a consequence of the disease but likely a cause [5].

PGC-1α knockout animals have mitochondrial deficiencies, are cold-sensitive, have increased body fat, abnormal heart rate, reductions in both FA and glucose oxidation, cannot restore normal blood flow after an ischaemic insult and exhibit numerous heart failure symptoms [69, 80-82]. Interestingly, they are also less susceptible to diet induced insulin resistance [81]. Furthermore, PGC-1α knock out animals present with symptoms characteristic of neurodegenerative diseases such as Huntington's, Parkinson's and Alzeimer's [66]. Of note, a double knock-out mice (PGC-1 α /β (–/–) had reduced cardiac output, pronounced maturational defects in mitochondrial biogenesis and died shortly after birth. This suggests that PGC-1α and β have overlapping roles in heart maturation as well as metabolism [80].

2.5 Tissue-specific dysregulation

It is important to note that the relationship between PGC-1α expression and disease is not

always clear cut. Some diseases such as cancer and diabetes exhibit different PGC-1α expression states based on the type of tissue. For example, PGC-1α is reduced in breast, colon, ovary and liver tumours, but increased in endometrial and renal tumors [83]. In the skeletal muscle of patients with Type II diabetes, PGC-1α is down-regulated, but up-regulated in the liver of both Type I and Type II diabetic patients. A polymorphism of PGC-1α has also been linked to an increased risk of Type II diabetes [58]. These examples illustrate that the expression of PGC-1α is complex and is influenced by many genetic and environmental factors. Understanding the significance of PGC-1α expression levels in a disease state is a current challenge.

3.0 Substrate preference in the heart

3.1 The heart as a metabolic omnivore

The heart requires a constant supply of energy, which in normal conditions is stringently balanced between energy demand and fuel delivery [40]. The heart is metabolically flexible, and has the capacity to use nearly any cellular fuel including FA, carbohydrates, and ketone bodies to produce energy [84]. Different fuel substrates are different in size, chemical properties and storage locations. Each type has specific advantages and disadvantages depending on the physiological situation. It is for these reasons that the ability of the system to modulate the specific mixture of fuels is an essential requirement for survival and adaptation [85]. For example, in a situation where oxygen is limiting, it would be advantageous to utilize glucose as an energy source because it requires less oxygen to metabolize. See Section 3.2.

3.2 Comparative energy yields between fatty acids and glucose

Glucose metabolism yields approximately 31 molecules of ATP per one glucose molecule and 6 molecules of oxygen (O_2). This number can vary between 30-38 molecules of ATP depending on local conditions and efficiency of the system. The first stage of glucose metabolism occurs in the cytoplasm and is an anaerobic process called glycolysis. Glycolysis breaks down one molecule of glucose into two molecules of pyruvate and produces 2 net molecules of ATP in the process. The rest of the ATP is produced in the mitochondria by oxidation of pyruvate by a process called glucose oxidation (GO). In contrast, fatty acid oxidation (FAO) occurs in the mitochondria and produces 105 ATP molecules per oxidation of one palmitate molecule and 23 O_2. The phosphate/oxygen (P/O) ratio is the number of ATP molecules obtained from two electrons and the reduction of one atom of oxygen by the electron transport chain (ETC) in the mitochondria. There is a P/O ratio difference between FAO and GO, where GO is about 10% more efficient. However, since the difference in overall efficiency between FAO and GO is closer to 25% (GO being more efficient), there must be other factors which contribute to lowered cardiac efficiency of FAO [22, 86].

Mitochondria make up nearly 50% of the heart in mass, so it is unsurprising that the heart uses mostly oxidative phosphorylation to produce ATP [87]. Creatine kinase (CK), creatine (Cr) and phosphocreatine (PCr) are key players in ATP homeostasis. Enzymatic activity of CK reversibly interconverts Cr into PCr. If energy is in excess, CK can donate a phosphate group to Cr forming PCr, which stores the high energy phosphate for when it is needed. Alternatively, CK can remove a phosphate from PCr and donate it to ADP when energy is required. In this way, PCr serves as a buffer that maintains energetic

homeostasis by keeping a reserve pool of high energy phosphates. During increased cardiac work, stress, or as a result of heart disease, ATP levels can quickly become depleted. In those situations, the need for a high functioning CK/PCr system is absolutely necessary. [88, 89]. Studies show that preconditioning increases CK flux, while impairment of the CK/PCr system leads to contractile dysfunction [87].

As already mentioned, the cardiomyocyte has a huge population of mitochondria which are responsible for maintaining a constant ATP supply. Approximately 60-80% of ATP is produced via FAO. Though FAO is considered less efficient, it also generates more ATP (at the expense of more oxygen) [22]. This being said, glucose is still an extremely important substrate and is crucial when high levels of ATP must be produced quickly or when oxygen is a commodity [85].

3.3 Biochemistry of FAO and GO

FAO and GO exist in a competitive balance [90]. Metabolism is regulated in response to available substrates, hormone signalling, changes in fuel expenditure as well as oxygen supply [22]. Both the GO and FAO cycle end up producing acetyl CoA, which enters into the TCA cycle, the products of which enter the ETC. Which pathway predominates depends on a complex threadwork of competing signalling pathways. Glucose enters the cardiomyocyte via two main types of glucose transporters (GLUT). GLUT1 is responsible for insulin independent glucose transport while GLUT4 is responsible for insulin sensitive glucose transport. Glucose is phosphorylated by hexokinase-6-phosphate (G6P) and enters the glycolytic pathway which eventually yields pyruvate. Pyruvate

moves on to the mitochondria where it finally enters into the GO cycle and is oxidized to acetyl CoA which is catalyzed by the pyruvate dehydrogenase complex (PDC). Acetyl CoA then enters the TCA cycle followed by the ETC. FAs enter the cardiomyocyte cytoplasm via CD36, a multifunctional protein receptor responsible for lipid uptake [91]. Once in the cytoplasm, the FAO cycle hydrolyzes lipids into acyl CoA. Carnitine palmitoyltransferase 1 (CPT1) facilitates acyl CoA transport from the cytoplasm into the mitochondria. CPT1 is considered a rate limiting step in the FAO cycle [85, 92]. Once in the mitochondria, acyl CoA is converted to acetyl CoA, which then feeds into the TCA cycle and eventually the ETC.

3.4 Regulation of metabolism

Any factor which regulates the major control points and/or rate limiting steps in either GO or FAO cycle are especially important in determining the predominating cycle. For example, the complex responsible for catalyzing the GO cycle, pyruvate dehydrogenase complex (PDC), is repressed by pyruvate dehydrogenase kinase 4 (PDK4) [92]. PDK is activated by high acetyl CoA and NADH/NAD ratios, while pyruvate has the opposite effect and inhibits PDK. PDK is an important control point and interacts with numerous factors. PDK4 is also known to upregulate PPARα, which is responsible for mediating many enzymes in the FAO pathway [92, 93]. There are also multiple factors which regulate PDK4, including E2F1, PPARα, PGC-1α, Foxo, estrogen-related receptors, FA and AMPK [92, 94]. Overall, the presence of PDK4 has a repressive effect on GO while inducing FAO. On the other hand, CPT1 (the facilitator for acyl CoA transport into the mitochondria) is inhibited by malonyl-CoA, an intermediate in FAO cycle [95]. Hypoxia inducible factor 2 (HIF-2α) also has a suppressive effect on FAO [95, 96]. Furthermore,

sirtuins have the ability to coordinate the switch from GO to FAO during inflammatory responses [97, 98]. AMPK also plays an important role in fuel selection, and can regulate both GO and FAO. AMPK is a sensor for cellular energy balance. It is activated by AMP, signalling a low energy status. AMPK activation leads to a cascade of events leading to restoring energy levels [99]. Depending on substrate availability and physiologic requirements, AMPK activation can either lead to GLUT4 or CD36 translocation to the sarcolemma (enhancing either glucose or FA uptake respectively) [100]. Epinephrine stimulates lypolysis, while insulin and adenosine inhibit it [90, 101]. TNFα on the other hand induces glucose oxidation, while ghrelin maintains glucose homeostasis by repressing insulin [102, 103]. Interestingly, a paper by Wolfe points out that GO is mainly regulated by glucose availability. In contrast, FAO, is not regulated by availability of FA but by changes in the rate of oxidation [90].

PPAR α and PGC-1α are crucial in regulating this energy production network, especially with regard to FAO [40, 84]. PGC-1α regulates both FA and glucose burning pathways. PGC-1α can regulate the activity of the GLUT1 transporter as well as genes of glycolysis [104]. Alternatively, increased PGC-1α and β levels have been shown to increase CD36 and CPT1 [97, 105]. Furthermore, cAMP/PKA pathway has also been shown to activate the Sirt1-PGC1α transcriptional complex which upregulates FAO [106]. Despite being able to affect both pathways, PGC-1α is more commonly associated with upregulation of *FAO* [2].

4.0 Oxygen and/or nutrient deprivation

4.1. Ischemia and hypoxia

Blood is an invaluable substance that provides necessary nutrients and oxygen to cells. In a situation where blood flow is interrupted, this homeostasis is disrupted. Hypoxia refers to an inadequate oxygen supply to tissues. Ischemia is a more complex situation, and describes an insufficient supply of blood to the tissue. This leads to a deficiency in metabolic substrates, waste build-up, acidosis and tissue hypoxia [I] [2]. Ischemia always leads to hypoxia, but hypoxia can exist without the presence of ischemia. Hypoxia can occur as a result of lung disorders, cancer or stroke [107]. Ischemia can occur in any disease that reduces blood supply to the heart. Typically, it is because of a physical obstruction (such as a plaque or stenosis) or pressure overload which prevents sufficient blood from reaching the heart [I][108]. A tissue chronically undersupplied with blood will become hypoxic as well as ischemic. Once the partial pressure of oxygen falls to a hypoxic level, mitochondrial respiration and oxidative metabolism also decrease, leading to cell death if not corrected. [I] Interestingly, both hypoxia and ischemia induce secretion of angiogenic factors like vascular endothelial growth factor (VEGF), suggesting a stress induced repair mechanism [82, 109].

4.2 Ischemia/hypoxia and its effects on substrate preference

Under normal conditions, the hearts use of FAO and GO exists in a homeostasis, with the majority of ATP produced via FAO. Ischemic conditions cause a disruption in this balance [22]. There are numerous groups which study how oxygen deprivation affects

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[I] http://cvphysiology.com/CAD/CAD005.htm

FAO and GO pathways; however no consensus exists at this time. Some studies report that an ischemic heart experiences upregulation of glycolysis while FAO and GO are downregulated [110-112]. An increase in glycoclysis during ischemia can be seen as an adaptation to decreased oxidative capacity. However, in combination with GO dysregulation and ischemic conditions, proton accumulation (acidosis) can ensue. Neely et al differentiate between hypoxic and ischemic conditions. They state that while hypoxia upregulates glucose and glycogen utilization, ischemia downregulates them. They also state that ischemia reduces FAO. Other studies report an increase in the rate of FAO under hypoxic conditions [112]. Interestingly, reperfusion post-ischemia causes FAO upregulation, however GO remains downregulated. [II] [III]

Several studies report that presence of FA during ischemia further uncouples GO and FAO cycles [22]. Folmes et al show that during mild ischemia, FAO is the main source of oxidative metabolism. This is accompanied by reduced cardiac function and efficiency [113]. It *is* generally accepted that an increase in FAO corresponds to an overall lower cardiac efficiency. Studies in healthy humans as well as pigs show that elevation of plasma FFA causes a drop in cardiac mechanical efficiency [22, 114]. It has also been reported that high levels of FA can induce arrhythmia, decrease contractility and uncouple oxidative phosphorylation [112, 115, 116]. The same lack of consensus exists in studies examining the shift between FAO and GO in heart failure models. This is discussed in further detail in the section 5.3.

[II] http://www.ncbi.nlm.nih.gov/books/NBK21624/,

[III] http://www.heartandmetabolism.com/download/53/8.pdf

4.3 PGC-1α's role in ischemia and hypoxia

Numerous studies have shown that PGC-1α is involved in the response to both hypoxia and ischemia. A recent study from our lab showed that hypoxia represses PGC-1α mRNA expression in rat neonatal cardiomyocytes in a time dependant manner. A significant decrease occurred at 9 hours of hypoxia, reaching 50% inhibition at 12 hours. Beyond 12 hours, nutrients dwindle and ischemic conditions set in. At 24 hours hypoxia, we observed a significant up-regulation of PGC-1α levels. Importantly, re-feeding the cells at 12 hours while maintaining hypoxic conditions until 24 hours brought PGC-1α expression back down to levels seen at 12 hour hypoxia. Using a glucose free medium in combination with extended hypoxia also caused an up-regulation of PGC-1α. An *in vivo* study also showed that hypoxic but non ischemic adult rats had significantly reduced ventricular PGC-1α mRNA and protein expression [4]. From these studies, it appears that PGC-1α expression exists in a biphasic state, where its expression is differently influenced by exposure to either hypoxia or ischemia. When glucose is available but oxygen is limiting, PGC-1α expression is reduced. When both oxygen and glucose are limiting, PGC-1α is up-regulated [4, 15]. In line with our data, Vigano et al. report PGC-1α reduction in the mouse proteome during acute (48 hours) and chronic (10 days) hypoxia [107]. Rimbaud et al. also found that severe hypoxia downregulates PGC-1α levels in the cardiomyocyte [80]. Another study in the cardiomyocyte by Huss et al. showed that hypoxia inhibits PPARα activity [117]. Since PGC-1α coactivates PPARα to induce FA oxidation, repression of PPARα suggests a similar downregulation of PGC-1α may occur [118]. A study in the rat liver showed a similar decrease in PGC-1α expression in response to hypoxia [119].

In contrast to our findings, one report indicated that exposure to normobaric hypoxia (21 days) made no difference in PGC-1α protein levels in rat skeletal muscle [120]. Other groups showed that hypoxia increased PGC-1α expression in H9c2 cells, primary myocardial cells and mouse brain tissue [121, 122]. The study by Zhu et al. reported that 24 hour hypoxia increased PGC-1α expression in H9c2 cells and primary rat cardiomyocytes [121]. The fact that hypoxia was maintained for 24 hours suggests that the cell media may have become limiting, causing ischemia to set in, which we demonstrated induces PGC-1α up-regulation [4]. However, they also correlated PGC-1α expression with the severity of cyanosis in patients with Tetralogy of Fallot (TOF), a disease characterized by chronic hypoxia [121]. Mammalian hibernation is used as a model for some types of hypoxia and can induce a preference for FA oxidation by mediating PPARγ/PGC-1. One study showed that hibernation increases expression of PGC-1 in skeletal muscle [123]. A possible limitation of the hibernation model is despite the low blood oxygen content, hibernating animals also have a drastically reduced metabolic rate to match it, and therefore are not experiencing oxygen starvation to the same extent as a truly hypoxic animal.

In 2008, a paper by Bruce Spiegelman's group showed that PGC-1α expression is induced by nutrient and oxygen deprivation (i.e.: ischemia) in C2C12 myotubes, primary skeletal muscle cells, striatal neurons, hepatocytes and fibroblasts. Interestingly, but in contrast to our findings, they found that either removal of nutrients *or* oxygen could individually induce PGC-1α, though a combination of the two lead to the biggest increase

[82]. A study by Fabregat-Andreas et al. provided subsequent evidence that reduced blood flow and reduced oxygen both induce PGC-1α in *in vitro* cell assays as well as animal models [124].

Since PGC-1α is a key factor in mitochondrial biogenesis, a review paper by Shoag and Arany discussed the concept that it makes little sense to up-regulate mitochondrial genes when the cell is undergoing ischemia. They refer to an unpublished study where they observe that ischemia represses mitochondrial genes in cultured myotubes [2]. In contrast, another group reports an induction of mitochondrial biogenesis in rat neonates with cerebral ischemic injury. Quite surprisingly, they found PGC-1α mRNA and protein levels were unchanged [125].

4.4 PGC-1α as an activator of angiogenesis

Though the exact role of PGC-1α in ischemia and hypoxia is unclear, there are numerous studies which unambiguously demonstrate that PGC-1α is a potent activator of angiogenesis. The above-noted paper by Spiegleman's group showed that PGC-1α stimulated induction of VEGF. They also noted that PGC-1α knockout animals experienced blunted blood flow return in previously ablated arteries in comparison to wildtype animals. In contrast, PGC-1α overexpressed in skeletal muscle resulted in significantly faster blood flow return in comparison to wildtype. As a result of these findings, they proposed that PGC-1α plays a protective role in ischemia by initiating angiogenesis [82]. Subsequent studies report that PGC-1α can promote angiogenesis in ischemic mesenchymal stem cells, retinal cells, skeletal muscle and cardiac tissue [126-130]. Interestingly, PGC-1β is also considered a potent activator of angiogenesis [131].

One study contradicts the angiogenic role for PGC-1α and by showing that transient up-regulation of PGC-1α during ischemia reperfusion actually had adverse effects and slowed tissue recovery in mouse hearts [132].

The exact role of PGC-1α in ischemia and hypoxia is debatable considering the number of contradicting studies. However, the majority of studies do suggest that hypoxia reduces PGC-1α while ischemia upregulates it. Since PGC-1α predominantly affects FAO, it can be extrapolated that hypoxic conditions may reduce FAO while ischemic conditions upregulate FAO. However, this extrapolation cannot be confirmed at this time, considering the conflicting studies discussed in section 4.3. On the other hand, PGC-1α's pro-angiogenic role has been strongly documented. Though the exact details remain unknown, it can be said with certainty that PGC-1α is a crucial factor in metabolic homeostasis, substrate energy utilization, stress response, healing and disease.

4.5 PGC-1α as a marker for disease progression and recovery

PGC-1α has also been suggested as a marker for disease progression. In two separate papers, Fabregat-Andreas et al. showed that PGC-1α mRNA levels may serve as a valuable indicator of cardiac recovery [73, 124]. One study showed that idiopathic pulmonary arterial hypertension (IPAH) patients who had higher levels of PGC-1α exhibited greater improvement in heart and lung function compared to those with lower levels [73]. They also showed that PGC-1α blood levels were higher in individuals who experienced ST-Elevation Myocardial Infarction (STEMI). This second paper was different in that they found that increased PGC-1α levels correlated with a larger necrotic area and myocardium dysfunction, suggesting that induction of PGC-1α may be an

important component of recovery [124]. Another study showed that PGC-1α up-regulation had positive effects on the heart after traumatic haemorrhage [133]. As already mentioned in the previous section, the study by Zhu et al. reported a higher mitochondrial count and an up-regulation of PGC-1α expression in patients with TOF. They propose that PGC-1α up-regulation may be an adaptive attempt to increase ATP and reduce damage [121].

