NUCLEAR RESPIRATORY FACTOR 1: A NOVEL INHIBITOR OF THE HUMAN GENE PROMOTER OF THE CARDIAC ISOFORM OF ACETYL-COENZYME A CARBOXYLASE

TASNEEM ADAM

Submitted for the Degree of Master of Science in Medicine

Faculty of Medicine, University of Cape Town

August, 2008

Supervisor: Prof. L.H. Opie MD; DPhil; FRCP

(Hatter Cardiovascular Research Institute, University of Cape Town)

Co-supervisor: Prof. M.F. Essop PhD

(Department of Physiological Sciences, University of Stellenbosch)

Declaration

I, Tasneem Adam, hereby declare that the work on which this thesis is based is my original work (except where acknowledgment indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature

14/11/08

Date

ACKNOWLEDGEMENTS

First and foremost, my sincere thanks to Prof. M. Faadiel Essop whose support, guidance and intellectual input were invaluable in enabling me to complete this Masters degree. I am very fortunate to have a supervisor who is knowledgeable, motivated, patient, understanding and has a positive attitude.

I am grateful to Prof. Lionel H. Opie for his excellent scientific advice and helpful discussions. His dedication is inspirational.

A huge thank you to Victor P. Claasen and Noel P. Markgraaff (Animal unit) for their technical assistance with the animals used in this study.

I am indebted to Petro Engelbrecht for showing me the cardiomyocyte isolation method.

I thank Dr. Martin E. Young for performing the quantitative Real-Time PCR experiments.

To Lydia Lacerda, sharing her expertise on tissue culture was greatly appreciated.

I acknowledge all my colleagues at the Hatter Cardiovascular Research Institute for their cooperation and valuable technical and administrative assistance.

A special thank you to Anna W-Y. Chan and Makhosazane Zungu for their friendship and support.

Finally, a heartfelt thank you to my husband and parents for their love, care, support and encouragement. This thesis is dedicated to them.

TABLE OF CONTENTS

TABLE OF CONTENTS Page No.

LIST OF FIGURES AND TABLES

Figure		Page No.
	Introduction	

Hypothesis and objectives

Results

Discussion

Conclusion

Appendix

Table

ABBREVIATIONS

ACCβ	Acetyl-coenzyme A carboxylase beta
ACO	Acyl-coenzyme A oxidase
ACS	Acyl-coenzyme A synthetase
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMP	Adenosine monophosphate
Amp^+	Ampicillin
AMPK	5'-AMP-activated protein kinase
AMPKK	5'-AMP-activated protein kinase kinase
ATP	Adenosine triphosphate
β-GPA	Beta-guanadinopropionic acid
bHLH	Basic helix-loop-helix
BMI	Body mass index
CaMKK	Calmodulin-dependent protein kinase kinase
cAMP	Cyclic adenosine monophosphate
CAT	Carnitine acyltranslocase
cDNA	Complementary deoxyribonucleic acid
ChREBP	Carbohydrate response element binding protein
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CPT-1	Carnitine palmitoyltransferase-1
CVD	Cardiovascular diseases
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dnNRF-1	Dominant negative nuclear respiratory factor-1
E. coli	*Escherichia coli*
ETC	Electron transport chain
FA	Fatty acids
FABP	Fatty acid binding proteins
F-1,6-BP	Fructose-1,6-bisphosphate
F-2,6-BP	Fructose-2,6-bisphosphate
$FADH_2$	Reduced flavin adenine dinucleotide
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport proteins

FCS	Foetal calf serum
Fi ori	Filamentous phage origin of replication
F-6-P	Frutose-6-phosphate
G-6-P	Glucose-6-phosphate
GLUT	Glucose transporters
HA	Hemagglutinin
HDL	High-density-lipoproteins
H_2O	Water
IDF	International Diabetes Federation
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrates
LC	Long-chain
LDH	Lactate dehydrogenase
LDL	Low-density-lipoproteins
LKB1	Tumour suppressor kinase
L-PK	Liver-type pyruvate kinase
LPL	Lipoprotein lipase
Luc^+	Luciferase
MCAD	Medium-chain acyl-coenzyme A dehydrogenase
MCD	Malonyl-coenzyme A decarboxylase
MCS	Multiple cloning site
MCT	Monocarboxylate transporter
MO25α	Mouse protein 25 alpha
MRF4	Muscle regulatory factor 4
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
mtTFA	Mitochondrial transcription factor A
NAD^+	Reduced nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NBS	Newborn calf serum
NCEP	National Cholesterol Education Program
NEFA	Nonesterified fatty acids
NH_2	Amino
NRFs	Nuclear respiratory factors
Ori	Origin of replication
PI	Promoter one

PII	Promoter two
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PFK-1	Phosphofructokinase-1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
Pi	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PPARα	Peroxisome proliferator-activated receptor alpha
qRT-PCR	Quantitative real-time polymerase chain reaction
RARα	Retinoic acid receptor alpha
RL	*Renilla reniformis*
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RXR	Retinoic X receptor
S14	Spot 14
SREBP	Sterol regulatory binding protein
ssDNA	Single-stranded deoxyribonucleic acid
STRADα	Ste20-related adaptor protein alpha
SV40	Simian virus 40
TCA	Tricarboxylic acid
TE	Tris-Ethylene diamine tetra-acetic acid
TG	Triglycerides
TZD	Thiozolidinedione
UCPs	Uncoupling proteins
USF1	Upstream stimulatory factor 1
VLDL	Very-low-density-lipoproteins
WHO	World Health Organisation

ZDF	Zucker Diabetic Fatty

UNITS OF MEASUREMENTS

ANOVA	analysis of variance
°C	degree Celsius
cm	centimetre
cm^2	square centimetre
=	equals sign
g	gram
x g	gravitational force
>	greater-than sign
≥	greater-than or equal to
kDA	kilodalton
kg	kilogram
<	less-than sign
l	litre
m^2	square metre
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
mmHg	millimetres of mercury
mM	millimolar
mmol	millimoles
M_r	molecular mass
ng	nanogram
nm	nanometre
%	percentage sign
SE	standard error
U	unit

ABSTRACT

Abstract

Introduction: Nuclear respiratory factor-1 (NRF-1) is a pivotal transcriptional modulator controlling the expression of nuclear genes encoding mitochondrial proteins. Activation of 5'-AMP-activated protein kinase (AMPK) has been associated with enhanced NRF-1 gene promoter binding activity. Furthermore, NRF-1 has been linked with increased gene expression of carnitine palmitoyltransferase-1 (CPT-1), the rate-limiting mitochondrial fatty acid (FA) transfer enzyme. Upstream, the cardiac-enriched isoform of acetyl-CoA carboxylase (ACCβ) synthesises malonyl-CoA, a potent inhibitor of CPT-1 and FA β-oxidation. Since ACCβ induction elevates malonyl-CoA levels, we hypothesised that AMPK activates NRF-1 thereby inhibiting ACCβ expression in the heart to ultimately increase FA β-oxidation.

Methods: A 1,317 bp human ACCβ gene promoter-luciferase construct (pPIIβ-1317/+65-Luc) was transiently transfected into rat neonatal cardiomyocytes ± the following expression constructs: NRF-1 (pSG5-NRF-1) and dominant negative NRF-1 (pCMV-HA-dnNRF-1). To elucidate whether AMPK is involved in the transcriptional regulation of ACCβ gene, the transfected cardiomyocytes were treated with AMPK activators, i.e. AICAR or metformin for 24 hours. Transfections were also performed with upstream stimulatory factor 1 (USF1), a known transactivator of the human ACCβ gene promoter. In addition, the effect of NRF-1 on endogenous USF1 transcriptional activity was evaluated with a luciferase construct containing multiple copies of USF1-specific enhancer elements (pUSF1-Luc).

Results: NRF-1 overexpression reduced ACCβ gene promoter activity by 56 ± 8.8% ($p<0.001$ vs. pPIIβ-1317/+65-Luc). Cotransfection with the dnNRF-1 construct abrogated this effect. Addition of AICAR dose-dependently decreased ACCβ gene promoter activity ($p<0.05$ vs. untreated control). In contrast, NRF-1 attenuation of ACCβ gene promoter activity was not altered by AICAR treatment. Exposure to metformin did not affect the ACCβ gene promoter activity at baseline, and in response to NRF-1. NRF-1 inhibited the USF1-dependent upregulation of ACCβ gene promoter activity by 58 ± 7.5% ($p<0.001$ vs. pPIIβ-1317/+65-Luc + USF1), an effect reversed with the dnNRF-1 construct. In addition, NRF-1 reduced endogenous USF1 transcriptional activity by 55 ± 6.2% ($p<0.001$ vs. pUSF1-Luc). This effect was abolished with the dnNRF-1 construct. The NRF-1-mediated decrease in endogenous USF1

transcriptional activity was independent of AMPK activation with AICAR. The ACCβ mRNA expression level was unaffected at baseline, and by NRF-1 overexpression. However, AICAR (2 mM) treatment markedly reduced the ACCβ mRNA expression level ($p<0.05$ vs. vehicle) at baseline, but had no effect in response to NRF-1 overexpression.

Conclusion: Our data reveal a unique, inhibitory role for NRF-1 in the transcriptional regulation of human ACCβ gene in the mammalian heart, independent of AMPK activation.

A. INTRODUCTION

1. GLOBAL BURDEN OF CARDIOVASCULAR DISEASE

1.1. Definition

Cardiovascular diseases (CVD) are described as a spectrum of disorders with structural and/or functional abnormalities of the heart or blood vessels.[1] For example, coronary artery disease, hypertension, stroke, rheumatic heart disease, congenital heart disease, arrhythmia, cardiomyopathy and heart failure.

1.2. Prevalence and causes

CVD rank as the leading cause of global morbidity and mortality.[2] By 2030, CVD is projected to affect nearly 23.3 million people worldwide.[3] The global burden of CVD is strongly associated with key risk markers distinct from genetics, attributable to socio-economic, psychosocial, lifestyle, physiological and pathophysiological factors (Table 1). Moreover, Yusuf et al[4] reported that the risk factors related to over 90% of the risk of an acute myocardial infarction and were common for gender, age and ethnicity in most populations around the world.

Table 1. Risk factors for CVD

Socioeconomic:
1. Unemployment
2. Poverty
3. Low education
4. Low literacy skills
Psychosocial:
1. Stress
2. Depression
Lifestyle:
1. Tobacco use
2. Alcohol consumption
3. Unhealthy diet
4. Physical inactivity

Physiological and Pathophysiological:

1. Abdominal obesity (BMI ≥30 kg/m^2 or waist girth >102 in men, >88 cm in women)
2. High LDL cholesterol (>2.6 mmol/l), low HDL cholesterol (<1.0 mmol/l in men, <1.3 mmol/l in women), high triglycerides (≥1.7 mmol/l)
3. Hypertension (blood pressure ≥130/85 mmHg)
4. Diabetes (fasting plasma glucose ≥6.1 mmol/l)
5. Elevated homocysteine
6. Elevated prothrombotic (fibrinogen, PAI-1) and proinflammatory factors

Table 1 adopted and modified from Yusuf et al[5, 6-9].

The incidence of CVD is escalating alarmingly in developing countries (low and middle income countries). In South Africa, a middle income country, the South African National Burden of Disease study 2000 estimated that CVD is responsible for 17% of all deaths.[10] The underlying causes of this phenomenon is ascribed to ongoing transitions that are taking place in politics, economics, industrialisation and urbanisation.[11] As a result, there is an increase in unhealthy dietary patterns, e.g. consumption of foods that are high in saturated fat, sugar and sodium. In parallel, there is a low intake of fibre, unrefined carbohydrates, fruit and vegetables, often linked to the adoption of sedentary lifestyles. These changes in social status, environment and behaviour also contribute to the pandemic rise in diabetes mellitus.

2. DIABETES MELLITUS

2.1. Definition

Diabetes mellitus is characterised as a chronic disease with hyperglycaemia. There are two types of diabetes[12]:

1. Type 1 diabetes is caused by insufficient insulin production due to the destruction of the pancreatic islet β-cells by the immune system.
2. Type 2 diabetes is the result of impaired insulin sensitivity and/or defective insulin secretion. It is the most common form of diabetes.

2.2. Diabetes and CVD

The number of people diagnosed with diabetes has dramatically increased during the last few decades.[13-15] At present, ~246 million people worldwide have diabetes and this figure is predicted to rise to ~366 million by 2030.[16,17] Diabetic patients are at heightened risk for developing CVD.[18-23] Since cardiovascular complications in the diabetic patient represent a global threat to human health, it is important to understand the link between these two pathophysiological conditions.

Emerging evidence has shown that derangements in cardiac substrate metabolism plays a crucial role in the aetiology of diabetes and CVD.[24] In light of this, I will now review cardiac substrate metabolism under physiological and pathophysiological states.

3. CARDIAC SUBSTRATE METABOLISM

Contractility of the heart is driven by a continuous supply of energy in the form of adenosine triphosphate (ATP) dynamically synthesised in the mitochondrion as a result of the metabolism of a variety of circulating fuel substrates. The selection of myocardial fuel substrates is dependent on substrate availability, workload, developmental and hormonal conditions.[25-27] Under physiological conditions, carbohydrates and fatty acids (FA) are the principal fuel substrates for the mammalian heart.

3.1. Carbohydrate metabolism

Carbohydrates, i.e. glucose and lactate provide ~20% of ATP in the healthy postnatal mammalian heart.[28] The carbohydrate supply for the heart is derived from both exogenous and endogenous (e.g. glycogen[29]) sources. Glucose uptake into cardiomyocytes relies on a transmembrane glucose gradient and on the content of glucose transporters (GLUT) on the plasma membrane (Figure 1).[30,31] Although twelve GLUT isoforms have been identified[32], GLUT 1 and GLUT 4 are the major isoforms expressed in the heart. GLUT 1 is the foetal isoform and primarily facilitates basal glucose uptake.[33] GLUT 4 is the adult isoform and mediates glucose uptake in response to several signals, such as insulin, contraction, ischaemia and 5'-AMP-activated protein kinase (AMPK).[34-38] Intracellular signalling cascades promote the translocation of GLUT 4 from the intracellular vesicles to the plasma membrane. Lactate uptake occurs via the monocarboxylate transporter (MCT).[39]

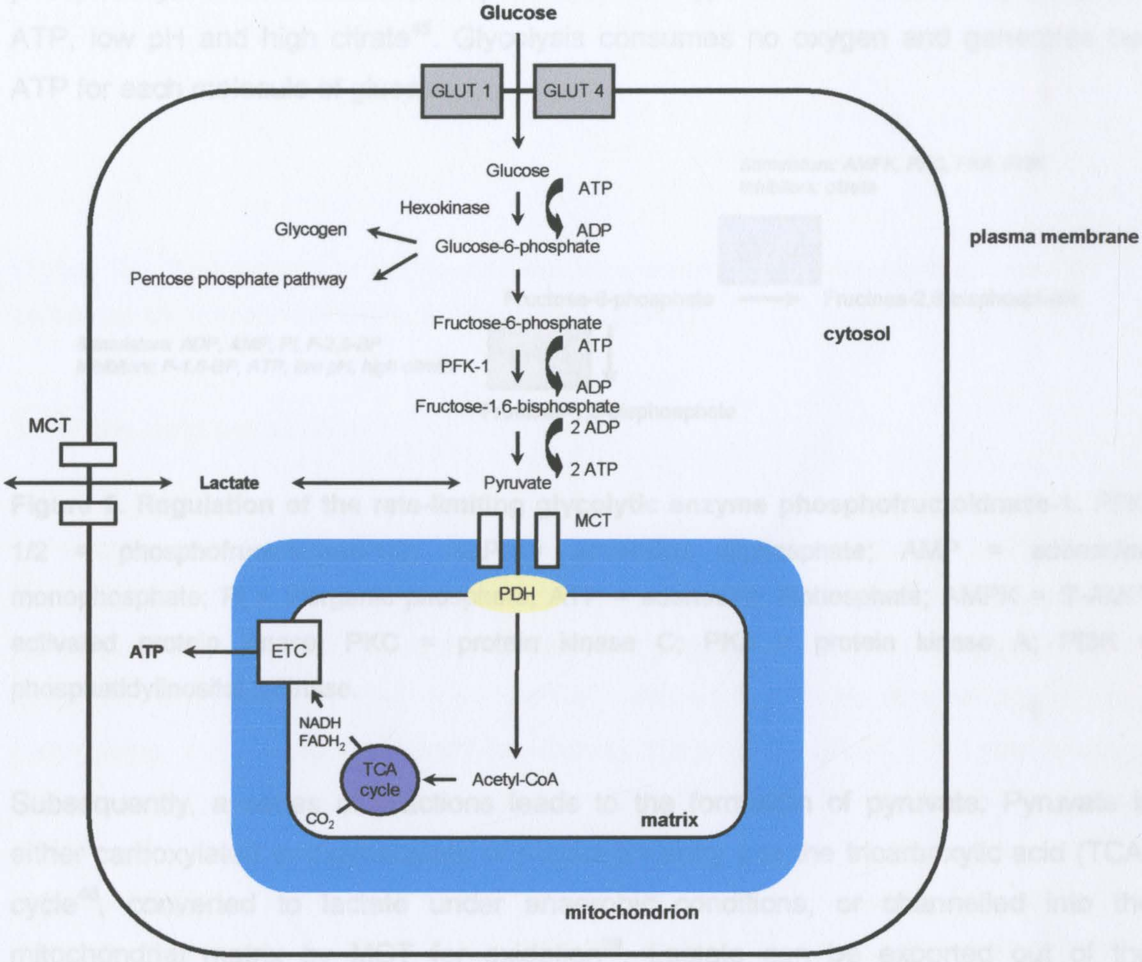


Figure 1. Schematic representation of carbohydrate utilisation in the cardiomyocyte. GLUT 1/4 = glucose transporters 1/4; PFK-1 = phosphofructokinase-1; MCT = monocarboxylate transporter; PDH = pyruvate dehydrogenase; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate; ADP = adenosine diphosphate.

