Transcriptional regulation of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) promoter

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List of Abbreviations

Acetyl-CoA	Acetyl-Coenzyme A
Acyl-CoA	Acyl-Coenzyme A
Ad-ERRα	Adenovirus Encoding ERRα
Ad-GFP	Adenovirus Encoding Green Fluorescent Protein
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanine Monophosphate
ChIP	Chromatin Immunoprecipitation
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay
ER	Estrogen Receptor
ERR	Estrogen Related Receptor
ERE	Estrogen-Response Element
ERRE	Estrogen Related Receptor Response Element
FBS	Fetal Bovine Serum
GABPA	GA-Binding Protein Alpha
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase

HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl) Piperazine-1 Ethanesulfonic Acid
HS	Horse Serum
MAP	Mitogen-Activated Protein
MCAD	Medium-Chain Acyl-Coenzyme A Dehydrogenase
MEF	Myocyte Enhancer Factor
mtDNA	Mitochondrial DNA
NP40	Nonyl Phenoxylpolyethoxylethanol
NRF	Nuclear Respiratory Factor
PPAR	Peroxisome Proliferator-Activated Receptor
PBS	Phosphate Buffered Saline
PGC-1α	PPARγ Coactivator-1α
PKA	Protein Kinase A
PMSF	Phenylmethylsulfonyl Fluoride
PRC	PGC-Related Coactivator
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TCA	Tricarboxylic Acid
Tfam	Mitochondrial DNA Replication Transcription Factor
TSA	Trichostatin A

I Abstract

PGC-1α regulates cardiac mitochondrial biogenesis and energy metabolic gene expression, thus transcriptional regulation of PGC-1α gene expression is of great importance in understanding metabolic gene expression in cardiac health and disease. We provide evidence that estrogen related receptor α (ERRα), which also plays a role in cardiac energy metabolism, regulates expression of the PGC-1α gene via direct interaction with the PGC-1α gene promoter. In the presence of an inverse agonist to ERRα, PGC-1α gene expression was significantly decreased, while over-expression of ERRα increased PGC-1α gene expression. We have also demonstrated that expression of PGC-1α was down regulated in hypoxic cardiomyocytes due to histone deacetylation. Our data identify ERRα as a novel regulator of cardiac PGC-1α gene expression, and suggests that promoter deacetylation in hypoxia plays a role in reduced PGC-1α expression. These results reveal a new mechanism that may contribute to energetic derangement in the heart during ischemia and/or failure.

II Introduction

The heart is a muscular organ responsible for delivering blood to the body. Heart failure occurs when the heart is unable to supply adequate blood flow to meet the body's needs. [1] This continuously working organ requires more energy than any other organ in the body. Energy metabolism in the heart is regulated at a molecular level by signaling pathways that control gene expression. Transcription factors are molecular regulators that can either enhance or inhibit gene expression. They are affected by many factors including the binding affinity of the protein to its DNA binding site and by interactions with other transcription factors bound to sites nearby.[2]

The nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family are transcription factors involved in the regulation of cardiac lipid metabolism.[3,4] There are three isoforms including PPARα, PPARβ and PPARγ however, it is PPARα that is directly involved in the switch from fatty acid oxidation to glucose oxidation, resulting in the depletion of cardiac energy stores under pathophysiological conditions.[5] It has been shown in both animal models and in human patients with cardiac hypertrophy, a cardiomyopathy that involves energy depletion of the myocardium, the expression of PPARα was decreased and in turn a proportional decrease in fatty acid oxidation was observed.[6,7,8] This finding supports the hypothesis that down-regulation of PPARα plays a major role in the switch from fatty acid metabolism to glucose metabolism in hypertrophied hearts.

PPARγ coactivator-1α (PGC-1α) is a transcriptional coactivator of the PPAR superfamily and a regulator of mitochondrial metabolic function. PGC-1α itself is regulated by the transcription factor myocyte enhancer factor-2C (MEF2C) and repressed

by histone deacetylase (HDAC) 5.[9] PGC-1α has regulatory effects in brown fat, muscle and liver tissue according to metabolic requirements.[10] Increased expression of PGC-1α was observed during exercise in muscle tissue.[11] Increased expression of PGC-1α also occurred during fasting states in the liver, demonstrating its role in metabolic regulation.[11] In a cardiac specific PGC-1α over-expression mouse model, an increase in mitochondrial proliferation in cardiomyocytes was observed, suggesting that PGC-1α is a regulator of mitochondrial biogenesis.[12] In a mouse knockout model of nuclear transcription factor PGC-1α developed by Lin et al., the mice were viable and fertile, and a normal mitochondrial phenotype was observed.[13] However, a decrease in cardiac ATP production and reduced oxygen consumption in hepatocytes was noted.[14, 13] In another study using a PGC-1α knock out mouse model, cardiac stress was induced via transverse aortic constriction.[15] Rapid onset of cardiac dysfunction was observed in these mice suggesting that repression of PGC-1α contributes to heart failure.[15] It is evident that PGC-1α is a key integrator of signals involved in the regulation of cardiac energy metabolism. Studying PGC-1α will enable us to achieve a better understanding of how pathological stimuli may influence gene expression resulting in energetic derangement. We have identified a putative binding site for estrogen related receptor α (ERRα) within the PGC-1α promoter. This suggests that ERRα may be involved in the regulation of PGC-1α gene expression.

It has been shown that ERRα interacts with PGC-1α to upregulate the PPARα gene to increase fatty acid uptake and oxidation.[16, 17] However the transcription of ERRα is in turn regulated by PGC-1α resulting in the formation of a feedback loop. ERRα and

its relatives ERRβ and ERRγ are orphan nuclear receptors that have sequence similarity with the steroid estrogen receptor, although they do not bind to estrogen. ERRα levels are high in oxidative tissue such as the heart. ERRα plays a role in fatty acid oxidation by regulating the promoter of medium-chain acyl-coenzyme A dehydrogenase.[18]

Current evidence suggests that impaired substrate metabolism contributes to contractile dysfunction and left ventricular remodeling. By studying the transcriptional regulation of the PGC-1α promoter, we will be able to better understand how PGC-1α is expressed and under what conditions. A clear understanding of the role of PGC-1α in cardiac energetics, regulating the mitochondrial function and cardiac energy demand following a cardiac event may be possible to improve a patient's prognosis and quality of life.

III Statement of Hypothesis

We hypothesize that estrogen related receptor ERRα, a central regulator of cardiac energy metabolism, regulates expression of the PGC-1α gene via direct interaction with the PGC-1α gene promoter.

IV Objectives

We will investigate the role of ERRα in the regulation of PGC-1α expression. We will also investigate how pathophysiological stress (hypoxia) affects the regulation of PGC-1α expression.

In order to determine whether ERRα directly binds to the PGC-1α promoter, an EMSA and ChIP assay was used in vitro and in vivo respectively. A luciferase reporter assay was performed on the PGC-1α promoter to investigate whether ERRα regulates the activation of the promoter. Promoter mutations were generated by changing two to three base pairs within the potential binding sites of ERRα. Once the deletions are introduced, ERRα will no longer be able to bind to its respective binding site and activation from the luciferase reporter assay should not be detected. We also looked at mRNA expression of the PGC-1α gene using quantitative real-time polymerase chain reaction (qRT-PCR). PGC-1α expression levels will be measured in cardiomyocytes where ERRα is inhibited, over-expressed, exposed to hypoxia or treated with TSA.

V Literature Review

1.0 Heart Failure

Congestive heart failure is a condition in which the heart can no longer adequately pump blood and therefore cannot deliver oxygen and nutrients to the body sufficient to meet its needs. Heart failure develops once the heart becomes damaged or gradually becomes weak due to other underlying diseases, such as ischemic heart disease, hypertension or diabetes. Within five years of being diagnosed with heart failure, 40-50% of all cases in Canada are fatal. [I] One of the most common causes of heart failure is a myocardial infarction (heart attack). This occurs when arteries, the blood vessels that supply the heart with blood, become occluded and therefore the working heart muscle is deprived of oxygen and can no longer generate adequate energy to function. It is estimated that every year approximately 70 000 Canadians experience myocardial infarctions, resulting in approximately 17,000 deaths due to these incidences. [I] With more attention given to the warning signs and more successful treatments, Canadians are living longer after experiencing myocardial infarctions. This also means that these individuals are more susceptible to heart failure, with an estimated 400,000 Canadians currently living with this condition.[I] In addition to the adverse affects of heart attacks, cardiovascular diseases, which include heart disease, vascular diseases and stroke accounted for 31% of all deaths in Canada, making cardiovascular disease the number one cause of death versus any other disease in the year 2005.[I]

[I] http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3483991/k.34A8/Statistics.htm#chf

1.1 Underlying Causes of Heart Failure

Heart disease can develop as a result of genetic inheritance or can be idiopathic in nature. There are many different types of heart disease; some examples include congenital heart disease, coronary heart disease, ischemic cardiomyopathy, hypertrophic cardiomyopathy, valvular cardiomyopathy, hypertensive cardiomyopathy or dilated cardiomyopathy.[19] Common risk factors among patients with heart disease are hypertension, elevated cholesterol, smoking, family history of premature coronary artery disease, physical inactivity, obesity and diabetes. In 2005, of all cardiovascular deaths in Canada, 53% were due to ischemic cardiomyopathies, which is defined as a weakening of the heart muscle due to inadequate oxygen supply.[I] No matter what the cause of any particular cardiomyopathy, once it progresses to heart failure, the end result is a depression in cardiac function, loss of adrenergic support and a depletion of energy stores. Cardiac remodeling involves changes at the cellular and molecular level that alters size and function of the heart to compensate for any damage it has experienced. In a diseased state, myocardial energy demand increases due to compensatory increases in muscle mass or increases in wall stress. Increased fibrosis, increased myocyte size and/or decreases in mitochondrial function also contribute to decreases in the energy supply available to the heart. The overall metabolic inefficiency of the heart that occurs in heart failure results in failure to meet the entire body's metabolic demand.

1.2 Metabolic Abnormalities in Humans

Various deficiencies in humans give rise to metabolic abnormalities that can be observed in the individual's cardiac phenotype.[20] These cases give some insight to different components of metabolic pathways that control cardiac function. The following are human inborn errors of metabolism and their corresponding metabolic deficiency. Systemic carnitine deficiency leads to a defect in carnitine synthesis which affects transmembrane transport and intestinal uptake of fatty acids. This deficiency results in a dilated cardiomyopathy, cardiac arrest and cardiomegaly.[21] Malonyl carboxylase deficiency causes higher levels of malonyl-CoA which is an inhibitor of carnitine palmitoyl transferase 1 and results in a dilated cardiomyopathy, decreased contractility and heart failure.[22] Impaired mitochondrial transport of acyl-CoA is caused by a deficiency of carnitine palmitoyl transferase 2. The result of this impaired transport is hypertrophy of the heart, fatal cardiomegaly and dysrhythmias.[23] Errors in various short-chain, medium-chain, long-chain and very long-chain acyl-CoA dehydrogenases leads to the dysfunction of fatty acid β-oxidation. Short-chain acyl-CoA dehydrogenase errors lead to mild left ventricular dysfunction and biatrial hypertrophy. Medium-chain acyl-CoA dehydrogenase errors have rare cardiac involvement, therefore no phenotypical differences are observed. Long and very long-chain acyl-CoA dehydrogenase errors leads to severe dilated or hypertrophic cardiomyopathy.[24] Finally, MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), Barth syndrome (mutation of mitochondrial DNA) and Leigh's syndrome (mutation of mitochondrial or nuclear DNA) causes various deficiencies in respiratory chain complexes and lead to dilated

cardiomyopathy, hypertrophic cardiomyopathy, conduction defects and ventricular ectopy. [24,25]

1.3 Treatment of Heart Failure Due to Metabolic Disturbances

Heart failure induces a metabolic myopathy by decreased oxidative capacity, shift in substrate utilization and altered energy transfer. Exercise has been shown to improve oxygen and substrate delivery, increase lipid utilization, and improve energy production and utilization.[26] In an animal model of heart failure, left ventricle function was improved by glucagon-like peptide1 that increased glucose utilization.[27] In a study of human patients, intracoronary injections of pyruvate improved cardiac function in the short term.[28] In a mouse model, creatine transport function was improved by increasing concentrations of creatine and phosphocreatine.[29] It has also been shown in an animal model that by activating genes responsible for glucose transporters cardiac function can inprove.[30] Increased consumption of dietary ω-3 fatty acids has a beneficial effect on cardiovascular health.[31] These treatments show that by studying the regulation and identifying the molecules that are critical in the proper functioning of the heart, the outcome of patients can be improved.

2.0 Cardiac Energy Metabolism

The heart utilizes more energy than any other organ in the body. Energy is released from fuels through metabolic pathways that break down substrates such as carbohydrates and fatty acids and stores the energy released in the high energy bonds of

ATP.[32] Approximately 6 kg of ATP is used by the human heart every day. Mechanical failure of the heart will occur if adequate ATP is not supplied to the continuously working heart muscle.[20] Heart failure can be the result of structural and functional problems that disrupt the normal filling or ejection of the ventricles due to metabolic changes in the myocardium.[33]

Cardiac energy metabolism consists of three main components: substrate utilization, oxidative phosphorylation and ATP utilization.[20,33] The mitochondrion is the organelle where chemical energy is produced. In cardiomyocytes, mitochondria take up approximately 25 to 35 % of cell volume and store and transform 90% of the energy within the cells.[34] It has been observed that in failing hearts cardiac mitochondria increase in number and show abnormalities in their structure.[35] Mitochondria contain the machinery, including enzymes and intermembrane transporters, which are involved in metabolic processes such as glucose and fatty acid transportation and oxidation, tricarboxylic acid (TCA) cycle enzymes and electron transport chain complexes. The rate at which substrates are catabolized and electrons transferred in the mitochondria is controlled by substrate to product ratios, enzyme activity and by the level of expression of metabolic proteins. The mechanisms in place for the regulation of metabolic pathways allow the heart to adapt to any changes in energy requirements such as exercise, fasting or ischemia.

The supply of fuels to the working myocardium is delivered by the coronary circulation. In a fasted state blood fatty acid levels are high. In a fed state (after a carbohydrate rich meal) energy production is mostly from the oxidation of carbohydrates.[19] The majority of metabolism in the heart is aerobic, however if there is a

lack of oxygen to the myocardium, such as in coronary artery disease or during exercise, the breakdown of glucose by glycolysis is favored over oxidation of fatty acids. Anaerobic glycolysis produces lactate which enters the TCA cycle to produce only two molecules of ATP via oxidative phosphorylation.[36] Both glycolysis and fatty acid oxidation produce reduced electron carriers (NADH/NADPH or $FADH_2$) that proceed to the mitochondria in order to generate ATP. Mitochondrial oxidative phosphorylation occurs when NADH (or NADPH) and $FADH_2$ that are generated from dehydrogenation reactions from the TCA cycle donate electrons to the electron transport chain. This creates a hydrogen gradient across the membrane and generates ATP via membrane bound ATP synthase with oxygen being the final electron acceptor.[33] These reduction-oxidation reactions release energy, which is used to form ATP.