From this brief overview of research, it is clear that PGC-1α plays a pivotal role in ischemic/ hypoxic conditions. Low oxygen and low fuel trigger unique global responses, but due to the discord between publications, the exact role of PGC-1α remains unclear. However, there are several ways to interpret the differences observed in the behavior of PGC-1α. First, hypoxia and ischemia affect PGC-1α differently depending on the tissue type. Second, it is important to consider that ischemic and hypoxic conditions are not necessarily independent of each other. Especially in *in vitro* studies, gradual fuel depletion during hypoxia treatment as a result of cells consuming nutrients from media will eventually lead to ischemia. Third, PGC-1α is capable of functioning within numerous signaling axes and many factors tailor the final response of PGC-1α. Furthermore, hypoxia or ischemia affect only a subset of PGC-1α regulated genes [2]. It follows that any number of particular combinations of upstream/downstream signalling events could converge on PGC-1α, taking into account not only oxygen or glucose deprivation but all cellular events. For example, the metabolic changes or accumulation of ischemic bi-products (as a result of insufficient waste removal) could eventually affect PGC-1α levels differently than at the start of ischemia. Finally, PGC-1α expression can

be modified at both the transcriptional and post-translational level [2]. This suggests that in the experimental set up, different periods of hypoxia/ischemia, type of tissue used and even different media formulations can impart a different PGC-1α activity profile. Therefore, it is likely that individual studies contain small snapshots of reality under highly specific conditions.

5.0 The hallmark metabolic switch from fatty acids to glucose

5.1 The metabolic switch

The heart is capable of responding to a wide array of situations, and even in a disease state will attempt to adapt in order to meet the body's energy demand. Beginning in the late 1960s, many groups began to observe the now 'hallmark' shift from fatty acids to glucose in various failing heart animal models, including hypertension, hypertrophy, congestive heart failure and right ventricular pressure overload [134-140]. In 1996, Dan Kelly's group showed that FA oxidation enzymes were down-regulated in humans with left ventricular heart failure. They proposed that this down-regulation occurs at pre- and post-translational levels during the development of heart failure [141]. Subsequent studies in humans showed a similar pattern in idiopathic dilated cardiomyopathy and correlated increased LV mass to a decrease in the rate of FA oxidation in hypertrophic hearts [142, 143].

The fetal heart relies mostly on glucose and lactate until a pronounced shift to FA occurs in the early perinatal period [144]. Interestingly, one study showed that inducing hypertrophy in newborn piglets delayed the onset of cardiac FA oxidation metabolism

[145]. A diseased heart is inefficient at pumping blood and struggles to provide enough oxygen and fuel to the body. The switch from FA to glucose could reflect an increase in energy demand, defects in oxygen delivery, defects in mitochondrial energy production or a combination of these factors. The significance of the substrate shift and whether it is adaptive or maladaptive remains unclear [36].

5.2 PGC-1α downregulation accompanying metabolic shift in HF models

Down-regulation of PGC-1α is considered key in initiating the switch from FA to glucose. Considering that the same metabolic shift is observed in chronic heart disease, it is tempting to speculate that PGC-1α is associated with this shift in a diseased heart [2]. Indeed, there are many studies which support this. Several groups independently showed that PGC-1α levels were significantly down-regulated in various rodent models of myocardial infarction, hypertrophy and congestive heart failure [55, 146-149]. Of note, Rimbaud et al. were able to differentiate between compensated and decompensated cardiac hypertrophy, and showed that PGC-1α down-regulation occurred only in the decompensated state [80]. To provide a human perspective, a study by Sihag et al. in failing human hearts showed a down-regulation of PGC-1α as well as a subset of its downstream targets [150].

5.3 Is the metabolic shift from FA to glucose a hallmark of HF?

A number of studies question whether the switch to a fetal metabolic profile is truly a hallmark of hypertrophy and cardiac failure. Several studies show inconsistent glucose utilisation in heart failure conditions. Some report a decrease in glucose uptake in

hypertrophic models, while others report no change in glucose utilization in MI models [148, 151, 152]. Some groups observe a decrease in both FA *and* glucose oxidation in cardiac hypertrophy and heart failure models [148], [153]. Other groups see no change at all in FA oxidation during compensated hypertrophy [154, 155]. Others still propose that FA uptake or FA oxidation may be up-regulated during heart failure [156-158]. Interestingly, ketone bodies have the ability to inhibit FA uptake and oxidation, meaning that a normal FA uptake level in heart failure patients could be explained by potentially higher concentrations of ketone bodies [22, 159]. Another study showed that after the initial drop in FA oxidation in an MI mice model, FA oxidation genes returned to normal levels 3 months post MI, though the animals still developed congestive heart failure [160]. The above studies challenge the idea that hypertrophied or failing hearts exhibit a fetal gene pattern of metabolic protein expression. Although the substrate switch from FA to glucose clearly does occur in some cases, it is becoming clear that FA and glucose oxidation levels are not always connected to disease progression.

Importantly, some studies report an initial down-regulation of metabolism as a whole, which is subsequently followed by the hallmark fetal gene program in later stages of cardiac hypertrophy or congestive heart failure [153, 160]. Dan Kelly's group also reported that although their hypertrophic rat model did see an eventual down-regulation of FA oxidation genes, enzyme/protein levels did not decrease immediately, but only when heart failure ensued [141].

Despite the conflicting data, most research agrees that the hypertrophic/failing heart becomes progressively more dependent on glucose [36]. However, it is also clear that both glucose and FA uptake and utilization levels depend on the molecular profile and severity of the particular disease. Some propose that the switch from FA to glucose may not occur until the end stage of heart failure [80, 153, 160]. If that is true, then it is not clear why the switch to glucose would occur so late in disease progression, considering that this switch conserves oxygen. However, prolonged use of glucose also leads to reduced energy production, functional deterioration and eventually decompensated hypertrophy [36, 161]. Making the switch to glucose so late in disease progression may explain the maladaptive component of the substrate switch, as well as the progressive health decline seen at end stage disease. Differences between individual studies may also account for some discrepancy. Type, intensity and duration of stimulus to induce disease and the neurohormonal status of experimental animals before the procedure could play a role in creating different molecular profiles [80].

6.0 The connection between inflammation and metabolism

6.1 NF-κB

In addition to metabolic dysfunction, another important feature of cardiovascular disease is chronic inflammation [6]. A central component of the inflammatory response is the NF-κB transcription factor family, and numerous studies report its up-regulation in various forms of heart disease [35]. Furthermore, the NF-κB family also plays a key role in response to stress, injury, protein turn-over, exercise and immunity [35, 162]. NF-κB has the capacity to directly interact with over 150 genes, including those of cytokines and

regulators of metabolism, redox status, immunity and apoptosis [163]. NF-κB is induced by pro-inflammatory cytokines, ROS, angiotensin II, endothelin I, growth factors, lipopolysacharides, hyperglycemia, MAPK and intracellular calcium [164, 165].

The NF-κB family consists of five members: p65/Rel A, RelB, c-Rel, p100/p52 and p105/p50. p65 is particularly important because it is the only subunit that can function as a transcriptional activator. p65 has weak DNA binding ability and can be regulated via ubiquitination, nitrosylation, acetylation, prolyl isomerization, monomethylation and phosphorylation [166-171]. p65 most commonly exists as a heterodimer with p50, though other combinations are possible [3]. There are also several NF-κB splice variant isoforms that been identified. The various combinations of hetero/homodimers each have a specific repertoire of genes under their influence. NF-κB hetero/homo dimers can also undergo post-translational modifications which add further specificity to its downstream activity [IV] [164]. NF-κB dimer complexes are inactive in the cytoplasm as a result of interaction with an inhibitor called IκB. In response to external stimuli, IκB kinase (IKK) is activated and phosphorylates IκB , which causes its degradation. This releases the NF-κB heterodimer which then travels into the nucleus and alters gene regulation by binding to target genes [67, 164].

[IV] http://www.phosphosite.org/proteinAction.do?id=1012&showAllSites=true

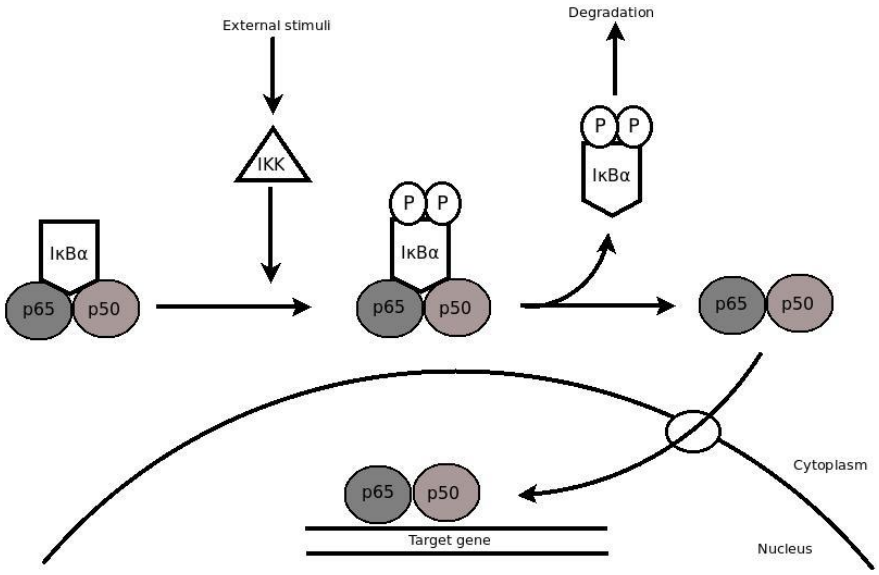

Figure 3: Mechanism of NF-κB heterodimer (p65/p50) activation

Once IκB kinase (IKK) is activated, it phosphorylates IκBα. This causes it to dissociate from the p65/p50 complex. Once the p65/p50 is free from IKB alpha it travels into the nucleus and imparts changes on target genes. The NF-κB complex is represented as the heterodimer p65/p50. It is important to note that other NF-κB complexes are possible; p65/p50 was selected because it is the most abundant form of NF-κB.

NF-κB plays a crucial process in normal function as well as disease states. NF-κB has the capacity to be cytoprotective and has both pro- and anti-apoptotic properties [172, 173]. For example, exercise can activate NF-κB in skeletal muscle, and induces muscle regeneration and provides resistance to future oxidative stress in skeletal muscle [6]. Interestingly, NF-κB also appears to have a link to metabolism. NF-κB may mediate metabolic changes by interacting with IL-6, a cytokine involved in mediating fat metabolism in humans [6, 174]. Another study highlighted a somewhat surprising function of NF-κB. They showed that an alternative non-canonical NF-κB signaling pathway stimulates mitochondrial biogenesis and slow twitch muscle fiber specificity by direct transcriptional activation of PGC-1β [175].

As already mentioned, the NF- κB family is an important player in homeostasis in cardiac physiology. However, abnormal NF-κB signalling is associated with disease, often exacerbating inflammation in already chronically stressed tissues. Up-regulation of NF-κB has been linked to obesity, insulin resistance, cachexia, cancer, myocarditis, cardiac hypertrophy and heart failure [3, 162, 165, 176, 177]. Interestingly, down-regulation of NF-κB is also associated with diseases involving functional deterioration [178]. Severity and duration of stress, as well as specificity of NF-κB dimer recruitment may in part explain the broad range of NF-κB signalling [165]. Considering the spectrum on which NF-κB can act, it is important to look at NF-κB as a control point. Numerous signalling pathways converge on NF-κB thereby facilitating crosstalk between the pathways and generating an informed appraisal of the microenvironment [179].

6.2 Connection between p65 and PGC-1α

Both inflammation and metabolism are crucial to maintaining homeostasis. Chronic stress dysregulates both processes so it is important to consider the crosstalk between the two pathways as it can play a role in disease outcome. Since NF-κB and PGC-1α are key regulators of their respective pathways, it is unsurprising that they are interconnected. Numerous studies describe this relationship**.**

Manuel Vasquez-Carrera's group from the University of Barcelona has published a series of papers studying the relationship between NF-κB and PGC-1α. In 2006 they showed that NF-κB is involved in PGC-1α and PPAR down-regulation in C2C12 myoblasts [180]. In 2009, they found that PGC-1α expression was reduced as a result of TNFα (Tissue Necrosis Factor-α, an endogenous activator of NF-κB) both in the human cardiac

model AC16 cell line and a TNFα over-expressing cardiac-specific mouse model [35]. A year later, they published another paper which showed that the p65 subunit and PGC-1α directly interact in human and mouse cardiac cells. They also showed that this interaction disrupts glucose oxidation. They suggest that the increased interaction between PGC-1α and NF-κB is in part responsible for the metabolic changes seen in diseases like dilated cardiomyopathy, insulin resistance and obesity [181]. Other groups have also contributed to the knowledge available about PGC-1α and NF-κB interaction. In 2011, Dan Kelly's group showed that NF-κB is a necessary component for lipopolysaccharide-mediated repression of PGC-1α in mouse hearts [182]. Wang et al. have also shown that PGC-1α is constitutively associated with NF-κB in bone marrow stromal cells [183].

A recent 2013 paper studying the loss of skeletal muscle oxidative phenotype found that NF-κB reduced PGC-1α mRNA levels *in vitro*, as well as disrupted downstream PGC-1α signalling events in skeletal muscle [184]. An earlier paper by the same group showed chronic obstructive pulmonary disease patients had markedly reduced PGC-1α mRNA levels [79]. Palomer et al. also showed that NF-κB activation down-regulated PGC-1α in AC16 cardiac cells [185]. Since PGC-1α is a co-activator for PPARs, and PPARs are similarly down-regulated in heart failure, repression of PPAR by NF-κB has also been documented [185, 186].

While there is a significant amount of data supporting the repressive role of p65 on PGC-1α, there are several studies which suggest that PGC-1α can have a repressive effect on NF-κB. PGC-1α can play an anti-inflammatory role by supressing NF-κB expression in

human aortic smooth muscle, endothelial cells and skeletal muscle [3, 187]. One study also showed that muscle-specific PGC-1α transgenic mice had lower levels of p65 phosphorylation [188]. Phosphorylation of the p65 subunit is important in initiating transcription, protein interactions and ubiquitination of NF-κB interacting proteins [189-192]. However, another group reported the opposite effect on p65 in their PGC-1α transgenic model [193]. Lastly, PPARγ has also been shown play a role in the inflammatory response by inhibiting NF-κB [194].

These different studies illustrate that much like with metabolism, the exact role of PGC-1α in inflammation is unclear. PGC-1α itself is regulated during inflammation, and it is likely that PGC-1α and NF-κB have the capacity to affect each other. Which series of events occurs depends on the finely-tuned balance of environmental and molecular cues.

VI Materials and Methods

In silico analysis and mutagenesis

Two putative p65 sites were found on the PGC-1α promoter using the Transfac database. Mutagenesis of each site was performed using the Agilent Technologies QuikChange XL Site-Directed Mutagenesis Kit using custom-synthesized PCR primers (See Table 3) as per manufacturer's directions. A Sac I site was introduced as the mutation in order to make identification of mutants possible by digestion reaction.

p65 site #1: 5' GGGACTTT 3'

p65 site #2: 5' GGGACTGT 3'

p65 consensus sequence (mouse): GGGACTTTCC

Building the truncated PGC-1α promoters

Five truncations of the PGC-1α promoter were created via sequential deletion. To create each truncated version, the previously published ~3.1 kb PGC-1α promoter underwent five separate sets of double restriction digests [15]. Each digest reaction was run on a 1% agarose DNA gel and the appropriate linear fragment was excised and purified, using the Qiaquick Gel Extraction Kit. If the truncated linear promoter fragment was blunt ended, it was treated with Klenow DNA Polymersae (NEB Biolabs) prior to ligation. All promoters were then ligated using T4 DNA Ligase (Invitrogen). Ligated truncated promoters were transformed using α–select Competent Gold Efficiency cells (Bioline) and DNA purified using a modified Qiagen Miniprep protocol. The truncated promoters were digested and run on a 1% gel to confirm correct size and sequenced as a final step.

Transfection and luciferase assays

NIH 3T3 and cos7 cells were plated in 6-well plates at 800,000 cells/well. Neonatal cardiomyocytes were plated in 6-well plates at 1 million cells/well. A total of 1.005 μg DNA was co-transfected into each well in OptiMem medium (500 ng appropriate vector with 5 ng Renilla (pRL) transfection control vector (Promega). The PGC-1α promoter is contained in a pGL3 basic backbone vector, whose luciferase gene is located downstream of the promoter. A pCMX-based vector encoding p65 was employed for cell transfection. NIH 3T3 cells were washed and fed with DMEM media (HyClone) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% FBS after 24 hours. After 48 hours, NIH 3T3 cells were isolated. In contrast, transfected neonatal cardiomyocytes were exposed to either normoxia or hypoxia and isolated after 12 hours. 250 μl of 1x passive lysis buffer (Promega) was used to isolate both cardiomyocytes and 3T3 cells. After three freeze/thaw cycles, luciferase and renilla activity was concomitantly assayed using the Promega Dual Luciferase Reporter Assay System and a Promega Glomax MultiPlus Luminometer.

Electrophoretic mobility shift assay (EMSA)

Cos7 cells were used to isolate total nuclear proteins. Untreated cos7 cells, cos7 cells transfected with p65 in pCMX vector and cos7 cells treated with 10, 50 or 100 ng/μl rrTNFα (R&D Systems) for 4 hours were used in EMSA experiments. Nuclear protein was extracted using the Pierce NE-PER Nucleic and Cytoplasmic Extraction Kit. Biotin-labelled and cold probes were made for the wildtype PGC-1α promoter and obtained from Integrated DNA Technologies. See Table 3 for the probe sequences. Thermo

Scientific Lightshift EMSA Optimization and Control Kits were used to set up the control, shift, cold competition and supershift reactions1 μl of 50% glycerol, 100 mM $MgCl_2$ and NP-40 were added to all EMSA reactions in addition to the core set of reagents. The NFκB p65 antibody (Millipore) was used for the supershift reactions. Allsamples were then run on a 6% resolving acrylamide gel and transferred to a 0.45um Biodyne nylon membrane (Pall Life Sciences), crosslinked and developed with a Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

Cardiomyocyte isolation

Primary cardiomyocytes were isolated from Sprague-Dawley neonatal (1 day) rat pups as previously reported [4]. They were maintained in a DMEM medium (HyClone) supplemented with 16.6% M199, 1% FBS, 10% horse serum, 1% penicillin/streptomycin and 1% l-glutamine for 1-2 days after isolation. Prior to experiments, the neonatal rat cardiomyocytes were starved for 12-18 hours in DMEM medium supplemented with 16.6% M199, 0.5% FBS, 1% penicillin/streptomycin and 1% l-glutamine.