After uptake into the cardiomyocyte, glucose is swiftly phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase. G-6-P has 3 major fates: conversion to glycogen for storage, the pentose phosphate pathway, or catabolism by glycolysis. In the glycolytic pathway, the rate-limiting enzyme phosphofructokinase-1 (PFK-1) catalyses the phosphorylation of fructose-6-phosphate (F-6-P) into fructose-1,6-bisphosphate (F-1,6-BP) (Figure 2). PFK-1 is activated by adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inorganic phosphate (Pi).[40] Fructose-2,6-bisphosphate (F-

2,6-BP) also stimulates PFK-1. Its synthesis from F-6-P is catalysed by PFK-2.[41] PFK-2 activity is enhanced by AMPK, protein kinase A (PKA), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K).[42-44] Conversely, PFK-1 is inhibited by F-1,6-BP, ATP, low pH and high citrate[45]. Glycolysis consumes no oxygen and generates two ATP for each molecule of glucose.

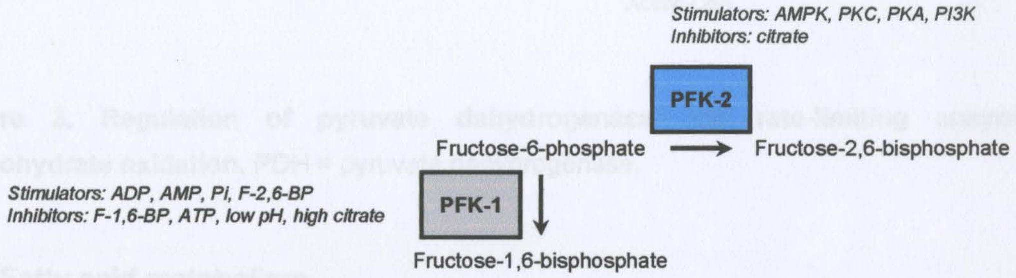


Figure 2. Regulation of the rate-limiting glycolytic enzyme phosphofructokinase-1. PFK-1/2 = phosphofructokinase-1/2; ADP = adenosine diphosphate; AMP = adenosine monophosphate; Pi = inorganic phosphate; ATP = adenosine triphosphate; AMPK = 5'-AMP-activated protein kinase; PKC = protein kinase C; PKA = protein kinase A; PI3K = phosphatidylinositol 3-kinase.

Subsequently, a series of reactions leads to the formation of pyruvate. Pyruvate is either carboxylated to oxaloacetate or malate for entry into the tricarboxylic acid (TCA) cycle[46], converted to lactate under anaerobic conditions, or channelled into the mitochondrial matrix by MCT for oxidation[39]. Lactate can be exported out of the cardiomyocyte or converted to pyruvate via the enzyme lactate dehydrogenase (LDH). For carbohydrate oxidation, the multienzyme complex pyruvate dehydrogenase (PDH) decarboxylates pyruvate into acetyl-CoA. PDH is the rate-limiting enzyme for carbohydrate oxidation and is controlled by two enzymes: PDH kinase (PDK) which phosphorylates and inactivates it, and PDH phosphatase which dephosphorylates and activates it (Figure 3).[47,48] The resulting acetyl-CoA enters the TCA cycle which releases CO_2 and produces reducing equivalents, NADH and $FADH_2$, used by the electron transport chain (ETC) for ATP generation by oxidative phosphorylation to sustain normal cardiac contractile function.

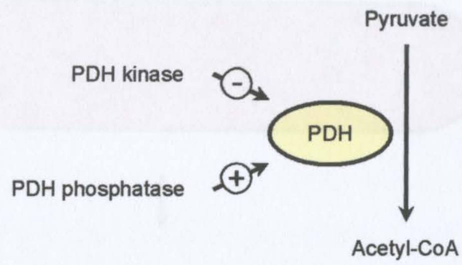


Figure 3. Regulation of pyruvate dehydrogenase, the rate-limiting enzyme of carbohydrate oxidation. PDH = pyruvate dehydrogenase.

3.2. Fatty acid metabolism

FA (mainly oleate and palmitate) constitute the major fuel substrate of the healthy postnatal mammalian heart, contributing 60-70% of ATP produced.[28] FA are stored in the adipose tissue and released into circulation by lipolysis. The heart acquires FA from circulation in the nonesterified form (NEFA), albumin-bound, as triglyceride (TG)-rich lipoproteins, chylomicrons, or very-low-density lipoproteins (VLDL) that are released through hydrolysis by the enzyme lipoprotein lipase (LPL).[49] LPL resides on the luminal surface of coronary endothelial cells and on the surface of cardiomyocytes.[50] In agreement, forced expression of LPL in the heart leads to increased FA uptake.[50] In contrast, a cardiac-specific deletion of LPL shifted the myocardial fuel substrate preference from FA to glucose.[51,52]

FA are transported into cardiomyocytes by passive diffusion and FA transporter proteins (Figure 4).[53] At present, three FA transporter proteins have been described: fatty acid binding protein found in the plasma membrane (FABPpm) and in the cytosol (FABPc)[54], fatty acid transport protein (FATP)[55] and fatty acid translocase (FAT/CD36)[56,57,58] AMPK is also thought to mediate FA uptake since its activation resulted in FAT/CD36 mobilisation to the plasma membrane and stimulated FA uptake in cardiomyocytes.[59]

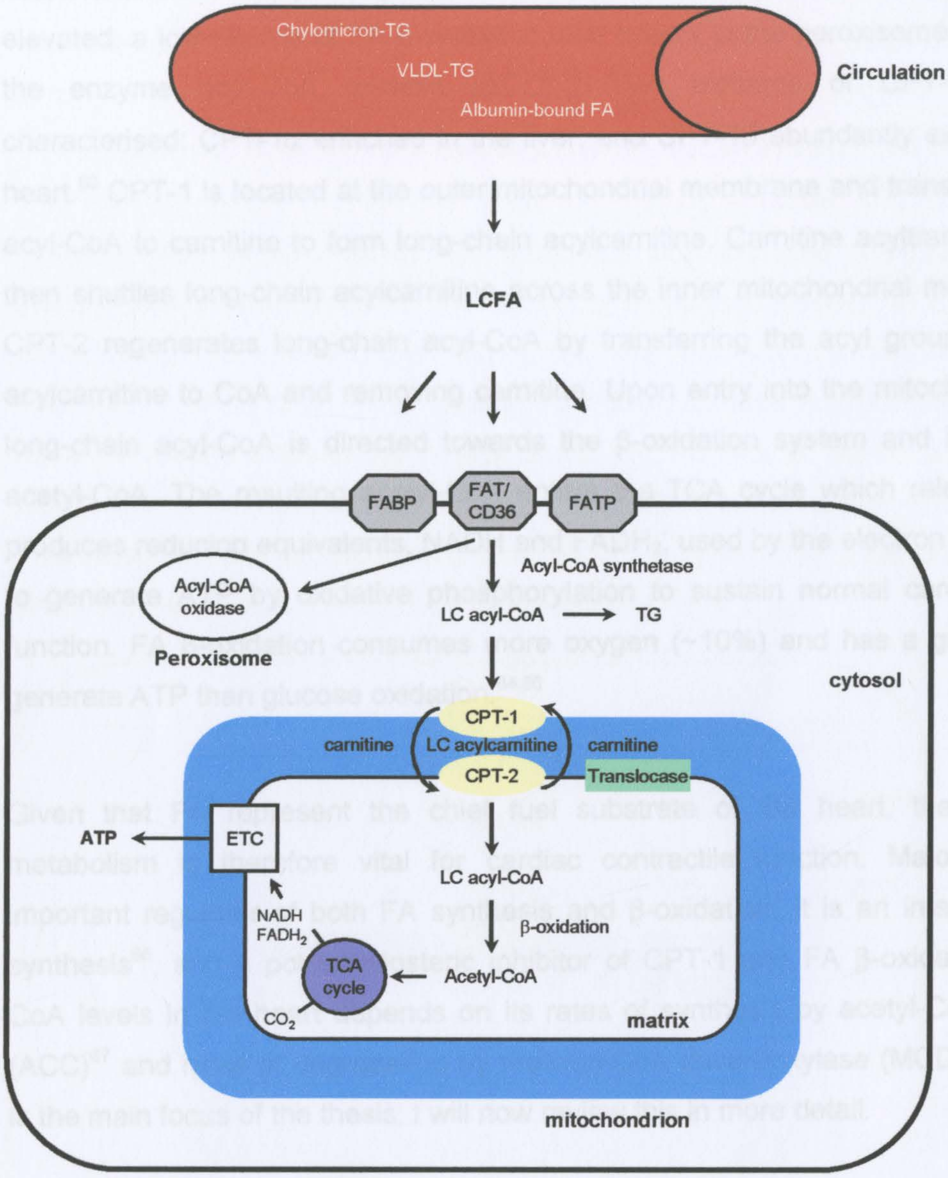


Figure 4. Schematic representation of fatty acid utilisation in the cardiomyocyte. VLDL = very-low-density lipoprotein; TG = triglyceride; LCFA = long-chain fatty acid; FABP = fatty acid binding protein; FAT/CD36 = fatty acid translocase; FATP = fatty acid transport protein; CPT-1/2 = carnitine palmitoyltransferase-1/2; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate.

Once in the cytosol, acyl-CoA synthetase (ACS) esterifies long-chain FA into long-chain acyl-CoA. Furthermore, ACS interacts with FAT/CD36 or FATP, which implicates ACS in FA uptake.[60] Long-chain acyl-CoA is then used for TG synthesis or imported into the mitochondrion for β-oxidation via the carnitine palmitoyltransferase (CPT) system[61]. The mitochondrion is the primary site for FA β-oxidation. However, when FA levels are elevated, a low degree of FA β-oxidation also occurs in the peroxisomes, catalysed by the enzyme acyl-CoA oxidase (ACO).[62] Two isoforms of CPT-1 have been characterised: CPT-1α enriched in the liver, and CPT-1β abundantly expressed in the heart.[63] CPT-1 is located at the outer mitochondrial membrane and transfers long-chain acyl-CoA to carnitine to form long-chain acylcarnitine. Carnitine acyltranslocase (CAT) then shuttles long-chain acylcarnitine across the inner mitochondrial membrane. Here CPT-2 regenerates long-chain acyl-CoA by transferring the acyl group of long-chain acylcarnitine to CoA and removing carnitine. Upon entry into the mitochondrial matrix, long-chain acyl-CoA is directed towards the β-oxidation system and is converted to acetyl-CoA. The resulting acetyl-CoA enters the TCA cycle which releases CO_2 and produces reducing equivalents, NADH and $FADH_2$, used by the electron transport chain to generate ATP by oxidative phosphorylation to sustain normal cardiac contractile function. FA β-oxidation consumes more oxygen (~10%) and has a greater ability to generate ATP than glucose oxidation.[64,65]

Given that FA represent the chief fuel substrate of the heart, the control of its metabolism is therefore vital for cardiac contractile function. Malonyl-CoA is an important regulator of both FA synthesis and β-oxidation. It is an intermediate in FA synthesis[66], and a potent allosteric inhibitor of CPT-1 and FA β-oxidation[63]. Malonyl-CoA levels in the heart depends on its rates of synthesis by acetyl-CoA carboxylase (ACC)[67] and rates of degradation by malonyl-CoA decarboxylase (MCD)[68]. Since ACC is the main focus of the thesis, I will now review this in more detail.

3.2.1. ACC: a pivotal regulator of FA metabolism

3.2.1.1. Structure and function

ACC catalyses the carboxylation of cytosolic acetyl-CoA to form malonyl-CoA (Figure 5). Mammals express two isoforms of ACC, i.e. ACCα (M_r = 265 kDa) and ACCβ (M_r = 280 kDa). These isoforms are encoded by separate genes, with ACCα mapped to human chromosome 17q21[69] and ACCβ mapped to human chromosome 12q23[70]. The nucleotide and deduced amino acid sequences of human ACCα and ACCβ share a 60% and 75% identity, respectively.[70]

The ACC isoforms display distinct tissue distribution and structural features, indicating their different functions. ACCα is expressed in lipogenic tissues such as the adipose tissue, liver and mammary gland. Cloning and sequencing of complementary deoxyribonucleic acids (cDNA) encoding human ACCα revealed that the first 50 amino acid residues of the NH_2-terminal region is highly hydrophilic and is localised in the cytosol. These characteristics are consistent with the role of ACCα in producing malonyl-CoA as a substrate for FA synthesis, a cytosolic process. In contrast, ACCβ is expressed in oxidative tissues such as the heart and skeletal muscle where there is very little or no *de novo* FA synthesis.[67] Moreover, human ACCβ uniquely contains an extra 114 amino acids in the NH_2-terminus and the first 20 amino acid residues are highly hydrophobic, suggesting that it anchors ACCβ to the mitochondrial membrane.[71] Therefore, ACCβ is implicated in the control of FA β-oxidation, a mitochondrial process, providing malonyl-CoA, a negative modulator of CPT-1.

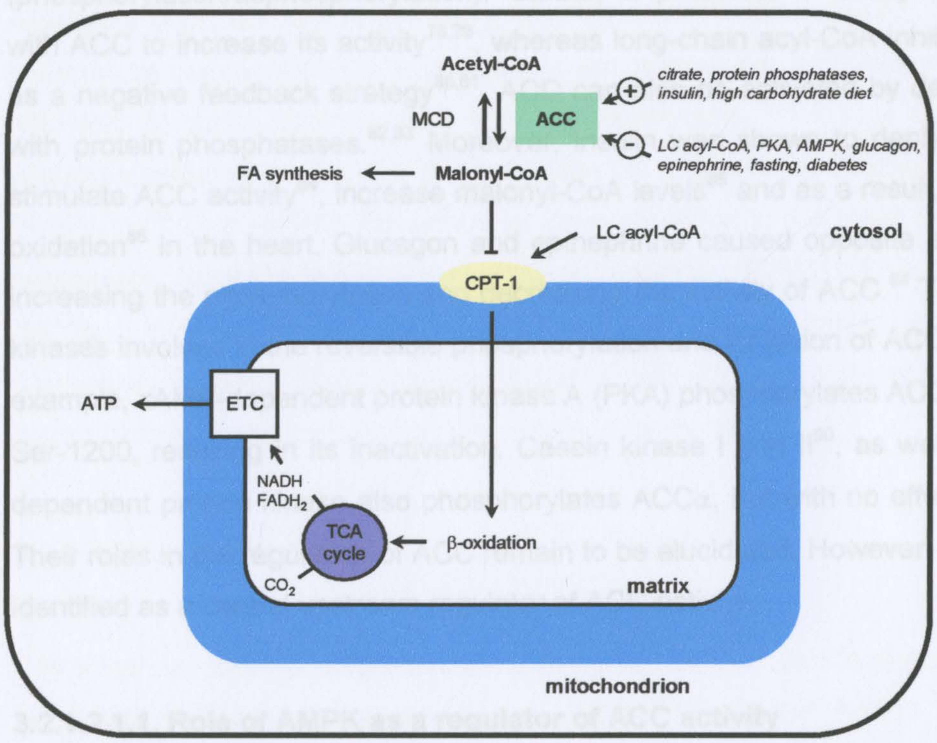


Figure 5. Schematic representation of the role of acetyl-CoA carboxylase in the regulation of FA synthesis and β-oxidation. ACC = acetyl-coenzyme A carboxylase; MCD = malonyl-coenzyme A decarboxylase; FA = fatty acid; LC = long-chain; CPT-1 = carnitine palmitoyltransferase-1; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate; PKA = protein kinase A; AMPK = 5'-AMP-activated protein kinase.

3.2.1.2. Regulation

Since ACC plays a significant role in controlling FA metabolism, it must be subjected to strict regulatory mechanisms. Physiologically, the half-life of ACC is 1 to 3 days.[72] Therefore, the activity and level of ACC is regulated by short-term (seconds to minutes) and long-term (hours to days) mechanisms, and in response to hormones and diet (Figure 5).[73-77] I will now discuss these mechanisms.

3.2.1.2.1. Short-term regulation

Short-term regulation controls the catalytic activity of ACC. This occurs by allosteric metabolites (citrate, long-chain acyl-CoA) and covalent modification (phosphorylation/dephosphorylation). Citrate, a precursor of acetyl-CoA, associates with ACC to increase its activity[78,79], whereas long-chain acyl-CoA inhibits ACC activity as a negative feedback strategy[80,81]. ACC can also be activated by dephosphorylation with protein phosphatases.[82,83] Moreover, insulin was shown to dephosphorylate and stimulate ACC activity[84], increase malonyl-CoA levels[85] and as a result, suppress FA β-oxidation[86] in the heart. Glucagon and epinephrine caused opposite effects to insulin, increasing the phosphorylation and decreasing the activity of ACC.[84] There are several kinases involved in the reversible phosphorylation and inhibition of ACC activity.[87-89] For example, cAMP-dependent protein kinase A (PKA) phosphorylates ACCα at Ser-77 and Ser-1200, resulting in its inactivation. Casein kinase I and II[90], as well as calmodulin-dependent protein kinase also phosphorylates ACCα, but with no effect on its activity. Their roles in the regulation of ACC remain to be elucidated. However, AMPK has been identified as a central upstream regulator of ACC activity.