Fatty acid oxidation yields more ATP when complete oxidation of the substrate occurs. However, when energy requirements of the heart are increased, glucose oxidation contributes to the production of additional ATP.[37] Energy requirements of the heart change depending on the work required from the heart, such as during exercise. Coronary blood flow supplies the heart with oxygen to meet these energetic needs. Regulation of coronary flow occurs by the release of vasoconstrictors or vasodilators, which are in turn regulated by the local metabolic need of the myocardium. As a result of ischemia (restriction in blood supply), adenosine, a vasodilator is formed. Vasodilatation allows more oxygen to be delivered to the mitochondria of the myocardial cell thus contributing to oxidative phosphorylation and ATP production.[38]

2.1 Carbohydrate Metabolism

Glycolysis is the process that breaks down glucose-6-phosphate to two molecules of pyruvate. Under aerobic conditions pyruvate is converted to acetyl-coenzyme A (acetyl-CoA), which enters the TCA cycle. Under anaerobic conditions pyruvate is converted to lactate. The healthy heart is a net consumer of lactate.[39,40] In cases of ischemia or poorly controlled diabetes there is a net build up of lactate.[41,41] Intracellular glycogen stores are also sources of glucose-6-phosphate. Glycogen stores are relatively small (30 μmol/g) and have a fairly rapid turnover rate.[42] Glycogenolysis is activated by increased concentrations of cyclic adenosine monophosphate (cAMP) and Ca^{2+}, usually associated with ischemia or exercise.[43]

Uptake of glucose is activated in the fed state, with increased work by the heart or by hypoxia or ischemia. It is inhibited in the fasted state, when blood fatty acid levels are high or with severe diabetes mellitus. Glucose uptake is promoted by the hormone insulin. Insulin acts by inhibiting the release of fatty acids from adipose tissue (thus removing the inhibition of glucose uptake by fatty acids) and by increasing the number of glucose transporters, primarily GLUT-4. Once inside a cardiac cell, glucose is phosporylated to glucose-6-phosphate and broken down to two molecules of pyruvate. Aerobic respiration yield two molecules of acetyl-CoA and anaerobic respiration yields two molecules of lactate.

Glucose is taken up from the bloodstream and into the cells of the heart by glucose transporters GLUT-1 and GLUT-4. GLUT-1 transports glucose into cardiomyocytes and GLUT-4 is responsible for insulin-mediated transport of glucose.[44] Theses transport proteins are stereospecific (meaning they only transport glucose) and do

not require energy as glucose is transported down its concentration gradient, from high concentrations in the extracellular space to the cytosol of the cardiomyocyte.[19, 45] Recent studies have shown that AMP-activated protein kinase may play a role in GLUT-4 translocation during ischemia in cardiomyocytes.[46] When reduced blood flow occurs, it was observed that more glucose is transported into the cell. Nitric oxide also plays a role in myocardial metabolism via the secondary messenger cyclic guanine monophosphate (cGMP) by inhibiting the transport of glucose into cardiomyocytes.[47] An increase in nitric oxide production decreases myocardial glucose utilization that can in turn limit heart work.[48, 49]

2.2 Fatty Acid Metabolism

At rest, when blood flow and oxygen supply is normal, cardiomyocytes depend on fatty acid oxidation for 60-70% of their energy requirements.[50] The oxidation of a six-carbon fatty acid yields 44 molecules of ATP than that of the breakdown of glucose, which yields 38 molecules of ATP.[51] Fatty acids enter cardiomyocytes through the sarcolemmal membrane where it is converted to acyl-coenzyme A (acyl-CoA). The carnitine carrier system is required for acyl-CoA to enter the mitochondrial membrane. Acyl-CoA enters the myocardial cell as acylcarnitine.[52] Once inside the mitochondria, β-oxidation of the fatty acid occurs and the fatty acid is sequentially broken down to acetyl-CoA. Acetyl-CoA enters the TCA cycle that takes place within the mitochondrial matrix and functions to produce ATP by oxidative phosphorylation.[53] The primary products of fatty acid oxidation are NADH and $FADH_2$ that enter the electron transport chain to form ATP.[33] Malonyl-CoA is a key regulator of fatty acid oxidation in the heart. A decrease in

concentration of malonyl-CoA increases fatty acid uptake and oxidation.[54] Malonyl-CoA is synthesized by the enzyme acetyl-CoA carboxylase and is inhibited by adenosine monophosphate (AMP) protein kinase.[19]

It has been observed that in the early stages of heart failure, fatty acid oxidation is increased and the level of glucose oxidation is lower. As heart failure progress and becomes more severe, fatty acid oxidation decreases and levels of glucose uptake and oxidation increases.[33] A decrease in overall mitochondrial oxidative capacity and a decrease in the production of enzymes involved in fatty acid oxidation contribute to the switch from fatty acid metabolism to glucose metabolism. Inhibition of PPARα also down-regulates fatty acid oxidation.[33] Once fatty acid oxidation is lowered, intermediates involved in fatty acid oxidation accumulate in the myocardium causing lipotoxicity.[55] During periods of starvation or diabetes, ketone bodies such as β-hydroxybutyrate and acetoacetate can accumulate in the myocardium.[56] These elevated levels of ketone bodies can inhibit the β-oxidation of fatty acid. High plasma levels of ketones will also inhibit the uptake of glucose and lactate.[19]

2.3 ATP and Creatine Phosphate

ATP is chemical energy that is used by the heart for heat production, muscle contraction and calcium uptake by the sarcoplasmic reticulum, all important processes that allow the heart to function. Smaller amounts of ATP are also used for phosphorylation of proteins involved in the production of cAMP, mitochondrial calcium uptake and protein synthesis.[19] Energy can also be stored as creatine phosphate, although it is ATP that is required by myocardial cells to contract. Energy can be transferred from

creatine phosphate to ATP by the enzyme creatine kinase. Creatine phosphate is rapidly diffused from the mitochondria to myofibrils. In a study performed by Ye *et al*., myocardial protein levels of creatine kinase were studied using a porcine model.[57] It was found that ATP synthesized in the mitochondria was exported out of the mitochondria and converted to creatine phosphate in the cytosol by the mitochondrial isoform of creatine kinase. Their model also suggests that in heart failure or hypertrophy, there is a decrease in the levels of mitochondrial creatine kinase.[57] Other studies using porcine models have also shown that ATP concentrations decrease in failing hearts.[58,59] Creatine phosphate transfers energy to ATP at the site of use and it has been shown that if transport of creatine phosphate is inhibited cardiac contractions are affected.[19,57] Creatine phosphate acts as an energy buffer in the myocardium. It was shown in a rat model that myofibrillar creatine phosphate is decreased up to 50% in a failing heart, in turn causing a decline in ATP transfer within the myocardium.[60] When energy demand is greater than energy supply, creatine phosphate levels fall but ATP levels remain constant. Adenosine diphosphate (ADP) levels drop causing cardiac myocyte contractions to be hindered.[20]

High levels of creatine phosphate in the blood from leakage through the sarcolemma due to ischemia can be a diagnostic measure for myocardial infarction.[19] Cardiac function can be improved during ischemia and reperfusion by increasing ATP production through glycolysis.[61]

When work is increased by the heart, more ATP is required. This can be achieved by stimulating glycolysis and fatty acid oxidation to produce more acetyl-CoA to enter the TCA cycle. The breakdown of ATP to ADP and further to AMP serves as a trigger to increase ATP production. AMP protein kinase (activated by AMP) inhibits malonyl-CoA.

Malonyl-CoA is a molecule that inhibits mitochondrial uptake of acetyl-CoA and increases translocation of glucose transporters, thus increasing glucose uptake.[19]

2.4 Molecular Regulators of Energy Production

Energy demand by the heart can change throughout development or even minute by minute depending on functional requirements. Energy production is regulated at a molecular level by mechanisms that control gene expression.[20,4] Transcriptional factors are molecules that affect the expression of genes, either by repressing the gene or by activating it. The nuclear receptors of the PPAR family are one of the most studied transcription factors involved in cardiac lipid metabolism. There are three isoforms PPARα, PPARβ and PPARγ. PPARα is the isoform that is directly involved in fatty acid oxidation. It has been shown in animal models and in humans with cardiac hypertrophy that expression of PPARα is down-regulated and therefore decreases fatty acid utilization.[20] PGC-1α is a regulator of metabolic function specific to the mitochondria. PGC-1α coactivates the PPAR superfamily and itself is regulated by coactivators MEF2C and HDAC5.[9] Decreases in gene expression of PGC-1α resulted in deficiencies in cardiac energy reserves.[15] It has been shown that the transcription factor ERRα interacts with PGC-1α to upregulate PPARα genes to increase fatty acid uptake and oxidation.[16,17]

2.5 Metabolic Gene-Knockouts in Mouse Models

With the use of mouse models, metabolic gene knockouts can be created in order to study the metabolic change and cardiac phenotype of the test animal.[20] In a knockout

mouse model of PPARα deletion, metabolism shifted from fatty acid utilization to glucose and lactate as substrates for metabolism. This in turn reduced cardiac energy stores during inotropic challenge.[5] Defective glucose transporter 4 in mice increased basal glucose transport but inhibited insulin-stimulated glucose transport. The result of this knockout was cardiac hypertrophy.[62] Mutations to PGC-1α gene resulted in reduced fatty acid oxidation as well as oxidative phosphorylation which caused a loss of contractile reserve in the mice.[63] In knockout mice of a heart specific mitochondrial DNA replication transcription factor, Tfam, glucose utilization was increased, whereas fatty acid oxidation as well as the activity of the respiratory chain was decreased. The result was cardiac hypertrophy, heart failure and conduction defects.[64] Cardiac hypertrophy also occurred in knockout mice of the adenine nucleotide translocase 1 gene, which caused a decrease in ADP stimulated mitochondrial respiration.[65] A mutation to the gene coding for mitochondrial and myofibrillar creatine kinase caused a shortened diffusion distance between mitochondrial and myofibrils resulting in hypertrophy and impaired contractile reserves.[66,67] Knockout mice for the gene coding for guanidine acetate methyl transferase, decreased the reaction velocity of creatine kinase and creatine synthesis and cause accumulation of guanidine acetate. This caused the heart to be more susceptible to ischemia and reperfusion injury and decreased inotropic reserves.[68] These gene-knockout mice models have demonstrated the importance of the expression of a single gene on metabolic function in the heart. By studying the effects and regulation of genes at the molecular level we will gain a better understanding of the role of small regulatory proteins in the overall physiological function of the heart.

3.0 Transcription Factors

Transcription factors are proteins that bind to specific DNA sequences in order to control the transcription of genetic information from DNA to mRNA. They can act as an activator (by up-regulating gene expression) or as a repressor (by down regulating gene expression) and perform these actions by attaching to specific sequences of DNA, upstream of the transcriptional start site of a gene to be transcribed. The structure of a transcription factor typically contains a DNA-binding domain, a transcriptional activation domain and a signal-sensing domain. The DNA-binding domain is where the transcription factor attaches to specific sequences of DNA located in either the enhancer or promoter sequence adjacent to the gene under regulation. The transcriptional activation domain contains binding sites for other proteins that impact the regulation of transcription. Signal sensing domains or ligand binding domains sense and transmit external signals to the transcriptional complex, further affecting the regulation of transcription.[2] Other proteins such as coactivators, corepressors, chromatin remodelers, and a variety of enzymes such as kinases and methylases also play a role in gene regulation, however are not considered transcription factors because they lack DNA-binding domains. The steroid hormone estrogen is an example of a ligand that regulates transcription by binding to the estrogen receptor (ER) transcription factor. Estrogen is primarily secreted by the ovaries. [51] This hormone can then travel to other recipient cells and diffuses through the cell's lipid bilayer into the cell's cytoplasm where it binds to estrogen receptors. The estrogen receptor then moves into the cell's nucleus where it binds to its DNA-binding sites to regulate transcription of associated genes.[69]

In addition to recruiting other ligands and proteins, transcription factors control transcription by several mechanisms. These include stabilizing or blocking the binding of RNA polymerase (the enzyme that transcribes DNA into RNA) to DNA or by catalyzing the acetylation or deacetylation of histone proteins.[70] Acetylation of histone proteins weakens the association between histones and DNA, allowing a strand to become more accessible for transcription. This process occurs with the help of enzymes called histone acetyltransferases (HATs). Deacetylation of histone proteins strengthens the association between histones and DNA, favoring chromatin condensation and in turn down regulating transcription. HDAC is the class of enzyme that removes the acetyl group from histones.[71]

3.1 Structure and Function of Nuclear Receptors

An intricate network of intracellular responses coupled to extracellular signaling molecules creates an elaborate signaling system which allows cells to recognize and respond to environmental and biological stimuli in order to control gene expression. The human genome encodes over 2000 proteins involved in the transcriptional regulation of genes. Transcription factors are often classified according to either their regulatory function or based on sequence similarities to other transcription factors.[71] Nuclear receptors are defined as a group of transcription factors with similar structures that bind to lipophilic ligands such as steroids and thyroid hormones to control gene expression within certain cells [72]. However it was also observed that there were certain nuclear receptors that had no identifiable ligands even though they were structurally similar to nuclear receptors with known ligands. This class of receptors was termed orphan nuclear

receptors. ERRα also known as nuclear receptor subfamily 3, group 1 (NR3B1) and Estrogen Realted Receptor beta ERRβ also known as nuclear receptor subfamily 3, group 2 (NR3B2) were the first orphan nuclear receptors identified.[73] Human ERRα was found to be 68% structurally similar to human ER. The ERR family contain three isoforms ERRα, ERRβ and ERRγ, none of which bind estrogen as a ligand, although the putative steroid-binding domain is 36% similar to ER.[73] All isoforms contain a less conserved N-terminal domain, a conserved C-terminal domain which contains the putative ligand-binding site and a zinc finger DNA-binding domain.[74] The majority of the N-terminal domain is conserved among the three ERR isoforms.[75] In ERRα and ERRγ, this region has been shown to be post-transcriptionally modified via phosphorylation and sumoylation in order to regulate the transcriptional activity of these ERRs. This mechanism was further induced with the presence of the coactivator, PGC-1α.[76] Through analysis of the crystal structure, the ligand-binding domain of ERRα was shown to contain a conserved activation function-2 motif that can be positioned in the active conformation without a ligand present. It was also shown through analysis of the crystal structure that PGC-1α binds to the ligand-binding domain of ERRα, suggesting that ERRs are able to bind to coactivators in a ligand-independent manner. [77] PGC-1α interacts with other nuclear receptors via a sequence-specific leucine-rich nuclear receptor interacting motif (LXXLL), however it interacts with ERRα via an atypical reverse (LLKYL) motif within its protein structure.[78] When bound to PGC-1α, ERRα has been shown to become a stronger transcriptional activator.[11] Receptor interacting protein 140 (RIP140) contains nine LXXLL motifs and one atypical LXXML motif and has been

shown to physically interact with ERRα, however it is unknown exactly which motif interacts with ERRα.[79] This interaction between ERRα and RIP140 inhibits the transcriptional activity of ERRα.[75] The DNA-binding domain typically recognizes the DNA sequence TNAAGGTCA, which is referred to as the ERR response element (ERRE).[80] ERRα binds to this sequence in the human lactoferrin promoter.[81] ERRs have also been shown to bind to estrogen-response elements (EREs), which also bind ERs. The DNA sequence of the ERE is AGGTCA, where ERs and ERRs bind to this region as a dimer. Binding of ERRs to the ERRE preferentially occurs as a monomer, however binding to this region by ERRs as a homodimer or heterodimer (two different ERR isoforms) has also been observed.[74,82]

3.2 ERRα Expression in Tissue

ERRs are expressed in tissues of the central nervous system (eye, brainstem, cerebellum, cerebrum, corpus striatum, olfactory bulb, spinal cord, hypothalamus, and pituitary), endocrine system (adrenal, thyroid, and pancreas), gastroenteric system (tongue, stomach, duodenum, jejunum, ileum, colon, and gall bladder), metabolic system (liver, kidney, brown and white adipose, and muscle), immune system (spleen and thymus), reproductive system (ovary, uterus, epididymus, preputial gland, prostate, seminal vesicles, testis, and vas deferens), cardiovascular system (aorta, heart, and lungs) and in structural components (bone and skin).[83] More specifically, ERRα is ubiquitously expressed in all tissues and is expressed to a larger extent than the other two isoforms, particularly in tissues that have high metabolic needs such as the heart, kidneys, intestinal

tract, skeletal muscle and brown adipose tissue.[83] It has also been noted that in white adipose tissue and in the liver, ERRα is expressed rhythmically according to the circadian clock.[84] In response to acute endurance training in human patients, there is an increase in PGC-1α and ERRα mRNA levels in skeletal muscle.[85] PGC-1α positively regulates the expression of ERRα, therefore expression of ERRα is highest in tissues that highly express PGC-1α. Physiological stimuli such as fasting in liver or cold exposure in brown adipose tissue and muscle increase expression of PGC-1α and ERRα.[11, 86] These findings suggest that ERRα plays an important role in regulating metabolic demand in these tissues.