Neonatal rat cardiomyocyte drug treatments

Neonatal cardiomyocytes were treated with 40, 5, 1 or 0.5 μM parthenolide (Sigma), a p65 inhibitor. However, 40 μM proved to be toxic to the cells and resulted in wide spread cell death. Neonatal cardiomyocytes were alternatively treated with 10, 50 or 100 ng/μl tumour necrosis factor alpha (rrTNFα, R&D Systems) for 6 hours, an endogenous activator of p65. Lastly, rrTNFα and parthenolide were used in conjunction. rrTNFα was

kept at a constant concentration of 50ng/μl , while parthenolide was tried at three different concentrations: 5 μM, 1 μM and 0.5 μM.

Hypoxia

Cardiomyocytes were subjected to hypoxia via an air-tight hypoxia incubator containing 95% N_2 and 5% CO_2 at 37°C. All cells were maintained in the hypoxia chamber for 12 hours unless otherwise stated.

Western blots

Total protein from neonatal rat cardiomyocytes was isolated using 250 μl of RIPA Lysis Buffer containing 1 mM PMSF, 0.1 mM DTT and 1x protein inhibitor (Thermo Scientific). After one minute of incubation, plates were scraped, incubated on ice for 15 minutes and centrifuged at 14,000 rpm at 4°C for 15 minutes. Nuclear and cytoplasmic protein was extracted using the Pierce NE-PER Nucleic and Cytoplasmic Extraction Kit. Thermo Scientific Pierce protein assay was used to assay protein concentrations. Samples were run on a 12% resolving polyacrylamide gel and transferred to PVDF membrane. Table 1 outlines the conditions used for each western experiment.

Antibody	Block Conditions	Primary Antibody	Secondary Antibody and horse radish peroxidase (HRP)
NFκB p65 (Rel A) (Millipore)	5% milk in PBST (0.1% Tween) for 1 hour	1% milk in PBST, 1/500 dilution for 1 hour	1% milk in PBST, 1/5,000 dilution of Goat anti Mouse and HRP for 1 hour
PGC-1α (Calbiochem)	5% milk in TBST (0.1% Tween) for 1 hour	5% TBST, 1/1000 dilution for 3 hours	1% milk in TBST, 1/10,000 dilution of Goat anti Mouse and HRP for 1 hour
α -tubulin (DSHB)	5% milk in PBST (0.1% Tween) for 1 hour	1% PBST, 1/5000 dilution for 1 hour	1% milk in PBST, 1/10,000 dilution of Goat anti Mouse and HRP for 1 hour

Table 1: Western conditions used for NFκB p65, PGC-1α and α–tubulin antibodies

Three washes of TBST/PBST (5 minutes) were completed after the primary antibody incubation. After the secondary antibody incubation, blots were washed twice with TBST/PBST (10 minutes) followed by a wash with TBS/PBS (5 minutes). The Immuno Cruz Western Blotting Luminol reagent Kit (Santa Cruz) was used for band visualization. All experiments were normalized against α-tubulin, and band intensity was measured using Bio-Rad Quantity One software.

qPCR

RNA was isolated from neonatal rat cardiomyocytes using the Thermo Scientific GeneJet RNA Purification Kit. 25 ng RNA was used for all real time PCR reactions. The reactions were set up using a qScript One Step qRT PCR Kit (Quanta Biosciences) and a Bio-Rad iQ5 real-time PCR thermocycler was used for amplification. See Table 2 for qPCR program details. PGC-1α primers specific for rat/mice were used for all qPCR reactions. See Table 3 for primer sequences.

Cycle	**Repeats**	**Dwell time**	**Temperature**	
1	1	10 minutes	50 °C	
2	1	5 minutes	95 °C	
3	45	10 seconds	95 °C	
		30 seconds	60 °C	*Real time acquisition*
4	1	1 minute	95 °C	
5	1	1 minute	55 °C	
6	81	10 seconds	55 °C	*Melt Curve*
		Temperature change at 0.5 °C		
		End temperature 95 °C		

Table 2: qPCR program used on neonatal rat cardiomyocyte RNA with PGC-1α primers

All data was normalized to GAPDH, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ formula.

Cell viability

Neonatal cardiomyocyte viability following hypoxia or normoxia has been previously reported [4]. Treatment with TNFα or parthenolide in either hypoxic or normoxic conditions was assessed using Trypan blue exclusion, where non-viable cells uptake blue dye. Neonatal rat cardiomyocytes were plated in 6 well dishes at 1 million cells/well. 12 hours after drug administration, cells were washed once with PBS and harvested by incubating cells with 200 μl Tryple Express (HyClone) for 5 minutes, followed by gentle scraping. Cells were re-suspended in feeding medium and then mixed 1:1 with 0.4% trypan blue (Sigma Aldrich) and incubated for 5 min at room temperature. A hemocytometer was used to count the number of non-viable blue cells in comparison to the total number of cells. Viability was expressed as a percentage of the ratio between non-viable and viable cells.

Primers

Purpose	Primer name	Primer sequence	Notes
EMSA	PGC-1α promoter p65 site #2 forward probe	5'GCTTTGTCATGTGACTGGGGACTGTAGTAAGACA GGTGCCTTCAG 3	Underlined region represents the p65 binding site
	PGC-1α promoter p65 site #2 reverse probe	CTGAAGGCACCTGTCTTACTACAGTCCCCAGTCACA TGACAAAGC 3‘	
Mutagenesis for site #2	PGC-1α promoter p65 binding site #2 (forward)	5’ GCTTTGTCATGTGACTG**GaGctc**GTAGTAAGACAGGT GCCTTCAG 3’	Bolded region represents the p65 binding site. Lower case letters represent changes made
	PGC-1α promoter p65 binding site #2 (reverse)	3’ CTGAAGGCACCTGTCTTACTAC**gagCtC**CAGTCACAT GACAAAGC 5’	
qPCR	GAPDH (forward)	5’ TGCACCACCAACTGCTTAGC 3’	Used to normalize data
	GAPDH (reverse)	3’ GGCATGGACTGTGGTCATGAG 5’	
	PGC-1α (forward)	5’ AAGTGTGGAACTCTCTGGAACTG 3’	
	PGC-1α (reverse)	3’ GGGTTATCTTGGTTGGCTTTATG 5’	

Table 3: EMSA, mutagenesis and qPCR primers used in this study

Statistical analysis

Origin 9 was used to construct graphs and perform statistical analysis. The two sample Student’s t-test and one-way ANOVA with post-hoc Tukey analysis (unless otherwise stated) were used to determine significance, with $p<0.05$.

VII Results

1.0 Two putative p65 binding sites on the PGC-1α promoter

Using visual inspection and the Transfac database, we completed an *in silico* analysis and found two putative p65 sites on the PGC-1α promoter.

ATTTGGGAATCCTCTATACAAAGTTGGAAGAAGTGAGAGGCAGGCTGCACACACACACACACACACACACA
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AAAATCCAAGGTTGGTGAGCAACTAACAATTTAAATTCTCTTGAGAAGAGCAAAAAGCTGGACAGAAGAGG
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TACCCCTTTTTATGAAGGTAGAGGACAGAGTGGCTGTTTTAAGCAGATCATTAGCTTCATGGATGTGCTGG
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GCCAGGATTGCTTGATAATGATGAATAAACATCGACTCTTTATAGCTAAGTGGCCAGCATTATTTCACAGT
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AAAAAAAGAATCTCATGAAAATGTATCACATGAGGAGCGCTTGCTTCAGTTCCAAGCTGAGTCTGGGGCTA
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TTTGCTATTTGCCTGTTTTGGATGGAAAATAAATTTAAAAAAAAAAGATTGCAGGAGATTTGAGTTATTAT
GTGAGCAGGGCTCCGGTTTAGAGTTGGTGGCATTCAAAGCTGGCTTCAGTCACAGTGTGATGCTTGAAGCC
TCCCAAAGGCCAAGTGTTTCCTTTTCTTTCTTCTATTTTTTTTTTCCTCTCTCTCTAAGCGTTACTTCACT
GAGGCAGAGGGCTGCCTTGGAGTGACGTCAGGAGTTTGTGCAGCAAGCTTGCACAGGAGAAGGGAGGCTGG
GTGAGTGACAGCCCAGCCTACTTTTTAATAGCTTTGTCATGTGACTGGGGACTGTAGTAAGACAGGTGCCT
TCAGTTCACTCTCAGTAAGGGGCTGGTTGCCTGCATGAGTGTGTGCTGTGTGTCAGAGTGGATTGGAGTTG
AAAAAGCTTGACTGGCGTCATTCGGGAGCTGG

Figure 4: The two putative p65 binding sites on the PGC-1α promoter

The PGC-1α promoter is 3112 bp long. The two putative p65 binding sites on the promoter are marked in yellow. The first site is located at 1564-1571 bp and reads: 5' GGGACTTT 3'. The second site is located from 2959-2966 bp and reads: 5' GGGACTGT 3'. These nucleotide positions are relative to the ATG translational start codon. For comparison, the p65 consensus sequence for mice is 5' GGGACTTTCC 3'.

2.0 The PGC-1α promoter is repressed by p65

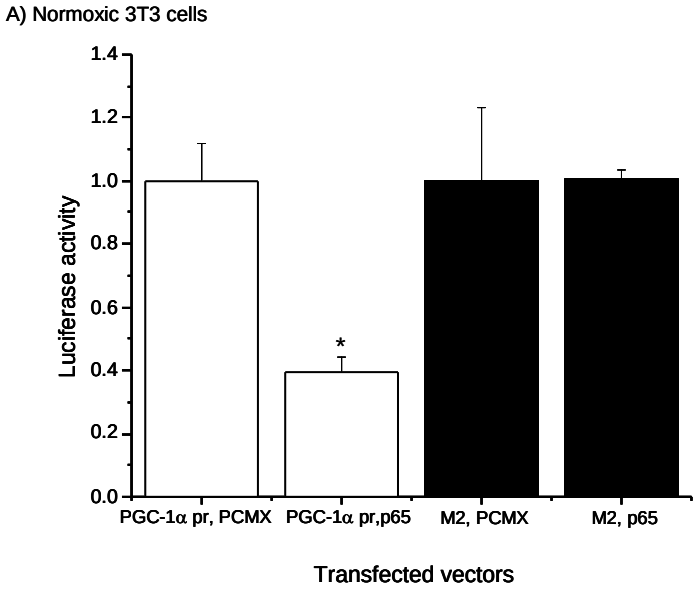
2.1 Mutated PGC-1α promoter is not repressed by p65

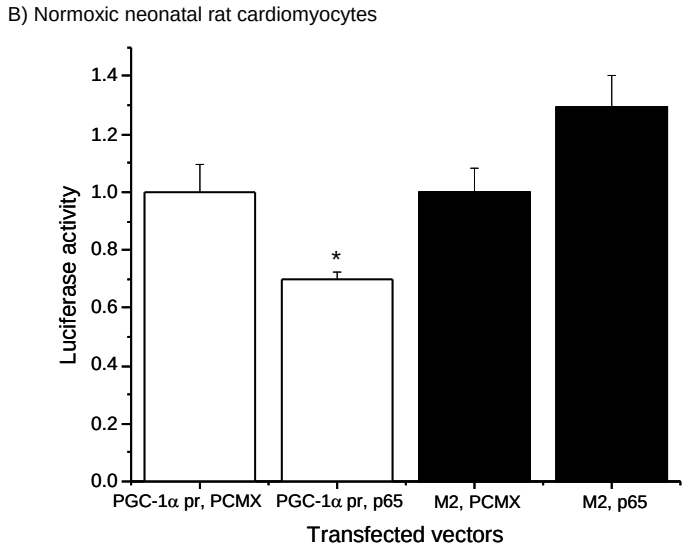
The PGC-1α promoter in the pGL3 luciferase vector was transfected in combination with the p65 in pCMX vector into 3T3 cells, normoxic and hypoxic neonatal rat cardiomyocytes. p65 was found to have a repressive effect on the transactivation of the wildtype PGC-1α promoter in both cell types.

Each vector was co-transfected at a concentration of 500 ng with the exception of the internal control, renilla which was co-transfected at 5 ng. All experiments were assessed using the Promega Glomax MultiPlus Luminometer. Results were normalized to the empty vector, pCMX.

Two putative binding sites were identified in PGC-1α promoter which read as: p65 site #1: 5' GGGACTTT 3' and p65 site #2: 5' GGGACTGT 3'. A mutation was introduced into both putative p65 binding sites on the PGC-1α promoter to elucidate whether either of these sites is important for p65 binding. Of the two sites, only the second p65 site was successfully mutated. This mutated PGC-1α promoter in pGL3 was used in transfection experiments identical to the wildtype PGC-1α promoter described above. In contrast to

the wildtype PGC-1α promoter, the mutated PGC-1α promoter was found to be unresponsive to the repressive effects of p65. This suggests that the second p65 binding site on the PGC-1α promoter is important in p65 mediated repression of PGC-1α.





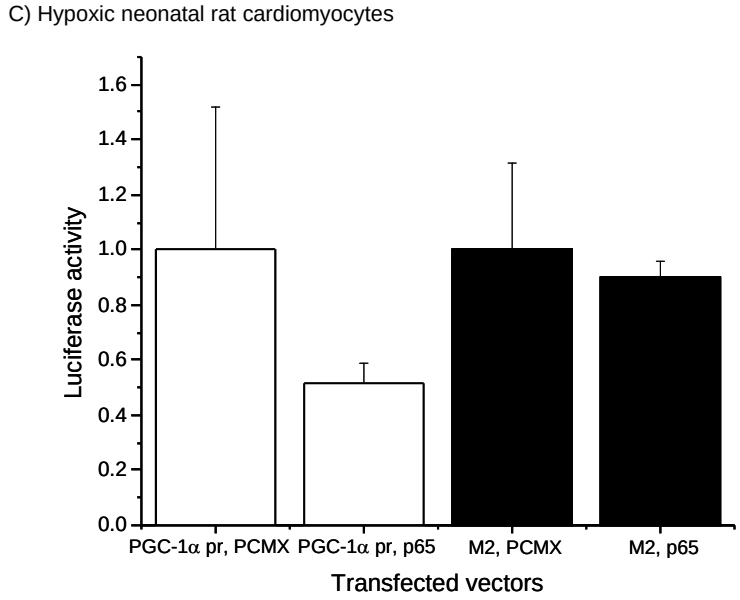


Figure 5: Effect of p65 on wildtype and mutant PGC-1α promoter in 3T3 and normoxic/hypoxic neonatal rat cardiomyocytes

A) *White bars represent luciferase activity of 3T3 cells transfected with the wildtype PGC-1α promoter with either pCMX (backbone vector for p65) or p65. Black bars represent the luciferase activity of cells transfected with the PGC-1α promoter mutated at putative p65 binding site #2 (M2) with either pCMX or p65. n=3 *p<0.05 against backbone vector* ***B)*** *White bars represent luciferase activity of normoxic neonatal rat cardiomyocytes transfected with the wildtype PGC-1α promoter with either pCMX (backbone vector for p65) or p65. Black bars represent the luciferase activity of cells transfected with the PGC-1α promoter mutated at putative p65 binding site #2 (M2) with either pCMX or p65. n=5 *p<0.05 against backbone vector. C****)*** *White bars represent luciferase activity of hypoxic neonatal rat cardiomyocytes transfected with the wildtype PGC-1α promoter with either pCMX (backbone vector for p65) or p65. Black bars represent the luciferase activity of cells transfected with the PGC-1α promoter mutated at putative p65 binding site #2 (M2) with either pCMX or p65. n=2.*

2.2 Truncated PGC-1α promoter is less repressed by p65 than the wildtype PGC-1α promoter

The PGC-1α promoter was sequentially deleted five separate times in an attempt to confirm the mutagenesis work (as described in section 2.0) as well as determine whether

putative p65 site #1 as well other regions are important for p65 binding. SS, SN, SP, NX and SX PGC-1α promoter were named after first letter of the restriction enzyme sites used to truncate them. Each of these truncated PGC-1α promoters were transfected in combination with the p65 in pCMX vector in 3T3 cells. Each vector was co-transfected at a concentration of 500 ng with the exception of the internal control, renilla which was co-transfected at 5 ng. All experiments were assessed using the Promega Glomax MultiPlus Luminometer. NX, SX and SP PGC-1α promoters showed the least repression by p65, suggesting that they no longer contained the region(s) important for p65 mediated repression of the PGC-1α promoter.

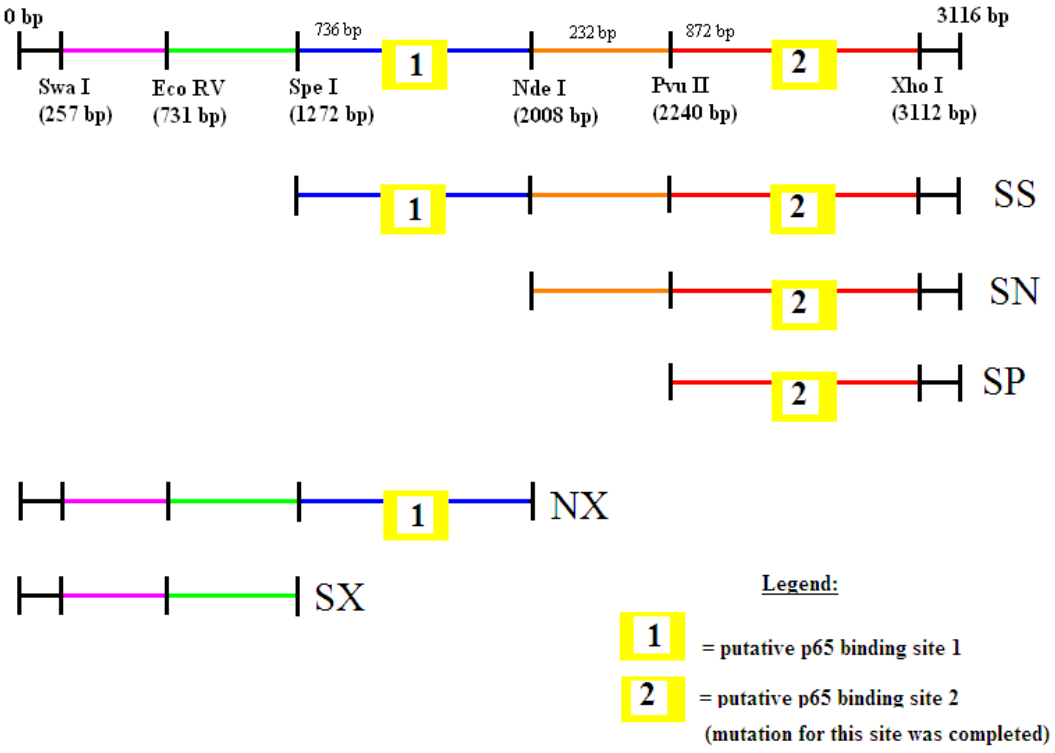
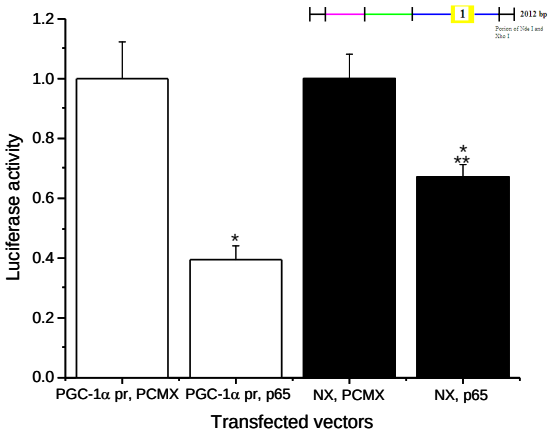


Figure 6: Outline of sequentially deleted PGC-1α promoters

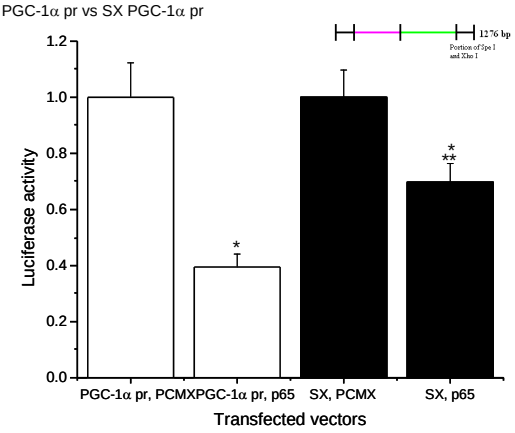
The full PGC-1α promoter is shown in comparison to the five truncated PGC-1α promoters created by double digest. The name of each truncated PGC-1α promoter refers to the restriction enzymes used to truncate them. Yellow boxes represent the two putative

p65 binding sites. SS contains both putative p65 binding sites and is missing a small portion of its 5' end. SN and SP are both missing putative binding site #1. NX is missing putative p65 binding site #2 (serving as a control for mutagenesis work on putative p65 binding site #2 from section 2.0), while SX is missing both putative p65 binding sites.