3.2.1.2.1.1. Role of AMPK as a regulator of ACC activity

3.2.1.2.1.1.1. Structure and function of AMPK

AMPK is a serine/threonine kinase often described as the 'cellular fuel gauge'. It responds to energetic stress by switching on ATP generating catabolic pathways and switching off ATP consuming anabolic pathways (Figure 6).[91] Energetic stress signals such as myocardial ischaemia[92,93], contraction[94,95], exercise[96-98] and hypoglycaemia[99] cause an increase in AMPK activity in order to enhance the cell's bioenergetic capacity.

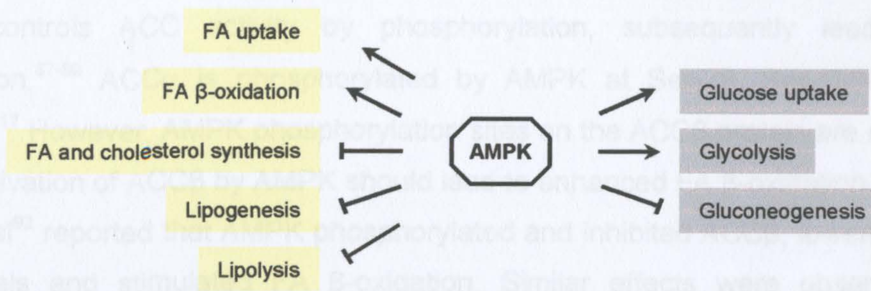


Figure 6. Metabolic pathways activated or inhibited by AMPK. AMPK = 5'-AMP-activated protein kinase; FA = fatty acid.

AMPK is a conserved heterotrimeric enzyme consisting of a catalytic α (α_1 and α_2) and regulatory β (β_1 and β_2) and γ (γ_1, γ_2 and γ_3) subunits. Specifically, the α_2, β_2 and γ_2 isoforms are predominantly expressed in the human heart.[100] AMPK is allosterically activated by high intracellular 5'-AMP concentrations.[101,102] It is also activated by phosphorylation of the Thr-172 residue within the α-subunit by upstream AMPK kinases (AMPKK).[103] Two AMPKK have thus far been identified in the heart, i.e. LKB1 (tumour suppressor kinase):Ste20-related adaptor proteinα (STRADα):mouse protein 25α (MO25α) complex[104] and calmodulin-dependent protein kinase kinase (CaMKK)[105]. Conversely, AMPK is allosterically inhibited by creatine phosphate and ATP.[106] It is also inactivated by dephosphorylation via protein phosphatase 2C.[107]

3.2.1.2.1.1.2. Pharmacological activators of AMPK

AMPK can be pharmacologically activated by AICAR[108,109], and anti-diabetic drugs such as metformin[110] and thiazolidinediones (TZD)[111,112]. AICAR enters the cells and is phosphorylated by adenosine kinase to form the AMP mimetic, ZMP, which allosterically activates AMPK and also by facilitating its phosphorylation through AMPKK. Both metformin and TZD are suggested to indirectly activate AMPK through inhibition of respiratory chain complex 1.[113-115] These pharmacological agents are therefore useful tools to investigate the functional role of AMPK in substrate energy metabolism.

3.2.1.2.1.1.3. ACC: an AMPK target

AMPK controls ACC activity by phosphorylation, subsequently leading to its inactivation.[87-89] ACCα is phosphorylated by AMPK at Ser-79, Ser-1200 and Ser-1215.[116,117] However, AMPK phosphorylation sites on the ACCβ protein are still unclear. The inactivation of ACCβ by AMPK should lead to enhanced FA β-oxidation. In support, Kudo et al[92] reported that AMPK phosphorylated and inhibited ACCβ, lowered malonyl-CoA levels and stimulated FA β-oxidation. Similar effects were observed during exercise and skeletal muscle contraction, with the net result an increase in energy production.[118,97,95] AMPK-mediated increases in FA β-oxidation was also associated with reduced TG accumulation in the skeletal muscle and liver.[110,119] Therefore, it appears that increasing FA β-oxidation via AMPK activation and the subsequent inhibition of ACCβ may be beneficial in pathophysiological states such as type 2 diabetes, which are often characterised by excess intracellular lipid accumulation.

3.2.1.2.1.1.4. Additional AMPK targets

AMPK also influences other enzymes involved in FA metabolism. Recent evidence showed that AICAR-induced AMPK activation elevated FA transport in cardiomyocytes by significantly increasing the protein expression and the plasma membrane content of FAT/CD36 and FABP.[120] Moreover, C2C12 mouse skeletal muscle cells treated with AICAR increased gene expression of PPARα (ligand-activated transcription factor and nuclear receptor which regulates FA metabolism enzyme-encoding genes) and several of its target genes (FABP3, ACO, CPT-1, peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α)).[121] This was paralleled by enhanced FA β-oxidation. These mechanisms would likely lower plasma NEFA and TG levels and improve insulin sensitivity since high levels impair insulin secretion from the pancreatic islet β cells and insulin-mediated suppression of lipolysis in the adipose tissue.

AMPK also controls FA and cholesterol synthesis, lipogenesis and lipolysis. Isolated rat hepatocytes incubated with AICAR caused the inactivation of ACC and 3-hydroxy-3-methylglutaryl-CoA reductase (a rate-limiting enzyme of cholesterol synthesis).[122] This led to the inhibition of FA and cholesterol synthesis. Furthermore, FA synthesis was inhibited by the expression of constitutively active AMPK in isolated rat hepatocytes, which downregulated the expression of lipogenic genes such as ACC, fatty acid synthase (FAS), liver-type pyruvate kinase (L-PK) and Spot 14 (S14), when exposed to high glucose levels.[123,124] Lipolysis and lipogenesis were also dramatically reduced in response to AICAR stimulation in isolated rat adipocytes.[125] This occurred via increased phosphorylation and inactivation of ACC. Another study showed that activation of AMPK stimulated lipolysis in 3T3-L1 mouse adipocytes.[126] This effect is consistent for a role of AMPK in increasing FA availability since there is also support that AMPK activation enhances cardiac heparin-releasable lipoprotein lipase activity.[127] However, further studies are required to elucidate AMPK actions on lipolysis. The changes documented here are essential to conserve energy.

3.2.1.2.1.1.5. Role of AMPK in glucose metabolism

AMPK also regulates glucose metabolism by increasing both its uptake and glycolysis.[128,129] In this setting, Russell et al[38] demonstrated that *in vivo* administration of AICAR in rats activated myocardial AMPK and resulted in the recruitment of GLUT 4 to the plasma membrane of cardiomyocytes, thereby enhancing glucose uptake. In support, the expression of constitutively active AMPK elevated glucose uptake in H-2K^b mouse skeletal muscle cells.[130] This was suggested to occur by increasing GLUT 1, GLUT 4 and hexokinase II protein expression. However, the expression of dominant negative AMPK attenuated the AICAR-mediated increase in glucose uptake. These results clearly establish that AMPK is involved in promoting glucose uptake. AMPK is also likely to enhance myocardial glycolysis by activation of PFK-2, with a subsequent rise in F-2,6-BP levels.[42] The ability of AMPK to increase glycolysis for energy production is important in energetic stress conditions such as ischaemia and cardiac hypertrophy.[131]

Collectively, the described actions of AMPK undoubtedly emphasises its versatile role as an energy sensor and regulator of FA and glucose metabolism.

3.2.1.2.1.1.6. AMPK: a transcriptional modulator

The profound effects of AMPK on metabolic enzymes raised the possibility that it may also have important functions in the transcriptional activation and/or repression of nuclear proteins. A number of nuclear proteins have been identified as direct downstream targets of AMPK-mediated transcriptional modulation.[132] For example, AICAR treatment upregulated GLUT 4 gene transcriptional activity in murine skeletal muscle.[133] This may contribute to its increased gene expression and the resulting rise in glucose uptake following endurance exercise. Recently, AMPK was also shown to phosphorylate and inhibit the transcriptional activity of carbohydrate response element binding protein (ChREBP).[134] ChREBP is a glucose-responsive transcription factor which regulates lipogenic enzyme-encoding genes in the liver.[135,136] This effect could be responsible for controlling hepatic gene expression in response to glucose.[123,124] Additional targets of AMPK also include the gluconeogenic enzymes, phosphoenolpyyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Here the expression of both enzyme-encoding genes was blocked in rat hepatoma cell lines (H4IIE, HL1C) treated with AICAR.[137] This finding suggests that AMPK is a necessary component in the control of hepatic gluconeogenesis. Also, *in vivo* infusion of AICAR into rat skeletal muscle was found to accelerate CPT-1 gene transcription, a possible approach for increasing FA β-oxidation.[138]

Thus these data give compelling evidence that AMPK plays a central role in regulating the activity of metabolic enzymes as well as providing a link between cellular energy status and the control of gene transcription. However, the question of whether AMPK is involved in the regulation of ACCβ gene at a transcriptional level as a potential mechanism to increase FA β-oxidation must still be answered and was therefore explored in our study.

3.2.1.2.2. Long-term regulation

Long-term regulation of ACC controls the quantity of ACC and is determined by transcriptional and translational mechanisms.

3.2.1.2.2.1. Transcriptional regulation of ACCα gene

ACCα has been extensively characterised at the gene and protein level.[139,77,140] The ACCα gene contains two promoters, designated as PI and PII, with different effects. PI activity is enhanced by stimulation of lipogenesis in the liver and adipose tissue induced by a fat-free carbohydrate-rich diet.[141,142] PII activity is affected by insulin[143], cAMP[143] and tumour necrosis factor[144]. Glucose also increases PII activity through the binding of the transcription factor Sp1 to glucose responsive elements and glucose-mediated dephosphorylation of Sp1 by type 1 phosphatase.[145]

3.2.1.2.2.2. Transcriptional regulation of ACCβ gene

Transcription of the ACCβ gene is also controlled by two promoters: PI and PII. However, its regulation is not fully understood. Myogenic regulatory factors such as MyoD, myogenin, muscle regulatory factor 4 (MRF4), Myf5 and Myf6 are basic helix-loop-helix (bHLH) transcription factors that play a role in myogenic differentiation. Oh et al[146] demonstrated that MyoD and MRF4 could enhance PI activity in NIH3T3 mouse fibroblast cells. In contrast, Lee et al[147] showed that MyoD and Myf6 markedly induced PII activity. The effects of myogenic regulatory factors on ACCβ transcriptional activity were also shown to be differentially regulated by retinoic acid receptors (RARα, RXRα).[146,148] In addition, ACCβ protein expression increased during the differentiation of H9C2 rat cardiac-derived cells from myoblasts into myotubes.[149] These results provide support for a role of ACCβ in myogenic differentiation. Another study reported that PI but not PII was activated in C2C12 mouse skeletal muscle cells by the transcription factors, cardiac-specific homeobox protein Csx/Nkx2.5 and the zinc finger protein GATA4.[146] These transcription factors activate cardiac genes and are involved in cardiac development. Together, these findings demonstrate that ACCβ gene expression can be differentially regulated in different tissues by the alternative usage of its two promoters.

3.2.1.2.2.2.1. Glucose: a transcriptional modulator of ACCβ gene

Recent advances have also highlighted the role of glucose as a signalling molecule regulating the transcription of the ACCβ gene. The effects of glucose are suggested to occur via glucose-responsive transcription factors that bind to the promoter region of metabolic enzyme-encoding genes. Two examples are sterol regulatory binding protein (SREBP)[150,151] and upstream stimulatory factors (USFs)[152,153]. SREBP-1 belongs to the bHLH/leucine zipper family of transcription factors. It mediates the transcriptional regulation of several lipogenic enzymes in the liver in response to diet and insulin.[154,155]

Observations by Oh et al[151] revealed that a fat-free high carbohydrate diet enhanced ACCβ gene PII activity in the liver. This result was associated with increased SREBP-1 binding to the ACCβ gene PII. Of considerable interest to our study are the USFs, which we regard as strong candidate factors that regulate ACCβ gene expression. In light of this, I will now summarise USF transcriptional function.

3.2.1.2.2.2.1.1. Upstream stimulatory factors

USFs are also members of the bHLH family of transcription factors. To date, two USF polypeptides have been identified, i.e. USF1 (M_r = 43 kDa) and USF2 (M_r = 44 kDA), that are encoded by two distinct genes.[156] These genes are ubiquitously expressed, but the quantity of USF proteins differs in a tissue-specific manner. USF1 and USF2 bind as homodimers or heterodimers to the consensus DNA binding motif 5'-CANNTG-3', termed E-boxes on the promoter regions of several metabolic enzyme-encoding genes (Figure 7).[157-160]

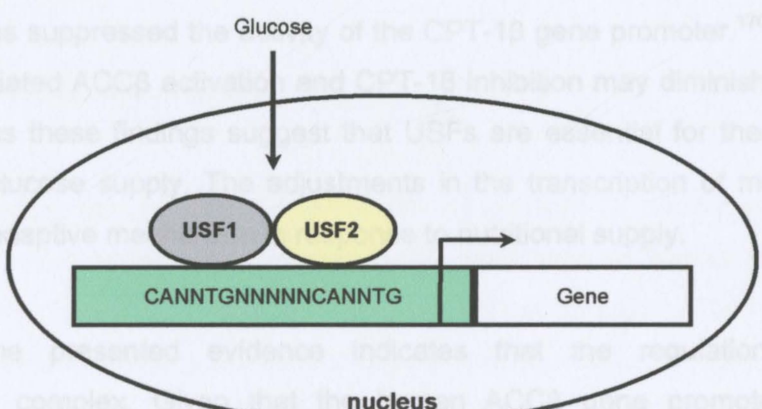


Figure 7. Illustration of transcriptional regulation in response to glucose. Glucose may transcriptionally regulate metabolic enzyme-encoding genes through the binding of USF1 and USF2 to the corresponding recognition sites located within their promoter region. USF1/2 = upstream stimulatory factor 1/2; N = variant nucleotides.

Consistent with the role of USFs in glucose response, Wang and Sul[161] reported an increase in USF1 protein levels in the liver of rats refed a carbohydrate-enriched diet after an acute fast. The lack of both USF1 and USF2 in mice stimulated with glucose decreased hepatic FAS gene expression.[162] Likewise, USF2-deficient mice that were refed a high carbohydrate diet following a 24 hour fast displayed reduced L-PK and S14 gene expression in the liver.[163] Interestingly, Vallet et al[164] demonstrated that USF2 compensated for the reduced glucose response of hepatic L-PK and S14 genes in USF1-knock-out mice.

In the heart USFs are expressed in cardiomyocytes and are involved in the transcriptional regulation of α B-crystallin enhancer[165]. In addition, it regulates contractile proteins such as myosin light chain-2[166] and α-myosin heavy chain[167,168]. A role for USF1 in the transcriptional regulation of ACCβ gene was investigated by Makaula et al[169]. This study revealed that USF1 overexpression transactivated the human ACCβ gene PII in neonatal rat cardiomycytes via an E-box cis-element located near to the transcription start site. Moreover, USF1 further enhanced the transcriptional activity of human ACCβ gene PII in CV-1 monkey kidney fibroblast cells exposed to high glucose levels. In the same study, upregulated cardiac ACCβ gene and protein expression was also observed in mice that fasted and were fed a high carbohydrate diet for 48 hours. These data provide further support for the notion that ACCβ is glucose responsive. On the other hand, forced expression of USF1 and USF2 in neonatal rat cardiomyocytes suppressed the activity of the CPT-1β gene promoter.[170] We speculate that USF-mediated ACCβ activation and CPT-1β inhibition may diminish cardiac FA β-oxidation. Thus these findings suggest that USFs are essential for the transcriptional response to glucose supply. The adjustments in the transcription of metabolic genes represent an adaptive mechanism in response to nutritional supply.

Combined, the presented evidence indicates that the regulation of ACC is extraordinarily complex. Given that the human ACCβ gene promoter[70] has been recently cloned and its characterisation is largely unknown, it is important to understand the transcriptional regulation of ACCβ gene expression in the heart, and was therefore the main focus of our study.

I have reviewed carbohydrate and FA metabolism of the heart. I now wish to focus on mitochondrial energy production, as the end product of these metabolic pathways. Understanding the gene regulatory programs for mitochondrial energy production is fundamental for normal cardiac function because the heart depends on an abundant supply of ATP in order to drive a myriad of specialised cellular processes, such as contractility and ion homeostasis.[64]

3.3. Mitochondrial energy production

Due to the high energy needs of the mammalian heart, cardiomyocytes have copious mitochondria and a high bioenergetic capacity. Additionally, the heart has the ability to adapt to myocardial energy demands by modulating mitochondrial respiratory function, and the number and content of mitochondria (referred to as mitochondrial biogenesis). For example, postnatal development[171,172], exercise training[173] and electrical stimulation[174] enhanced mitochondrial proliferation and bioenergetic capacity. However, perturbations in the adaptive response to myocardial energy demands as a result of mutations in mitochondrial DNA (mtDNA) or the nuclear genome (FA transport proteins or β-oxidation enzymes) can cause defective mitochondrial respiratory function and manifest in a variety of inherited and acquired diseases. Examples include childhood cardiomyopathy, atrioventricular conduction abnormalities, heart failure and diabetes mellitus.[175-185] Therefore, mechanisms regulating mitochondrial energy metabolism involve the coordinate expression of nuclear- and mitochondrial-encoded genes that are under stringent transcriptional regulation.[186,187]

3.3.1. Nuclear respiratory factors

Nuclear respiratory factors-1[188] and -2[189] (NRF-1 and NRF-2) have been identified as transcriptional modulators of nuclear-encoded mitochondrial genes involved in the regulation of mitochondrial biogenesis and respiratory function (Figure 8).[190,191] The importance of NRF-1 in this process was highlighted by Huo et al[192] demonstrating that a targeted disruption of NRF-1 gene in mice caused a dramatic reduction of mtDNA levels and led to premature mortality during embryonic development.