In an ERRα–null mouse model, animals were viable, fertile and did not display any apparent gross anatomical changes.[87] However with no change in food consumption or energy expenditure compared to the wild type mice, ERRα-null mice did have a lower body weight and less peripheral fat deposits.[87] These mice were also resistant to high fat diet-induced obesity. In a microarray study on the white adipose tissue of these mice, known gene targets of ERRα, lactotransferrin and medium-chain acyl-coenzyme A dehydrogenase (MCAD) were down-regulated. Other genes involved in lipid metabolism and energy metabolism including fatty acid synthase, stearoyl-coenzyme A desaturase 2, fatty acid coenzyme A ligase long-chain 5, acetyl-coenzyme A dehydrogenase, acetyl-coenzyme A dehydrogenase long chain, somatic cytochrome c, carnitine acetyltransferase, mitochondrial creatine kinase 2, and mitochondrial uncoupling protein 1, were altered in these mice, creating an imbalance between enzymes involved in fatty acid metabolism and energy production. [87] Enterocytes isolated from the intestines of these ERRα knock-out mice had a lower capacity for β-oxidation and had altered

expression of genes involved in lipid digestion and absorption, including pancreatic lipase-related protein 2 and fatty acid-binding protein 1 and 2. It was also identified that ERRα is a direct regulator of the apolipoprotein A-IV promoter, a protein involved in intestinal fat absorption, indicating that ERRα plays a role in dietary lipid handling.[88]

In another study, mRNA from brown adipose tissue was analyzed from ERRα knock out mice and it was found that the expression of mitochondrial genes involved in oxidative phosphorylation and fatty acid metabolism were down-regulated by 30-50% in these mice compared to wild type mice. This down-regulation included a 2-fold decrease in expression of PPARα and PGC-1α. The brown adipose tissue from the ERRα knock-out mice contained a lower mitochondrial density and 37% less mitochondrial DNA (mtDNA) copies compared to wild type mice. These mice lacking ERRα could not maintain their body temperature when exposed to cold temperatures, suggesting that a loss of ERRα activity leads to a decrease in mitochondrial function, a reduced capacity for energy production and a defect in adaptive thermogenesis in order to deal with metabolic stress such as cold exposure.[89]

During embryonic development, the expression of the ERRα was confirmed in the bones of mice.[90] This finding was of interest in that bone formation is affected by estrogen. It is known that upon menopause and a decrease in estrogen levels, the development of osteoporosis is more prevalent, therefore structural similarities between ER and ERRα make ERRα an intriguing factor to study in the regulation of bone formation and loss.[74] This may give insight to potentially link between estrogen signaling pathways and ERRα signaling pathways despite its inability to bind estrogen.

3.3 Transcriptional Regulation by ERRα

In a genome-wide profiling experiment, ERRα and ERRγ were immunopreciptitated while bound to putative DNA binding sites via chromatin immunoprecipitation assays (ChIP). These samples were then hybridized to a custom microarray containing 1000 base pair segments of 18,657 mouse genes, starting 800 bp upstream of their transcriptional start sites. ERREs were found in 69% of promoters bound by ERRα and 74% of promoters bound by ERRγ.[82] ERRα and ERRγ were shown to bind to promoters of genes involved in the uptake and utilization of energy substrates, production and transport of ATP, regulation of phosphocreatine production, calcium contractile work and cellular energy sensing mechanisms.[82] It was also identified that ERRα and ERRγ directly regulate transcription factors involved in biological pathways in the adult mouse heart. Some examples include GA-binding protein alpha chain (GABPA), retinoic acid receptor alpha (RARa), transformation related protein 53 (Trp53) and cofactor required for Sp1 activation (Crsp3).[82] GABPA (also known as nuclear respiratory factor 2 or NRF-2) regulates the biogenesis of mitochondria by activating mitochondria transcription factor A.[91,92] Both RARa (a member of the nuclear hormone receptor family) and Sp1 are transcription factors that are involved in the regulation of cell growth, differentiation, homeostasis and apoptosis.[93,94] Sp1 has been shown to interact with HDACs, thus playing a role in chromatin remodeling.[94] It has also been shown that the promoter of the MCAD gene contains an ERRE and therefore is responsive to transcriptional control by ERRα. MCAD is responsible for the first reaction in the mitochondrial β-oxidation of fatty acids and is considered a key regulator of this

pathway.[95] In a study done by Seth *et al.*, a myotube cell line lacking expression of RIP104 and treated with the ERRα inverse agonist XCT790 noted a decrease in MCAD gene expression, further confirming the role of ERRα in its regulation. Additional genes involved in fatty acid oxidation and import, mitochondrial respiratory chain components, glucose metabolism, cholesterol metabolism and other metabolic pathways were confirmed an experiment performed in rat neonatal cardiomyocytes that were infected with an ERRα over-expression virus. RNA isolated from these cells were analyzed via microarray to observe changes in gene expression. A significant increase in 90 genes involved in cellular energy metabolism was observed. A few examples of the genes identified are: the MCAD gene (Acadm), Fabp3 (fatty acid binding protein, heart) and Cycs (mitochondrial respiratory chain component cytochrome c).[16] This study also identified that ERRα is a key component in PGC-1α regulation of mitochondrial metabolism.

Apart from metabolic genes, ERRs in general had been identified to regulate the expression of the pS2 gene. The pS2 gene is a human breast cancer prognostic marker and is known to be estrogen inducible.[96] The pS2 promoter contains binding sites for both ERRs and ERs, suggesting that these two transcription factors may have overlapping regulatory pathways. This study also demonstrated identified that transcriptional activity of ERs and ERRs can be regulated by diethylstilbestrol, a synthetic estrogen molecule that is an agonist for ERs and antagonist of ERRs. It is also important to note that it is non-selective towards all ERRs. The inhibitory action of diethylstilbestrol on cell proliferation in cancer cells makes it a useful drug in targeting breast cancer.[96] Although the mechanism of action through ERs and ERRs is not fully understood, the use of ERR

antagonists as a therapeutic agent to treat breast cancer is encouraging in utilizing small molecules to regulate transcription of genes in diseases.

3.4 PGC-1α

As mentioned previously, PGC-1α is a regulator of mitochondrial biogenesis and function. The PGC-1 family of transcriptional coactivators are proteins that act through protein-protein interactions to enhance transcription. PGC-1α was discovered in the mitochondria-rich tissue of murine brown fat cells.[97] Brown adipose tissue serves a role in thermogenesis and therefore requires a lot of energy generation. PGC-1α expression was increased upon cold exposure, demonstrating its role in adaptive thermogenesis and in turn energy homeostasis.[97] The PGC-1 family also consists of PGC-1β and PGC-related coactivator (PRC). PGC-1β contains an N-terminal activation domain that is 40% homologous to PGC-1α, a 35% homologous central regulatory domain and a 48% homologous C-terminal RNA binding domain and is highly expressed in brown fat cells and in the heart.[98] In contrast, PRC contains an N-terminal domain of 28% homology to PGC-1α and a C-terminal domain of 44% homology. [99] It is important to note that PGC-1 proteins are not able to bind to DNA directly, therefore their control of transcription occurs through interactions with other transcription factors and recruitment of other coactivators that remodel chromatin for transcription.[100] PGC-1α is a 92kDa protein and contains a specific LXXLL nuclear hormone-binding motif, an activation domain as well as RNA processing factors.[14] The protein acetyl transferase p300 acetylates histones in response to PGC-1α binding to PPARγ. PGC-1α also directly interacts with the

TRAP/DRIP complex to attract the RNA polymerase II complex to initiate transcription.[101] RNA polymerase II can be phosphorylated via the binding of a protein called ménage-a-trois 1 (a component of cyclin-dependent kinase 7) to PGC-1α, therefore further regulating transcription.[102] Through the nuclear receptor-binding domain PGC-1α is able to bind to the NRF-1, NRF-2, ERRα and PPARα.[14] Binding of NRF-1 to PGC-1α activate genes involved in mitochondrial biogenesis.[103] Part of this regulatory pathway involves activation of Tfam, a factor required for mitochondrial DNA replication. In addition to Tfam activation, NRF-1 and NRF-2 bind to the promoters of mitochondrial transcription specificity factors TFB1M and TFB2M thus promoting mitochondrial biogenesis.[104] The promoters of many genes involved in oxidative phosphorylation contain many NRF-1α (GABPA) and ERRα binding sites for these transcription factors.[105] The transcriptional activity of ERRα has been noted to be dependent on the presence of PGC-1α, suggesting that PGC-1α can be considered a protein ligand for the activation of ERRα.[77] It is also interesting to note that activation of genes by ERRα is also dependent on the presence of PPARα.[16]

3.5 Regulation of PGC-1α Expression

PGC-1α is upregulated by the transcription factor myocyte enhancer factor 2 MEF2. There are two sites on the PGC-1α promoter where MEF2C binding occurs to directly regulate transcription of the gene. This activation of the PGC-1α gene by MEF2C is attenuated by HDAC5. It was shown that the down-regulation of the PGC-1α gene by HDAC5 was due to deacetylation of histones at the distal MEF2C binding site.[9]

PGC-1α is also activated through phosphorylation by p38 kinases. Increases cAMP levels activates p38 mitogen-activated protein (MAP) kinase, which in turn phosphorylates PGC-1α.[106] Furthermore, cAMP activates protein kinase A (PKA) that phosphorylates cAMP response element binding protein (CREB) in order to regulate PGC-1α expression.[10]

In mouse muscle cells, it was observed that PGC-1α forms a double positive feedback loop with ERRα and GABPA on the expression of genes involved in oxidative phosphorylation. A promoter database of the mouse genome was studied to observe ERRα and GABPA binding in response to PGC-1α expression. The database consisted of 2000 base pairs centered around the transcriptional start site of 5034 mouse genes. It is important to note that the ERRα binding motifs of TGACCTTG and TGACCTT were observed in this experiment.[105] In a microarray study, human RNA samples were taken from 59 patients with heart failure due to either ischemic heart disease or idiopathic dilated cardiomyopathy. These samples were compared to non-failing heart samples using a microarray to observe which genes are upregulated or downregulated due to heart failure.[107] It was found that there was a significant down-regulation in PGC-1α and ERRα gene expression as well as many of their gene targets. This is a good indication that an energy starved failing heart undergoes a change in gene expression. Studying the mechanism that leads to this down regulation in genes involved in energy metabolism may provide a potential treatment via regulation of these genes in the treatment of heart failure.

As previously discussed, ERRα activates many genes involved in oxidative metabolism. ERRα is activated by PGC-1α and both are expressed in response to

physiological stimuli such as exercise, thermogenesis and fasting. PGC-1α is also a know co-regulator for PPARα and GABPA transcription factors in order to activate metabolic genes. There is however a gap in the literature on the potential regulation of PGC-1α by ERRα (Figure 1). The regulation of PGC-1α by ERRα under physiological (normoxic) and pathophysiological (hypoxic) conditions is the current topic for study.

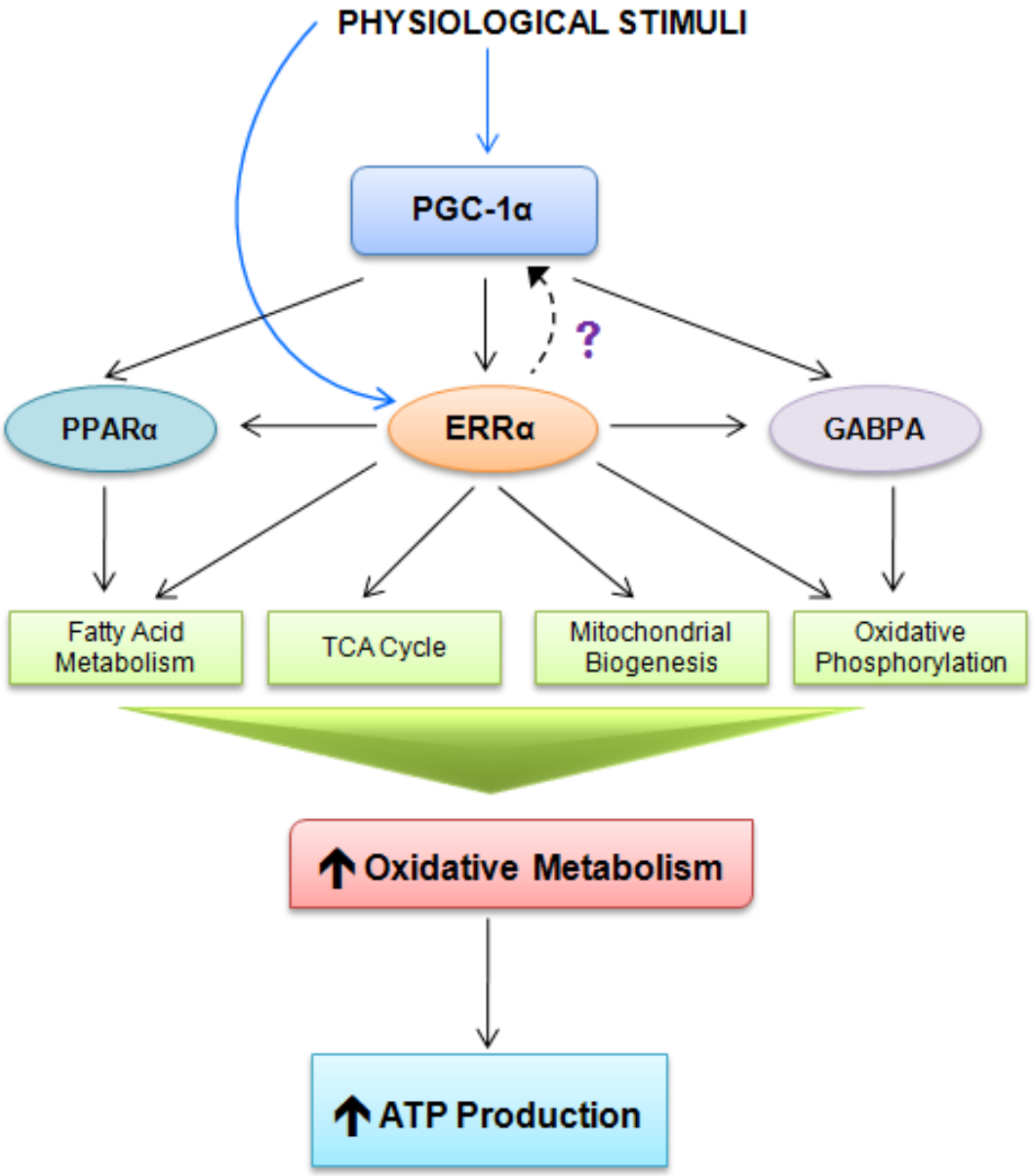


Figure 1: PGC-1α Gene Regulatory Cascade. This flow chart depicts the regulatory actions of PGC-1α on the activation of the transcription factors PPARα, ERRα and GABPA. Together these transcriptional regulators activate genes involved in oxidative metabolism (adapted from previous diagrams). [80,108]