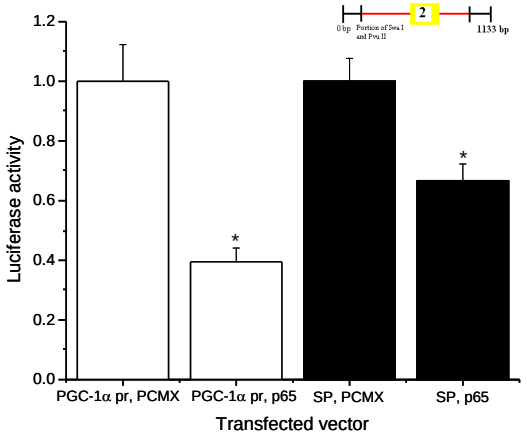
A) PGC-1α pr vs NX PGC-1α pr



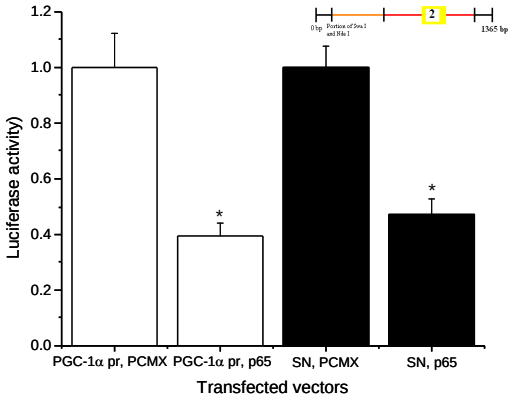
B) PGC-1α pr vs SX PGC-1α pr



C) PGC-1α pr vs SP PGC-1α pr



D)PGC-1α pr vs SN PGC-1α pr



E) PGC-1α pr vs SS PGC-1α pr

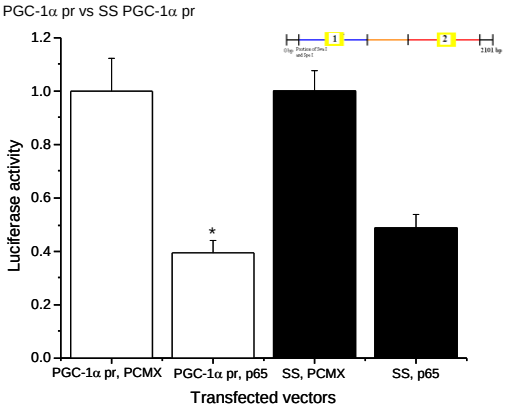


Figure 7: Effect of p65 on truncated PGC-1α promoters: SP, SN, SS, NX and SX in 3T3 cells

White bars represent luciferase activity of 3T3 cells transfected with the wildtype PGC-1α promoter with either pCMX or p65 in all four graphs. ***A)*** *The black bars represent luciferase activity of 3T3 cells transfected with the NX PGC-1α promoter with either pCMX or p65. NX PGC-1α promoter is missing p65 site #2, and is significantly less responsive to p65 than the wildtype promoter.* ***B)*** *The black bars represent luciferase activity of 3T3 cells transfected with the SX PGC-1α promoter with either pCMX or p65. The SX PGC-1α promoter is missing both p65 binding sites #1 and #2, and is the least downregulated promoter out of the five shown in this figure.* ***C)*** *The black bars represent luciferase activity of 3T3 cells transfected with the SP PGC-1α promoter with either pCMX or p65. SP PGC-1α promoter is missing p65 binding site #2 and is surprisingly comparable in activity to that of SX and NX PGC-1α promoter.* ***D)*** *The black bars represent luciferase activity of 3T3 cells transfected with the SN PGC-1α promoter with either pCMX or p65. SN PGC-1α promoter is comparable to the wildtype PGC-1α promoter.* ***E)*** *The black bars represent luciferase activity of 3T3 cells transfected with the SS PGC-1α promoter with either pCMX or p65. SS PGC-1α is repressed by p65 to a similar degree as SN and wildtype PGC-1α promoter.*
*Data was pooled for each of the graphs. n=20 for wildtype PGC-1α promoter, n=9 for NX PGC-1α promoter, n=7 for SX and SP PGC-1α promoter, n=6 for SN PGC-1α promoter, n=4 for SS PGC-1α promoter. *p<0.05 against each respective backbone vector. **p<0.05 against the wildtype PGC-1α promoter transfected with p65*

3.0 p65 binding site 2 on the PGC promoter is important for its interaction with p65

3.1 Direct interaction between p65 and PGC-1α promoter at the site of p65 binding site #2

We used EMSA to examine the interaction between the second p65 binding site on the PGC-1α promoter and nuclear p65 protein. Biotin labeled and cold probes of the wildtype PGC-1α promoter at the second putative binding site were obtained from Integrated DNA Technologies. Nuclear protein was isolated from cos7 cells transfected with the p65-pCMX vector. We found that the PGC-1α promoter probe at p65 binding site #2 and

nuclear p65 show a strong interaction, confirming the importance of the second p65 binding site (on the PGC-1α promoter) in p65 mediated repression of PGC-1α.

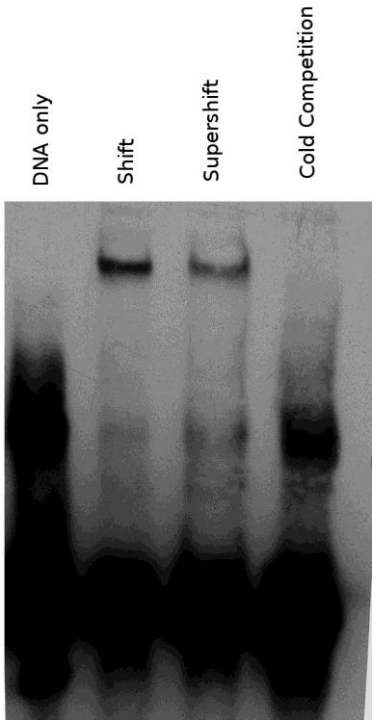


Figure 8: Interaction between wildtype PGC-1α promoter probe (at p65 site #2) and nuclear protein isolated from cos7 cells

This EMSA gel shows the interaction of the PGC-1α wildtype probe with nuclear p65 protein extract from cos7 cells. Lane 1 is a control reaction which contains only the biotin labeled wildtype PGC-1α promoter probe. Since the probe has nothing to bind to, the lane is expected to be empty. The band seen in lane 2 is the shift reaction and represents the interaction between the biotin labeled wildtype PGC-1α promoter probe and nuclear p65 protein. The third lane is the supershift reaction, which contains the biotin labeled wildtype PGC-1α promoter probe, p65 nuclear protein and a p65 antibody. The use of the p65 antibody in the supershift lane confirms that the nuclear protein interacting with the DNA probe is p65 and not a non-specific protein. The last lane is a cold competition reaction, which contains both labeled and unlabeled PGC-1α promoter probes and p65 nuclear protein. The labeled and unlabeled probes compete with each other for p65 binding. The cold competition reaction serves as a control to confirm DNA probe specificity.

3.2 TNFα increases the interaction between PGC-1α promoter probe and p65

Since TNFα is an endogenous activator of p65, we sought to determine whether nuclear protein extract from cos7 cells treated with TNFα would show increased interaction with the PGC-1α promoter probe. Nuclear protein was extracted after 4 hours of TNFα treatment. The greatest interaction is seen between the PGC-1α promoter probe and nuclear protein extracted from cos7 cells treated with 50 ng/μl of TNFα. Interestingly, shift and supershift reactions using nuclear protein extract from untreated cos7 cells did not show a shift or supershift band (data not shown). This is due to the short amount of time the cells were incubated in the 37°C incubator either in the presence or absence of TNFα (4 hours).

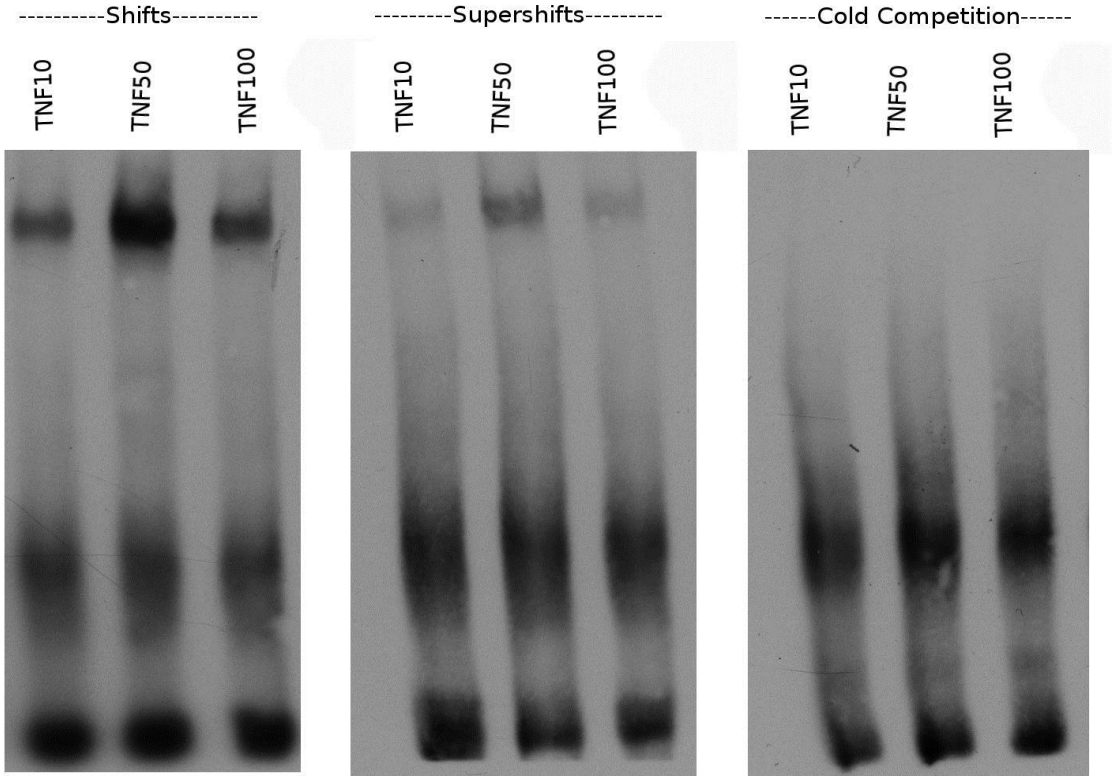


Figure 9: Interaction between wildtype PGC-1α promoter probe (at p65 site #2) and nuclear protein extract from cos7 cells treated with TNFα

Shift, supershift and cold competition reactions are shown in 3 separate panels. The interaction of the PGC-1α promoter probe with nuclear protein extracted from cos7 cells treated with 10, 50 or 100 ng/μl TNFα (TNF10, TNF50 or TNF100) can be seen in lanes 1, 2 and 3 of each panel.

4.0 Effect of TNFα and parthenolide on PGC-1α mRNA expression

4.1 TNFα decreases PGC-1α mRNA expression

To determine the effect of TNFα on PGC-1α mRNA, we treated normoxic neonatal rat cardiomyocytes with 10, 50 and 90 ng/μl TNFα for 6 hours. Total RNA was extracted and used for qPCR using primers listed in Table 3. PGC-1α expression was normalized to GAPDH. Progressive increase of TNFα levels corresponds to a decrease in PGC-1α mRNA levels, tapering off at 90ng/μl TNFα. TNFα increases endogenous levels of p65, which in turn binds to the PGC-1α promoter and decreases PGC-1α gene expression.

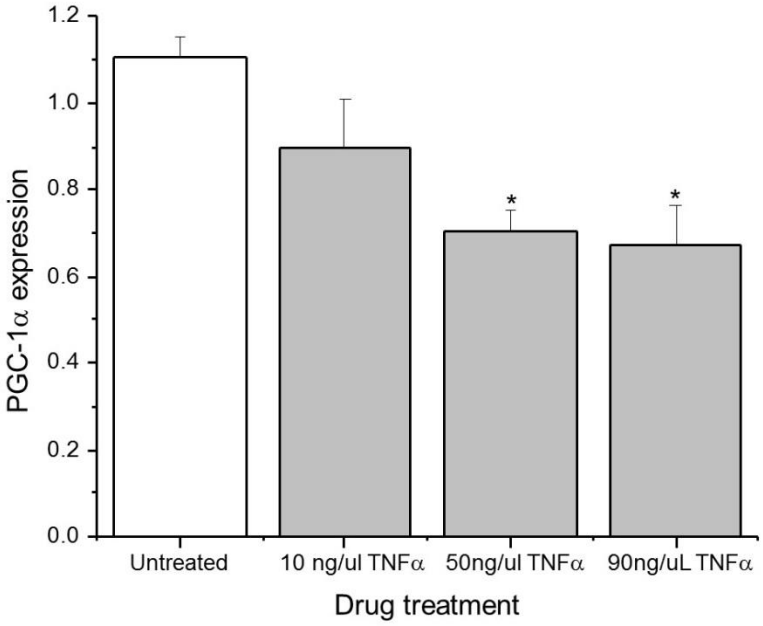


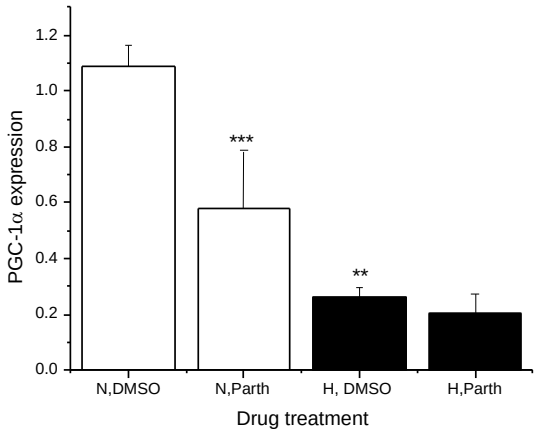
Figure 10: Effect of TNF α on PGC-1α RNA expression in normoxic rat neontatal cardiomyocytes

*The white bar represents PGC-1α activity in untreated neonatal rat cardiomyocytes and gray bars represent PGC-1α activity in neonatal cardiomyocytes treated with 10, 50 and 90 ng/μl TNFα respectively. n=3-5 *p<0.05 compared against untreated.*

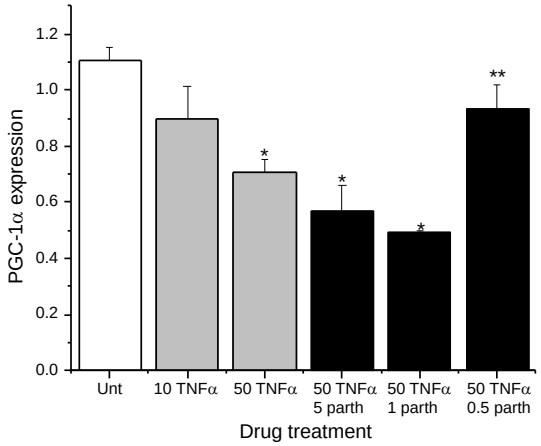
4.2 Parthenolide represses PGC-1α RNA and levels unless co-treated with TNFα

Parthenolide, a p65 inhibitor was introduced into the study as a method to prove that PGC-1α expression could be rescued by repression of p65. We expected to see an increase in PGC-1α mRNA in expression in response to parthenolide treatment. However, 5 μM of parthenolide caused a decrease in PGC-1α expression in both normoxic and hypoxic neonatal rat cardiomyocytes. We hypothesize that this was due to parthenolide induced cell cytotoxicity (see discussion for further information). We therefore examined whether a combination of TNFα and parthenolide could bypass the cytotoxic effects of parthenolide. By providing more p65 to begin with, we hoped that parthenolide would quench the extra p65 activated via TNFα rather than the p65 responsible for mediating cytoprotective pathways. Our data shows that 50ng/μl TNFα and 0.5μM of parthenolide could rescue PGC-1α RNA expression. Lastly, we performed a viability assay to assess how neonatal rat cardiomyocytes respond to either TNFα or parthenolide in comparison to untreated.