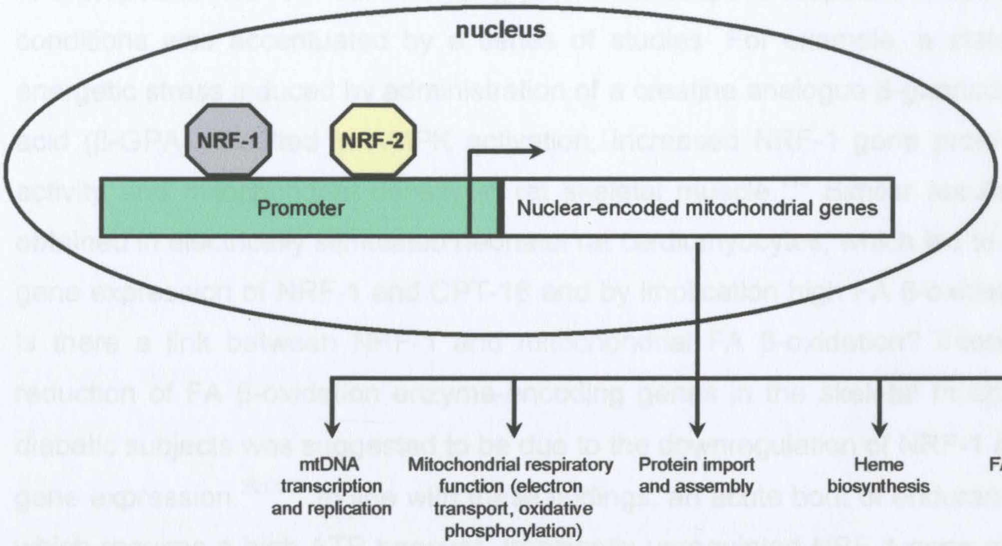


Figure 8. NRFs as regulators of mitochondrial function. NRF-1/2 = nuclear respiratory factor-1/2; mtDNA = mitochondrial deoxyribonucleic acid; FA = fatty acid.

NRF-1 binds as a homodimer to the GC-rich palindrome sequence 5'-(T/C)GCGCA(C/T)GCGC(A/G)-3', found in the promoter region of several genes.[187,190] The DNA binding and transcriptional activity of NRF-1 is increased by phosphorylation of serine residues within its NH_2-terminal domain.[193,194] NRF-1 controls genes encoding the subunits of five respiratory complexes, components of mtDNA transcription and replication machinery, enzymes involved in heme biosynthesis, constituents of mitochondrial protein import and assembly of respiratory apparatus.[190,191] In support, NRF-1 stimulates mtDNA transcription and replication by inducing the gene expression of mitochondrial transcription factor A (mtTFA), a nuclear-encoded transcription factor.[195] Recent evidence also indicates that NRF-1 can promote mitochondrial biogenesis through its direct interaction with the transcriptional coactivator PGC-1α. Here Wu et al[196] showed that PGC-1α induced mitochondrial biogenesis in C2C12 mouse skeletal muscle cells by robustly stimulating the gene expression of NRF-1 and NRF-2. This effect could be inhibited by dominant negative NRF-1. In addition, overexpression of PGC-1α in neonatal rat cardiomyocytes induced mitochondrial biogenesis and respiration through the upregulated expression of NRF-1 target genes, including genes of TCA cycle, electron transport, oxidative phosphorylation and mitochondrial FA β-oxidation enzymes.[197] There is evidence that NRF-1 also acts on genes that are not related to mitochondrial function.[189,198]

The proposed role for NRF-1 forming part of an adaptive response to energetic stress conditions was accentuated by a series of studies. For example, a state of chronic energetic stress induced by administration of a creatine analogue β-guanadinopropionic acid (β-GPA), resulted in AMPK activation, increased NRF-1 gene promoter binding activity and mitochondrial density in rat skeletal muscle.[199] Similar results were also obtained in electrically stimulated neonatal rat cardiomyocytes, which led to upregulated gene expression of NRF-1 and CPT-1β and by implication high FA β-oxidation rates.[200] Is there a link between NRF-1 and mitochondrial FA β-oxidation? Interestingly, the reduction of FA β-oxidation enzyme-encoding genes in the skeletal muscle of type 2 diabetic subjects was suggested to be due to the downregulation of NRF-1 and PGC-1α gene expression.[201,202] In line with these findings, an acute bout of endurance exercise, which requires a high ATP turnover, transiently upregulated NRF-1 gene expression in rat skeletal muscle. This supports its involvement in the exercise-induced increase in mitochondrial bioenergetic capacity.[203] Moreover, exercise increased FA β-oxidation in human skeletal muscle. This result was attributed to the inhibition of ACCβ activity and the subsequent lowering of malonyl-CoA levels.[204] Thus these data suggest that NRF-1 may be associated with FA β-oxidation enzymes-encoding genes, but a role for NRF-1 in the regulation of ACCβ gene expression as a mechanism of controlling cardiac FA β-oxidation must still be determined.

All of these results underscore the significance of transcriptional mechanisms regulating mitochondrial energy production as an adaptive response to myocardial energy demands. It also sheds light on the role of NRF-1 as a master regulator of the mitochondrial respiratory system.

I have reviewed several metabolic pathways regulating mitochondrial energy production of the normal mammalian heart. As discussed, these processes are exquisitely regulated and stringent control mechanisms ensure energetic homeostasis. However, alterations in cardiac substrate metabolism are considered to be part of an adaptive mechanism to increase energy efficiency in response to changing myocardial energy demands, e.g. the developing heart. Moreover, chronic stress signals may result in maladaptive cardiac metabolic remodelling, e.g. the diabetic heart, with detrimental consequences. I will now discuss examples of altered metabolism in the heart to illustrate both adaptive and maladaptive metabolic remodelling.

4. ALTERED CARDIAC SUBSTRATE METABOLISM

4.1. Developing heart

The foetal heart favours lactate oxidation and glycolysis as sources of ATP due to the low oxygen environment (Figure 9).[27,205,206] During gestation, lactate is enriched in the placenta[207] and is harnessed by the foetal heart as its main energy substrate. In contrast, plasma NEFA levels are low in the foetal heart[208] and therefore utilised to a reduced extent. After birth, plasma NEFA levels rise and the substrate preference switches to FA. As a result, postnatal hearts display a dramatic increase in FA β-oxidation with a concomitant decline in glycolysis and carbohydrate oxidation.[209]

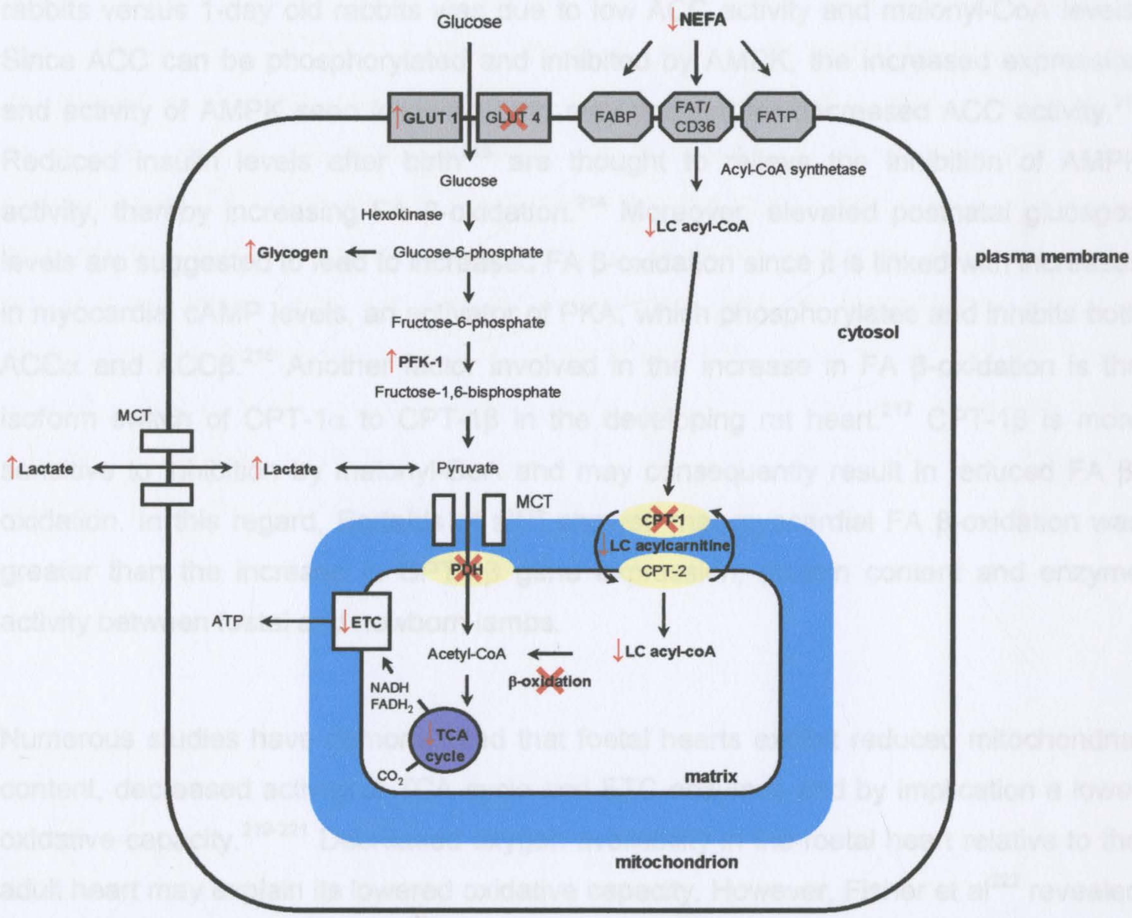


Figure 9. Alterations of carbohydrate and FA metabolism in the cardiomyocyte of foetal heart. GLUT 1/4 = glucose transporters 1/4; PFK-1 = phosphofructokinase-1; MCT = monocarboxylate transporter; PDH = pyruvate dehydrogenase; NEFA = nonesterified fatty acid; FABP = fatty acid binding protein; FAT/CD36 = fatty acid translocase; FATP = fatty acid

transport protein; CPT-1/2 = carnitine palmitoyltransferase-1/2; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate. Red cross indicates diminished flux through pathways.

This metabolic transition is accompanied by changes in the activity and gene expression of metabolic enzymes, as well as hormonal levels.[205,206] For example, glucose uptake mediated by the GLUT 1 isoform in foetal hearts is substituted by the insulin-sensitive isoform GLUT 4 in postnatal hearts.[210,211] Furthermore, the glycolytic enzyme PFK is less sensitive to inhibition by ATP and citrate, and more sensitive to activation by F-2,6-BP in foetal hearts.[212] These effects may be responsible for the high PFK activity and subsequent increase in glycolytic capacity observed in foetal hearts. Lopaschuk et al[213] reported that higher rates of FA β-oxidation in the hearts of 7-day old rabbits versus 1-day old rabbits was due to low ACC activity and malonyl-CoA levels. Since ACC can be phosphorylated and inhibited by AMPK, the increased expression and activity of AMPK seen following birth may account for decreased ACC activity.[214] Reduced insulin levels after birth[215] are thought to relieve the inhibition of AMPK activity, thereby increasing FA β-oxidation.[214] Moreover, elevated postnatal glucagon levels are suggested to lead to increased FA β-oxidation since it is linked with increases in myocardial cAMP levels, an activator of PKA, which phosphorylates and inhibits both ACCα and ACCβ.[216] Another factor involved in the increase in FA β-oxidation is the isoform switch of CPT-1α to CPT-1β in the developing rat heart.[217] CPT-1β is more sensitive to inhibition by malonyl-CoA and may consequently result in reduced FA β-oxidation. In this regard, Bartelds et al[218] showed that myocardial FA β-oxidation was greater than the increase in CPT-1β gene expression, protein content and enzyme activity between foetal and newborn lambs.

Numerous studies have demonstrated that foetal hearts exhibit reduced mitochondrial content, decreased activity of TCA cycle and ETC enzymes and by implication a lower oxidative capacity.[219-221] Decreased oxygen availability in the foetal heart relative to the adult heart may explain its lowered oxidative capacity. However, Fisher et al[222] revealed that myocardial oxygen consumption was similar in the foetal and the adult lamb. This finding was attributed to higher myocardial blood flow and oxygen extraction in the foetal lamb. Conversely, oxygen consumption in foetal guinea pig hearts was significantly different from adult guinea pig hearts.[223] These conflicting studies may be due to the differences in the animal species or the experimental protocols and require further investigation to resolve this issue.

The heart can return to the foetal program as an adaptive metabolic mechanism in a number of cardiac pathologies, including cardiac hypertrophy[224,225] and ischaemia[226,227]. Therefore it is important to further our knowledge of the metabolic transition in the developing heart.

4.2. Type 2 diabetic heart

A large body of evidence shows that a decrease in carbohydrate utilisation and the almost exclusive reliance on FA to generate ATP is a hallmark of the type 2 diabetic heart (Figure 10).[24,228,229,65,230] Notably, insulin appears to be ineffective in suppressing the release of FA from adipose tissue in type 2 diabetes. As a result plasma NEFA levels are markedly elevated, consistent with increased FA utilisation in the type 2 diabetic heart.[231,232] High plasma NEFA levels are thought to lead to intracellular lipid accumulation that attenuates insulin-mediated glucose uptake by inhibiting insulin receptor substrates (IRS) and PKB.[233] Furthermore, high fat feeding was shown to cause impaired glucose tolerance and decreased muscle insulin sensitivity.[234,235] These effects correspond with reduced carbohydrate utilisation in the type 2 diabetic heart. Alterations in gene expression in response to the metabolic environment may also account for the shift in fuel substrates for the type 2 diabetic heart.[236]

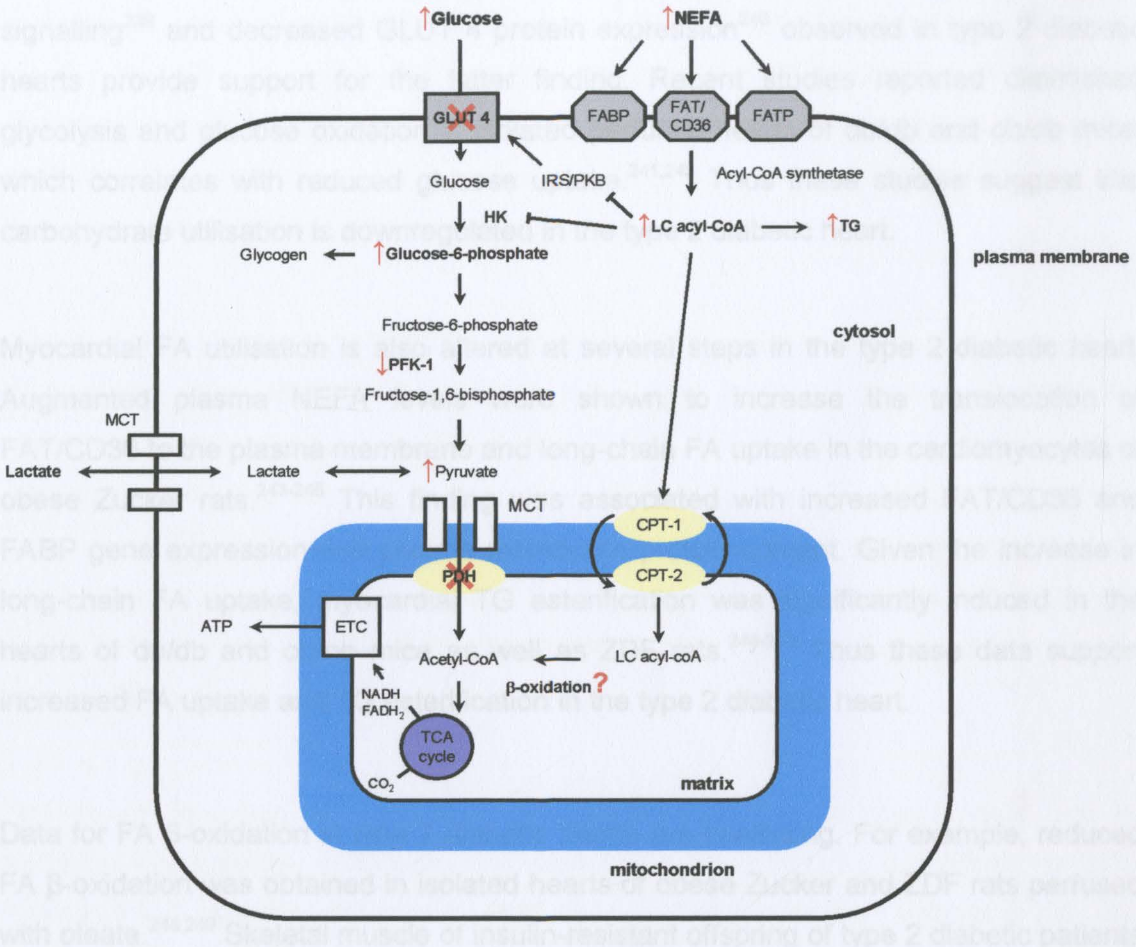


Figure 10. Alterations of carbohydrate and FA metabolism in the cardiomyocyte of the type 2 diabetic heart. GLUT 4 = glucose transporter 4; HK = hexokinase; PFK-1 = phosphofructokinase-1; MCT = monocarboxylate transporter; PDH = pyruvate dehydrogenase; NEFA = nonesterified fatty acid; FABP = fatty acid binding protein; FAT/CD36 = fatty acid translocase; FATP = fatty acid transport protein; TG = triglyceride; IRS = insulin receptor substrate; PKB = protein kinase B; CPT-1/2 = carnitine palmitoyltransferase-1/2; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate. Red cross indicates diminished flux through pathways.