VI Materials and Methods

1.0 Materials

Cell culture media (Dulbecco's Modified Eagle Medium , M199, OptiMEM, fetal bovine serum (FBS), horse serum (HS), L-glutamine and penicillin/streptomycin) were purchased from Hyclone (Logan, UT). Tissue culture plates and dishes were purchased from Fisher Scientific (Whitby, ON). Quickchange Site Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA). Lipofectamine 2000 Transfection Reagent was received from Invitrogen (Carlsbad, CA). The Luciferase Reporter Assay kit was purchased from Promega (Madison, WI). The biotin primer labeling kit, Biondine membrane and Electrophoretic Mobility Shift Assay (EMSA) kit were purchased from Pierce Biotechnology (Rockford, IL). Trichostatin A (TSA) was a gift from Dr. Lorrie Kirshenbaum's Lab. XCT790, Trypan blue and The GenElute Mammalian Total RNA Miniprep Kit was purchased from Sigma-Aldrich (Oakville, ON), while the B-R 1 Step SYBR Green qRT-PCR kit was purchased from Quanta Bioscience (Gaithersburg, MD). The ERRα antibody and rabbit IgG antibody used for the ChIP assay was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The acetyl histone H3 antibody used for ChIP assays were purchased from Cell Signaling (Danvers, MA). Protein A agarose beads was purchased from Thermo Scientific (Rockford, IL), while the EconoTaq DNA Polymerase was purchased from Lucigen (Middleton, WI). Phenol-chloroform 1:1 was attained from EMD Chemicals Inc. (Gibbstown, NJ), glycogen from USB (Cleveland, OH) and Complete Protease Inhibitor Cocktail Tablets from Roche (Mannheim,

Germany). ERRα expressing adenovirus was a gift from Dr. Dan Kelly's lab (Sanford-Burnham Medical Research Institute, Lake Nona, Orlando).

2.0 Methods

2.1 Cell Culture

COS7 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine (cell media). Primary cardiomyocytes were isolated from 1-day-old neonatal rat pups. Hearts were extracted from the pups and digested for 10 minutes with pancreatin in 1x phosphate buffered saline (PBS). Cells were then collected by centrifuging at 2000 rpm for 2 minutes and resuspended in DMEM supplemented with 16.6% M199, 1% FBS, 10% HS 1% penicillin/streptomycin and 1% L-glutamine (DMEM-M199). 18 digestion cycles were performed. Cells were then pre-plated and incubated for 1 hour on polystyrene tissue culture plates. Unattached cells were then collected and plated on 0.1% gelatin-coated plates. Animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and the University of Manitoba Animal Protocol Management and Review Committee.

2.2 Treatment of Neonatal Rat Cardiomyocytes

Primary neonatal rat cardiomyocytes were maintained in DMEM-M199 supplemented media. 1 million cells were plated per well on 6 well plates and 8 million cells were plated on 10 cm plates. Prior to any treatments, cardiomyoctes were incubated for 24 h in DMEM-M199 media (1% HS 1% penicillin/streptomycin and 1% L-

glutamine) in the absence of serum. This starvation media was then replaced with serum-supplemented DMEM-M199 media and incubated for 18 hours prior to any treatments. Cardiomyocytes were incubated under hypoxic conditions (0% O_2) for 3, 6, 9 or 12 hours. Cardiomyocytes were also treated with 100 nM TSA, 10 mM XCT790 or dimethyl sulfoxide (DMSO) (as a vehicle control) as required and incubated under hypoxic conditions. Some cardiomyocytes were also infected with either an adenovirus encoding green fluorescent protein (Ad-GFP) or ERRα (Ad-ERRα) using a multiplicity of infection of 100 prior to hypoxia. Cells were harvested following treatments for total RNA, ChIP assays, or for trypan blue cell viability assays.

2.3 Nuclear Extract

COS7 cells were plated on 10 cm cell culture plates 24 h prior to transfection and used at ~70-80% confluence. Prior to transfection, media was changed to OptiMEM. Each well was transiently co-transfected with 1μg ERRα expression vector pCMX-ERRα. Transfection was performed using Lipofectamine 2000 Transfection Reagent as per the manufacturer's instructions. Cells were incubated for 18 hours at 37^{o}C. The media was changed back to cell media and further incubated at 37^{o}C for 24 hours. Transfected cells were harvested by adding 500 ml lysis buffer A (10 mM 4-(2-hydroxyethyl) piperazine-1 ethanesulfonic acid (HEPES), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.05% nonyl phenoxylpolyethoxylethanol (NP40), 1x protease inhibitor (Roche), 1 mM phenylmethylsulfonyl (PMSF), pH 7.9) to the culture plate on ice and incubated for 10 minutes. Cells were then scraped thoroughly, collected and

centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the pellet containing the nuclei was resuspended on ice in 374 μl lysis buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 26% glycerol (v/v), pH 7.9) and 26 μl 4.6 M NaCl to give a final high NaCl concentration of 300 mM, which helps lyse membranes and forces DNA into solution. The pellet plus lysis buffer was then homogenized using a Dounce homogenizer (20 full strokes) on ice. The homogenate was incubated on ice for 30 minutes. Samples were then centrifuged at max speed (14 000 rpm) for 25 minutes. The supernatant containing the nuclear extract was then collected and stored at -80^{o}C.

2.4 Electrophoretic Mobility Shift Assay

Assay was performed with intact or mutated oligonucleotides. Oligonucleotides were made bearing the specific sequence of the PGC-1α promoter site to which ERRα may bind. These oligonucleotides were labeled with biotinylated dNTPs using a Pierce kit as per manufacturer's instructions. Nuclear extracts were isolated from COS7 cells transfected with the expression vector for ERRα (pCMX-ERRα). This purified nuclear extract was hybridized with the labeled oligonucleotides using the Pierce EMSA kit, as per manufacturer's instructions. The protein-DNA complexes were separated from free (unbound) DNA by electrophoresis through a nondenaturing 6% native polyacrylamide gel. The protein-DNA complex slows the mobility of the DNA fragments to which it binds. The DNA fragments were then transferred to a positively charged nylon membrane (Pierce), UV cross-linked, probed with streptavidin-HRP conjugate, incubated

with the chemiluminescent substrate and developed using X-ray film to visualize the shift. Free DNA migrates faster through the gel than the DNA-protein complex, displaying a shift when ERRα binds to the promoter.

2.5 Chromatin Immunoprecipitation Assay

Using neonatal rat cardiomyocytes, a ChIP assays were performed, precipitating cross-linked protein-DNA complexes using an antibody specific to ERRα (Santa Cruz) or acetyl histone 3 (Upstate). One 10 cm plate of cardiomyocytes was used per condition. After treatment of cardiomyocytes, proteins were cross linked to DNA by adding 270 μl 37% formaldehyde into the 10 ml media on plate (final concentration of 1%) and incubated for 10 minutes at 37^{o}C. Media was then aspirated off and cells were washed twice using ice cold 1x PBS with protease inhibitors (1x) and PMSF (1 mM). Cells were scraped off and centrifuged for 4 minutes at 2000 rpm at 4^{o}C. The cell pellet was resuspended in 200 μl sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, plus protease inhibitors (1X) and PMSF (1 mM)). Cell lysate was sonicated on ice (3 repetitions for 10 second pulse) to sheer DNA to 500 to 200 base pair fragments. Samples were then centrifuged for 10 minutes at 13 000 rpm at 4^{o}C. The supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl plus 1x protease inhibitor and 1 mM PMSF). This was done by adding 1800 μl ChIP dilution buffer to the 200 μl sonicated cell supernatant for a final volume of 2 ml in each immunoprecipitation condition. 1% of the diluted sample was kept for use as the input

control. To reduce nonspecific background, samples were pre-cleared with 75 μl protein A agarose/ BSA/herring Sperm DNA (0.5mg/ml BSA, 200 μg/ml herring sperm DNA, protein A agarose (to make up the rest of the volume)) for 1 hour at 4^{o}C with rotation. Agarose complexes were then centrifuged for 1 minute at 1000 rpm at 4^{o}C and the supernatant fraction was collected. 10 mg of immunoprecipitating antibody (either ERRα or acetyl histone H3) was added to the samples and incubated overnight at 4^{o}C with rotation. For a negative control, 10 mg non-specific IgG antibody was used in a separate sample. 60 μl of protein A agarose/ herring sperm DNA slurry was then added to the samples and incubated for one hour at 4^{o}C with rotation to collect the antibody/protein/DNA complex. The complexes were then centrifuged (1000 rpm at 4^{o}C for 1 minute) and the supernatant was carefully removed. The agarose/antibody/protein/DNA complex was then washed for 3 minutes on a rotating platform with 1 ml of the following buffers: low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl immune complex wash buffer (250 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), Tris-EDTA (TE) buffer (two washes). After each wash, agarose/antibody/protein/DNA complex was centrifuged and the buffer was carefully removed. Protein was eluted from the antibody by adding 250 μL elution buffer (1% SDS, 0.1M $NaHCO_3$) to the pelleted complex and incubated at room temperature for 15 minutes with rotation. Agarose was spun down and supernatant was carefully collected. This elution step was repeated and the total volume of combined eluates was 500 μL. To

reverse protein-DNA crosslinks, 20 µl 5 M NaCl was added to the eluates with incubation at 65^{o}C for 4 hours. Samples were then cleared with 10 µl 0.5 M EDTA, 20 µl 1 M Tris-HCl pH 6.5 and 2 µl 10 mg/mL proteinase K and incubate for one hour at 45^{o}C. DNA was recovered by phenol/chloroform extraction. 500 µl phenol:chloroform (1:1) was added to samples then centrifuged for 5 minutes at 2000 rpm. The supernatant was collected and 500 µl chloroform was added to samples followed by centrifugation for 5 minutes at 2000 rpm. The supernatant was collected and 1 ml of 100% ethanol, 50 µl sodium acetate and 1 µl glycogen was added to each sample, then incubated at -80^{o}C for 30 minutes. Samples were then centrifuged for 25 minutes at room temperature at 14 000 rpm. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and then resuspended in 30 µl double-distilled H_2O. Polymerase chain reactions (PCR) were performed using primers specific to the putative binding region on the rat PGC-1α promoter to amplify a 199 base pair region (Table 1). EconoTaq Polymerase, as per manufacturer's instructions, was used to amplify the 30 ml isolated sample amplicons were resolved on a 2% agarose gel.

2.6 Luciferase Reporter Assay

COS7 cells were plated in 6-well cell culture plates 24 hours prior to transfection and used at ~70-80% confluence. Prior to transfection media was changed to OptiMEM. Each well was transiently co-transfected with either 500 ng empty expression vector pCMX (control) or an ERRα expression vector, plus 500 ng pGL3 basic, reporter

plasmid pGL3-PGC-1α (mouse *PGC-1α* 3.1 kb promoter driving luciferase) or the PGC-1α promoter with mutations in the putative ERRα binding site (ATGACCTT to GGTACGCT). Mutations to the promoter were generated from the parent vector pGL3-PGC-1α by nested PCR, primers used are listed in Table 1. All samples were transfected with 5 ng renilla luciferase expression plasmid (pRL) for normalization. Transfection was performed using Lipofectamine 2000 Transfection Reagent as per the manufacturer's instructions. Cells were incubated for 24 hours and then media was changed back to cell media. At this time XCT790 was added to some cells as a treatment. Cells were then incubated for another 24 hours. Samples were lysed and assayed using the Luciferase Reporter Assay Kit from Promega. The luciferase activity of each sample was measured on a TD20/20 luminometer (Turner BioSystems, USA).

2.7 Quantitative Real-Time PCR

RNA was isolated from neonatal rat cardiomyocytes using a GenElute Mammalian Total RNA Miniprep Kit as per the manufacturer's instructions. RNA was quantified spectrophotometrically and 25 ng RNA was used for real-time PCR reactions, which were performed using the Quanta Biosciences B-R 1 Step SYBR qRT-PCR kit as per manufacturer's instructions. The primers used for the amplification of the PGC-1α, ERRα, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes are listed in Table 1 and were ordered from Sigma. A Bio-Rad iQ5 real-time PCR machine was used for the amplification reactions. The following thermocycler program conditions were used: an initial denaturing step at 50°C for 10 minutes to establish persistent well factors,

a denaturing step at 95°C for 5 minutes, 45 cycles of 10 seconds denaturing at 95°C and 30 seconds of annealing at 60°C (data collection point), 1 minute at 95°C and an annealing step of 1 minutes at 55°C. Melt curve data was collected from 81 cycles of an initial annealing at 55°C followed by a temperature increase of 0.5°C with each succeeding cycle. The cycling and melt curve parameters were consistent with the B-R 1 Step SYBR qRT-PCR kit. Data was normalized to GAPDH and was calculated using the $2^{-\Delta\Delta CT}$ method.

2.8 Trypan Blue Exclusion Test of Cell Viability

After hypoxic treatments as mentioned above (exposure to hypoxia, plus treatment with TSA or XCT790), neonatal rat cardiomyocytes were washed twice with 1x PBS and harvested from the culture plates by gentle scraping. Cells were centrifuged for 1 minute at 1000 rpm at room temperature and the supernatant was discarded. Cells were then resuspended in 1 ml PBS. One part cell suspension was then mixed with one part 0.4% trypan blue and incubated for 3 minutes at room temperature. Unstained cells (viable cells) and stained (nonviable cells) were counted using a hemocytometer. Percent viability was then calculated by dividing total number of unstained cells by total number of cells.

2.9 Primers

Table 1: Sequences of forward (fwd) and reverse (rev) primers and their usage for various applications.