A)


PGC-1α expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
N,DMSO
N,Parth
H, DMSO
H,Parth
Drug treatment

B)


PGC-1α expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
**
Unt
10 TNFα
50 TNFα
50 TNFα 5 parth
50 TNFα 1 parth
50 TNFα 0.5 parth
Drug treatment

C)

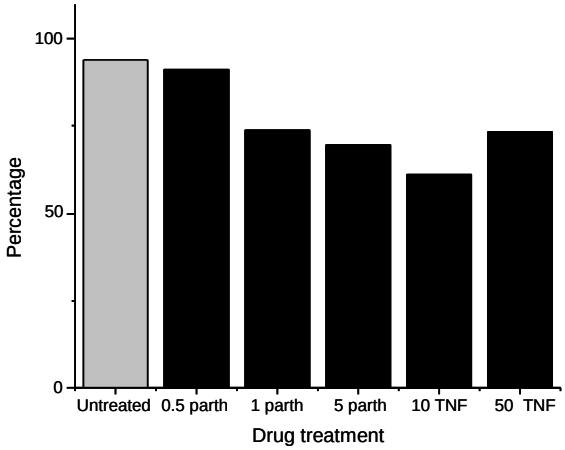

Percentage
100
50
0
Untreated
0.5 parth
1 parth
5 parth
10 TNF
50 TNF
Drug treatment

Figure 11: Effect of parthenolide and/or TNFα on PGC-1α RNA levels in normoxic/hypoxic neontatal rat cardiomyocytes

A) *White bars represent PGC-1α mRNA activity in cardiomyocytes under normoxic conditions treated with either DMSO (vehicle) or 5 uM parthenolide. Black bars represent PGC-1α mRNA activity in hypoxic cardiomyocytes treated with either DMSO or 5 uM parthenolide. n=3-6. **p<0.05 between normoxic DMSO and hypoxic DMSO ***p<0.05 between normoxic parthenolide and normoxic DMSO using post-hoc Fisher test* **B)** *All experiments were done under normoxic conditions. White bars represent PGC-1α mRNA activity in untreated cardiomyocytes. Gray bars represent PGC-1α mRNA activity in cardiomyocytes treated with either 10ng/μl or 50ng/μl TNFa. Lastly, black bars represent PGC-1α mRNA activity in cardiomyocytes treated with 50ng/μl TNFa with progressively decreasing concentrations of parthenolide (5uM, 1uM or 0.5uM). n=3-6 *P<0.05 compared to untreated **p<0.05 compared to 50 TNFa and 1uM parthenolide.* **C)** *This graph represents the viability of neonatal rat cardiomyocytes treated with 0.5uM, 1uM or 5uM of parthenolide (0.5 parth, 1 parth or 5 parth) and 10ng/μl or 50ng/μl TNFa (10 TNFa or 50 TNFa). Viability assay for untreated neonatal cardiomyocytes was done previously, and is marked in gray.*

5.0 Hypoxia increases levels of p65 and the interaction between p65 and PGC-1α

5.1 Interaction between p65 and PGC-1α promoter at the site of p65 binding site #2 increases under hypoxic conditions

After confirming by EMSA that wildtype PGC-1α promoter probe and nuclear p65 protein interact (Figure 8), we wanted to observe relationship between the p65 protein and the PGC-1α promoter probe under hypoxic conditions. We exposed neonatal rat cardiomyocytes to 12 hours of either normoxia or hypoxia and isolated nuclear protein from both. Comparison between the normoxic shift and 12 hour hypoxic shift band strongly suggests that interaction between the PGC-1α promoter probe and nuclear p65 is increased under hypoxic conditions. A stronger interaction under hypoxic conditions suggests that NF-κB is upregulated during low oxygen conditions in order to inhibit FAO metabolism via PGC-1α repression.

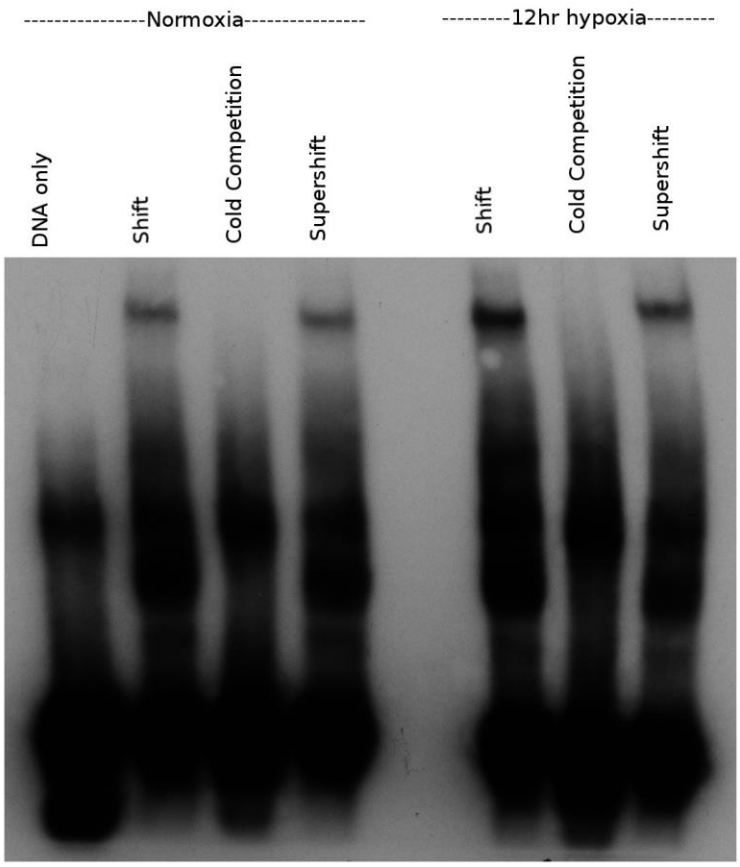


Figure 12: Interaction between wildtype PGC-1α promoter (at p65 site #2) and nuclear protein extract from hypoxic and normoxic neonatal rat cardiomyocytes

This EMSA gel compares the interaction between PGC-1α wildtype probe and nuclear protein extracted from either 12 hour hypoxic or 12 hour normoxic cardiomyocytes. Lane 1 is a control reaction which contains only the biotin labeled wildtype PGC-1α promoter probe. Lane 2,3 and 4 are shift, cold competition and supershift reactions containing the p65 protein obtained from normoxic cardiomyocytes. Lane 5 is an empty space. Lane 6, 7 and 8 are shift, cold competition and supershift reactions completed with p65 protein obtained from 12 hour hypoxic cardiomyocytes.

5.2 p65 protein levels are upregulated in hypoxic nucleic rat neonatal cardiomyocytes

We assessed p65 and PGC-1α protein levels in nucleic and cytoplasmic protein fractions in hypoxic and normoxic conditions to better understand their relationship and other cellular events which may occur. We exposed neonatal rat cardiomyocytes to either

normoxia or hypoxia for 12 hours, isolated cytoplasmic and nuclear protein and ran Western blots probing with either p65 or PGC-1α antibodies. All data was normalized to tubulin. We saw a dramatic increase in p65 in hypoxic nucleic fractions; however PGC-1α protein levels did not exhibit an obvious trend. An increase in p65 nuclear protein during hypoxia suggests that p65 is specifically activated during low oxygen conditions and traveling into the nucleus to impart changes on target genes, for example, PGC-1α.

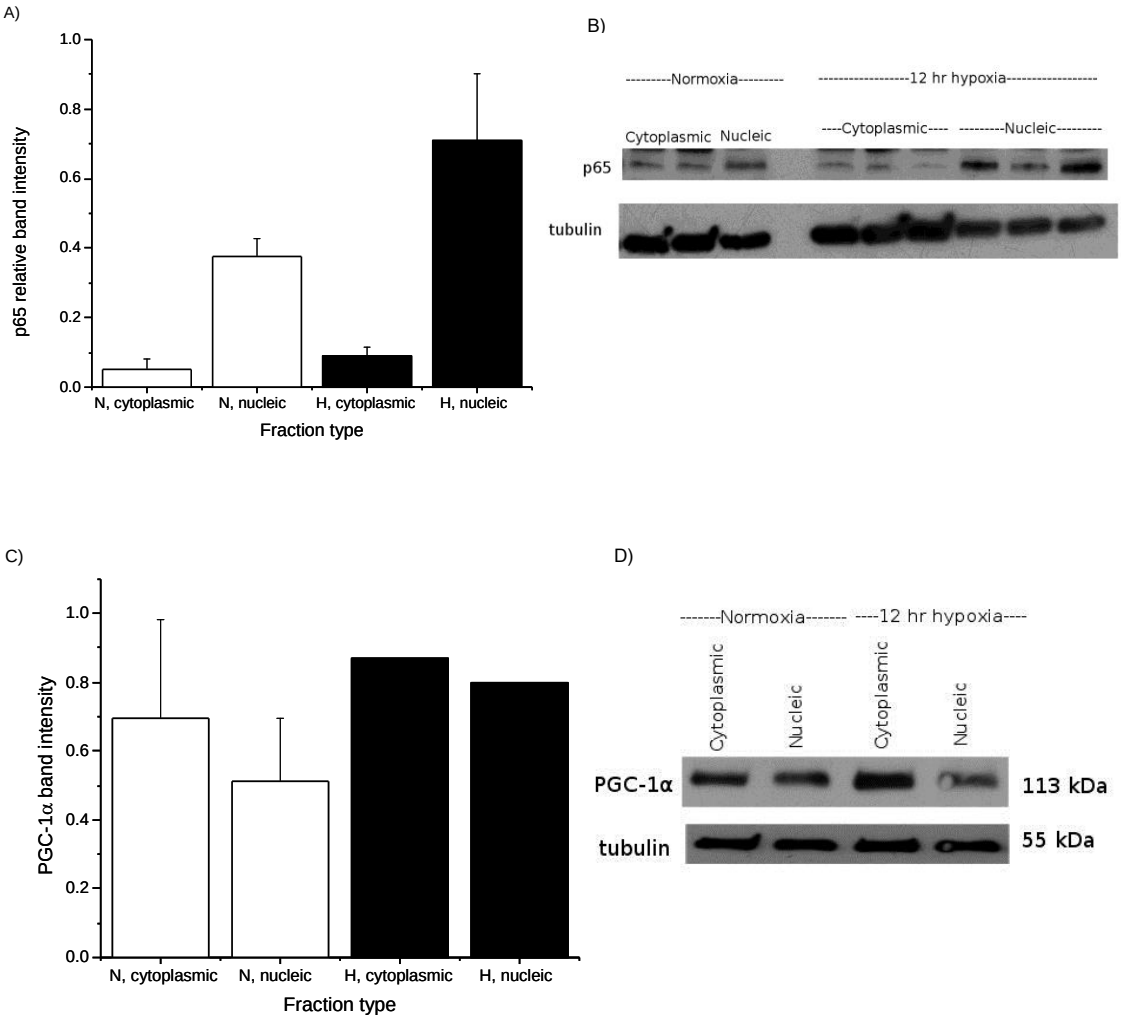


Figure 13: PGC-1α and p65 protein levels in cytoplasmic vs nuclear protein isolated from normoxic and hypoxic neonatal rat cardiomyocytes

A) *Graphical representation of the trend of p65 protein expression (normalized to tubulin) under normoxic and hypoxic conditions. White bars represent normoxic conditions (N) and black bars represent 12 hour hypoxic conditions (H). p65 is highly active in the nucleus, becoming nearly double under hypoxic conditions. n=5* ***B)*** *Western blot probed with p65 and tubulin antibodies. Normoxic samples are broken down into two cytoplasmic fractions and one nucleic fraction. 12 hour hypoxic samples are broken down into three fractions of cytoplasmic and three fractions of nucleic protein.* ***C)*** *Graphical representation of the trend of PGC-1α protein expression (normalized to tubulin) under normoxic and hypoxic conditions. White bars represent normoxic conditions (N) and black bars represent 12 hour hypoxic conditions (H). Normoxic samples have an n=2, while hypoxic samples have an n=1 (not enough to do statistical analysis).* ***D)*** *Western blot probed with both PGC-1α and tubulin antibodies. In both normoxia and hypoxia, PGC-1α protein appears to be more prevalent in the cytoplasmic fraction.*

VIII Discussion

There are numerous studies showing that PGC-1α is repressed by p65 in various cell types [35, 180, 181]. Since it is already known that p65 can directly interact with the PGC-1α promoter, we examined the PGC-1α promoter for possible p65 binding regions and found two potential sites. Through mutagenesis, sequential deletions and EMSA, we can say with relative confidence that the PGC-1α promoter has at least one p65 binding site. Using EMSA and ChIP, we observed an increased interaction between p65 and PGC-1α promoter at the p65 binding site under hypoxic conditions. Endogenous activation of p65 via TNFα increased interaction between p65 binding site on the PGC-1α promoter and p65, while decreasing overall PGC-1α mRNA expression. Inhibition of p65 via parthenolide was found to paradoxically decrease PGC-1α mRNA levels. However, a combination of parthenolide with TNFα was able to rescue PGC-1α mRNA expression.

The PGC-1α promoter has at least one p65 binding site

Transfection experiments in 3T3 cells and neonatal rat cardiomyocytes consistently show that the intact PGC-1α promoter is downregulated by p65 (Figure 5). Using Transfac software, we found that the PGC-1α promoter has two putative p65 binding sites (Figure 4). We attempted to mutate both p65 binding sites, however only the second site was successfully mutated. It was originally expected for site #1 to have more potential considering it is more similar to the murine p65 consensus sequence. It is unknown why putative p65 binding site #1 was resistant to undergoing mutagenesis. However, p65 binding site #2 on the PGC-1α promoter was mutated, used in transfection experiments

and was shown to be unresponsive to p65 in both 3T3 cells and neonatal rat cardiomyocytes under normal oxygen conditions (Figure 5). A minor modification significantly impaired the ability of p65 to bind to the PGC-1α promoter, strongly implicating this area as a real p65 binding site. When the transfection was completed under hypoxic conditions, the results obtained follow a similar trend but exhibited a large standard error. The sample size for hypoxia treated cardiomyocytes was small (n=2) and a larger sample size would likely correct that.

Several truncated versions of the PGC-1α promoter were constructed as a means to replace the failed mutagenesis of p65 binding site #1, as well as detect other regions along the promoter which may aid in p65 binding (Figure 6). As expected, the truncation of the PGC-1α promoter lead to varying degrees of repression by p65 (Figure 7). The SS PGC-1α promoter is the closest to the wildtype version, missing approximately 1 Kb from its 5' end. Unsurprisingly, since the SS PGC-1α promoter has both p65 binding sites intact, it behaved in a similar fashion to the wildtype PGC-1α promoter in the presence of p65. However, this data did not reach significance. The NX PGC-1α promoter lacks p65 binding site #2 and serves as a control for our previous mutagenesis work. Similar to the mutated PGC-1α promoter, NX was less responsive to the repressive effect of p65. Importantly, the NX PGC-1α promoter was still somewhat repressed by p65, while the mutated form of PGC-1α promoter activity was comparable to basal levels across cell types and oxygen conditions. The SX PGC-1α promoter lacks both p65 binding sites and appears the least susceptible to the repressive effects of p65. However, its activity is only slightly higher than that of NX PGC-1α promoter in the presence of p65. This suggests that although p65 binding site #1 could be involved in p65 binding, its role is likely

minor. The SN and SP PGC-1α promoters are both missing p65 site #1 and were created as an alternative to introducing a mutation. The SN PGC-1α promoter is 232 bp longer on its 5' end (see Figure 6), but otherwise SN and SP are identical. For this reason, it was expected for these promoters to behave similarly. SN PGC-1α promoter was repressed by p65, but to our surprise the SP PGC-1α promoter displayed a striking lack of repression by p65. The lack of repression was similar to those seen in SX and NX promoters which are lacking either both sites, or p65 binding site #2. Considering the only difference between them is the 232 bp region on the 5' end, it is possible this region plays a role in the three dimensional interaction of p65 with the PGC-1α promoter. Constructing a promoter that is missing that region could provide further information regarding the importance of this region.

CATATGAGAAAAGAAATAAGGGGTGGGGGCAGGTGAGTAGCTAAGCTGTTTCAGGGATGGCAGCAGCAATT
GTATTTTCTAGCATTTGTTTTCTGGGAGCCTATGAGATCCACGGAAAGAATCATGAGGGGGAACCCAAGAG
TCTAGGGTGTTGTGGCTTGCTTGCTTTACAAGGAGCAAGGCAAACTGCAGTAACAGTTTAGGAGACTGCAT
TCTCTACTGCCAAGGAGACAGCTG

Figure 14: 232 bp region of the PGC-1α promoter potentially important for p65 binding

Purple represents the recognition sequence for Nde I, and green for Pvu II. The 232 bp region in between them is the region missing in the SP PGC-1α promoter. Since the promoter which is missing this region (SP) is repressed by p65, this region may be important for modulating p65 activity.

Based on the results we obtained from our mutagenesis and transfection work in 3T3 cells and neonatal rat cardiomyocytes, we strongly suspect that putative p65 binding site #2 is crucial to p65 binding. To test this hypothesis, we constructed biotin labeled DNA probes at the p65 binding site #2 on the PGC-1α promoter and used nuclear protein extracts and p65 specific antibodies to observe the protein/probe interactions. Our EMSA

data showed that the biotin labeled PGC-1α promoter probe (at p65 binding site #2) repeatedly interacts with nuclear p65 protein. We are able to say with confidence that the nuclear protein we see interacting with the DNA is p65 (and not a non-specific protein) due to the supershift reaction. A supershift reaction contains the biotin labeled probe, nuclear protein extract and p65 antibody. Since we observe a supershift, we know the p65 antibody was able to bind to the protein/DNA probe complex. Supershift reactions typically run higher than the shift reaction due to the increased molecular weight added by the p65 antibody. In the case of Figure 8, we do not observe a typical supershift reaction. Both shifted and supershifted bands are at the same level, but the supershift band is significantly fainter. This is an important distinction. First of all, it suggests that the p65 antibody did bind to a subset of the p65/ PGC-1α probe complexes, explaining why there are fewer complexes seen in the shift band. The supershift may not be visible due suboptimal amounts of p65 antibody. Secondly, and more likely, it is possible the p65 antibody is interfering with the binding between p65 and the PGC-1α probe, thereby inhibiting their interaction and reducing visibility of the shifted band. The p65 antibody we obtained from Millipore binds to the C-terminal of the p65 subunit, possibly in close proximity to where p65 binds to the PGC-1α probe. Therefore it would be worth exploring whether another p65 antibody could interact with the p65 protein through a different location.