Although the metabolic profile of enhanced FA utilisation and reduced carbohydrate utilisation in type 2 diabetic hearts is accepted, several studies investigating this shift in substrate metabolism are conflicting. This is possibly due to the variation in human studies and animal models, i.e. db/db mice, ob/ob mice, obese Zucker rats and Zucker diabetic fatty (ZDF) rats. For example, humans with type 2 diabetes exhibited insulin-responsive myocardial glucose uptake.[237,238] In contrast, the cardiomyocytes of db/db mice displayed reduced insulin-stimulated glucose uptake.[239] Impaired insulin signalling[239] and decreased GLUT 4 protein expression[240] observed in type 2 diabetic hearts provide support for the latter finding. Recent studies reported diminished glycolysis and glucose oxidation in isolated perfused hearts of db/db and ob/ob mice, which correlates with reduced glucose uptake.[241,242] Thus these studies suggest that carbohydrate utilisation is downregulated in the type 2 diabetic heart.

Myocardial FA utilisation is also altered at several steps in the type 2 diabetic heart. Augmented plasma NEFA levels were shown to increase the translocation of FAT/CD36 to the plasma membrane and long-chain FA uptake in the cardiomyocytes of obese Zucker rats.[243-245] This finding was associated with increased FAT/CD36 and FABP gene expression and plasma membrane protein content. Given the increase in long-chain FA uptake, myocardial TG esterification was significantly induced in the hearts of db/db and ob/ob mice as well as ZDF rats.[246-248] Thus these data support increased FA uptake and TG esterification in the type 2 diabetic heart.

Data for FA β-oxidation in type 2 diabetic hearts are conflicting. For example, reduced FA β-oxidation was obtained in isolated hearts of obese Zucker and ZDF rats perfused with oleate.[248,249] Skeletal muscle of insulin-resistant offspring of type 2 diabetic patients also displayed a similar effect.[250] In agreement with these findings, the skeletal muscle of type 2 diabetic patients exhibited increased ACCβ gene expression[232] and malonyl-CoA levels[251,252]. This result provides support for the idea that ACCβ or malonyl-CoA may be involved in the inhibition of FA β-oxidation in type 2 diabetes. Others proposed that decreased gene expression of PGC-1α, NRF-1 and its target genes accounts for the reduced FA β-oxidation in the skeletal muscle of type 2 diabetic subjects.[201,202] It was also suggested that reduced skeletal muscle mitochondrial density observed in insulin-resistant offspring of type 2 diabetic patients may be responsible for low FA β-oxidation rates.[253] Conversely, enhanced instead of reduced FA β-oxidation was reported in isolated hearts of db/db and ob/ob mice perfused with palmitate.[241,242] Upregulated gene expression of PPARα and its target genes (PDK4, medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase, very long-chain acyl-CoA dehydrogenase, uncoupling protein 2 (UCP2) and UCP3) in both db/db and ob/ob mice may explain this increase in FA β-oxidation.[254] Elevated gene expression of CPT-1β in the hearts of ob/ob mice[247] and ZDF rats[255] may also infer increased FA β-oxidation. Although these animal studies show increased FA β-oxidation in type 2 diabetic hearts, we speculate that it does not fully compensate for the excess NEFA supply. Taken together, the results of FA β-oxidation in the type 2 diabetic heart is inconclusive. This may be related to human versus animal studies and methodologies employed to determine substrate utilisation. Additional studies are therefore needed to clarify this contentious issue.

Based on the Randle hypothesis[256], increased FA β-oxidation is capable of inhibiting glucose utilisation. High FA β-oxidation rates raise cytosolic citrate concentrations, which can inhibit glycolysis at PFK-1 and PFK-2. G-6-P subsequently accumulates, suppressing HK and resulting in decreased glucose uptake. Elevated FA β-oxidation rates also decrease glucose oxidation by increasing mitochondrial acetyl-CoA/free CoA and $NADH/NAD^+$ ratios, which activate PDK that inhibits PDH complex activity as well as flux through PDH complex.[37] In support, hearts from ZDF rats displayed reduced flux through PDH complex.[257]

Conversely, inhibition of FA β-oxidation can promote carbohydrate uptake and its oxidation. For example, an increase in malonyl-CoA levels induced by inhibition of MCD reduced FA β-oxidation and stimulated glucose oxidation in both *ex vivo* rat and *in vivo* pig hearts.[258] This effect was also observed in hearts treated with dichloroacetate, a PDH activator. Here dichloroacetate decreased FA β-oxidation by increasing the activity of ACCβ and raising both malonyl-CoA and acetyl-CoA levels.[259,260] Furthermore, CPT-1 inhibitors such as perhexiline[261], etomixir[262] and oxfenicine[263] suppress cardiac FA β-oxidation and is associated with increased glucose oxidation. Also, carnitine administration to isolated working rat hearts perfused with palmitate resulted in accelerated glucose oxidation and decreased FA β-oxidation.[264] This was suggested to occur following the increase in PDH activity as a result of the decrease in mitochondrial acetyl-CoA/free CoA ratios. The addition of trimetazidine, an inhibitor of FA β-oxidation (long-chain-3-ketoacyl-CoA-thiolase), increased PDH activity and glucose oxidation in the isolated working rat heart.[265]

Thus the oversupply of NEFA and the theory proposed by Randle et al[256] is in favour of enhanced FA utilisation and reduced carbohydrate utilisation in the type 2 diabetic heart. Although this shift in myocardial substrate utilisation in type 2 diabetes emphasises the metabolic flexibility of the heart, chronically it can adversely impact the heart, such as diabetic cardiomyopathy.[228]

4.2.1. Diabetic cardiomyopathy

The metabolic profile of the type 2 diabetic heart is associated with the development of diabetic cardiomyopathy, which is described as a clinical condition of ventricular dysfunction that is not attributable to coronary artery disease and hypertension.[266-269] Indeed, impaired diastolic and systolic function have been demonstrated in the hearts of type 2 diabetic patients[270-272] and rodent models[241,273-276]. Multifactorial mechanisms have been proposed for initiating diabetic cardiomyopathy, including increased oxidative stress, impaired mitochondrial function and lipotoxicity, and will now be explained (Figure 11).

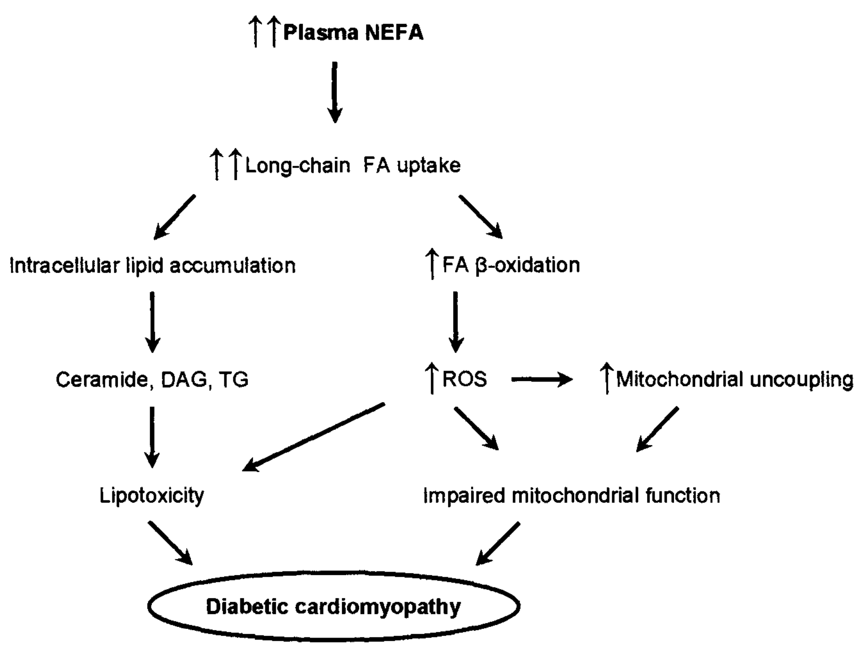


Figure 11. Potential mechanisms involved in the development of diabetic cardiomyopathy. In type 2 diabetes, excessively high plasma NEFA levels activate PPARα, which upregulates the transcription of several FA β-oxidation enzyme-encoding genes. High FA β-oxidation increases ROS production and may induce mitochondrial uncoupling, thereby leading to impaired mitochondrial function. High plasma NEFA levels also promote long-chain FA uptake and intracellular lipid accumulation (sarcolemmal FA uptake >> FA β-oxidation). This may lead to the production of toxic lipid intermediates (ceramide, DAG, TG), and result in

lipotoxicity. These effects may be associated with the development of diabetic cardiomyopathy. NEFA = nonesterified fatty acids; ROS = reactive oxygen species; DAG = diacylglycerol; TG = triglyceride.

4.2.1.1. Increased oxidative stress

Accumulating evidence implicate increased ROS generation in the pathogenesis of diabetic cardiomyopathy.[277,278] In type 2 diabetes, prolonged exposure to excessively high plasma NEFA levels activates PPARα, which upregulates the transcription of several FA β-oxidation enzyme-encoding genes. High FA β-oxidation augments ROS production, which can activate maladaptive signalling pathways that lead to cardiomyocyte damage or death.[279] This in turn may contribute to the development of diabetic cardiomyopathy. Increased ROS production is indeed observed in type 2 diabetic hearts.[248,277,278] Diabetes induced cardiomyopathy was prevented by overexpression of antioxidants, e.g. metallothionein[280], catalase[281] and manganese superoxide dismutase[282] in the heart. Therefore, interventions that reduce ROS or enhance ROS scavenging may be useful approaches against the development of diabetic cardiomyopathy.

4.2.1.2. Impaired mitochondrial function

Impaired mitochondrial function may be a contributing factor in the development of diabetic cardiomyopathy because it reduces the efficiency of ATP production. Kuo et al[283] reported decreased mitochondrial respiration (state 3) in db/db mouse hearts, which indicates impaired mitochondrial function. Moreover, high palmitate concentrations increased myocardial oxygen consumption and decreased cardiac efficiency in isolated perfused hearts from ob/ob mice[242] and isolated working hearts from db/db mice[284]. In ZDF rat hearts, myocardial oxygen consumption and cardiac efficiency was unchanged even though FA β-oxidation was high.[285] These inconsistencies may be due to the differences in animal models. However, the former finding suggests that oxygen wasting can potentially occur in the type 2 diabetic heart.

The precise mechanisms for this concept are unclear and are proposed to occur via uncoupling proteins (UCPs).[284,286,287] UCPs are located in the inner mitochondrial membrane and are thought to uncouple mitochondrial oxidative phosphorylation and ATP synthesis.[288] Other authors suggested that mitochondrial uncoupling may be an adaptive mechanism to alleviate FA-induced ROS damage inside the mitochondrial matrix.[289,290] When the mitochondrial FA supply is high, mitochondrial thioesterase-1 catalyses the hydrolysis of long-chain acyl-CoA to generate FA anions and CoA. Himms-Hagen and Harper[291] postulated that UCP3 exports FA anions from the mitochondrial matrix to the intermembrane space and cytosol, and releases CoA. These actions are proposed to promote FA β-oxidation, as well as reduce ROS production by decreasing the proton-motive force and increasing the activity of the ETC. There are two UCP isoforms expressed in the heart, i.e. UCP2 and UCP3. Augmented protein expression of UCP2 and UCP3 was obtained in isolated working hearts from ob/ob and db/db mice.[254] This result was associated with increased mitochondrial oxygen consumption and reduced cardiac efficiency. This increase also correlated positively with raised plasma NEFA levels, particularly in obese and type 2 diabetic patients with heart failure.[292] In contrast, a recent study by Boudina et al[286] reported that UCP protein levels were not altered in db/db hearts with reduced cardiac efficiency. The findings of Wilson et al[293] also showed that Wistar rats challenged with a high fat diet maintained their contractile function even though they exhibited increased cardiac UCP3 gene expression and myocardial oxygen consumption. This was suggested to be a positive adaptation by possibly reducing ROS production. In light of these discrepancies, further examination is required to determine the functional role of UCPs in the type 2 diabetic heart.

4.2.1.3. Cardiac lipotoxicity

Cardiac lipotoxicity may also be a potential mechanism involved in the development of diabetic cardiomyopathy.[294] It results from excessive FA uptake by the heart exceeding FA β-oxidation capacity, leading to myocardial lipid accumulation.[248,294,295] This in turn may lead to the synthesis of toxic lipid intermediates, such as ceramide, diacylglycerol (DAG) and TG, which is thought to trigger apoptosis and affect signalling cascades implicated in the development of skeletal muscle insulin resistance.[296,297,233,298,299] For example, ceramide upregulates inducible nitric oxide (iNOS) expression, thereby increasing nitric oxide production and the formation of peroxynitrite, thus inducing apoptosis via caspase 3 activation and cytochrome c release from mitochondria. Ceramide also directly associates with cytochrome c to promote apoptosis.[300] Elevated

DAG levels is presumed to activate PKCs (PKCβ2), which was shown to play a role in the development of cardiomyopathy.[301] Higher DAG levels are also implicated in impaired GLUT 4 translocation to the sarcolemma. A recent study suggested that the channelling of long-chain acyl-CoA into TG stores is protective against lipotoxicity.[302]

Insight into cardiac lipotoxicity was drawn from both human studies and animal models. For example, Sharma et al[255] detected intramyocardial lipid accumulation in explanted hearts of type 2 diabetic patients with non-ischaemic heart failure (Figure 12). Non-invasive magnetic resonance spectroscopy confirmed the elevation of myocardial TG content in humans with impaired glucose tolerance and type 2 diabetes.[303] Moreover, depressed contractile function was evident in the hearts of overweight and obese subjects with higher myocardial TG levels.[304] Similarly, the hearts of ZDF rats exhibited cardiac hypertrophy and contractile dysfunction, which was associated with elevated myocardial TG content.[248] The latter is presumably due to reduced myocardial FA β-oxidation, since there was downregulated gene expression of CPT-1α, ACO and PPARα. Increases in DNA laddering, ceramide levels and iNOS gene expression also occurred in the same study, suggesting apoptosis. Administration of the TZD troglitazone, attenuated these complications. Thus the evidence documented here supports a strong link between lipid accumulation, lipotoxicity and the development of diabetic cardiomyopathy.

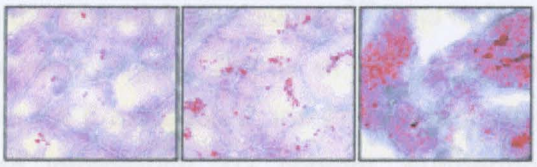


Figure 12. Intramyocardial lipid accumulation. Photomicrograph depicting low, moderate and high intramyocardial lipid accumulation in type 2 diabetic hearts with nonischaemic heart failure. Intramyocardial lipid accumulation was determined by Oil red O staining. Adopted the figure from Sharma et al[255].

The next question that arises is whether intramyocardial lipid accumulation in type 2 diabetic hearts occurs due to increased long-chain FA uptake or decreased FA β-oxidation? This question will be now addressed.

4.2.1.3.1. Role of increased FA uptake in intramyocardial lipid accumulation

With type 2 diabetes, plasma NEFA levels are elevated and are thought to promote excessively high long-chain FA uptake. This in tum may exceed FA β-oxidation rates in the mitochondrion and lead to intramyocardial lipid accumulation. Indeed, transgenic mice overexpressing long-chain ACS in the heart caused increased NEFA uptake, and resulted in significant cardiomyocyte TG deposition, increased myocardial ceramide content and the release of cytochrome c from mitochondria.[305] This led to the development of dilated cardiomyopathy, and resulted in premature death. Similar effects were also observed in mice with a cardiac-restricted overexpression of PPARα, which upregulates several FA uptake, esterification and β-oxidation enzyme-encoding genes.[306,307] TG accumulation in the hearts of these mice was proposed to occur as a result of increased FA uptake, since lipotoxic cardiomyopathy was completely attenuated in mice lacking FAT/CD36.[308] These data provide strong evidence that increased FA uptake leads to intramyocardial lipid accumulation.

4.2.1.3.2. Role of decreased FA β-oxidation in intramyocardial lipid accumulation

Intramyocardial lipid accumulation can also result from a reduction in FA β-oxidation. For example, obese Zucker rat hearts were unable to increase FA β-oxidation following fasting, which elevated the plasma NEFA supply.[249] This led to augmented ectopic lipid accumulation within cardiomyocytes. Befroy et al[250] and Petersen et al[309] showed reduced skeletal muscle FA β-oxidation in insulin-resistant offspring of type 2 diabetic patients with increased intracellular TG content. Furthermore, cardiomyocyte TG accumulation was induced in PPARα-deficient mice with a reduced capacity for myocardial FA β-oxidation and by treatment with the pharmacological CPT-1 inhibitor etomoxir.[310,311] These studies point towards intramyocardial lipid accumulation occurring from reduced FA β-oxidation.