Primer	Sequence	Usage
ERRα binding site Mutation (fwd)	GACTGTGTGGAAAGTAGAGCCCGGTACCGCTGTCCTGAATTTTAATAG	Mutagenesis
ERRα binding site Mutation (rev)	CTATTAAAATTCAGGACAGCGGTACCGGGCTCTACTTTCCACACAGTC	Mutagenesis
ERRα binding site (fwd)	GGAAAGTAGAGCCCATGACCTTTGTCCTGAATTTTAATAGT	EMSA
ERRα binding site (rev)	ACTATTAAAATTCAGGACAAAGGTCATGGGCTCTACTTTCC	EMSA
Mutated ERRα binding site (fwd)	GGAAAGTAGAGCCCGGTACCGCTGTCCTGAATTTTAATAGT	EMSA
Mutated ERRα binding site (rev)	ACTATTAAAATTCAGGACAGCGGTACCGGGCTCTACTTTCC	EMSA
ERRα binding site (fwd)	GTATCAGTTACCATCAGGATGCC	ChIP
ERRα binding site (rev)	CTTACTCTGTACTCCCACAAAGAAACAC	ChIP
PGC-1α (fwd)	AAGTGTGGAACTCTCTGGAACTG	RT-PCR
PGC-1α (rev)	GGGTTATCTTGGTTGGCTTTATG	RT-PCR
ERRα (fwd)	ACTGCCACTGCAGGATGAG	RT-PCR
ERRα (rev)	CACAGCCTCAGCATCTTCAA	RT-PCR
GAPDH (fwd)	TGCACCACCAACTGCTTAGC	RT-PCR
GAPDH (rev)	GGCATGGACTGTGGTCATGAG	RT-PCR

2.10 Statistical Analysis

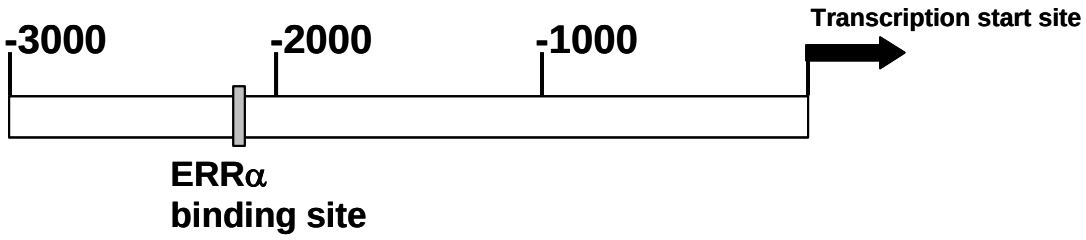
Values were calculated with standard errors of the mean. Statistical significance of data collected was determined by the Student's t-test, or by Student-Neuman-Keuls post hoc analysis of variance (ANOVA). $p<0.05$ was considered statistically significant.

VII Results

1.0 ERRα binds to the PGC-1α Promoter

We hypothesize that estrogen related receptor ERRα, a central regulator of cardiac energy metabolism, regulates expression of the PGC-1α gene via direct interaction with the PGC-1α gene promoter. Through *in silico* analysis we have identified the sequence, AAGGTCA (reverse complement of TGACCTT) as a potential binding site for ERRα as it related to the ERRE consensus sequence, TNAAGGTCA (Figure 2).[80] To identify whether ERRα binds to the putative binding site within the PGC-1α promoter, an EMSA was performed to visualize a band shift *in vitro* where ERRα binds to the specific sequence. This putative binding site was also mutated to test whether this would cause a loss of ERRα binding to the sequence. Biotin-labeled oligonucleotides of the putative binding site (intact and mutated) were incubated with nuclear extract from ERRα-transfected COS7 cells. Complexes were electrophoresed on a 6% native acrylamide gel. DNA was transferred to a nylon membrane, UV cross-linked, probed with streptavidin-HRP conjugate and incubated with a chemiluminescent substrate to visualize the shift. It was observed that incubation of nuclear extract from ERRα over-expressing COS7 cells with labeled intact oligonucleotide caused a band shift when compared to labeled oligonucleotide alone. When the nuclear extract was incubated with labeled oligonucleotide and an excess of unlabeled oligonucleotide, no band shift was observed due to competition with the labeled oligonucleotide. No band shift was observed when the oligonucleotide containing the mutated binding site was used (Figure 3A). A ChIP assay was performed on neonatal rat cardiomyocytes using an antibody

specific to ERRα to test whether ERRα binds to the putative site on the PGC-1α promoter *in vivo*. A non-specific IgG was used as a negative control. PCR was used to amplify a 199 base pair region around the PGC-1α promoter ERRα binding site. Genomic DNA was used as an input control. This ChIP assay shows that ERRα binds to the PGC-1α promoter *in vivo* (Figure 3B).

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Human      CACAGTTGATCGTT--GAGGGCAGAGCCAATGACCTTTGTCCTGAATTTTAATAATTTAC -1978
Mouse      CACAGTTGACTGTGTGGAAAGTAGAGCCCATGACCTTTGTCCTGAATTTTAATAGTTTAC -2130
Rat        CACAGTTGACTGTGTGGAGGGTAGAGCCAATGACCTTTGTCCTGAATTTTAATAGTTTAC -2394
           *********  **   **  * ****** ************************ *****
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Figure 2: Map of PGC-1α promoter (3.1 kb). Upper panel, schematic of the mouse PGC-1α promoter, with distance relative to the transcription start site indicated in bases. The gray box represents the putative ERRα binding site. Lower panel, alignment of putative ERRα binding sites in PGC-1α gene promoters from humans, mice and rats. Numbers indicate distance in base pairs from transcription start site.

2.0 ERRα Transactivates the PGC-1α Promoter

To determine whether ERRα activates the PGC-1α promoter by directly binding to it, a luciferase reporter assay was performed. In this assay, COS7 cells were transiently co-transfected with empty expression vector pCMX (control) or an ERRα expression

vector (pCMX-ERRα), plus the previously cloned mouse PGC-1α 3.1 kb promoter containing the putative ERRα binding site cloned into the luciferase reporter pGL3 basic.[9] A renilla luciferase expression plasmid, which emits light at a different wavelength as compared to the firefly luciferase gene present in the PGC-1α reporter construct, was used as a transfection control. Results were normalized to empty vector (pCMX). ERRα significantly transactivated the PGC-1α promoter (Figure 4A). To further confirm the site of ERRα binding to the PGC-1α promoter, the putative binding site was mutated (ATGACCTT to GGTACGCT) and a luciferase assay was performed. COS7 cells were transfected either with empty pGL3 basic (pGL3), intact PGC-1α promoter, or PGC-1α promoter bearing a mutated ERRα binding site. All samples were co-transfected with an ERRα expression vector. A renilla luciferase expression plasmid was used as a transfection control. Activation of the promoter bearing a mutated ERRα binding site was significantly decreased (Figure 4B), indicating that ERRα was unable to bind to the altered sequence and in turn activation of the promoter was down-regulated. Activity of the promoter was not completely lost, suggesting that ERRα may interact with other sites on the promoter.

3.0 ERRα Mediates Cardiomyocyte PGC-1α Expression

We measured changes in PGC-1α gene expression in response to ERRα over-expression in neonatal rat cardiomyocytes. Cardiomyocytes were transiently transfected with either empty expression vector pCMX or an ERRα expression vector, pCMX-

ERRα, then incubated for 24 hours. Total RNA was isolated and used for quantitative real-time PCR to assay expression of ERRα and PGC-1α mRNA, using GAPDH as an internal control. As expected, ERRα mRNA expression significantly increased in the cells transfected with the ERRα expression vector (Figure 5A). There was also a significant increase in PGC-1α mRNA expression, suggesting that ERRα up–regulates PGC-1α gene expression (Figure 5B).

To observe whether blocking the activity of ERRα has an effect on transactivation of the PGC-1α promoter, the ERRα inverse agonist XCT790 was added to the luciferase reporter assay. COS7 cells were transiently co-transfected with an ERRα expression vector plus pGL3-PGC-1α. A renilla expression plasmid was used as a transfection control. Cells were treated with 5 or 10 μM XCT790 for 24 hours following transfection. Control cells were treated with an equivalent volume of DMSO, as a vehicle control. XCT790 significantly decreased the transactivation of the PGC-1α promoter (Figure 6A). To measure the effect of XCT790 on PGC-1α gene expression, neonatal rat cardiomyocytes were incubated with 5, 10 or 20 μM XCT790 for 24 hours. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA. GAPDH was used as an internal control. 10 or 20 μM XCT790 significantly decreased the expression of PGC-1α mRNA (Figure 6B), indicating that by inhibiting the activity of ERRα with the use of an inverse agonist, PGC-1α expression is down-regulated, further confirming ERRα's role in PGC-1α gene regulation.

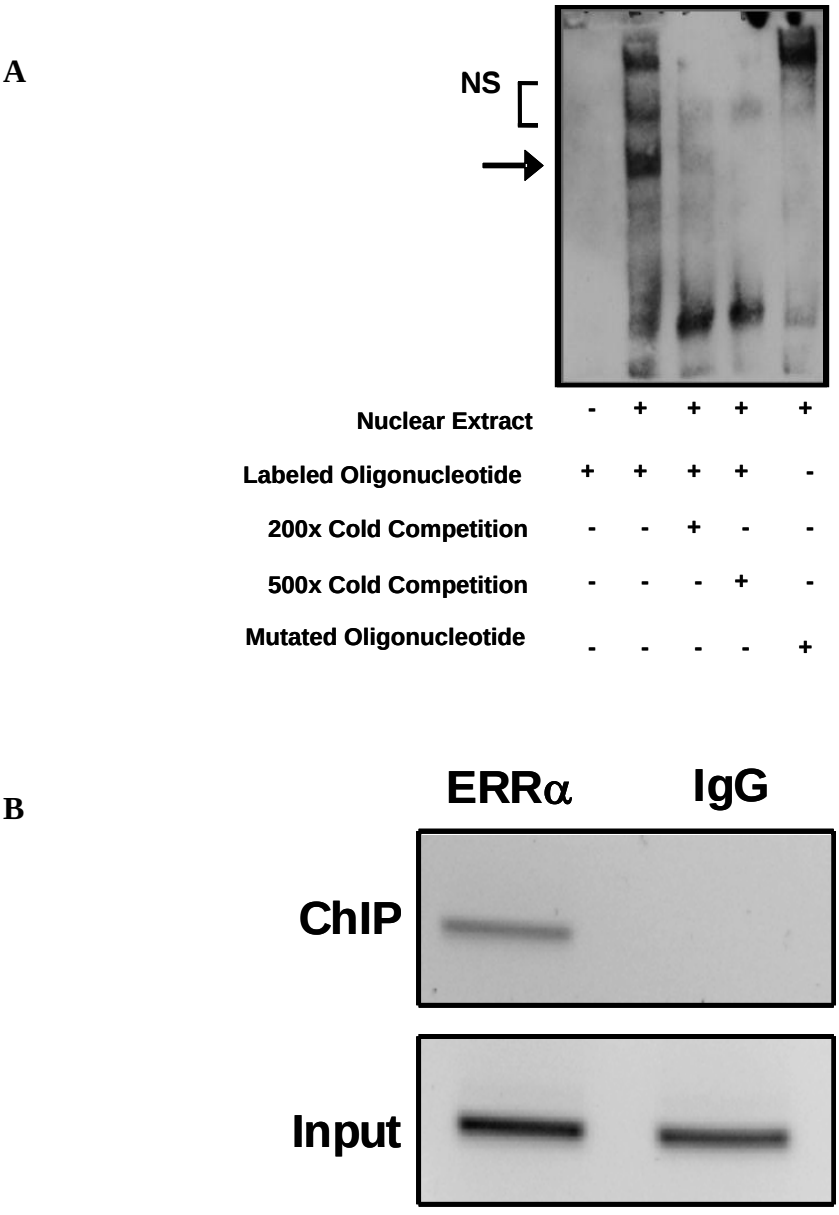


Figure 3: A) Electrophoretic mobility shift assay demonstrating ERRα binding to its putative binding site. Biotin-labeled oligonucleotides were incubated with nuclear extract from ERRα-transfected COS7 cells. Complexes were electrophoresed on a 6% native acrylamide gel. DNA was transferred to a nylon membrane, UV cross-linked, probed with streptavidin-HRP conjugate and incubated with a chemiluminescent substrate to visualize the shift. NS, non-specific binding. **B) Chromatin immunoprecipitation (ChIP) demonstrating ERRα binding to the PGC-1α promoter *in vivo*.** A ChIP assay was performed on neonatal rat cardiomyocytes using an antibody specific to ERRα or a non-specific IgG as negative control. PCR was used to amplify a 199 base pair region encompassing the PGC-1α promoter ERRα binding site. Genomic DNA was used as an input control.

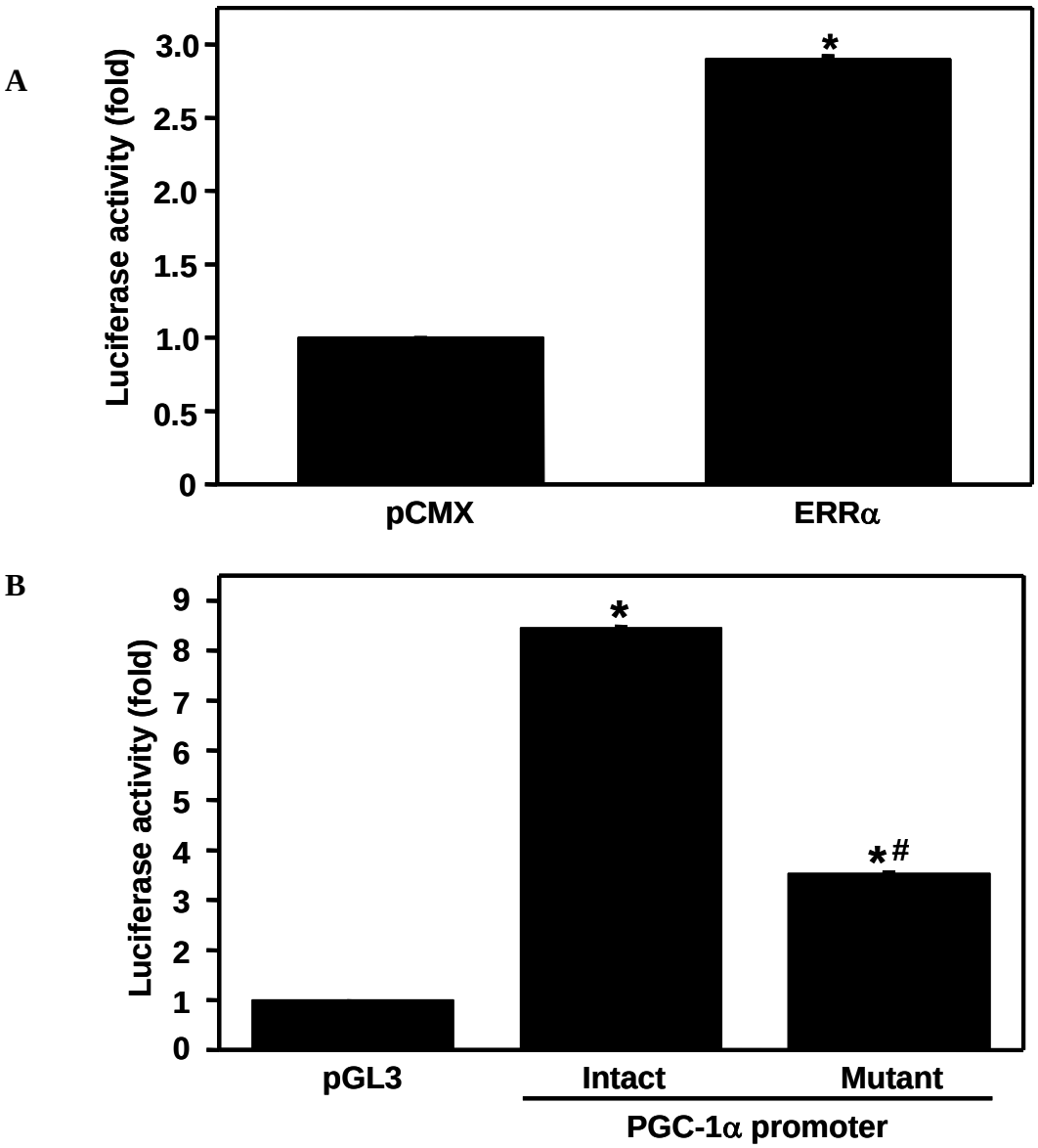


Figure 4: A) ERRα transactivates the PGC-1α promoter. COS7 cells were transiently co-transfected with empty expression vector pCMX (control) or an ERRα expression vector, plus the mouse PGC-1α 3.1 kb promoter cloned into the luciferase reporter pGL3 basic. A renilla expression plasmid was used as a transfection control. Results were normalized to empty vector (pCMX). ERRα significantly transactivated the PGC-1α promoter. n=3 *P<0.05 versus empty vector. **B) Mutation of the putative ERRα binding site attenuates PGC-1α promoter transactivation by ERRα.** COS7 cells were transfected either with empty pGL3 basic (pGL3), intact pGL3-PGC-1α promoter, or PGC-1α promoter bearing a mutated ERRα binding site (ATGACCTT to GGTACGCT). All samples were co-transfected with an ERRα expression vector. A renilla expression plasmid was used as a transfection control. Activation of the promoter bearing a mutated ERRα binding site was significantly decreased. n=3 *P<0.05 versus empty pGL3 basic vector; #P<0.05 versus intact PGC-1α promoter.