TNFα increases interaction between PGC-1α and p65 and decreases PGC-1α mRNA activity

TNFα is a pro-inflammatory cytokine and increases endogenous p65 by inducing the nuclear translocation of NF-κB dimers [165]. Numerous studies report that TNFα treatment results in downregulation of PGC-1α levels in cell types such as adipocytes, H9c2 cells and cardiomyocytes [103, 195, 196]. Based on these studies, we hypothesized that an increase in p65 would reduce PGC-1α mRNA and increase the interaction between p65 and p65 binding site #2 within the PGC-1α promoter probe. We used EMSA to study how TNFα affects the interaction between p65/ PGC-1α promoter. We treated cos7 cells with varying concentration of TNFα and used the resulting protein extract in our EMSA study. We found that 50ng/μl TNFα yielded the greatest increase in p65/PGC-1α promoter probe interaction in the span of four hours (Figure 9). A typical incubation time for cells used in previous EMSA experiments was two days, but a shorter time span was selected with TNFα treatments to prevent cardiomyocytes from undergoing apoptosis. Interestingly, untreated cos7 cells did not produce enough p65 protein to be visible on the EMSA gel (not shown). This highlights the fact that TNFα treatment greatly increased the amount of p65 in cos7 cells available to interact with the PGC-1α promoter probe. To elucidate whether TNFα would reduce PGC-1α mRNA levels, we treated neonatal rat cardiomyocytes with progressively increasing concentration of TNFα (Figure 10). We expected to see an increased concentration of TNFα correlate to a decrease in PGC-1α mRNA levels. This is exactly what we observed: PGC-1α mRNA levels decreased in response to 10ng/μl TNF-α. They decreased further in response to 50ng/μl TNFα, and tapered off at 90ng/μl. This data is in agreement with

other studies involving TNFα, and supports p65's repressive action on PGC-1α expression in neonatal rat cardiomyocytes. PGC-1α protein levels were also found to respond to TNFα in a similar manner (data not shown). However, before drawing any final conclusions, it is important to note that in addition to repressing PGC-1α, TNFα also induces apoptosis [165].

This is supported by our viability assay in Figure 11C, which shows a significant decrease in cell viability at 50ng/μl TNFα. Importantly, since we did not assay apoptosis specifically, we cannot be certain whether the cell death was due to apoptosis or necrosis. Neonatal rat cardiomyocytes experience a downregulation of PGC-1α mRNA (Figure 10 and 11B) and a substantial decrease in cell viability at 50ng/μl TNFα compared to untreated (Figure 11C). However, since our qPCR data was normalized to an internal control (GAPDH), we can be relatively certain that the downregulation of PGC-1α genes did not occur because of the observed cardiomyocyte death in the viability assay.

Parthenolide represses PGC-1α mRNA expression unless TNFα is present

Parthenolide is a sesquiterpene lactone that was first identified as an NF-κB inhibitor [197]. Due to this, we expected to see a positive correlation between increasing concentrations of parthenolide and increased PGC-1α levels. We hypothesized that inhibiting p65 would allow PGC-1α to be expressed due to the lack of p65 available to repress it. However, we did not account for the cytoprotective role of the NF-κB pathway and the resulting cytotoxicity that occurred as a result of p65 inhibition. In our first

parthenolide experiment, we isolated RNA from neonatal rat cardiomyocytes treated with 40 uM of parthenolide for 12 hours, which resulted in massive cell death (data not shown). In our next experiment, we reduced the concentration to 5 uM of of parthenolide. In comparing normoxic vs hypoxic vehicle treated cells, we observed a downregulation of PGC-1α, which is in line with our hypothesis. PGC-1α is downregulated during hypoxia due to limiting oxygen, thereby favoring a switch to glucose metabolism. However, 5 uM parthenolide decreased PGC-1α levels in both normoxic and hypoxic conditions as compared to vehicle (Figure 11A). There are several possible explanations. As already mentioned, NF-κB is known to have numerous cytoprotective roles [6],[172]. It is possible that inhibition of p65 ablated pro-survival cell pathways making it impossible to study expression of just PGC-1α expression. Second of all, there are numerous studies which show that parthenolide may initiate different pathways depending on its dose. Some studies suggest that 5-10 uM of parthenolide inhibits the NF-κB pathway. Others report that up to 5uM of parthenolide can induce anti-apoptotic events, while concentrations of 10-100 uM of parthenolide induce apoptosis. Inhibition of cell proliferation has also been reported at concentrations higher than 10 uM. Others still report that parthenolide can induce oxidative stress at concentrations equal to or greater than 5 uM. In summary, it appears that parthenolide can induce apoptosis and repress cell proliferation independently of its role in NF-κB inhibition [198-200]. Our data somewhat agrees. High concentrations of parthenolide are correlated to cell death, which is what we observed at 40 uM of parthenolide. However, we did not observe a protection from apoptosis at 5 uM of parthenolide, considering the cardiomyocyte viability was only 70%. It is possible that higher concentrations of parthenolide needed to be used to

observe the downstream effects of NF-κB inhibition (ie: PGC-1α upregulation). Lastly, it is interesting to note that parthenolide appears to have less effect on PGC-1α levels during hypoxic conditions. The difference in PGC-1α expression between normoxic cells treated with either DMSO or parthenolide is much larger than the difference between hypoxic cells treated with either DMSO or parthenolide. Since more p65 should be active during hypoxic conditions, it is possible that the 5 uM of parthenolide cannot repress all of the p65 and impart as much cytotoxic damage to the cell. Under hypoxic conditions, there may also be more drive to repress glucose metabolism and therefore more p65 is diverted to repress specifically PGC-1α. For this reason, the presence of parthenolide may result in a slight up-regulation of PGC-1α due to the repression of p65. However, it is still important to point out that despite the above; the end result is still a downregulation of PGC-1α.

Considering that our parthenolide experiments did not end up being overly telling, we hypothesized that a combination of TNFα with parthenolide may provide more revealing data. By providing more p65 (via TNFα) in the beginning, we hoped that we could bypass the seemingly apoptotic effects of parthenolide. Parthenolide could quench the p65 activated by TNFα rather than the p65 delegated for cytoprotective pathways. In the presence of 50ng/μl TNFα, 5 uM and 1 uM of parthenolide continue to have a repressive effect on PGC-1α activity. However, 0.5 uM of parthenolide in the presence of 50ng/μl TNFα increases PGC-1α expression to nearly basal levels. 0.5 uM of parthenolide not only rescued PGC-1α activity to the level of PGC-1α in cardiomyocytes treated with just 50ng/μl TNFα, but surpassed the level of PGC-1α present in cardiomyocytes treated with

only 10ng/μl TNFα (see Figure 11B). Though this is an interesting result, we cannot be sure why PGC-1α expression was upregulated. Considering the wide repertoire of parthenolide, we cannot say with certainty that PGC-1α increased as a result of p65 repression (since some studies report inhibition of NF-κB only in between 5 and 10uM of parthenolide). It is possible that the p65 in neonatal rat cardiomyocytes is sensitive to smaller doses of parthenolide, or perhaps PGC-1α upregulation occurred as a result of a cytoprotective pathway unrelated to NFκB. Since cell viability drastically improved at 0.5uM of parthenolide, an anti-apoptotic program was likely in play.

After observing that 0.5uM of parthenolide with 50ng/μl TNFα offset PGC-1α inhibition, we hoped that 0.5 uM parthenolide by itself may have less repressive effects on PGC-1α mRNA. qPCR using PGC-1α specific primers was completed using RNA isolated from neonatal cardiomyocytes treated with 0.5 uM parthenolide in normoxic conditions resulted in a similar downregulation of PGC-1α expression (results not shown). This further proved that because parthenolide affects cell signaling in numerous ways, its unpredictability makes it challenging to focus on a specific cell signaling pathway.

Hypoxia induces PGC-1α repression via activation of p65

Continuing with the EMSA work, we explored the interaction between p65 and PGC-1α promoter at p65 binding site #2 under hypoxic conditions. Comparison between the normoxic shift and 12 hour hypoxic shift band strongly suggests that interaction between the PGC-1α promoter probe and nuclear p65 is increased under hypoxic conditions (Figure 12). Both normoxic and hypoxic supershifts are fainter than their shift

counterpart, suggesting that the p65 antibody is binding to a subset of the probe/protein complexes. This confirms that the PGC-1α promoter probe is interacting specifically with p65 as opposed to a non-specific nucleic protein. We also exposed neonatal rat cardiomyocytes to either normoxia or hypoxia for 12 hours. Nucleic and cytoplasmic fractions were isolated and western blots were run probing with the p65 or PGC-1α antibody (Figure 13). p65 protein levels were upregulated in both hypoxic and normoxic nucleic fractions, being significantly higher levels under hypoxia. This is in line with previous data. Hypoxia is known to upregulate the NF-κB pathway by allowing the active form of the hetero/homodimer to pass into the nucleus. PGC-1α protein levels on the other hand were not as obvious in their behavior. PGC-1α protein levels appear similar between nuclear and cytoplasmic fractions from normoxic neonatal cardiomyocytes. In hypoxia however, PGC-1α experiences a dramatic downregulation in nucleic protein fractions as compared to cytoplasmic. This is in agreement with our hypothesis that PGC-1α is decreased under hypoxic conditions. However, hypoxia also appears to upregulate cytoplasmic PGC-1α protein levels in comparison to normoxic cytoplasmic and nuclear PGC-1α protein levels. It is not clear why there is such a drastic increase in cytoplasmic PGC-1α, considering PGC-1α's action is usually limited to the nucleus. It is possible that the increase in cytoplasmic PGC-1α protein during hypoxia represents an increase in mitochondria related functions. Why an increase in expression of mitochondrial genes would occur during oxygen deprivation is unclear. See section 4.3 of the literature review for a summary of work regarding hypoxia, ischemia and PGC-1α.

IX Conclusions

1. p65 represses the PGC-1α promoter in normoxic 3T3 cells and neonatal rat cardiomyocytes
2. p65 binding site #2 on the PGC-1α promoter is important for p65 binding
3. Endogenous activation of p65 via TNFα decreases PGC-1α mRNA expression
4. Parthenolide inhibits PGC-1α mRNA in neonatal rat cardiomyocytes unless treated with TNFα
5. Endogenous activation of p65 increases interaction between PGC-1α promoter p65 binding site #2 and p65
6. Hypoxic conditions increase interaction between PGC-1α promoter p65 binding site #2 and p65
7. Preliminary ChIP experiments show an increased interaction between p65 and PGC-1α promoter under hypoxic conditions

X Future work

Future work should include ChIP and IkB virus work to further elucidate the interaction between p65 and PGC-1α promoter. Trichostatin A (TSA), an HDAC inhibitor should be utilized in ChIP experiments to elucidate whether HDAC inhibition causes a decrease in the interaction between PGC-1α and p65. Furthermore, a 6^{th} truncated PGC-1α promoter should be constructed to explore whether the 232 bp region between Nde I and Pvu II on the PGC-1α promoter is important for p65 binding.

XI Methodical Limitations of this study

We completed a series of ChIP experiments exploring the interaction between p65 and PGC-1α promoter in hypoxic and normoxic conditions. Unfortunately, there was a number of technical difficulties which resulted in inconsistent results. However, our preliminary data did show that the interaction between PGC-1α and p65 increases under hypoxic conditions. Since ChIP explores the *in vivo* relationship between p65 and PGC-1α promoter (in comparison to EMSA, which uses an artificial PGC-1α probe), it is important to perfect this experiment in future studies. The use of parthenolide as a means to inhibit PGC-1α proved to be challenging considering the wide range of effects parthenolide can impart on a cell system. An IκB virus (IκB keeps p65 repressed) whose repressive effect would be limited to p65 may be a better option. Several experiments discussed in the discussion section would also benefit from a higher sample size. Not discussed in this thesis, we also attempted to build two other sets of PGC-1α promoter probes which did not work as expected. The first was a set of probes (forward and reverse) encoded the PGC-1α promoter mutated at p65 binding site #2. The second set encoded a PGC-1α promoter with p65 binding site #2 missing. Both of these EMSA experiments yielded shift bands in the presence of p65 nuclear protein, suggesting that the region we consider the p65 binding site #2 (5' GGGACTGT 3') may extend farther on either end.

XII References

1. Lehman JJ, Kelly DP: **Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart**. *Clinical and experimental pharmacology & physiology* 2002, **29**(4):339-345.
2. Shoag J, Arany Z: **Regulation of hypoxia-inducible genes by PGC-1 alpha**. *Arteriosclerosis, thrombosis, and vascular biology* 2010, **30**(4):662-666.
3. Eisele PS, Salatino S, Sobek J, Hottiger MO, Handschin C: **The peroxisome proliferator-activated receptor gamma coactivator 1alpha/beta (PGC-1) coactivators repress the transcriptional activity of NF-kappaB in skeletal muscle cells**. *The Journal of biological chemistry* 2013, **288**(4):2246-2260.
4. Ramjiawan A, Bagchi RA, Blant A, Albak L, Cavasin MA, Horn TR, McKinsey TA, Czubryt MP: **Roles of histone deacetylation and AMP kinase in regulation of cardiomyocyte PGC-1alpha gene expression in hypoxia**. *American journal of physiology Cell physiology* 2013, **304**(11):C1064-1072.
5. Finck BN, Kelly DP: **Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease**. *Circulation* 2007, **115**(19):2540-2548.
6. Kramer HF, Goodyear LJ: **Exercise, MAPK, and NF-kappaB signaling in skeletal muscle**. *Journal of applied physiology* 2007, **103**(1):388-395.
7. Irrcher I, Ljubicic V, Kirwan AF, Hood DA: **AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells**. *PloS one* 2008, **3**(10):e3614.
8. Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY *et al*: **Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes**. *Nature medicine* 2001, **7**(4):437-443.
9. Krivoruchko A, Storey KB: **Epigenetics in anoxia tolerance: a role for histone deacetylases**. *Molecular and cellular biochemistry* 2010, **342**(1-2):151-161.
10. Arsenault D, Brochu-Gaudreau K, Charbonneau M, Dubois CM: **HDAC6 deacetylase activity is required for hypoxia-induced invadopodia formation and cell invasion**. *PloS one* 2013, **8**(2):e55529.
11. Shi H, Chen L, Wang H, Zhu S, Dong C, Webster KA, Wei J: **Synergistic induction of miR-126 by hypoxia and HDAC inhibitors in cardiac myocytes**. *Biochemical and biophysical research communications* 2013, **430**(2):827-832.
12. Zhao L, Chen CN, Hajji N, Oliver E, Cotroneo E, Wharton J, Wang D, Li M, McKinsey TA, Stenmark KR *et al*: **Histone deacetylation inhibition in pulmonary hypertension: therapeutic potential of valproic acid and suberoylanilide hydroxamic acid**. *Circulation* 2012, **126**(4):455-467.
13. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN: **Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy**. *Cell* 2002, **110**(4):479-488.
14. Cavasin MA, Demos-Davies K, Horn TR, Walker LA, Lemon DD, Birdsey N, Weiser-Evans MC, Harral J, Irwin DC, Anwar A *et al*: **Selective class I histone deacetylase inhibition suppresses hypoxia-induced cardiopulmonary remodeling through an antiproliferative mechanism**. *Circulation research* 2012, **110**(5):739-748.

15. Czubryt MP, McAnally J, Fishman GI, Olson EN: **Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(4):1711-1716.
16. Knoll AH: **End of the Proterozoic eon**. *Scientific American* 1991, **265**(4):64-73.
17. Bishopric NH: **Evolution of the heart from bacteria to man**. *Annals of the New York Academy of Sciences* 2005, **1047**:13-29.
18. Deshmukh AB, Patel JK, Prajapati AR, Shah S: **Perspective in chronic kidney disease: targeting hypoxia-inducible factor (HIF) as potential therapeutic approach**. *Renal failure* 2012, **34**(4):521-532.
19. Edwards L, Wanless IR: **Mechanisms of liver involvement in systemic disease**. *Best practice & research Clinical gastroenterology* 2013, **27**(4):471-483.
20. Bauer TA, Brass EP, Hiatt WR: **Impaired muscle oxygen use at onset of exercise in peripheral arterial disease**. *Journal of vascular surgery* 2004, **40**(3):488-493.
21. Muers MF, Round CE: **Palliation of symptoms in non-small cell lung cancer: a study by the Yorkshire Regional Cancer Organisation Thoracic Group**. *Thorax* 1993, **48**(4):339-343.
22. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC: **Myocardial fatty acid metabolism in health and disease**. *Physiological reviews* 2010, **90**(1):207-258.
23. Javaheri S, Javaheri S, Javaheri A: **Sleep Apnea, Heart Failure, and Pulmonary Hypertension**. *Current heart failure reports* 2013.
24. Zhao G, Jeoung NH, Burgess SC, Rosaaen-Stowe KA, Inagaki T, Latif S, Shelton JM, McAnally J, Bassel-Duby R, Harris RA *et al*: **Overexpression of pyruvate dehydrogenase kinase 4 in heart perturbs metabolism and exacerbates calcineurin-induced cardiomyopathy**. *American journal of physiology Heart and circulatory physiology* 2008, **294**(2):H936-943.
25. Shah AM: **Ventricular Remodeling in Heart Failure with Preserved Ejection Fraction**. *Current heart failure reports* 2013.
26. Hunt SA, American College of C, American Heart Association Task Force on Practice G: **ACC/AHA 2005 guideline update for the diagnosis and management of chronic heart failure in the adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure)**. *Journal of the American College of Cardiology* 2005, **46**(6):e1-82.
27. Sindone A, Erlich J, Perkovic V, Suranyi M, Newman H, Lee C, Barin E, Roger SD: **ACEIs for cardiovascular risk reduction - Have we taken our eye off the ball?** *Australian family physician* 2013, **42**(9):634-638.
28. Strauss MH, Hall AS: **Angiotensin receptor blockers may increase risk of myocardial infarction: unraveling the ARB-MI paradox**. *Circulation* 2006, **114**(8):838-854.

29. Cheng JW, Rybak I: **Use of digoxin for heart failure and atrial fibrillation in elderly patients**. *The American journal of geriatric pharmacotherapy* 2010, **8**(5):419-427.
30. Maron BA, Leopold JA: **Aldosterone receptor antagonists: effective but often forgotten**. *Circulation* 2010, **121**(7):934-939.
31. Sharpe N: **Beta-blockers in heart failure. Future directions**. *European heart journal* 1996, **17 Suppl B**:39-42.
32. Yusuf S, Peto R, Lewis J, Collins R, Sleight P: **Beta blockade during and after myocardial infarction: an overview of the randomized trials**. *Progress in cardiovascular diseases* 1985, **27**(5):335-371.
33. Hoppe UC, Erdmann E: **Digitalis in heart failure! Still applicable?** *Zeitschrift fur Kardiologie* 2005, **94**(5):307-311.
34. Escher P, Braissant O, Basu-Modak S, Michalik L, Wahli W, Desvergne B: **Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding**. *Endocrinology* 2001, **142**(10):4195-4202.
35. Palomer X, Alvarez-Guardia D, Rodriguez-Calvo R, Coll T, Laguna JC, Davidson MM, Chan TO, Feldman AM, Vazquez-Carrera M: **TNF-alpha reduces PGC-1alpha expression through NF-kappaB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model**. *Cardiovascular research* 2009, **81**(4):703-712.
36. van Bilsen M, Smeets PJ, Gilde AJ, van der Vusse GJ: **Metabolic remodelling of the failing heart: the cardiac burn-out syndrome?** *Cardiovascular research* 2004, **61**(2):218-226.
37. Barger PM, Brandt JM, Leone TC, Weinheimer CJ, Kelly DP: **Deactivation of peroxisome proliferator-activated receptor-alpha during cardiac hypertrophic growth**. *The Journal of clinical investigation* 2000, **105**(12):1723-1730.
38. Kanda H, Nohara R, Hasegawa K, Kishimoto C, Sasayama S: **A nuclear complex containing PPARalpha/RXRalpha is markedly downregulated in the hypertrophied rat left ventricular myocardium with normal systolic function**. *Heart and vessels* 2000, **15**(4):191-196.
39. Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, Hintze TH, Lopaschuk GD, Recchia FA: **Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure**. *Circulation* 2002, **106**(5):606-612.
40. Aubert G, Vega RB, Kelly DP: **Perturbations in the gene regulatory pathways controlling mitochondrial energy production in the failing heart**. *Biochimica et biophysica acta* 2013, **1833**(4):840-847.
41. Campbell FM, Kozak R, Wagner A, Altarejos JY, Dyck JR, Belke DD, Severson DL, Kelly DP, Lopaschuk GD: **A role for peroxisome proliferator-activated receptor alpha (PPARalpha) in the control of cardiac malonyl-CoA levels: reduced fatty acid oxidation rates and increased glucose oxidation rates in the hearts of mice lacking PPARalpha are associated with higher concentrations of malonyl-CoA and reduced expression of malonyl-CoA decarboxylase**. *The Journal of biological chemistry* 2002, **277**(6):4098-4103.

42. Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD *et al*: **The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus**. *The Journal of clinical investigation* 2002, **109**(1):121-130.
43. Jamshidi Y, Montgomery HE, Hense HW, Myerson SG, Torra IP, Staels B, World MJ, Doering A, Erdmann J, Hengstenberg C *et al*: **Peroxisome proliferator--activated receptor alpha gene regulates left ventricular growth in response to exercise and hypertension**. *Circulation* 2002, **105**(8):950-955.
44. Burkart EM, Sambandam N, Han X, Gross RW, Courtois M, Gierasch CM, Shoghi K, Welch MJ, Kelly DP: **Nuclear receptors PPARbeta/delta and PPARalpha direct distinct metabolic regulatory programs in the mouse heart**. *The Journal of clinical investigation* 2007, **117**(12):3930-3939.
45. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM: **Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity**. *Cell* 2003, **113**(2):159-170.
46. Wang P, Liu J, Li Y, Wu S, Luo J, Yang H, Subbiah R, Chatham J, Zhelyabovska O, Yang Q: **Peroxisome proliferator-activated receptor {delta} is an essential transcriptional regulator for mitochondrial protection and biogenesis in adult heart**. *Circulation research* 2010, **106**(5):911-919.
47. Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y *et al*: **Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy**. *Nature medicine* 2004, **10**(11):1245-1250.
48. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM: **A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis**. *Cell* 1998, **92**(6):829-839.
49. Madrazo JA, Kelly DP: **The PPAR trio: regulators of myocardial energy metabolism in health and disease**. *Journal of molecular and cellular cardiology* 2008, **44**(6):968-975.
50. Son NH, Park TS, Yamashita H, Yokoyama M, Huggins LA, Okajima K, Homma S, Szabolcs MJ, Huang LS, Goldberg IJ: **Cardiomyocyte expression of PPARgamma leads to cardiac dysfunction in mice**. *The Journal of clinical investigation* 2007, **117**(10):2791-2801.
51. Yin KJ, Deng Z, Hamblin M, Zhang J, Chen YE: **Vascular PPARdelta protects against stroke-induced brain injury**. *Arteriosclerosis, thrombosis, and vascular biology* 2011, **31**(3):574-581.
52. Honda T, Kaikita K, Tsujita K, Hayasaki T, Matsukawa M, Fuchigami S, Sugiyama S, Sakashita N, Ogawa H, Takeya M: **Pioglitazone, a peroxisome proliferator-activated receptor-gamma agonist, attenuates myocardial ischemia-reperfusion injury in mice with metabolic disorders**. *Journal of molecular and cellular cardiology* 2008, **44**(5):915-926.
53. Shiomi T, Tsutsui H, Hayashidani S, Suematsu N, Ikeuchi M, Wen J, Ishibashi M, Kubota T, Egashira K, Takeshita A: **Pioglitazone, a peroxisome proliferator-activated receptor-gamma agonist, attenuates left ventricular remodeling and failure after experimental myocardial infarction**. *Circulation* 2002, **106**(24):3126-3132.

54. Yue Tl TL, Chen J, Bao W, Narayanan PK, Bril A, Jiang W, Lysko PG, Gu JL, Boyce R, Zimmerman DM *et al*: **In vivo myocardial protection from ischemia/reperfusion injury by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone**. *Circulation* 2001, **104**(21):2588-2594.
55. Young ME, Laws FA, Goodwin GW, Taegtmeyer H: **Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart**. *The Journal of biological chemistry* 2001, **276**(48):44390-44395.
56. Puigserver P, Spiegelman BM: **Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator**. *Endocrine reviews* 2003, **24**(1):78-90.
57. Ventura-Clapier R, Garnier A, Veksler V: **Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha**. *Cardiovascular research* 2008, **79**(2):208-217.
58. Liang H, Ward WF: **PGC-1alpha: a key regulator of energy metabolism**. *Advances in physiology education* 2006, **30**(4):145-151.
59. Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM: **Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1**. *Molecular cell* 2001, **8**(5):971-982.
60. Jager S, Handschin C, St-Pierre J, Spiegelman BM: **AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(29):12017-12022.
61. Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P: **Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha**. *The EMBO journal* 2007, **26**(7):1913-1923.
62. Teyssier C, Ma H, Emter R, Kralli A, Stallcup MR: **Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation**. *Genes & development* 2005, **19**(12):1466-1473.
63. Rytinki MM, Palvimo JJ: **SUMOylation attenuates the function of PGC-1alpha**. *The Journal of biological chemistry* 2009, **284**(38):26184-26193.
64. Monsalve M, Wu Z, Adelmant G, Puigserver P, Fan M, Spiegelman BM: **Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1**. *Molecular cell* 2000, **6**(2):307-316.
65. Lin J, Handschin C, Spiegelman BM: **Metabolic control through the PGC-1 family of transcription coactivators**. *Cell metabolism* 2005, **1**(6):361-370.
66. Handschin C, Spiegelman BM: **Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism**. *Endocrine reviews* 2006, **27**(7):728-735.
67. Knutti D, Kralli A: **PGC-1, a versatile coactivator**. *Trends in endocrinology and metabolism: TEM* 2001, **12**(8):360-365.
68. Russell LK, Mansfield CM, Lehman JJ, Kovacs A, Courtois M, Saffitz JE, Medeiros DM, Valencik ML, McDonald JA, Kelly DP: **Cardiac-specific induction of the transcriptional coactivator peroxisome proliferator-activated**

receptor gamma coactivator-1alpha promotes mitochondrial biogenesis and reversible cardiomyopathy in a developmental stage-dependent manner. *Circulation research* 2004, **94**(4):525-533.

69. Arany Z, He H, Lin J, Hoyer K, Handschin C, Toka O, Ahmad F, Matsui T, Chin S, Wu PH *et al*: **Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle**. *Cell metabolism* 2005, **1**(4):259-271.
70. Puigserver P, Adelmant G, Wu Z, Fan M, Xu J, O'Malley B, Spiegelman BM: **Activation of PPARgamma coactivator-1 through transcription factor docking**. *Science* 1999, **286**(5443):1368-1371.
71. Hollinger K, Gardan-Salmon D, Santana C, Rice D, Snella E, Selsby JT: **Rescue of dystrophic skeletal muscle by PGC-1alpha involves restored expression of dystrophin-associated protein complex components and satellite cell signaling**. *American journal of physiology Regulatory, integrative and comparative physiology* 2013, **305**(1):R13-23.
72. Handschin C, Kobayashi YM, Chin S, Seale P, Campbell KP, Spiegelman BM: **PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy**. *Genes & development* 2007, **21**(7):770-783.
73. Mata M, Sarrion I, Milian L, Juan G, Ramon M, Naufal D, Gil J, Ridocci F, Fabregat-Andres O, Cortijo J: **PGC-1alpha induction in pulmonary arterial hypertension**. *Oxidative medicine and cellular longevity* 2012, **2012**:236572.
74. Kuznetsov AV, Winkler K, Wiedemann FR, von Bossanyi P, Dietzmann K, Kunz WS: **Impaired mitochondrial oxidative phosphorylation in skeletal muscle of the dystrophin-deficient mdx mouse**. *Molecular and cellular biochemistry* 1998, **183**(1-2):87-96.
75. Swoboda W, Turnheim E, Frisch H, Spona J: **[A new antigonadotropin in the treatment of precocious puberty and pubertal gynaecomastia (author's transl)]**. *Padiatrie und Padologie Supplementum* 1977(5):109-120.
76. Arany Z: **PGC-1 coactivators and skeletal muscle adaptations in health and disease**. *Current opinion in genetics & development* 2008, **18**(5):426-434.
77. Angus LM, Chakkalakal JV, Mejat A, Eibl JK, Belanger G, Megeney LA, Chin ER, Schaeffer L, Michel RN, Jasmin BJ: **Calcineurin-NFAT signaling, together with GABP and peroxisome PGC-1{alpha}, drives utrophin gene expression at the neuromuscular junction**. *American journal of physiology Cell physiology* 2005, **289**(4):C908-917.
78. Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP: **Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis**. *The Journal of clinical investigation* 2000, **106**(7):847-856.
79. Remels AH, Schrauwen P, Broekhuizen R, Willems J, Kersten S, Gosker HR, Schols AM: **Peroxisome proliferator-activated receptor expression is reduced in skeletal muscle in COPD**. *The European respiratory journal* 2007, **30**(2):245-252.
80. Rimbaud S, Garnier A, Ventura-Clapier R: **Mitochondrial biogenesis in cardiac pathophysiology**. *Pharmacological reports : PR* 2009, **61**(1):131-138.

81. Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C *et al*: **PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis**. *PLoS biology* 2005, **3**(4):e101.
82. Arany Z, Foo SY, Ma Y, Ruas JL, Bommi-Reddy A, Girnun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM *et al*: **HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha**. *Nature* 2008, **451**(7181):1008-1012.
83. Deblois G, St-Pierre J, Giguere V: **The PGC-1/ERR signaling axis in cancer**. *Oncogene* 2013, **32**(30):3483-3490.
84. Jaswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD: **Targeting fatty acid and carbohydrate oxidation--a novel therapeutic intervention in the ischemic and failing heart**. *Biochimica et biophysica acta* 2011, **1813**(7):1333-1350.
85. Kolwicz SC, Jr., Tian R: **Glucose metabolism and cardiac hypertrophy**. *Cardiovascular research* 2011, **90**(2):194-201.
86. Hinkle PC: **P/O ratios of mitochondrial oxidative phosphorylation**. *Biochimica et biophysica acta* 2005, **1706**(1-2):1-11.
87. Schlattner U, Tokarska-Schlattner M, Wallimann T: **Mitochondrial creatine kinase in human health and disease**. *Biochimica et biophysica acta* 2006, **1762**(2):164-180.
88. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: **Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis**. *The Biochemical journal* 1992, **281 (Pt 1)**:21-40.
89. ten Hove M, Lygate CA, Fischer A, Schneider JE, Sang AE, Hulbert K, Sebag-Montefiore L, Watkins H, Clarke K, Isbrandt D *et al*: **Reduced inotropic reserve and increased susceptibility to cardiac ischemia/reperfusion injury in phosphocreatine-deficient guanidinoacetate-N-methyltransferase-knockout mice**. *Circulation* 2005, **111**(19):2477-2485.
90. Wolfe RR: **Metabolic interactions between glucose and fatty acids in humans**. *The American journal of clinical nutrition* 1998, **67**(3 Suppl):519S-526S.
91. Xu S, Jay A, Brunaldi K, Huang N, Hamilton JA: **CD36 Enhances Fatty Acid Uptake by Increasing the Rate of Intracellular Esterification but Not Transport across the Plasma Membrane**. *Biochemistry* 2013, **52**(41):7254-7261.
92. Houten SM, Chegary M, Te Brinke H, Wijnen WJ, Glatz JF, Luiken JJ, Wijburg FA, Wanders RJ: **Pyruvate dehydrogenase kinase 4 expression is synergistically induced by AMP-activated protein kinase and fatty acids**. *Cellular and molecular life sciences : CMLS* 2009, **66**(7):1283-1294.
93. Forman BM, Chen J, Evans RM: **Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(9):4312-4317.

94. Zhang L, Mori J, Wagg C, Lopaschuk GD: **Activating cardiac E2F1 induces up-regulation of pyruvate dehydrogenase kinase 4 in mice on a short term of high fat feeding**. *FEBS letters* 2012, **586**(7):996-1003.
95. Stanley WC, Morgan EE, Huang H, McElfresh TA, Sterk JP, Okere IC, Chandler MP, Cheng J, Dyck JR, Lopaschuk GD: **Malonyl-CoA decarboxylase inhibition suppresses fatty acid oxidation and reduces lactate production during demand-induced ischemia**. *American journal of physiology Heart and circulatory physiology* 2005, **289**(6):H2304-2309.
96. Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q, Haase VH: **Hypoxia-inducible factor 2 regulates hepatic lipid metabolism**. *Molecular and cellular biology* 2009, **29**(16):4527-4538.
97. Liu TF, Vachharajani VT, Yoza BK, McCall CE: **NAD+-dependent sirtuin 1 and 6 proteins coordinate a switch from glucose to fatty acid oxidation during the acute inflammatory response**. *The Journal of biological chemistry* 2012, **287**(31):25758-25769.
98. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR *et al*: **SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation**. *Nature* 2010, **464**(7285):121-125.
99. Kahn BB, Alquier T, Carling D, Hardie DG: **AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism**. *Cell metabolism* 2005, **1**(1):15-25.
100. Luiken JJ, Coort SL, Willems J, Coumans WA, Bonen A, van der Vusse GJ, Glatz JF: **Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling**. *Diabetes* 2003, **52**(7):1627-1634.
101. Peters EJ, Klein S, Wolfe RR: **Effect of short-term fasting on the lipolytic response to theophylline**. *The American journal of physiology* 1991, **261**(4 Pt 1):E500-504.
102. Pradhan G, Samson SL, Sun Y: **Ghrelin: much more than a hunger hormone**. *Current opinion in clinical nutrition and metabolic care* 2013, **16**(6):619-624.
103. Gao F, Ni Y, Luo Z, Liang Y, Yan Z, Xu X, Liu D, Wang J, Zhu S, Zhu Z: **Atorvastatin attenuates TNF-alpha-induced increase of glucose oxidation through PGC-1alpha upregulation in cardiomyocytes**. *Journal of cardiovascular pharmacology* 2012, **59**(6):500-506.
104. O'Hagan KA, Cocchiglia S, Zhdanov AV, Tambuwala MM, Cummins EP, Monfared M, Agbor TA, Garvey JF, Papkovsky DB, Taylor CT *et al*: **PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(7):2188-2193.
105. Song S, Zhang Y, Ma K, Jackson-Hayes L, Lavrentyev EN, Cook GA, Elam MB, Park EA: **Peroxisomal proliferator activated receptor gamma coactivator (PGC-1alpha) stimulates carnitine palmitoyltransferase I (CPT-Ialpha) through the first intron**. *Biochimica et biophysica acta* 2004, **1679**(2):164-173.

106. Lim JH, Gerhart-Hines Z, Dominy JE, Lee Y, Kim S, Tabata M, Xiang YK, Puigserver P: **Oleic acid stimulates complete oxidation of fatty acids through protein kinase A-dependent activation of SIRT1-PGC1alpha complex**. *The Journal of biological chemistry* 2013, **288**(10):7117-7126.
107. Vigano A, Vasso M, Caretti A, Bravata V, Terraneo L, Fania C, Capitanio D, Samaja M, Gelfi C: **Protein modulation in mouse heart under acute and chronic hypoxia**. *Proteomics* 2011, **11**(21):4202-4217.
108. Giordano FJ: **Oxygen, oxidative stress, hypoxia, and heart failure**. *The Journal of clinical investigation* 2005, **115**(3):500-508.
109. Carmeliet P: **Angiogenesis in health and disease**. *Nature medicine* 2003, **9**(6):653-660.
110. Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC *et al*: **Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats**. *Nature genetics* 1999, **21**(1):76-83.
111. Aasum E, Hafstad AD, Severson DL, Larsen TS: **Age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from db/db mice**. *Diabetes* 2003, **52**(2):434-441.
112. Neely JR, Morgan HE: **Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle**. *Annual review of physiology* 1974, **36**:413-459.
113. Folmes CD, Sowah D, Clanachan AS, Lopaschuk GD: **High rates of residual fatty acid oxidation during mild ischemia decrease cardiac work and efficiency**. *Journal of molecular and cellular cardiology* 2009, **47**(1):142-148.
114. Korvald C, Elvenes OP, Myrmel T: **Myocardial substrate metabolism influences left ventricular energetics in vivo**. *American journal of physiology Heart and circulatory physiology* 2000, **278**(4):H1345-1351.
115. Kurien VA, Yates PA, Oliver MF: **The role of free fatty acids in the production of ventricular arrhythmias after acute coronary artery occlusion**. *European journal of clinical investigation* 1971, **1**(4):225-241.
116. Borst P, Loos JA, Christ EJ, Slater EC: **Uncoupling activity of long-chain fatty acids**. *Biochimica et biophysica acta* 1962, **62**:509-518.
117. Huss JM, Levy FH, Kelly DP: **Hypoxia inhibits the peroxisome proliferator-activated receptor alpha/retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O2-dependent modulation of mitochondrial fatty acid oxidation**. *The Journal of biological chemistry* 2001, **276**(29):27605-27612.
118. Vega RB, Huss JM, Kelly DP: **The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes**. *Molecular and cellular biology* 2000, **20**(5):1868-1876.
119. Chaung WW, Jacob A, Ji Y, Wang P: **Suppression of PGC-1alpha by Ethanol: Implications of Its Role in Alcohol Induced Liver Injury**. *International journal of clinical and experimental medicine* 2008, **1**(2):161-170.
120. Martinez-Bello VE, Sanchis-Gomar F, Nascimento AL, Pallardo FV, Ibanez-Sania S, Olaso-Gonzalez G, Calbet JA, Gomez-Cabrera MC, Vina J: **Living at high**

altitude in combination with sea-level sprint training increases hematological parameters but does not improve performance in rats. *European journal of applied physiology* 2011, **111**(6):1147-1156.