In light of these studies, it is imperative to elucidate the mechanisms responsible for intramyocardial lipid accumulation and lipotoxicity, since it may unlock novel therapeutic interventions for diabetic cardiomyopathy. Studies performed on ACCβ-deficient mice demonstrate low malonyl-CoA levels and high FA β-oxidation rates in the heart and skeletal muscle, as well as a decline in myocardial TG levels.[312,313] Reduced hepatic fat, decreased body weight and adipose tissue fat content, despite hyperphagia were also observed in the same study. Moreover, these mice were protected from obesity and type 2 diabetes when fed a high fat/high carbohydrate diet.[314] Savage et al[315] showed that inhibiting ACCα and ACCβ with antisense oligonucleotides increased FA β-oxidation, lowered malonyl-CoA and lipid levels (long-chain acyl-CoA, DAG, TG), and enhanced insulin sensitivity in isolated hepatocytes from rats challenged with a high fat diet. These data raise the possibility that increasing FA β-oxidation via inhibition of ACCβ may favourably affect the type 2 diabetic heart, by clearing detrimental intramyocardial lipid accumulation and consequent lipotoxicity. In light of this, we explored the transcriptional mechanisms modulating ACCβ gene expression in the heart.

B. HYPOTHESIS AND OBJECTIVES

NRF-1 is a pivotal transcriptional modulator of nuclear-encoded mitochondrial proteins.[190,191] A recent study demonstrated that activation of AMPK is associated with enhanced NRF-1 gene promoter binding activity.[199] Moreover, NRF-1 has been linked with increased gene expression of CPT-1, the rate-limiting mitochondrial FA transfer enzyme.[200] Upstream, ACCβ produces malonyl-CoA, a potent inhibitor of CPT-1 and FA β-oxidation. Interestingly, the hearts of ACCβ-deficient mice displayed reduced malonyl-CoA levels, increased FA β-oxidation, decreased TG levels and maintained normal cardiac function.[312,313] These mice were also protected from obesity and type 2 diabetes following a high fat/high carbohydrate diet.[314]

Hypothesis

We postulated that NRF-1 inhibits ACCβ gene promoter activity in the heart via AMPK activation. If proved true, we predict that future work will show that this would reduce malonyl-CoA levels and lead to enhanced mitochondrial FA uptake and β-oxidation, thus potentially lowering damaging intramyocardial lipid accumulation.

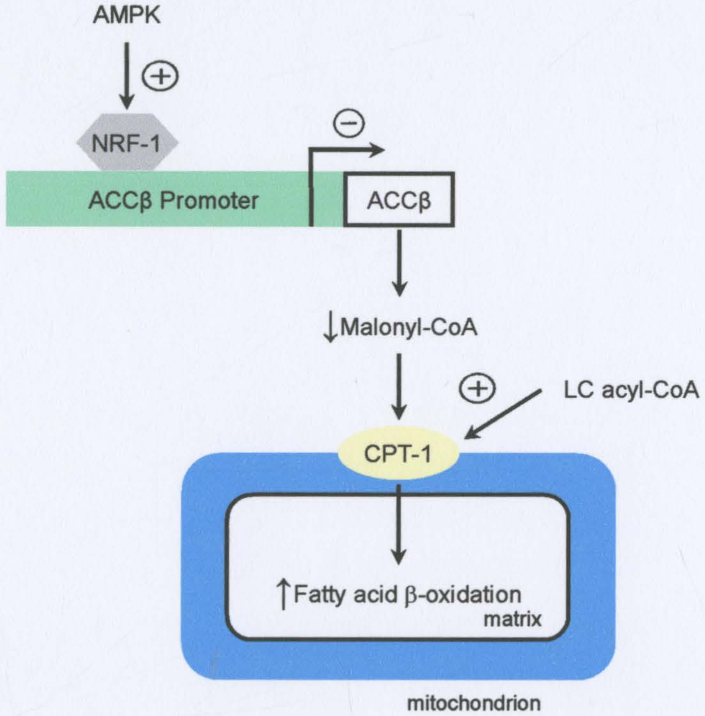


Figure 13. Schematic representation of hypothesis. AMPK = 5'-AMP-activated protein kinase; NRF-1 = nuclear respiratory factor-1; ACCβ = acetyl-coenzyme A carboxylase beta; CPT-1 = carnitine palmitoyltransferase-1; LC = long-chain.

Objectives

Using neonatal rat cardiomyocytes, the objectives of our study were to:

1. Investigate the regulation of the human ACCβ gene promoter by NRF-1, and whether the effect is dependent on AMPK activation.
2. Assess the effects of NRF-1 overexpression on USF1-mediated transactivation of the human ACCβ gene promoter.
3. Determine endogenous ACCβ gene and protein expression for Aims #1 and #2 (above).

C. MATERIALS AND METHODS

1. CELL CULTURE

1.1. Cardiomyocyte isolation

Neonatal rat cardiomyocytes are a widely applied experimental model that has been used routinely in cardiac research over the years.[316,317] Numerous studies have demonstrated that this model offers a valuable tool to explore cardiac gene regulation.[169,170] The isolation procedure is well-described in the literature, easy, and high quality and yields of cardiomyocytes are repeatedly obtained.[318-320] Another advantage is that the interference of other cell types in the heart, such as fibroblasts and endothelial cells, are excluded. Cultured cardiomyocytes also have a highly stable phenotype.[321] Importantly, ACCβ is abundantly expressed in the heart.[67] Moreover, Linssen et al[322] showed that the capacity for palmitate oxidation was higher in cardiomyocytes than in either fibroblast or endothelial cells. Therefore, cardiomyocytes was selected for our study.

Cardiomyocytes were isolated from heart tissue of 1 - 3 day old Wistar rats using a standard protocol with minor modifications.[318] Animals older than 3 days were not used since these cardiomyocytes display increased cell debris, low cell viability, and do not suitably adhere to the surface of tissue culture plates. Hearts were harvested aseptically, rinsed in 1 x ADS buffer (Appendix) to remove blood and debris, and minced finely. Cells were released by digesting the tissue in a collagenase (Worthington, Lakewood, NJ, USA)/pancreatin (Sigma-Aldrich, Steinheim, Germany) solution (Appendix) for 15 minutes in a 37°C shaking water bath (Labcon, South Africa). Digestion of the tissue was repeated several times until most or all of the tissue was digested. The cells were centrifuged (Harrier 18/80, MSE, UK) at 300 x g for 3 minutes, and collected in newborn calf serum (NBS) (Invitrogen, Paisley, Scotland, UK). Cardiomyocytes were subsequently purified on a Percoll (GE Life Sciences, Vienna, Austria) gradient (top layer 1.082 g/ml, middle layer 1.062 g/ml and bottom layer 1.050 g/ml) (Appendix) and centrifuged (Harrier 18/80, MSE, UK) at 1,000 x g for 20 minutes. Percoll separates cardiomyocytes from other cell types in the heart, i.e. cardiomyocytes appeared between the middle and bottom layer of the gradient. The cardiomyocytes were collected with a pasteur pipette, washed in 1 x ADS buffer, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Paisley, Scotland, UK) supplemented with 20% foetal calf serum (FCS) (Highveld Biological, South Africa) and 100 μg/ml penicillin/streptomycin (Highveld Biological, South Africa). Thereafter, the cardiomyocytes were incubated (SHEL LAB TC2323, Cornelius, OR, USA) at 37°C (5% CO_2, 95% air) for 72 hours.

1.2. Cell counting and viability

The cell count and viability for the cardiomyocytes was estimated by initially mixing an equal volume of cell suspension and 5.2 mM trypan blue dye (Sigma-Aldrich, Steinheim, Germany) (Appendix). Trypan blue is taken up and stains cells with damaged membranes.[323] The stained cells were then added to the chamber of a haemocytometer (LASEC, South Africa) and visualised with an Olympus light microscope (Wirsam Scientific, South Africa). The number of cells/ml was calculated by multiplying the average number of unstained cells (viable) by 2 (dilution factor) x 10^4. The cell count was adjusted to a concentration of 1 x 10^6 cells/ml. The cell viability is the number of viable cells divided by the total number of cells (viable and dead) and expressed as a percentage. A cell viability of >85% was used for all experiments.

2. PLASMID DNA PREPARATION

2.1. Plasmid DNA isolation

One nanogram of plasmid DNA was transformed into 50 μl of JM109 *Escherichia coli* competent cells (Promega, Madison, WI, USA), plated onto 2 x YT agar (Appendix) containing 100 μg/ml of ampicillin (Roche, Mannheim, Germany) (Appendix), and incubated (Labcon, South Africa) overnight at 37°C. The following day, a single colony was inoculated into 300 ml of 2 x YT broth (Appendix) containing 100 μg/ml of ampicillin, and incubated (Labcon, South Africa) at 37°C with shaking for 16 hours. Plasmid DNA isolation was subsequently carried out using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA pellets were resuspended in 300 μl of 1 x TE buffer (Appendix) and stored at -20°C.

2.2. Plasmid DNA quantification and purity

Plasmid DNA concentrations were determined by measuring the absorbance at a wavelength of 260 nm in a Shimadzu UV-1201 spectrophotometer (Shimadzu, Columbia, MD, USA), and was calculated as follows:

$$A_{260\ nm} \times 50\ \mu g/ml \times \text{dilution factor} = x \text{ amount of DNA in } \mu g/ml$$

One absorbance unit at 260 nm is equal to 50 µg/ml of double-stranded DNA. The plasmid DNA concentrations were adjusted to 1 µg/µl with sterile H_2O.

The purity of the plasmid DNA was evaluated from the ratio of the readings at 260 nm and 280 nm ($A_{260\ nm}/A_{280\ nm}$). Pure DNA has an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8 - 2. An $A_{260\ nm}/A_{280\ nm}$ ratio < 1.8 denotes contamination of proteins, and $A_{260\ nm}/A_{280\ nm}$ ratio > 2 denotes contamination with RNA in the preparation. Plasmid DNA with an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8 - 2 was routinely used.

2.3. Plasmid DNA constructs

The following plasmid DNA constructs (Appendix) were used in subsequent transient transfection experiments:

The luciferase construct constitutively expressed by SV40 promoter (pGL3-Control), was acquired from Promega (Madison, WI, USA). The *Renilla* (*Renilla reniformis*) luciferase construct containing the cytomegalovirus (CMV) immediate early enhancer/promoter region (pRL-CMV), was also acquired from Promega (Madison, WI, USA). The firefly (*Photinus pyralis*) luciferase construct for the full-length human ACCβ gene promoter (pPIIβ-1317/+65-Luc), which was cloned into the vector pGL3-Basic, was previously desciibed and generously provided by Dr. Kyung-Sup Kim (Yonsei University College of Medicine, Seoul, Korea).[147] The human NRF-1 expression construct (pSG5-NRF-1) was generated by Dr. Richard Scarpulla, and the dominant negative NRF-1 construct (pCMV-HA-dnNRF-1) was procured from Dr. Helmut Grasberger (University of Chicago, USA).[189,324] The human USF1 expression construct (pUC-SRα-USF1) was a kind gift from Dr. Tetsuya Kamataki (Hokkaido University, Japan).[325] A reporter construct containing multiple copies of USF1-specific enhancer elements (pUSF1-Luc) inserted into a translucent control luciferase vector (pControl-Luc), was purchased from Panomics (Redwood City, CA, USA).

3. TRANSIENT TRANSFECTION

Transfection is the transfer of foreign DNA into eukaryotic cells. The transfection protocol employed here is transient since the transfected DNA is not inserted into the nuclear genome and can be lost during cell mitosis. This approach is utilised for the study of gene regulation and function, and to monitor protein expression.

For the transient transfection experiments, cardiomyocytes were seeded at a density of 1 x 10^6 cells/12-well plates pre-coated with 5 μg/cm^2 of fibronectin (Roche, Penzberg, Germany) (Appendix).[326] The next day, cardiomyocytes were transfected using FuGene 6 reagent (Roche, Mannheim, Germany) following the supplier's instructions. FuGene 6 is a lipid-based reagent that was previously shown to generate a high transfection efficiency, almost no cytotoxicity, and a large number of contracting cardiomyocytes after 36 - 48 hours incubation.[327] In addition, FuGene 6 entails very little optimisation, performs well with or without serum, and the cells maintain the majority of their normal physiological functions.

Initially, optimisation of the transfection procedure was carried out at a ratio of 2:1 and 6:1 with amounts of 1.5 μl of FuGene 6 to 750 ng and 250 ng respectively, of plasmid DNA (pGL3-Control, pPIIβ-1317/+65-Luc). A ratio of 2:1 was chosen for subsequent assays.

To investigate the transcriptional regulation of the human ACCβ gene promoter by NRF-1, the transfection was performed with 375 ng of pPIIβ-1317/+65-Luc ± 125 ng of each of the expression constructs, pSG5-NRF-1 and pCMV-HA-dnNRF-1.

To delineate the potential role of AMPK activation on the transcriptional regulation of ACCβ gene promoter, cardiomyocytes were exposed to two AMPK activators. Here 0.5, 1 and 2 mM of AICAR (Sigma-Aldrich, Steinheim, Germany) (Appendix) in serum-free medium, or 1 mM of metformin (Sigma-Aldrich, Steinheim, Germany) (Appendix), were administered for 24 hours.

USF1 is a previously identified transactivator of the human ACCβ gene promoter.[169] Next, we assessed the NRF-1 response to USF1-mediated transactivation of the ACCβ gene promoter with 270 ng of pPIIβ-1317/+65-Luc and various combinations of 160 ng of pUC-SRα-USF1, pSG5-NRF-1, or pCMV-HA-dnNRF-1. In addition, 375 ng of pUSF1-Luc was cotransfected ± 188 ng of NRF-1 and pCMV-HA-dnNRF-1 to determine the effect of NRF-1 on endogenous transcriptional activity of USF1, and in response to AMPK activation by AICAR treatment as detailed earlier.

Total DNA concentrations were equalised with vector only (pGL3-Basic, pControl-Luc). Ten nanograms of pRL-CMV was included in the transfection reactions to control for differences in cell viability or transfection efficiency.

4. TRANSCRIPTIONAL ACTIVITY

4.1. Luciferase assay

The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) employs the bioluminescent enzyme luciferase as a reporter for the transcriptional activity of specific promoters. This technique simultaneously expresses and sequentially measures from a single lysate one reporter (firefly) containing the experimental promoter and a second reporter (Renilla) containing a constitutive promoter which serves as an internal control. Relative changes in the activity of the reporter are directly proportional to changes in the activity of the linked promoter.

Forty eight hours after transfection, cardiomyocytes were washed with 1 x PBS (Appendix). The cells were lysed by adding 200 µl of 1 x passive lysis buffer (Promega, Madison, WI, USA), scraped from the culture plates, and transferred into 1.5 ml microfuge tubes. The cell lysates were stored at -80°C and subjected to one freeze/thaw cycle to ensure complete cell lysis for the luciferase assay. To remove residual cell debris, cell lysates were centrifuged (Sanyo Micro Centaur, MSE, UK) at 12, 000 x g for 2 minutes at 4°C. Light units for firefly *(Photinus pyralis)* luciferase activity was obtained by adding 50 µl of luciferase assay reagent II (LAR II) into a white 96-well plate (Thermo Labsystems, Franklin, MA, USA) containing 10 µl of the cell lysate. Concurrently, the firefly reaction was quenched and light units for *Renilla* (*Renilla reniformis*) luciferase activity were achieved by adding 50 µl of Stop and Glo reagent. Luciferase activity was measured in a Veritas luminometer (Turner BioSystems, Sunnyvale, CA, USA).

4.2. Relative luciferase activity determination

The relative luciferase activity was calculated as follows: a) the firefly luciferase measurement was divided by the *Renilla* luciferase measurement to correct for differences in cell viability or transfection efficiency, and b) the corrected values for each replicate were divided by the average corrected value for vector only (pGL3-Basic or pControl-Luc). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only.

5. ENDOGENOUS ACCβ GENE AND PROTEIN EXPRESSION

5.1. RNA/Protein Isolation

In a separate set of experiments, total RNA and protein was simultaneously purified from transfected cardiomyocytes using the AllPrep RNA/Protein kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

5.2. RNA/Protein quantification

The concentration of the RNA and protein was determined using the Quant-iT assay (Invitrogen, Paisley, Scotland, UK) as described in the manufacturer's manual. A Quant-iT working solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. Next, two Quant-iT RNA standards (0 and 10 ng/µl) or three Quant-iT protein standards (0, 200 and 400 ng/µl) were diluted 1:20 in the Quant-iT working solution. 1 - 4 µl of the RNA or protein samples were also diluted in the Quant-iT working solution. All tubes were vortexed and after incubation at room temperature for 2 minutes for the RNA or 15 minutes for the protein, the tubes were measured in a Qubit fluorometer (Invitrogen, Paisley, Scotland, UK). The concentration of the samples was calculated by multiplying the QF value (value given by the Qubit fluorometer) by the dilution factor, and was given as ng/ml or µg/ml respectively, for the RNA and protein. The purified RNA and protein was stored at -80°C.

5.3. Quantitative Real-Time PCR (qRT-PCR)

The integrity of the RNA was evaluated by agarose gel electrophoresis (Appendix). qRT-PCR was performed in collaboration with Dr. Martin E. Young (Houston, TX, USA), and the method has been previously described.[328] mRNA transcript levels of the ACCβ gene were determined using the DNA primer and probe sequences shown in Table 2. Internal RNA standards were made using the T7 polymerase procedure (Ambion, Austin, TX, USA). The gene expression results are expressed as mRNA molecules per nanogram of total RNA.

Table 2. DNA primer and probe sequences for ACCβ gene

Forward primer	5'-TCATCGCCAATAATGGTATCG-3'
Reverse primer	5'-TAGCGCGTTCATTACGGAA-3'
Probe	5'-CGGTCAAGTGGATGCGCTCCA-3'

6. STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of the mean (SE) of experiments performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) and the Bonferroni post hoc test (Graph Pad Instat). Values were considered significant when $p<0.05$.