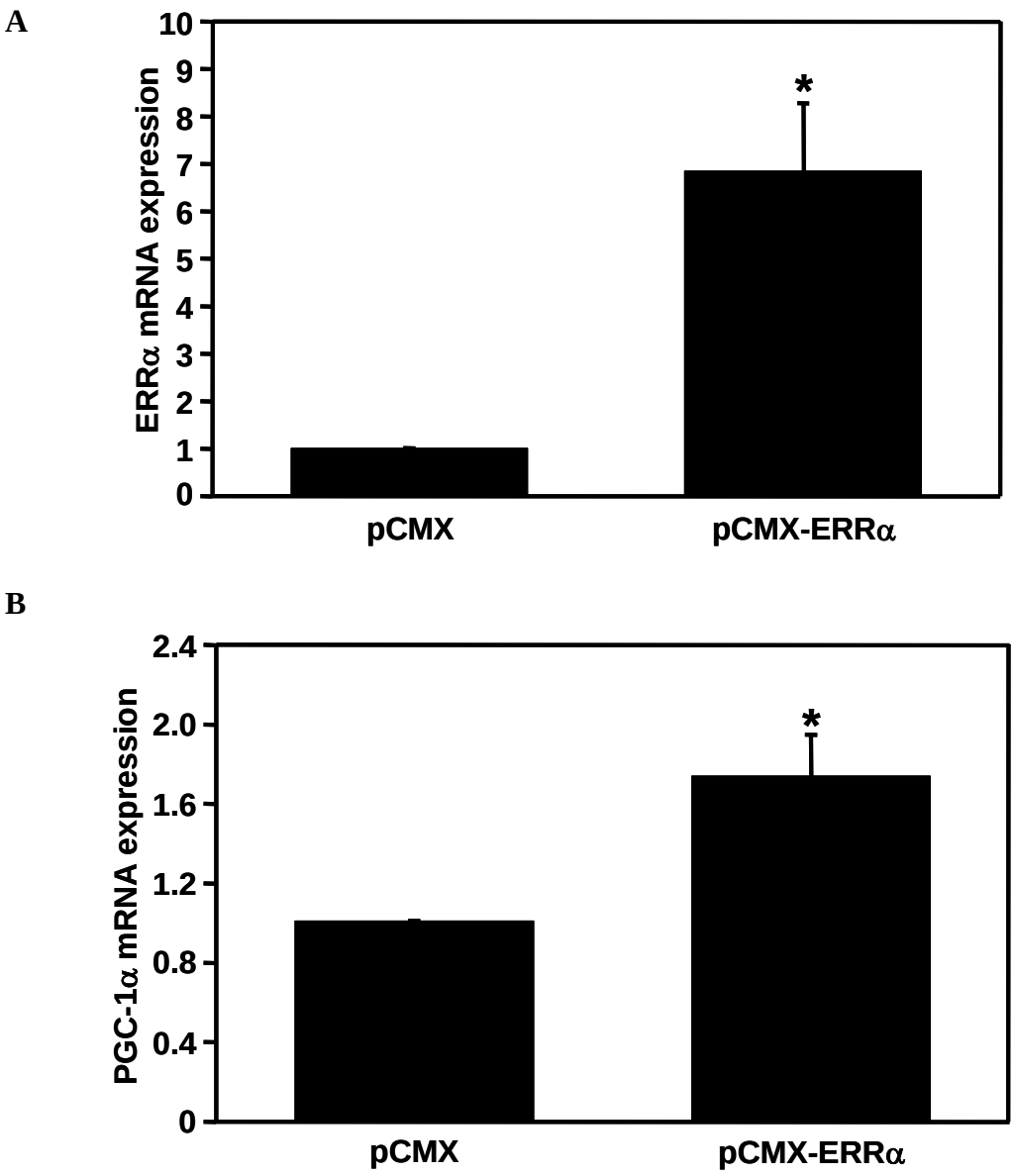


Figure 5: A) Over-expression of ERRα in cardiomyocytes. Cardiomyocytes were transiently co-transfected with either empty expression vector pCMX or an ERRα expression vector, pCMX-ERRα, then incubated for 24 hours. Total RNA was isolated and used for quantitative real-time PCR to assay expression of ERRα mRNA, using GAPDH as an internal control. *n=3* *P<0.05 versus control. **B) Expression of PGC-1α is up-regulated in cardiomyocytes by ERRα over-expression.** Cardiomyocytes were transiently co-transfected with either empty expression vector pCMX or an ERRα expression vector, pCMX-ERRα, then incubated for 24 hours. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA, using GAPDH as an internal control. *n=3* *P<0.05 versus control.

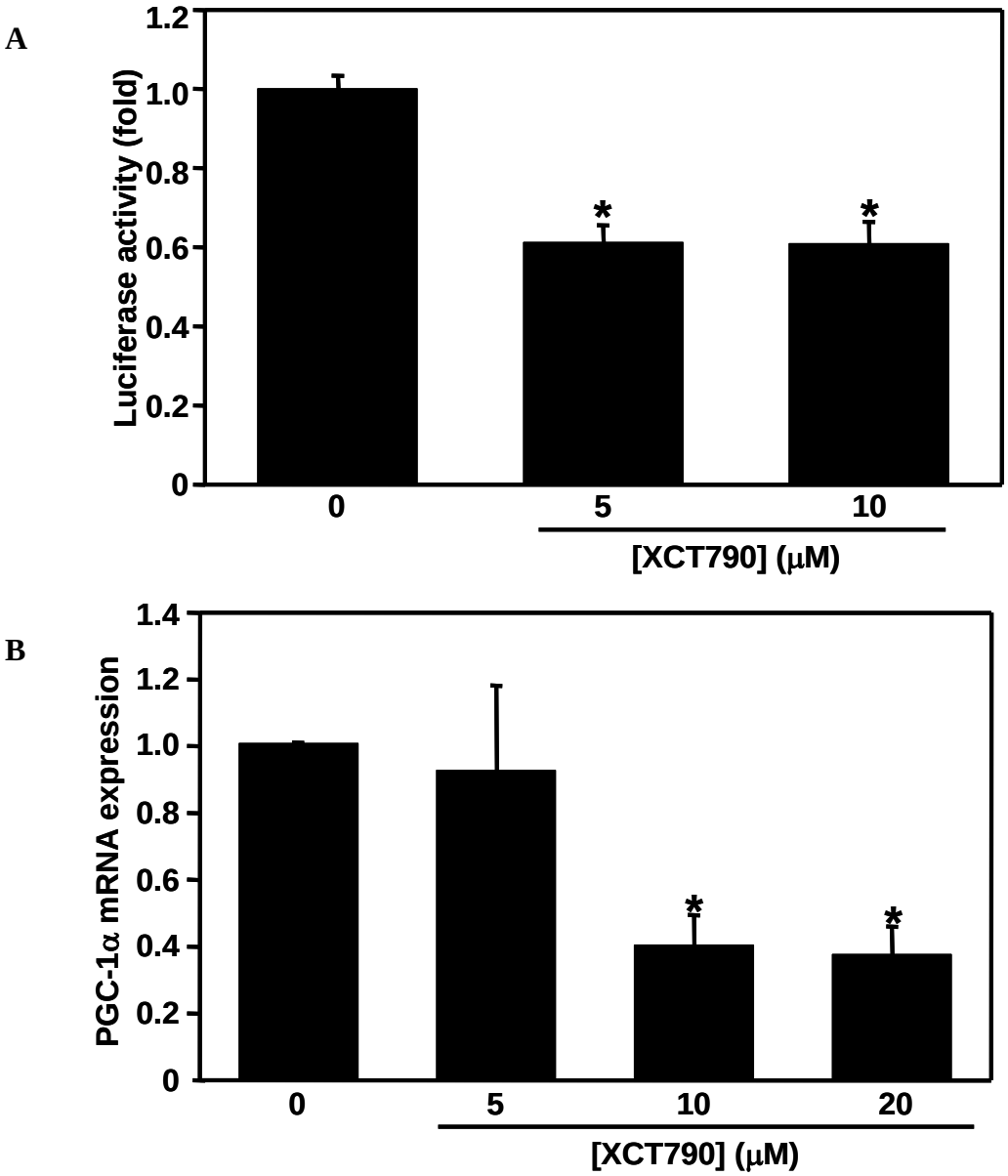


Figure 6: A) ERRα-mediated transactivation of the PGC-1α promoter is attenuated by the ERRα inverse agonist XCT790. COS7 cells were transiently co-transfected with an ERRα expression vector plus pGL3-PGC-1α. A renilla luciferase expression plasmid was used as a transfection control. Cells were treated with 5 or 10 μM XCT790 for 24 hours following transfection. XCT790 significantly decreased the transactivation of the PGC-1α promoter. *n=3* *P<0.05 versus vehicle. **B) XCT790 inhibits PGC-1α expression in cardiomyocytes.** Cardiomyocytes were incubated with 5, 10 or 20 μM XCT790 for 24 hours. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA. 10 or 20 μM XCT790 significantly decreased the expression of PGC-1α mRNA. *n=3* *P<0.05 versus vehicle.

4.0 Viability of Neonatal Rat Cardiomyocytes in Response to Hypoxia

We examined the effect of hypoxia on the expression and occupancy of the PGC-1α promoter. We created a model of an under-perfused myocardium by incubating neonatal rat cardiomyocytes under hypoxic conditions (0% O_2), without changing culture medium. To demonstrate that any changes in gene expression or occupancy of the promoter by ERRα was due to exposure to hypoxia and not cell death, we performed a cell viability assay using trypan blue exclusion. Hypoxia, with or without TSA (100 nM) or XCT790 (10 μM) did not adversely affect cell survival compared to normoxia (Figure 7).

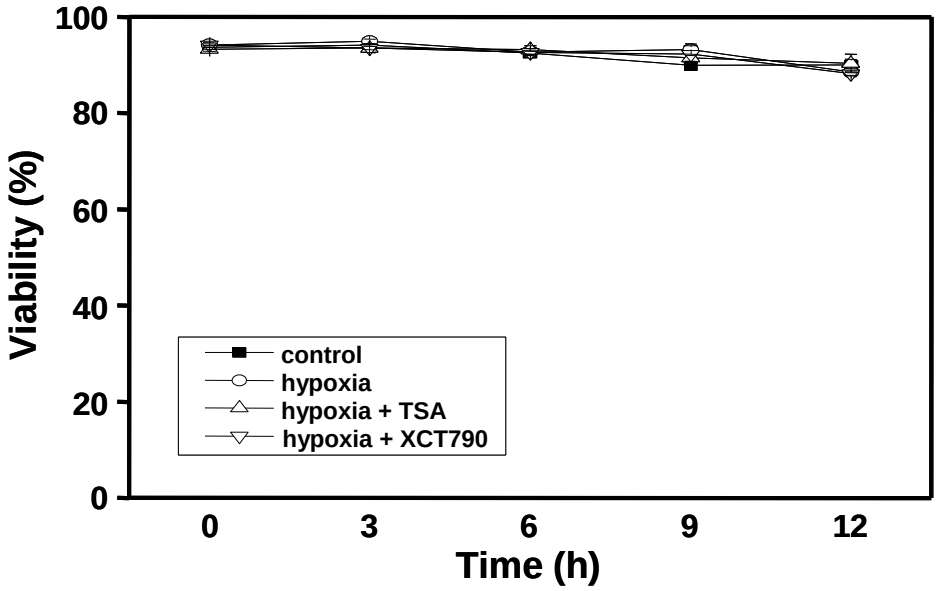
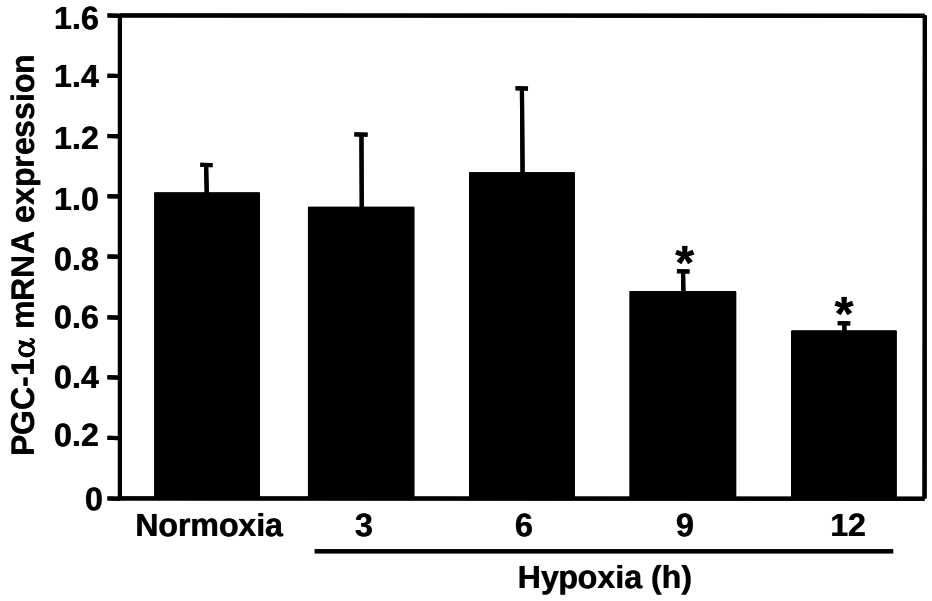


Figure 7: Figure 7: Viability of neonatal rat cardiomyocytes in response to hypoxia. Cell viability was assayed by trypan blue exclusion. Hypoxia, with or without TSA (100 nM) or XCT790 (10 μM) did not adversely affect cell survival compared to normoxia. n=3.

5.0 PGC-1α and ERRα Are Down-Regulated in Hypoxic Cardiomyocytes

Cardiomyocytes require oxygen to act as the final electron acceptor in the electron transport chain in order to produce ATP. If there is no oxygen available, there will be a loss of ATP production by the mitochondria. The heart may experience hypoxic conditions when arteries carrying oxygenated blood become occluded and blood flow is reduced. To study the effect of hypoxia on the expression of PGC-1α, neonatal rat cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours. Control cardiomyocytes were maintained under normoxic conditions. Total RNA was isolated from cells and PGC-1α and ERRα expression was measured by quantitative real-time PCR, using primers specific to the PGC-1α and ERRα genes. GAPDH was used as an internal control and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. After 9 hours of exposure to hypoxia PGC-1α mRNA expression was significantly decreased (Figure 8A). Conversely a significant decrease in ERRα mRNA expression was measured as early as 3 hours of exposure to hypoxia (Figure 8B). Since ERRα is down-regulated after 3 hours of hypoxia compared to PGC-1α down-regulation after 9 hours of hypoxia, this indicates that loss of ERRα expression may contribute to loss of PGC-1α expression.