121. Zhu L, Wang Q, Zhang L, Fang Z, Zhao F, Lv Z, Gu Z, Zhang J, Wang J, Zen K *et al*: **Hypoxia induces PGC-1alpha expression and mitochondrial biogenesis in the myocardium of TOF patients**. *Cell research* 2010, **20**(6):676-687.
122. Gutsaeva DR, Carraway MS, Suliman HB, Demchenko IT, Shitara H, Yonekawa H, Piantadosi CA: **Transient hypoxia stimulates mitochondrial biogenesis in brain subcortex by a neuronal nitric oxide synthase-dependent mechanism**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008, **28**(9):2015-2024.
123. Storey KB: **Mammalian hibernation. Transcriptional and translational controls**. *Advances in experimental medicine and biology* 2003, **543**:21-38.
124. Fabregat-Andres O, Tierrez A, Mata M, Estornell-Erill J, Ridocci-Soriano F, Monsalve M: **Induction of PGC-1alpha expression can be detected in blood samples of patients with ST-segment elevation acute myocardial infarction**. *PloS one* 2011, **6**(11):e26913.
125. Yin W, Signore AP, Iwai M, Cao G, Gao Y, Chen J: **Rapidly increased neuronal mitochondrial biogenesis after hypoxic-ischemic brain injury**. *Stroke; a journal of cerebral circulation* 2008, **39**(11):3057-3063.
126. Lu D, Zhang L, Wang H, Zhang Y, Liu J, Xu J, Liang Z, Deng W, Jiang Y, Wu Q *et al*: **Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) enhances engraftment and angiogenesis of mesenchymal stem cells in diabetic hindlimb ischemia**. *Diabetes* 2012, **61**(5):1153-1159.
127. Hsueh WA, Gupte AA: **PGC-1alpha: the missing ingredient for mesenchymal stem cell-mediated angiogenesis**. *Diabetes* 2012, **61**(5):979-980.
128. Saint-Geniez M, Jiang A, Abend S, Liu L, Sweigard H, Connor KM, Arany Z: **PGC-1alpha regulates normal and pathological angiogenesis in the retina**. *The American journal of pathology* 2013, **182**(1):255-265.
129. Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S, Arany Z: **The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(50):21401-21406.
130. Patten IS, Rana S, Shahul S, Rowe GC, Jang C, Liu L, Hacker MR, Rhee JS, Mitchell J, Mahmood F *et al*: **Cardiac angiogenic imbalance leads to peripartum cardiomyopathy**. *Nature* 2012, **485**(7398):333-338.
131. Rowe GC, Jang C, Patten IS, Arany Z: **PGC-1beta regulates angiogenesis in skeletal muscle**. *American journal of physiology Endocrinology and metabolism* 2011, **301**(1):E155-163.
132. Lynn EG, Stevens MV, Wong RP, Carabenciov D, Jacobson J, Murphy E, Sack MN: **Transient upregulation of PGC-1alpha diminishes cardiac ischemia tolerance via upregulation of ANT1**. *Journal of molecular and cellular cardiology* 2010, **49**(4):693-698.
133. Hsieh YC, Yang S, Choudhry MA, Yu HP, Rue LW, 3rd, Bland KI, Chaudry IH: **PGC-1 upregulation via estrogen receptors: a common mechanism of salutary effects of estrogen and flutamide on heart function after trauma-**

hemorrhage. *American journal of physiology Heart and circulatory physiology* 2005, **289**(6):H2665-2672.

134. Taegtmeyer H, Overturf ML: **Effects of moderate hypertension on cardiac function and metabolism in the rabbit**. *Hypertension* 1988, **11**(5):416-426.
135. Massie BM, Schaefer S, Garcia J, McKirnan MD, Schwartz GG, Wisneski JA, Weiner MW, White FC: **Myocardial high-energy phosphate and substrate metabolism in swine with moderate left ventricular hypertrophy**. *Circulation* 1995, **91**(6):1814-1823.
136. Feldman AM, Weinberg EO, Ray PE, Lorell BH: **Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding**. *Circulation research* 1993, **73**(1):184-192.
137. Christe ME, Rodgers RL: **Altered glucose and fatty acid oxidation in hearts of the spontaneously hypertensive rat**. *Journal of molecular and cellular cardiology* 1994, **26**(10):1371-1375.
138. Takeyama D, Kagaya Y, Yamane Y, Shiba N, Chida M, Takahashi T, Ido T, Ishide N, Takishima T: **Effects of chronic right ventricular pressure overload on myocardial glucose and free fatty acid metabolism in the conscious rat**. *Cardiovascular research* 1995, **29**(6):763-767.
139. Wittels B, Spann JF, Jr.: **Defective lipid metabolism in the failing heart**. *The Journal of clinical investigation* 1968, **47**(8):1787-1794.
140. Bishop SP, Altschuld RA: **Increased glycolytic metabolism in cardiac hypertrophy and congestive failure**. *The American journal of physiology* 1970, **218**(1):153-159.
141. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: **Fatty acid oxidation enzyme gene expression is downregulated in the failing heart**. *Circulation* 1996, **94**(11):2837-2842.
142. Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, Gropler RJ: **Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy**. *Journal of the American College of Cardiology* 2002, **40**(2):271-277.
143. de las Fuentes L, Herrero P, Peterson LR, Kelly DP, Gropler RJ, Davila-Roman VG: **Myocardial fatty acid metabolism: independent predictor of left ventricular mass in hypertensive heart disease**. *Hypertension* 2003, **41**(1):83-87.
144. Lopaschuk GD, Collins-Nakai RL, Itoi T: **Developmental changes in energy substrate use by the heart**. *Cardiovascular research* 1992, **26**(12):1172-1180.
145. Kantor PF, Robertson MA, Coe JY, Lopaschuk GD: **Volume overload hypertrophy of the newborn heart slows the maturation of enzymes involved in the regulation of fatty acid metabolism**. *Journal of the American College of Cardiology* 1999, **33**(6):1724-1734.
146. Sun CK, Chang LT, Sheu JJ, Wang CY, Youssef AA, Wu CJ, Chua S, Yip HK: **Losartan preserves integrity of cardiac gap junctions and PGC-1 alpha gene expression and prevents cellular apoptosis in remote area of left ventricular myocardium following acute myocardial infarction**. *International heart journal* 2007, **48**(4):533-546.

147. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R: **Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles**. *The Journal of physiology* 2003, **551**(Pt 2):491-501.
148. Doenst T, Pytel G, Schrepper A, Amorim P, Farber G, Shingu Y, Mohr FW, Schwarzer M: **Decreased rates of substrate oxidation ex vivo predict the onset of heart failure and contractile dysfunction in rats with pressure overload**. *Cardiovascular research* 2010, **86**(3):461-470.
149. Huss JM, Imahashi K, Dufour CR, Weinheimer CJ, Courtois M, Kovacs A, Giguere V, Murphy E, Kelly DP: **The nuclear receptor ERRalpha is required for the bioenergetic and functional adaptation to cardiac pressure overload**. *Cell metabolism* 2007, **6**(1):25-37.
150. Sihag S, Cresci S, Li AY, Sucharov CC, Lehman JJ: **PGC-1alpha and ERRalpha target gene downregulation is a signature of the failing human heart**. *Journal of molecular and cellular cardiology* 2009, **46**(2):201-212.
151. Zhabyeyev P, Gandhi M, Mori J, Basu R, Kassiri Z, Clanachan A, Lopaschuk GD, Oudit GY: **Pressure-overload-induced heart failure induces a selective reduction in glucose oxidation at physiological afterload**. *Cardiovascular research* 2013, **97**(4):676-685.
152. Amorim PA, Nguyen TD, Shingu Y, Schwarzer M, Mohr FW, Schrepper A, Doenst T: **Myocardial infarction in rats causes partial impairment in insulin response associated with reduced fatty acid oxidation and mitochondrial gene expression**. *The Journal of thoracic and cardiovascular surgery* 2010, **140**(5):1160-1167.
153. van den Bosch BJ, Lindsey PJ, van den Burg CM, van der Vlies SA, Lips DJ, van der Vusse GJ, Ayoubi TA, Doevendans PA, Smeets HJ: **Early and transient gene expression changes in pressure overload-induced cardiac hypertrophy in mice**. *Genomics* 2006, **88**(4):480-488.
154. Degens H, de Brouwer KF, Gilde AJ, Lindhout M, Willemsen PH, Janssen BJ, van der Vusse GJ, van Bilsen M: **Cardiac fatty acid metabolism is preserved in the compensated hypertrophic rat heart**. *Basic research in cardiology* 2006, **101**(1):17-26.
155. Chandler MP, Kerner J, Huang H, Vazquez E, Reszko A, Martini WZ, Hoppel CL, Imai M, Rastogi S, Sabbah HN *et al*: **Moderate severity heart failure does not involve a downregulation of myocardial fatty acid oxidation**. *American journal of physiology Heart and circulatory physiology* 2004, **287**(4):H1538-1543.
156. Miyamoto T, Takeishi Y, Tazawa S, Inoue M, Aoyama T, Takahashi H, Arimoto T, Shishido T, Tomoike H, Kubota I: **Fatty acid metabolism assessed by 125I-iodophenyl 9-methylpentadecanoic acid (9MPA) and expression of fatty acid utilization enzymes in volume-overloaded hearts**. *European journal of clinical investigation* 2004, **34**(3):176-181.
157. Paszko Z, Pronaszko A, Padzik H, Nowosielski J: **The influence of LH on synthesis of RNA, DNA, proteins and lipids and content of ascorbic acid and cholesterol in the luteinized rat ovary**. *Archivum immunologiae et therapiae experimentalis* 1972, **20**(1):149-156.

158. Taylor M, Wallhaus TR, Degrado TR, Russell DC, Stanko P, Nickles RJ, Stone CK: **An evaluation of myocardial fatty acid and glucose uptake using PET with [18F]fluoro-6-thia-heptadecanoic acid and [18F]FDG in Patients with Congestive Heart Failure**. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* 2001, **42**(1):55-62.
159. Forsey RG, Reid K, Brosnan JT: **Competition between fatty acids and carbohydrate or ketone bodies as metabolic fuels for the isolated perfused heart**. *Canadian journal of physiology and pharmacology* 1987, **65**(3):401-406.
160. de Brouwer KF, Degens H, Aartsen WM, Lindhout M, Bitsch NJ, Gilde AJ, Willemsen PH, Janssen BJ, van der Vusse GJ, van Bilsen M: **Specific and sustained down-regulation of genes involved in fatty acid metabolism is not a hallmark of progression to cardiac failure in mice**. *Journal of molecular and cellular cardiology* 2006, **40**(6):838-845.
161. Sano M, Schneider MD: **Energizer: PGC-1 alpha keeps the heart going**. *Cell metabolism* 2005, **1**(4):216-218.
162. Cai D, Frantz JD, Tawa NE, Jr., Melendez PA, Oh BC, Lidov HG, Hasselgren PO, Frontera WR, Lee J, Glass DJ *et al*: **IKKbeta/NF-kappaB activation causes severe muscle wasting in mice**. *Cell* 2004, **119**(2):285-298.
163. Pahl HL: **Activators and target genes of Rel/NF-kappaB transcription factors**. *Oncogene* 1999, **18**(49):6853-6866.
164. Palomer X, Salvado L, Barroso E, Vazquez-Carrera M: **An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy**. *International journal of cardiology* 2013.
165. Delhalle S, Blasius R, Dicato M, Diederich M: **A beginner's guide to NF-kappaB signaling pathways**. *Annals of the New York Academy of Sciences* 2004, **1030**:1-13.
166. Moreno R, Sobotzik JM, Schultz C, Schmitz ML: **Specification of the NF-kappaB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKK epsilon**. *Nucleic acids research* 2010, **38**(18):6029-6044.
167. Matthews JR, Botting CH, Panico M, Morris HR, Hay RT: **Inhibition of NF-kappaB DNA binding by nitric oxide**. *Nucleic acids research* 1996, **24**(12):2236-2242.
168. Chen L, Fischle W, Verdin E, Greene WC: **Duration of nuclear NF-kappaB action regulated by reversible acetylation**. *Science* 2001, **293**(5535):1653-1657.
169. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP: **Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA**. *Molecular cell* 2003, **12**(6):1413-1426.
170. Ea CK, Baltimore D: **Regulation of NF-kappaB activity through lysine monomethylation of p65**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(45):18972-18977.
171. Naumann M, Scheidereit C: **Activation of NF-kappa B in vivo is regulated by multiple phosphorylations**. *The EMBO journal* 1994, **13**(19):4597-4607.

172. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: **Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B**. *Nature* 1995, **376**(6536):167-170.
173. Tran H, Brunet A, Griffith EC, Greenberg ME: **The many forks in FOXO's road**. *Science's STKE : signal transduction knowledge environment* 2003, **2003**(172):RE5.
174. Petersen AM, Pedersen BK: **The anti-inflammatory effect of exercise**. *Journal of applied physiology* 2005, **98**(4):1154-1162.
175. Bakkar N, Ladner K, Canan BD, Liyanarachchi S, Bal NC, Pant M, Periasamy M, Li Q, Janssen PM, Guttridge DC: **IKKalpha and alternative NF-kappaB regulate PGC-1beta to promote oxidative muscle metabolism**. *The Journal of cell biology* 2012, **196**(4):497-511.
176. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M: **IKK-beta links inflammation to obesity-induced insulin resistance**. *Nature medicine* 2005, **11**(2):191-198.
177. Baracos VE: **Cancer-associated cachexia and underlying biological mechanisms**. *Annual review of nutrition* 2006, **26**:435-461.
178. Andersen NM, Tang R, Li L, Javan H, Zhang XQ, Selzman CH: **Inhibitory kappa-B kinase-beta inhibition prevents adaptive left ventricular hypertrophy**. *The Journal of surgical research* 2012, **178**(1):105-109.
179. Hamid T, Guo SZ, Kingery JR, Xiang X, Dawn B, Prabhu SD: **Cardiomyocyte NF-kappaB p65 promotes adverse remodelling, apoptosis, and endoplasmic reticulum stress in heart failure**. *Cardiovascular research* 2011, **89**(1):129-138.
180. Coll T, Jove M, Rodriguez-Calvo R, Eyre E, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M: **Palmitate-mediated downregulation of peroxisome proliferator-activated receptor-gamma coactivator 1alpha in skeletal muscle cells involves MEK1/2 and nuclear factor-kappaB activation**. *Diabetes* 2006, **55**(10):2779-2787.
181. Alvarez-Guardia D, Palomer X, Coll T, Davidson MM, Chan TO, Feldman AM, Laguna JC, Vazquez-Carrera M: **The p65 subunit of NF-kappaB binds to PGC-1alpha, linking inflammation and metabolic disturbances in cardiac cells**. *Cardiovascular research* 2010, **87**(3):449-458.
182. Schilling J, Lai L, Sambandam N, Dey CE, Leone TC, Kelly DP: **Toll-like receptor-mediated inflammatory signaling reprograms cardiac energy metabolism by repressing peroxisome proliferator-activated receptor gamma coactivator-1 signaling**. *Circulation Heart failure* 2011, **4**(4):474-482.
183. Wang LH, Yang XY, Zhang X, Farrar WL: **Inhibition of adhesive interaction between multiple myeloma and bone marrow stromal cells by PPARgamma cross talk with NF-kappaB and C/EBP**. *Blood* 2007, **110**(13):4373-4384.
184. Remels AH, Gosker HR, Bakker J, Guttridge DC, Schols AM, Langen RC: **Regulation of skeletal muscle oxidative phenotype by classical NF-kappaB signalling**. *Biochimica et biophysica acta* 2013, **1832**(8):1313-1325.
185. Palomer X, Alvarez-Guardia D, Davidson MM, Chan TO, Feldman AM, Vazquez-Carrera M: **The interplay between NF-kappaB and E2F1 coordinately regulates inflammation and metabolism in human cardiac cells**. *PloS one* 2011, **6**(5):e19724.

186. Planavila A, Laguna JC, Vazquez-Carrera M: **Nuclear factor-kappaB activation leads to down-regulation of fatty acid oxidation during cardiac hypertrophy**. *The Journal of biological chemistry* 2005, **280**(17):17464-17471.
187. Kim HJ, Park KG, Yoo EK, Kim YH, Kim YN, Kim HS, Kim HT, Park JY, Lee KU, Jang WG *et al*: **Effects of PGC-1alpha on TNF-alpha-induced MCP-1 and VCAM-1 expression and NF-kappaB activation in human aortic smooth muscle and endothelial cells**. *Antioxidants & redox signaling* 2007, **9**(3):301-307.
188. Wenz T, Rossi SG, Rotundo RL, Spiegelman BM, Moraes CT: **Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(48):20405-20410.
189. Zhong H, Voll RE, Ghosh S: **Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300**. *Molecular cell* 1998, **1**(5):661-671.
190. Neumann M, Naumann M: **Beyond IkappaBs: alternative regulation of NF-kappaB activity**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2007, **21**(11):2642-2654.
191. Schmitz ML, Mattioli I, Buss H, Kracht M: **NF-kappaB: a multifaceted transcription factor regulated at several levels**. *Chembiochem : a European journal of chemical biology* 2004, **5**(10):1348-1358.
192. Sasaki CY, Barberi TJ, Ghosh P, Longo DL: **Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway**. *The Journal of biological chemistry* 2005, **280**(41):34538-34547.
193. Olesen J, Larsson S, Iversen N, Yousafzai S, Hellsten Y, Pilegaard H: **Skeletal muscle PGC-1alpha is required for maintaining an acute LPS-induced TNFalpha response**. *PloS one* 2012, **7**(2):e32222.
194. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK: **The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation**. *Nature* 1998, **391**(6662):79-82.
195. Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, Qin DN, Kou CZ, Qin XH, Tong ML *et al*: **TNF-alpha induces mitochondrial dysfunction in 3T3-L1 adipocytes**. *Molecular and cellular endocrinology* 2010, **328**(1-2):63-69.
196. Valerio A, Cardile A, Cozzi V, Bracale R, Tedesco L, Pisconti A, Palomba L, Cantoni O, Clementi E, Moncada S *et al*: **TNF-alpha downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents**. *The Journal of clinical investigation* 2006, **116**(10):2791-2798.
197. Bork PM, Schmitz ML, Kuhnt M, Escher C, Heinrich M: **Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kappaB**. *FEBS letters* 1997, **402**(1):85-90.
198. Kurdi M, Bowers MC, Dado J, Booz GW: **Parthenolide induces a distinct pattern of oxidative stress in cardiac myocytes**. *Free radical biology & medicine* 2007, **42**(4):474-481.

199. Li-Weber M, Palfi K, Giaisi M, Krammer PH: **Dual role of the anti-inflammatory sesquiterpene lactone: regulation of life and death by parthenolide**. *Cell death and differentiation* 2005, **12**(4):408-409.
200. Herrera F, Martin V, Rodriguez-Blanco J, Garcia-Santos G, Antolin I, Rodriguez C: **Intracellular redox state regulation by parthenolide**. *Biochemical and biophysical research communications* 2005, **332**(2):321-325.