D. RESULTS

1. Cardiomyocyte isolation

Since ACCβ is predominantly expressed in the heart and skeletal muscle, our study was undertaken in neonatal rat cardiomyocytes.[67,77] Sufficient cardiomyocytes were obtained from heart tissue of 10 - 20 neonatal Wistar rats. Microscopic evaluation revealed a monolayer of cardiomyocytes that attached to the fibronectin-coated 12-well plates (Figure 14). More than 85% of the cardiomyocytes excluded trypan blue, indicating minimal cell loss. After 3 days of culture, spontaneously and synchronously beating cardiomyocytes were observed.

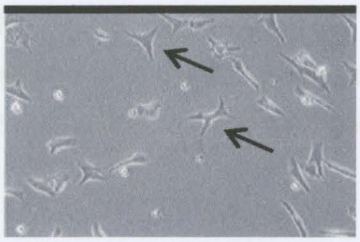


Figure 14. Microscopic representation of neonatal rat cardiomyocytes. The cardiomyocytes attached to the fibronectin-coated 12-well plates within one day. On day two, 10x magnification of the Olympus light microscope showed a monolayer of elongated cardiomyocytes with growing pseudopodia, and are indicated as arrows.

2. Optimisation of transient transfection in neonatal rat cardiomyocytes

Preliminary experiments were performed to optimise transient transfection conditions in neonatal rat cardiomyocytes. To accomplish this, we tested FuGene 6 to DNA ratios of 2:1 and 6:1, respectively. Firstly, we transiently transfected neonatal rat cardiomyocytes with the positive control reporter gene construct, pGL3-Control, which strongly expresses luciferase from an SV40 gene promoter. As shown in Figure 15A, both FuGene 6 to DNA ratios tested resulted in a robust induction of SV40 gene promoter activity. Secondly, we examined the transfection efficiency of the experimental reporter gene construct for the full-length human ACCβ gene promoter, pPIIβ-1317/+65-Luc. Likewise, transfection with the ACCβ gene promoter was successful and optimal transfection efficiency was obtained with the 2:1 FuGene 6 to DNA ratio (Figure 15B). Consequently, the 2:1 ratio of FuGene 6 to DNA was chosen for transient transfection assays.

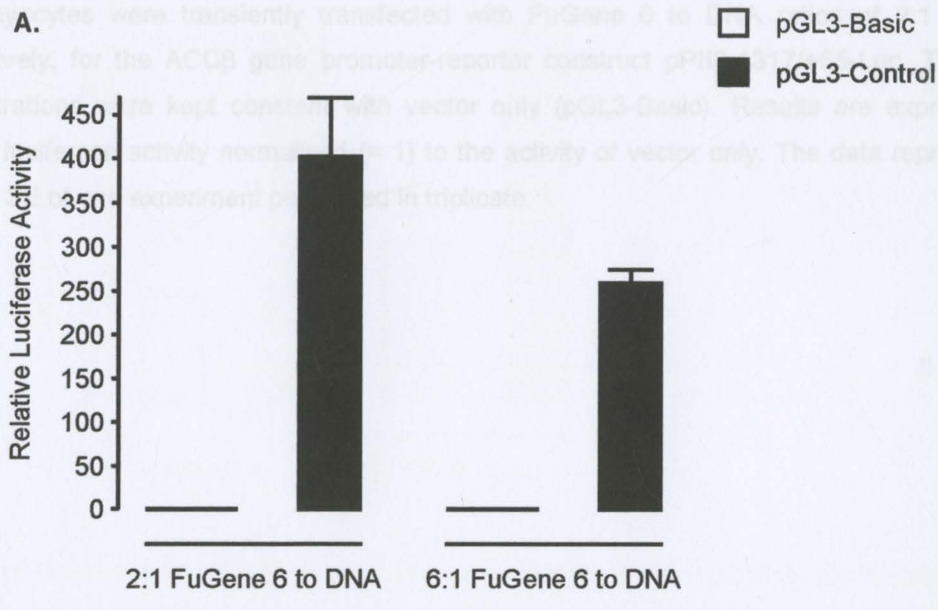


Figure 15A. Effect of FuGene 6 to DNA ratios on transfection efficiency. Neonatal rat cardiomyocytes were transiently transfected with FuGene 6 to DNA ratios of 2:1 and 6:1, respectively, for the SV40 gene promoter-reporter construct pGL3-Control. Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of one experiment performed in triplicate.

B.

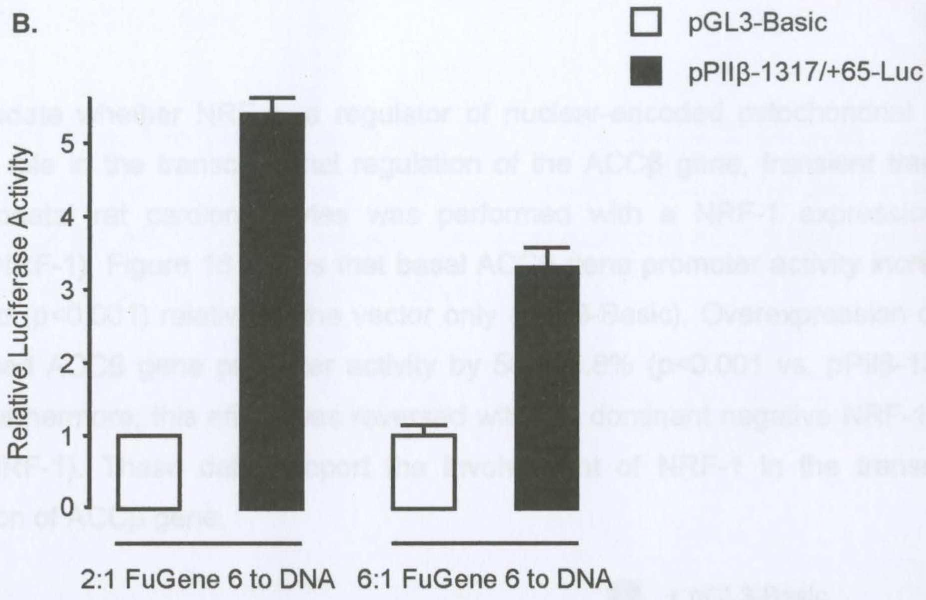


Figure 15B. Effect of FuGene 6 to DNA ratios on transfection efficiency. Neonatal rat cardiomyocytes were transiently transfected with FuGene 6 to DNA ratios of 2:1 and 6:1, respectively, for the ACCβ gene promoter-reporter construct pPIIβ-1317/+65-Luc. Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of one experiment performed in triplicate.

3. Role of NRF-1 as a transcriptional regulator of the ACCβ gene promoter

To elucidate whether NRF-1, a regulator of nuclear-encoded mitochondrial proteins, plays a role in the transcriptional regulation of the ACCβ gene, transient transfection into neonatal rat cardiomyocytes was performed with a NRF-1 expression vector (pSG5-NRF-1). Figure 16 shows that basal ACCβ gene promoter activity increased by ~2.5-fold ($p<0.001$) relative to the vector only (pGL3-Basic). Overexpression of NRF-1 decreased ACCβ gene promoter activity by 56 ± 8.8% ($p<0.001$ vs. pPIIβ-1317/+65-Luc). Furthermore, this effect was reversed with the dominant negative NRF-1 (pCMV-HA-dnNRF-1). These data support the involvement of NRF-1 in the transcriptional regulation of ACCβ gene.

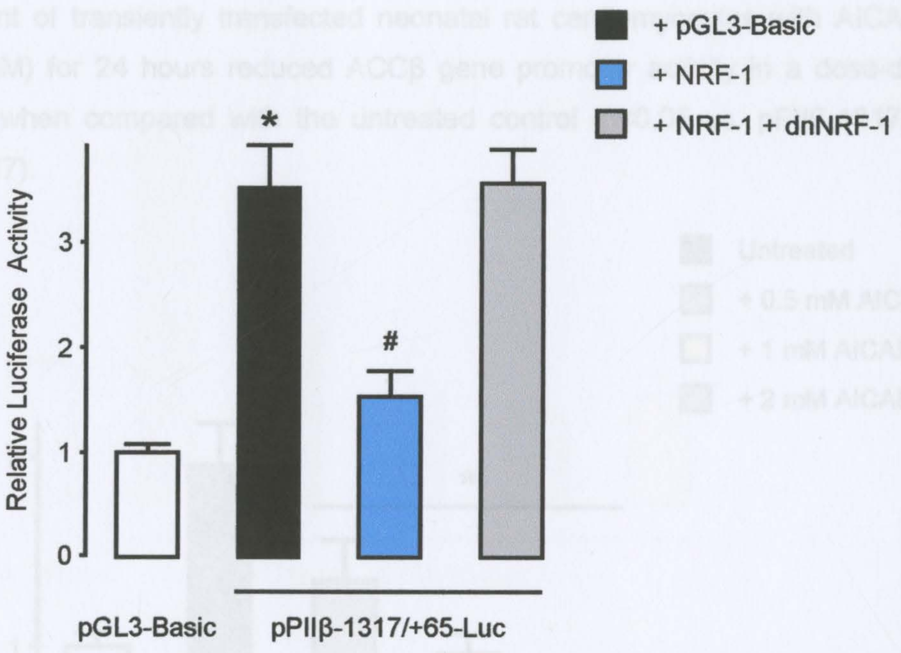


Figure 16. NRF-1 inhibits ACCβ gene promoter activity. The ACCβ gene promoter-reporter construct (pPIIβ-1317+65-Luc) was transiently transfected into neonatal rat cardiomyocytes ± each of the expression constructs, NRF-1 (pSG5-NRF-1) and dominant negative NRF-1(pCMV-HA-dnNRF-1). Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of two independent experiments performed in triplicate. *$p<0.001$ vs. pGL3-Basic, #$p<0.001$ vs. pPIIβ-1317/+65-Luc.

4. Delineation of the signalling pathway mediating the transcriptional regulation of ACCβ gene

The energy sensor AMPK, is a major regulator of ACC activity.[66] To further explore our hypothesis, we investigated the potential role of AMPK activation on the transcriptional regulation of the ACCβ gene at baseline, and in response to NRF-1. Here we employed two AMPK activators, i.e. AICAR and metformin.[108,329,110]

4.1. AMPK activation with AICAR dose-dependently reduces ACCβ gene promoter activity

Treatment of transiently transfected neonatal rat cardiomyocytes with AICAR (0.5, 1 and 2 mM) for 24 hours reduced ACCβ gene promoter activity in a dose-dependent manner when compared with the untreated control ($p<0.05$ vs. pPIIβ-1317/+65-Luc) (Figure 17).

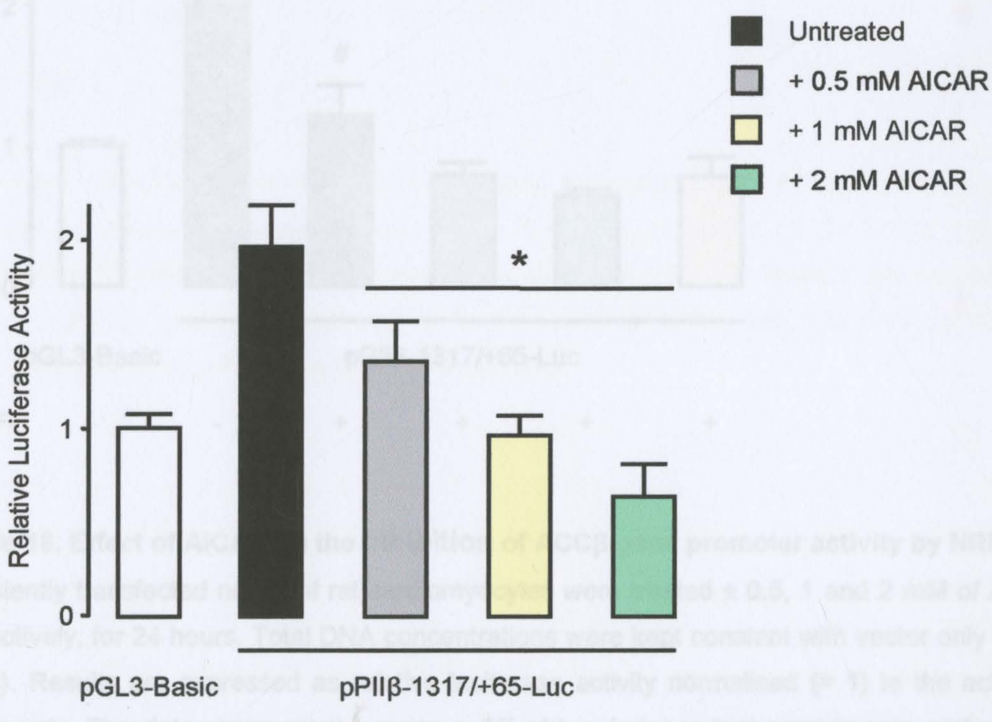


Figure 17. Effect of AICAR on ACCβ gene promoter activity. Transiently transfected neonatal rat cardiomyocytes were treated ± 0.5, 1 and 2 mM of AICAR, respectively, for 24 hours. Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data

represent the mean ± SE of two independent experiments performed in triplicate.*$p<0.05$ vs. pPIIβ-1317/+65-Luc (untreated control).

4.2. NRF-1 attenuation of ACCβ gene promoter activity is independent of AMPK activation with AICAR

In contrast, treatment of transiently transfected neonatal rat cardiomyocytes with AICAR (0.5, 1 and 2 mM) for 24 hours had no further effect on NRF-1-mediated inhibition of ACCβ gene promoter activity (Figure 18).

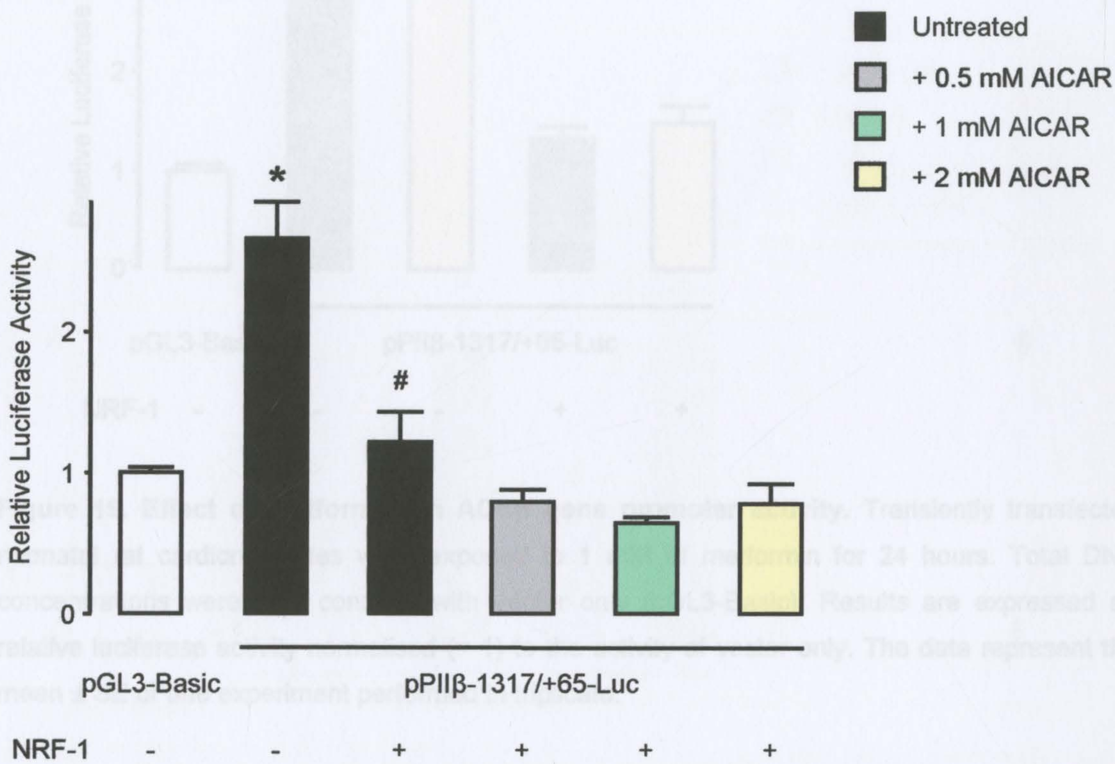


Figure 18. Effect of AICAR on the inhibition of ACCβ gene promoter activity by NRF-1. Transiently transfected neonatal rat cardiomyocytes were treated ± 0.5, 1 and 2 mM of AICAR, respectively, for 24 hours. Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of two independent experiments performed in triplicate.*$p<0.001$ vs. pGL3-Basic, #$p<0.001$ vs. pPIIβ-1317/+65-Luc (untreated control).

4.3. Metformin exerts no effect on ACCβ gene promoter activity

Transiently transfected neonatal rat cardiomyocytes exposed to metformin (1 mM) for 24 hours did not display a reduction of basal ACCβ gene promoter activity as predicted (Figure 19). Likewise, a similar effect was observed for the cardiomyocytes cotransfected with NRF-1.