A



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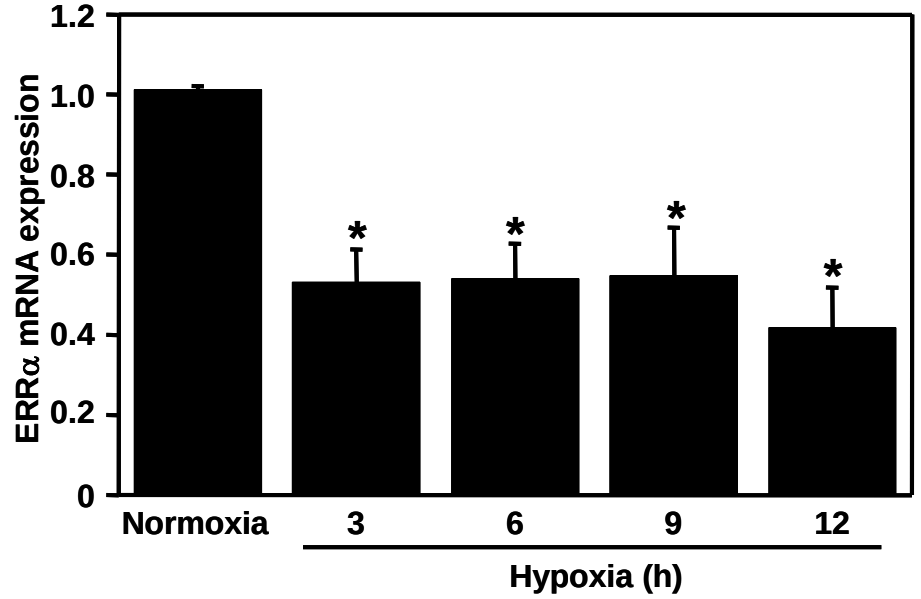

Figure 8: A) PGC-1α is down-regulated in cardiomyocytes exposed to hypoxia. Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours. Total RNA was isolated from cells and PGC-1α expression measured by quantitative real-time PCR, using GAPDH as an internal normoxia. *n=3* *P<0.05 versus control. **B) ERRα is down-regulated in cardiomyocytes exposed to hypoxia.** Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours. Total RNA was isolated from cells and ERRα expression measured by quantitative real-time PCR. *n=3* *P<0.05 versus normoxia.

6.0 ERRα Inverse Agonist XCT790 Accelerates Down-Regulation of PGC-1α Expression in Hypoxic Cardiomyocytes

By inhibiting the activity of ERRα with use of the ERRα inverse agonist XCT790, expression of PGC-1α was measured in neonatal rat cardiomyocytes after exposure to hypoxia. Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours in the presence of 10 μM XCT790 or vehicle (DMSO). Control cardiomyocytes were normoxic. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA, using GAPDH as an internal control. XCT790 appears to accelerate PGC-1α down-regulation after 6 hours of exposure to hypoxia (Figure 9).

7.0 PGC-1α Promoter ERRα Occupancy and Histone H3 Acetylation in Hypoxic Cardiomyocytes

A ChIP assay was performed on neonatal rat cardiomyocytes using an antibody specific to ERRα to test whether occupancy of the PGC-1α promoter by ERRα is altered under hypoxic conditions. Cardiomyocytes were incubated under hypoxic conditions for 3, 6, 9 or 12 hours. Normoxic cardiomyocytes were used as a control. A non-specific IgG was used as a negative control. PCR was used to amplify a 199 base pair region around the PGC-1α promoter ERRα binding site. Genomic DNA was used as an input control. This ChIP assay showed that occupancy of the PGC-1α promoter by ERRα does

decrease after 12 hours of hypoxia (Figure 10 upper panel). However, this does not correspond to the down regulation of PGC-1α expression after 9 hours of exposure to hypoxia. A ChIP assay using an antibody specific to acetylated histone H3 showed a decrease in acetylated histones surrounding the ERRα binding site on the PGC-1α promoter by 6 hours of hypoxia (Figure 10 lower panel).

8.0 Down-Regulation of PGC-1α and ERRα During Hypoxia is Attenuated by the Histone Deacetylase Inhibitor Trichostatin A

After observing a decrease in acetylated histones on the PGC-1α promoter around the putative ERRα binding site, we treated neonatal rat cardiomyocytes with the histone deacetylase inhibitor TSA during hypoxia to observe any changes in PGC-1α and ERRα gene expression. Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours in the presence of 100 nM TSA or vehicle (DMSO). Control cardiomyocytes were normoxic. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α and ERRα mRNA, using GAPDH as an internal control. Down-regulation of PGC-1α, after 9 hours of exposure to hypoxia was attenuated by TSA (Figure 11A). Similar results were observed for ERRα expression (Figure 11B).

9.0 Down-Regulation of PGC-1α by Hypoxia is Attenuated by Over-Expression of ERRα

To measure the effect of over-expression of ERRα on hypoxia-mediated PGC-1α down-regualtion, neonatal rat cardiomyocytes were infected with GFP- or ERRα-encoding adenovirus 18 hours prior to hypoxia (0% O_2) for 3, 6, 9, or 12 hours. Untreated cardiomyocytes were normoxic and uninfected. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of ERRα or PGC-1α mRNA, using GAPDH as an internal control (Figure 12). As expected, under normoxic conditions over-expression of ERRα lead to a significant increase in ERRα. This lead to a decrease in PGC-1α mRNA levels. After 12 hours of exposure to hypoxic conditions down-regulation of ERRα and PGC-1α was observed in the GFP-infected cardiomyocytes. This down-regulation was attenuated in cardiomyocytes in which ERRα was over-expressed, suggesting that loss of PGC-1α expression can by rescued by ERRα upon exposure to hypoxia.

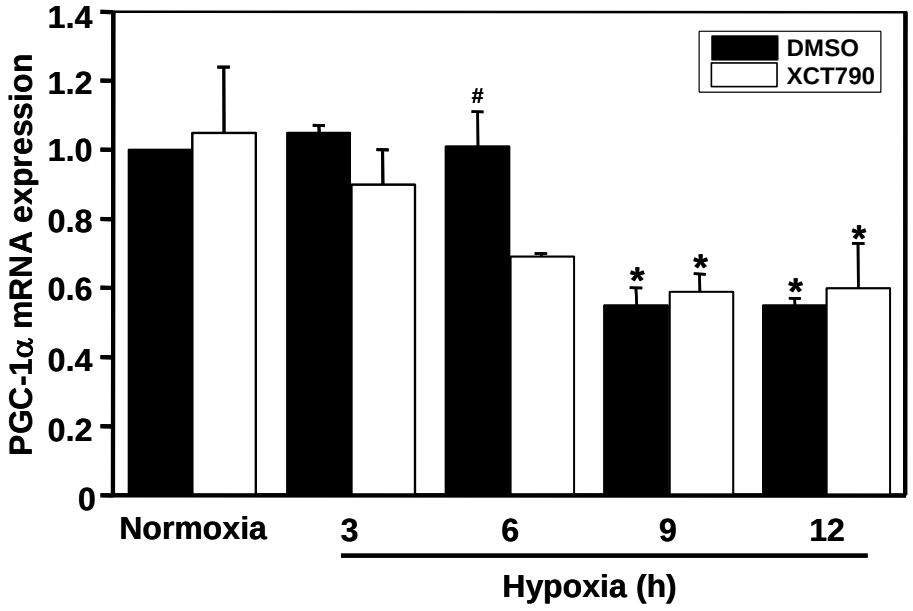

Figure 9: Effect of ERRα inverse agonist XCT790 on PGC-1α expression in hypoxic cardiomyocytes. Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours in the presence of XCT790 or vehicle (DMSO). Control cardiomyocytes were normoxic. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA, using GAPDH as an internal control. XCT790 appears to accelerate PGC-1α down-regulation. *n=3* *P<0.05 vs normoxic control, #P<0.05 vs 12 hours DMSO and XCT790 treatments.

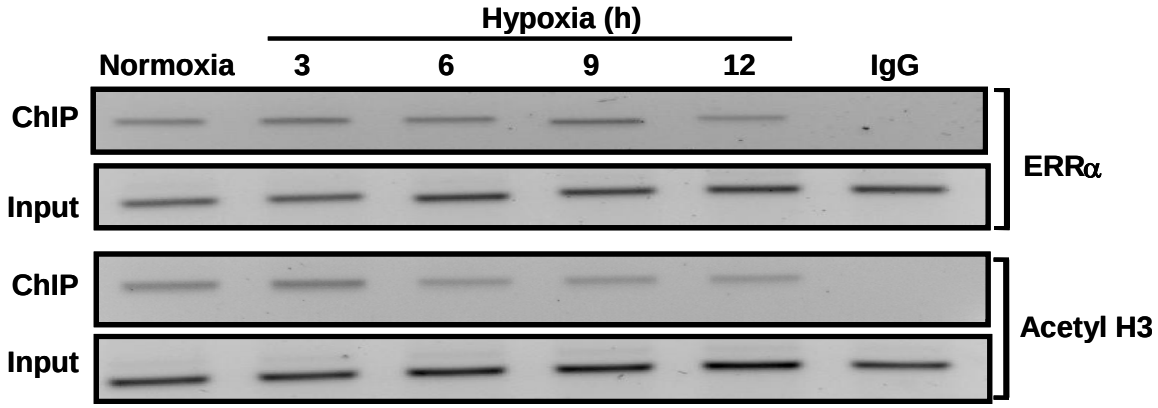

Figure 10: Figure 10: ChIP of the PGC-1α promoter during hypoxia. Upper panel, occupancy of the PGC-1α promoter by ERRα decreases at 12 hours of hypoxia. Lower panel, histone H3 acetylation surrounding the ERRα binding site of the PGC-1α promoter decreases with hypoxia.

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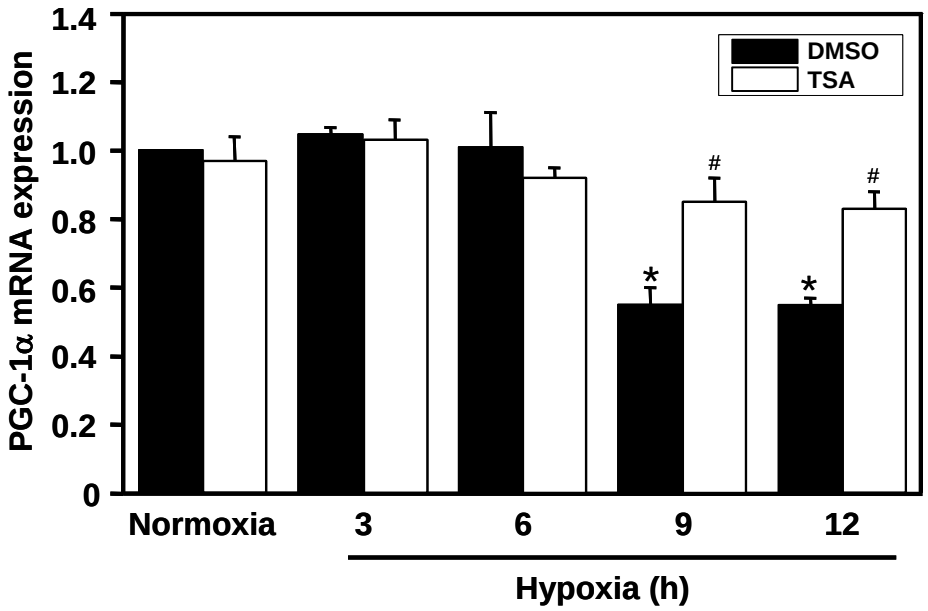

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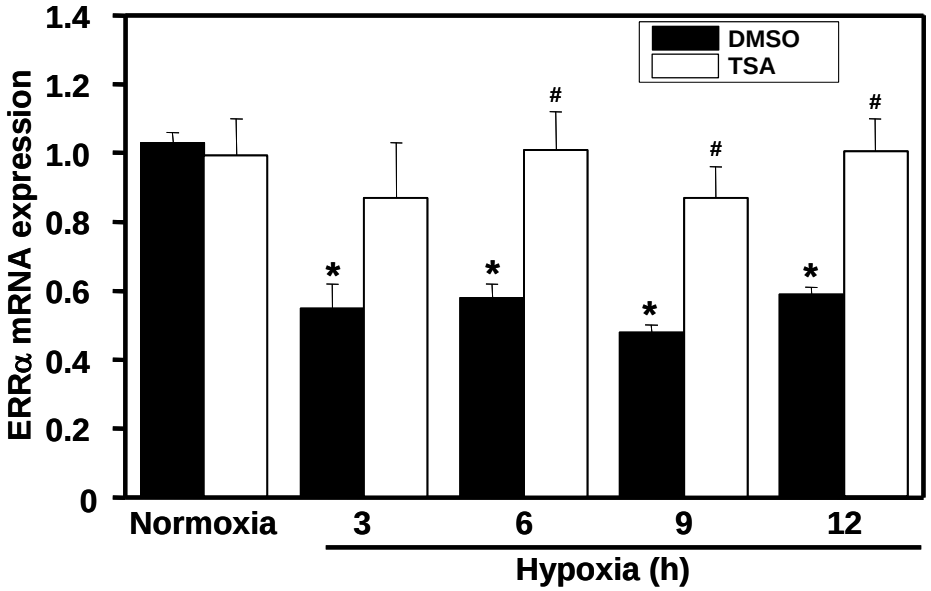

Figure 11: A) Down-regulation of PGC-1α during hypoxia is attenuated by the histone deacetylase inhibitor Trichostatin A. Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours in the presence of 100 nM TSA or vehicle (DMSO). Control cardiomyocytes were normoxic. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of PGC-1α mRNA, using GAPDH as an internal control. *n=3* *P<0.001 vs normoxic control, #P<0.05 vs DMSO. **B) Down-regulation of ERRα by hypoxia is attenuated by the histone deacetylase inhibitor Trichostatin A.** Cardiomyocytes were incubated in a hypoxia chamber (0% O_2) for 3, 6, 9, or 12 hours in the presence of 100 nM TSA or vehicle (DMSO). Control cardiomyocytes were normoxic. Total RNA was isolated from cells and used for quantitative real-time PCR to assay the expression of ERRα mRNA, using GAPDH as an internal control. *n=3* *P<0.01 vs normoxic control, #P<0.05 vs DMSO.

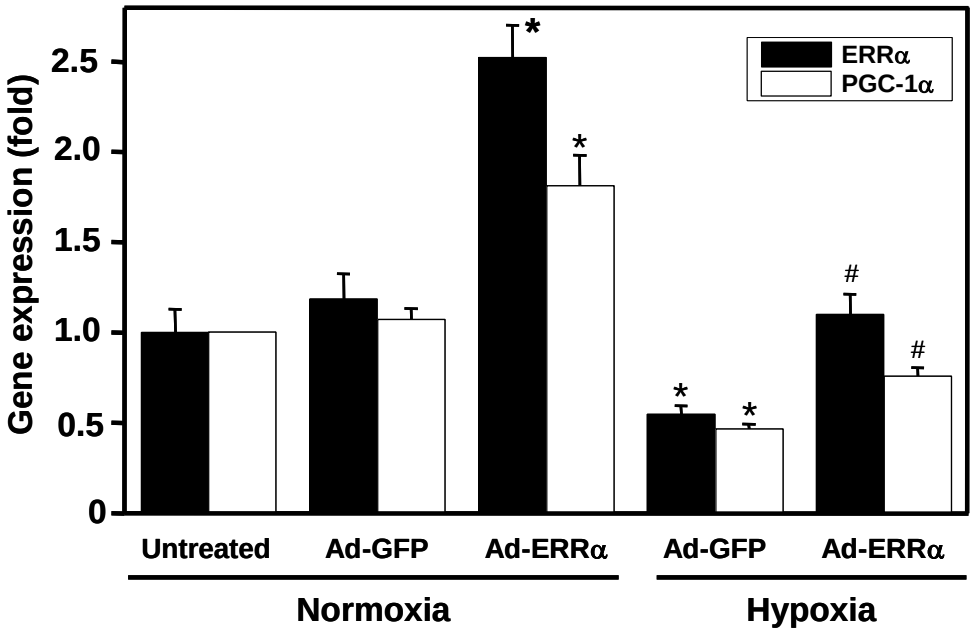

Figure 12: Down-regulation of PGC-1α by hypoxia is attenuated by over-expression of ERRα. Cardiomyocytes were infected with GFP- or ERRα-encoding adenovirus 18 hours prior to hypoxia (0% O_2) for 3, 6, 9, or 12 hours. Untreated cardiomyocytes were normoxic and uninfected. Total RNA was isolated from cells and used for quantitative real-time PCR to measure the expression of ERRα or PGC-1α mRNA, using GAPDH as an internal control. *n=3* *P<0.01 vs untreated normoxic control, #P<0.05 vs Ad-GFP infected hypoxic cardiomyocytes.

VIII Discussion

Both ERRα and PGC-1α are regulators of the expression of genes involved in fatty acid oxidation, TCA cycle components, oxidative phosphorylation enzymes and mitochondrial biogenesis. It is known that PGC-1α co-activates the expression of ERRα and together they transactivate many genes involved in the regulation of cardiac energy metabolism. In separate mouse models lacking expression of ERRα or PGC-1α, a change in mitochondrial metabolism and deregulation of many genes involved in energy metabolism was observed.[14,13,87] These results support the suggestion that these two transcription factors play an important role in maintaining the expression of energy metabolism genes. We investigated the interaction of these two proteins to understand how cardiac energy metabolism is regulated under altered pathophysiologic conditions.

1.0 ERRα as a Regulator of PGC-1α Promoter Activity

We have shown that ERRα regulates the expression of PGC-1α through direct interaction with its promoter. The putative ERRα binding site on the PGC-1α promoter that we have identified is located 1978 base pairs upstream from the transcriptional start site on the human promoter, 2130 base pairs upstream on the mouse promoter and 2394 base pairs on the rat promoter. The specific sequence of the binding site is conserved within the human, mouse and rat PGC-1α promoter (Figure 2). Through an EMSA assay we have shown that ERRα binds to this putative sequence in vitro and when point mutations were introduced to this site, ERRα was no longer able to bind to the sequence (Figure 3A). To confirm binding *in vivo*, a ChIP assay using an antibody specific to

ERRα and primers to amplify the putative binding site confirmed that ERRα does bind to the PGC-1α promoter in neonatal rat cardiomyocytes (Figure 3B). To measure activation of the promoter, a luciferase reporter assay showed that by overexpressing ERRα, the activity of the PGC-1α promoter is significantly increased (Figure 4A). By mutating the putative binding site on the promoter, activity of the promoter was significantly decreased, however activity was not completely lost suggesting that additional ERRα binding sites may exist (Figure 4B).

2.0 Altered Activity and Expression of ERRα Affects Expression of PGC-1α

Over-expression of ERRα in rat neonatal cardiomyocytes lead to a significant increase in PGC-1α gene expression, confirming ERRα's role in regulating the transcriptional activity of PGC-1α (Figure 5A and 5B). To further investigate ERRα's ability to activate the PGC-1α promoter the ERRα inverse agonist XCT790 was used in a luciferase assay and it was observed that XCT790 significantly decreased the activation of the PGC-1α promoter (Figure 6A). Expression of the PGC-1α gene in rat neonatal cardiomyocytes treated with XCT790 was also measured via qRT-PCR to observe any changes in transcriptional activity. XCT790 significantly decreased the expression of the PGC-1α gene (Figure 6B).

3.0 Hypoxia Induced Down-Regulation of PGC-1α and ERRα; Altered Promoter Occupancy

It has been observed in heart tissue samples from patients with various cardiomyopathies that the expression of both PGC-1α and ERRα genes are down regulated with the development of disease. This study included samples from various heart conditions and were divided into two groups, ischemic heart disease and idiopathic heart disease. These samples were pooled together and the expression of genes was then measured, therefore the gene expression data collected was not inclusive to one type of cardiomyopathy.[107] In cases of ischemic heart disease where metabolic substrates are inadequate to meet the demand of the tissue, one of the major components of this disease is hypoxia, or reduction of oxygen to the working heart muscle. The effects of this condition on metabolism in the heart include, blockade of the TCA cycle, inhibition of β-oxidation of fatty acids and a decrease in translocation of ATP from the mitochondria to the cytoplasm.[109] Genes involved in these pathways are down-regulated and many of these genes are under the regulation of PGC-1α and ERRα. This has led us to study the effect of hypoxia on the expression and transcriptional regulation of PGC-1α by ERRα. Our approach involved incubating cardiomyocytes under hypoxic conditions. To ensure any changes in gene expression were not due to cell death, a cell viability assay was performed using the various experimental treatments to show that cells remained viable throughout the experiments (Figure 7). Upon 9 hours of exposure to hypoxic conditions, PGC-1α gene expression in neonatal rat cardiomyocytes was significantly lower than control cardiomyocytes that were kept under normoxic conditions (Figure 8A). ERRα

gene expression was significantly lower after just 3 hours of exposure to hypoxia (Figure 8B). To further investigate the effect of ERRα on PGC-1α gene expression, cardiomyocytes were treated with the ERRα inverse against XCT790 and then incubated under the hypoxic condition. After 6 hours of exposure to hypoxia, PGC-1α mRNA levels were significantly lower than control cells as well as untreated cells at this time point (Figure 9). By inhibiting the activity of ERRα, the down regulation of the PGC-1α gene was accelerated under hypoxic conditions.

To assess whether any changes occurred in occupancy of the PGC-1α promoter by ERRα, a ChIP assay was performed. After 12 hours of hypoxia, less ERRα was bound to the promoter (Figure 10). This however does not account for the decrease in PGC-1α after 9 hours of exposure to hypoxia. If hypoxia mediated down-regulation of PGC-1α expression was dependent on ERRα disengaging from the promoter we would expect a loss of ERRα binding prior to the reduction of PGC-1α mRNA, suggesting there are other transcriptional regulators involved in the expression of the PGC-1α gene during hypoxia. Acetylation of DNA is a process that facilitates gene expression. Using a ChIP assay we examined the acetylation of histones around the putative ERRα binding site on the promoter. We found that after 6 hours of exposure to hypoxia, histones present on the promoter around this region were less acetylated, suggesting that transcription of the PGC-1α gene in hypoxic cardiomyocytes may be repressed by the deacetylation of histones on its promoter (Figure 10). These results suggests that down-regulation of PGC-1α expression upon exposure to hypoxia is the result of a decrease in histone acetylation surrounding the ERRα binding site on the promoter. This was followed by a

decrease in occupancy of the promoter by ERRα that may further contribute to a decrease in PGC-1α gene expression (Figure 13).

4.0 Rescue of PGC-1α Down-Regulation Due to Hypoxia

Deacetylation of histones occurs by HDACs and by inhibiting the activity of HDACs (with use of the inhibitor TSA), histones remain acetylated and therefore transcription of the desired gene occurs. Treatment of cardiomyocytes with TSA prior to exposure to hypoxia did not show any significant decrease in PGC-1α or ERRα mRNA levels when compared to normoxic cardiomyocytes (Figure 11A and 11B). This suggests that treatment with the HDAC inhibitor TSA attenuates the down-regulation of PGC-1α and ERRα in hypoxic cardiomyocytes. This finding further supports the idea that histone deacetylation is a critical event in the down-regulation of PGC-1α expression during hypoxia. This result is consistent with a previous report that over-expression of a constitutively active HDAC5 in transgenic mice resulted in a significant loss of PGC-1a expression .[9]

Finally we over-expressed ERRα in cardiomyocytes prior to exposure to hypoxia to observe changes in PGC-1α gene expression. Not only did ERRα over-expression result in an increase in PGC-1α expression in normoxic cardiomyocytes (Figure 5), ERRα over-expression also attenuated the down regulation of PGC-1α by hypoxia (Figure 12). In studying the genes that are deregulated in cardiac diseases, we can gain a better understanding of which genes are crucial in maintaining a healthy functioning heart. Inhibiting the loss of ERRα and PGC-1α under hypoxia could potentially be a

therapeutic target to regulate gene expression and in turn energy deregulation in the diseased heart.

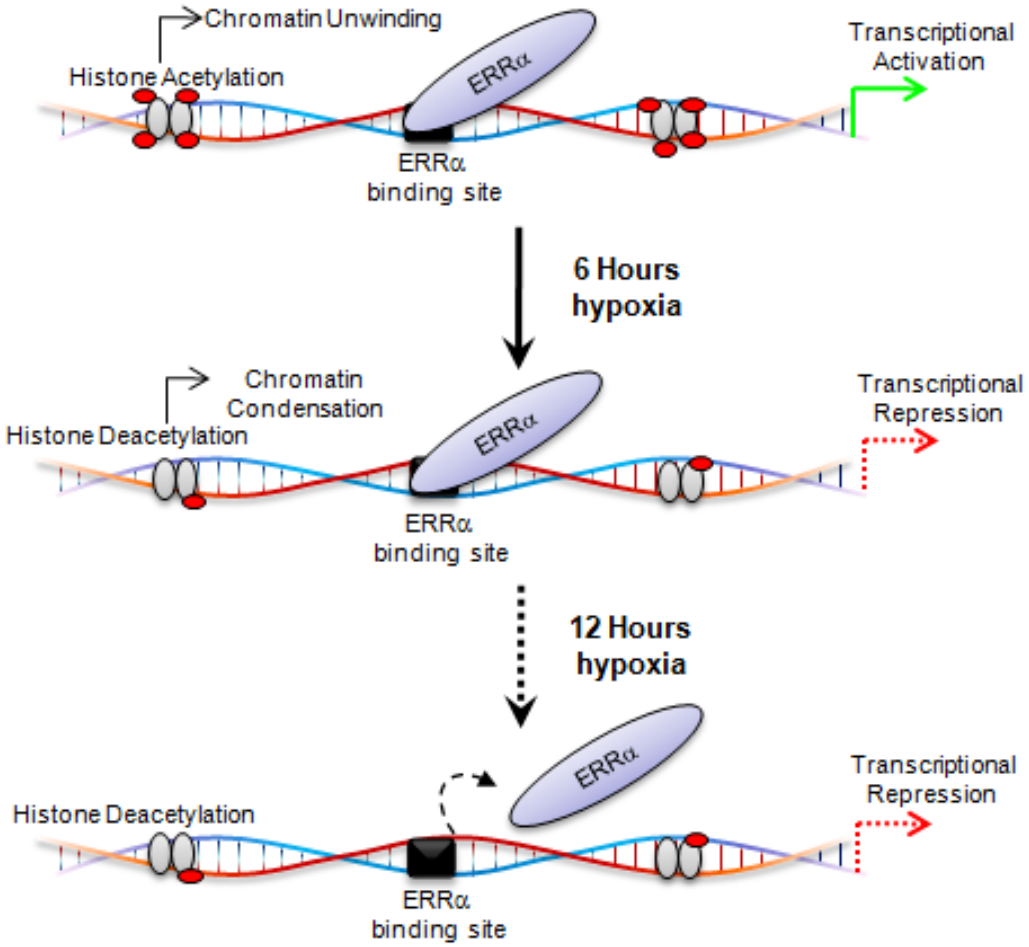

Figure 13: Hypoxia-induced changes in PGC-1α promoter acetylation and occupancy by ERRα. Upon 6 hours of exposure to hypoxia, there is a decrease in acetylation of the PGC-1α promoter surrounding the ERRα binding site. This was followed by a significant decrease PGC-1α gene expression. Furthermore, occupancy of the promoter by ERRα was decreased upon 9 hours of exposure to hypoxia.

IX Conclusions

1. ERRα binds to a putative site on the PGC-1α promoter *in vitro* and *in vivo* in neonatal rat cardiomyocytes. The sequence in which ERRα binds to the promoter is conserved within mice, rats and humans, and is highly similar to the previously reported ERR consensus binding sequence.
2. PGC-1α promoter activity is up regulated by the transcription factor ERRα. Promoter activity is attenuated when the putative ERRα binding site is mutated.
3. Promoter activity and gene expression of PGC-1α in neonatal rat cardiomyocytes is attenuated in the presence of the inverse agonist of ERRα, XCT790.
4. Over-expression of ERRα significantly increases the gene expression of PGC-1α in neonatal rat cardiomyocytes.
5. Expression of PGC-1α and ERRα is down regulated in neonatal rat cardiomyocytes when exposed to hypoxic conditions (0% O_2).
6. ERRα inverse agonist XCT790 accelerates down-regulation of PGC-1α expression in hypoxic neonatal rat cardiomyocytes.
7. Histone H3 acetylation and ERRα occupancy of the PGC-1α promoter is reduced in hypoxic neonatal rat cardiomyocytes.
8. Down-regulation of PGC-1α and ERRα during exposure to hypoxic conditions is attenuated by the histone deacetylase inhibitor TSA**.**
9. Down-regulation of PGC-1α in hypoxic neonatal rat cardiomyocytes is attenuated by over-expression of ERRα.

X Future Directions

We have considered a number of experiments by which we might extend the present findings. For example, a future project may involve the measurement of the effect of hypoxia on gene expression in adult rat cardiomyocytes. Human patients typically develop ischemic heart disease as adults and thus the study of gene regulation in adult rat models would be appropriate as a point of approach.

Another putative project could be structured around the effects of re-oxygenation of cardiomyocytes after exposure to hypoxia in order to mimic reperfusion after an episode of ischemia. Gene expression would be measured to observe whether down-regulation of PGC-1α and ERRα returns to normal levels or if there is a continual decline in gene expression.

Finally, an experiment designed to approach the question of promoter occupancy and gene expression in whole hearts during an ischemic event followed by reperfusion would be a useful to determine whether our current observations in isolated cells also occurs *in vivo*. Essentially, these studies would provide greater relevance to our basic investigations described in this thesis.

XI Methodological Limitations of the Current Study

The current study relied on the use of real-time RT-PCR to measure changes in gene expression levels. Even though RT-PCR is a reliable method for measuring mRNA expression, the changes we noted with mRNA may not strictly translate to changes in protein expression/function. Unfortunately, we were unable to obtain a reliable antibody against PGC-1α. Several antibodies were assayed in Western blots to examine PGC-1α protein expression, but none gave reliable results due to significant background binding. One antibody was pulled from general distribution during the experiments. Furthermore, the time period studied may not have been long enough to observe any changes in protein expression. We are currently in the process of trying another antibody against PGC-1α. The use of Western analysis to confirm real time-PCR data would strengthen key conclusions within the context of this thesis.

The hypoxia experiments were performed in a hypoxia chamber that contained 0% O_2. This was a very useful tool in measuring the effect of hypoxia on cardiomyocytes. Throughout all experiments the oxygen monitor read "0", however we did not have the equipment to measure dissolved oxygen content in the media of the cells throughout the experiments. By measuring dissolved oxygen content, we may have been able to better establish at what time point after exposure to hypoxia gene expression is altered.

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