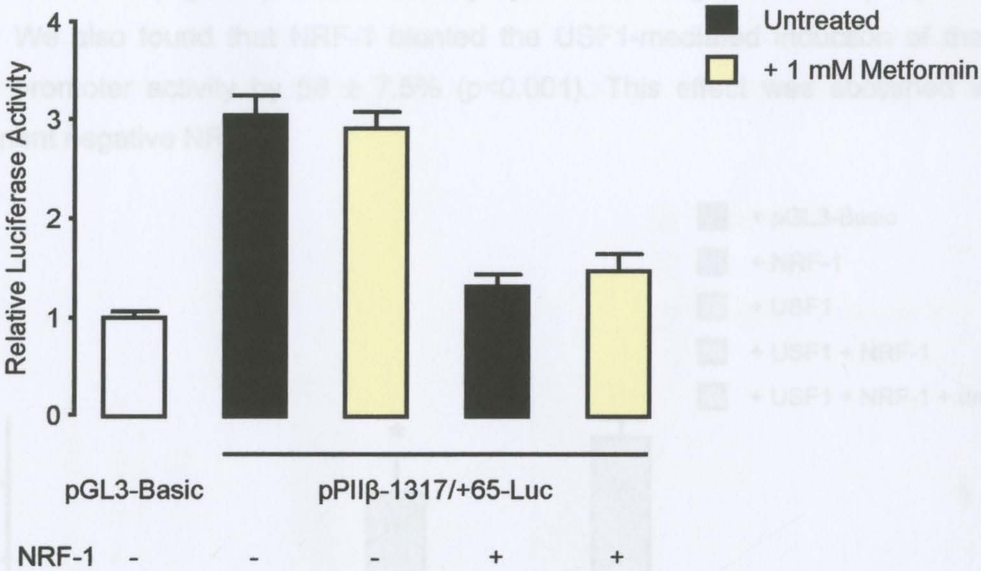


Figure 19. Effect of metformin on ACCβ gene promoter activity. Transiently transfected neonatal rat cardiomyocytes were exposed to 1 mM of metformin for 24 hours. Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of one experiment performed in triplicate.

5. Effect of NRF-1 on USF-1-mediated transactivation of the ACCβ gene promoter

We have previously identified USF1 as a transactivator of the human ACCβ gene promoter.[169] Next, we assessed the NRF-1 response to USF1-mediated transactivation of the ACCβ gene promoter. Figure 20 demonstrates that USF1 overexpression augmented ACCβ gene promoter activity by 55 ± 6.8% ($p<0.001$ vs. pPIIβ-1317/+65-Luc). We also found that NRF-1 blunted the USF1-mediated induction of the ACCβ gene promoter activity by 58 ± 7.5% ($p<0.001$). This effect was abolished with the dominant negative NRF-1.

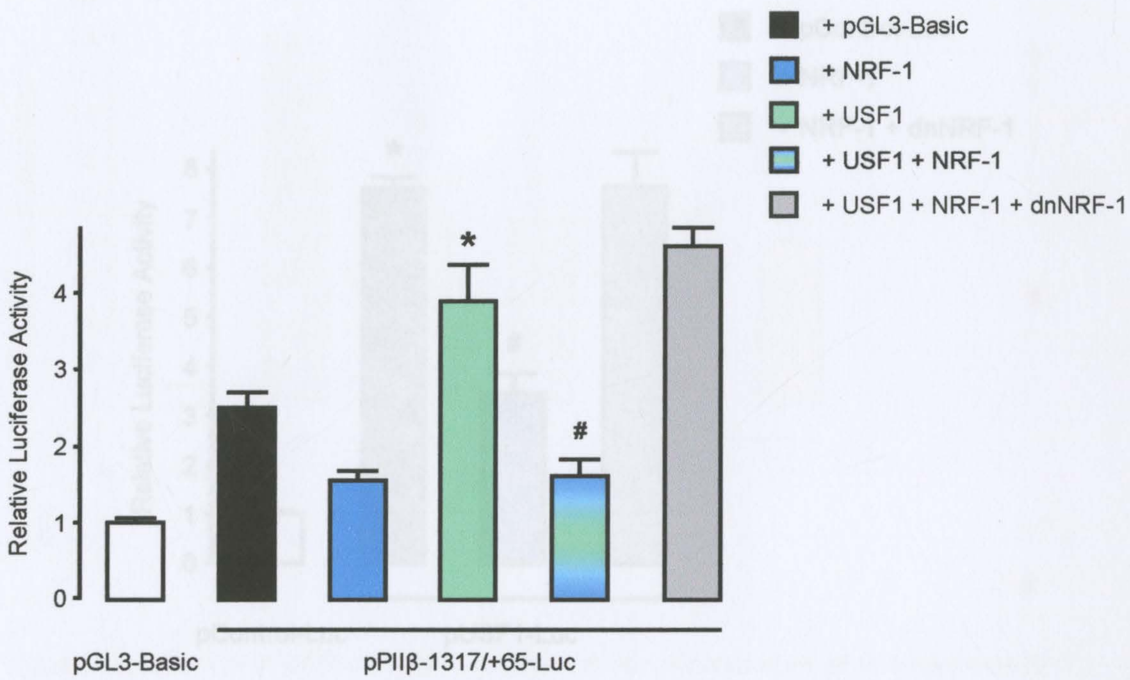


Figure 20. NRF-1 inhibits USF1-mediated transactivation of the ACCβ gene promoter. The ACCβ gene promoter-reporter construct (pPIIβ-1317/+65-Luc) was transiently transfected into neonatal rat cardiomyocytes with various combinations of USF1 (pUC-SRα-USF1), NRF-1 (pSG5-NRF-1), or dominant negative NRF-1 (pCMV-HA-dnNRF-1). Total DNA concentrations were kept constant with vector only (pGL3-Basic). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of 2 independent experiments performed in triplicate.*$p<0.001$ vs. pPIIβ-1317/+65-Luc, $^{\#}p<0.001$ vs. pPIIβ-1317/+65-Luc + USF1.

6. Determination of endogenous USF1 transcriptional activity in response to NRF-1

To determine whether endogenous USF1 transcriptional activity is controlled by NRF-1, neonatal rat cardiomyocytes were transiently transfected with a reporter construct containing multiple copies of USF1-specific enhancer elements (pUSF1-Luc). Here endogenous USF1 transcriptional activity was enhanced ~6.5-fold ($p<0.001$ vs. pControl-Luc) (Figure 21). Moreover, NRF-1 suppressed endogenous USF1 transcriptional activity by 55 ± 6.2% ($p<0.001$), while the dominant negative NRF-1 abrogated this.

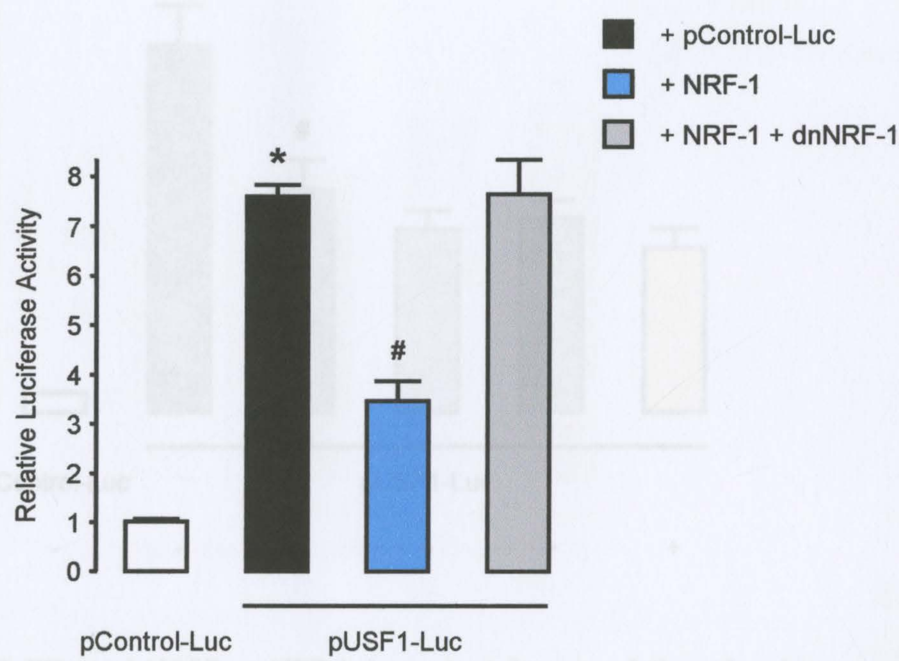


Figure 21. NRF-1 downregulates endogenous USF1 transcriptional activity. The USF1 reporter construct (pUSF1-Luc) was transiently transfected into neonatal rat cardiomyocytes ± NRF-1 (pSG5-NRF-1) and dominant negative NRF-1 (pCMV-HA-dnNRF-1). Total DNA concentrations were kept constant with vector only (pControl-Luc). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of two independent experiments performed in triplicate. *$p<0.001$ vs. pControl-Luc, #$p<0.001$ vs. pUSF1-Luc.

7. Role of AMPK activation on NRF-1-dependent downregulation of endogenous USF1 transcriptional activity

We also evaluated the effect of AICAR (0.5, 1 and 2 mM) treatment on NRF-1-mediated suppression of endogenous USF1 transcriptional activity. Here AICAR had no effect on endogenous USF1 transcriptional activity in response to NRF-1 (Figure 22).

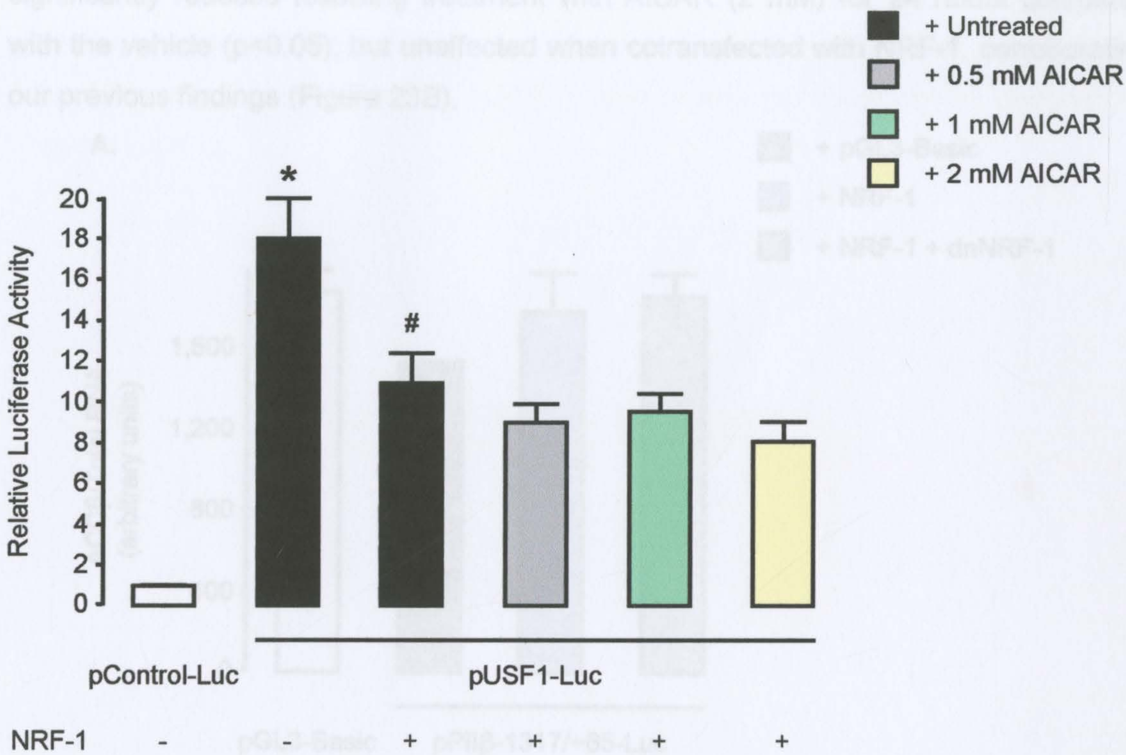
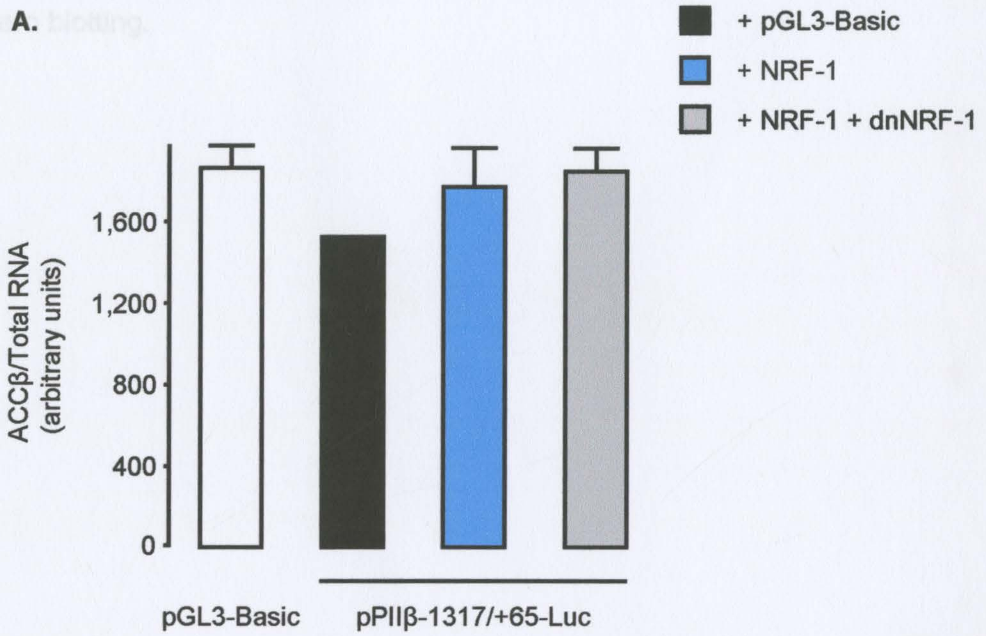


Figure 22. Effect of AICAR on NRF-1-dependent downregulation of endogenous USF1 transcriptional activity. The USF1 reporter construct (pUSF1-Luc) was transiently transfected into neonatal rat cardiomyocytes with NRF-1 (pSG5-NRF-1) and treated with 0.5, 1 and 2 mM of AICAR, respectively, for 24 hours. Total DNA concentrations were kept constant with vector only (pControl-Luc). Results are expressed as relative luciferase activity normalised (= 1) to the activity of vector only. The data represent the mean ± SE of two independent experiments performed in triplicate. *p<0.001 vs. pControl-Luc, #p<0.001 vs. pUSF1-Luc.

8. Endogenous ACCβ gene expression

To further study the mechanisms of the transcriptional regulation of ACCβ gene, qRT-PCR was carried out to ascertain endogenous ACCβ mRNA expression. As shown in Figure 23A, the mRNA expression level of ACCβ did not alter at baseline, and by NRF-1 overexpression. On the other hand, the ACCβ mRNA expression level was significantly reduced following treatment with AICAR (2 mM) for 24 hours compared with the vehicle ($p<0.05$), but unaffected when cotransfected with NRF-1, corroborating our previous findings (Figure 23B).

A.



B.

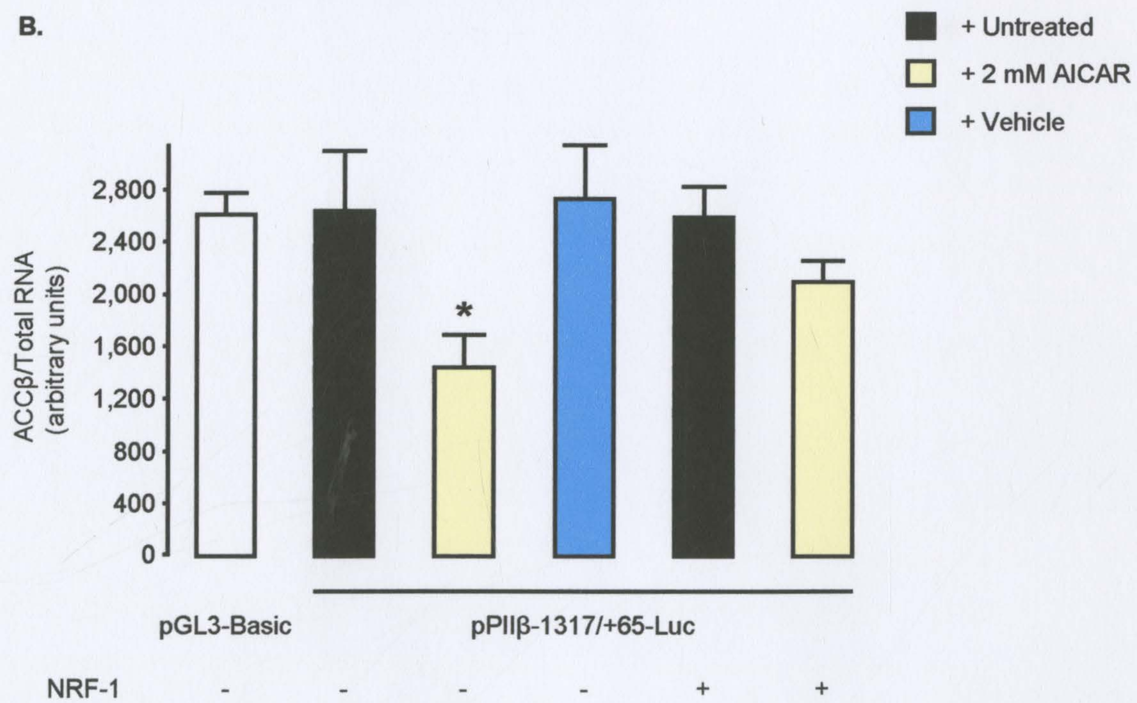


Figure 23. Endogenous ACCβ gene expression. Total RNA was isolated from transiently transfected neonatal rat cardiomyocytes, and ACCβ mRNA expression analysed by qRT-PCR. The data represent the mean ± SE of two independent experiments performed in triplicate. *$p<0.05$ vs. vehicle.

9. Endogeous ACCβ protein expression

Inadequate amounts of total protein were obtained from transiently transfected neonatal rat cardiomyocytes and therefore ACCβ protein expression could not be detected by Western blotting.

E. DISCUSSION

Type 2 diabetes is recognised as an important risk factor in the development of CVD.[18-20,23] Given the impact of CVD on global morbidity and mortality[330], we concentrated on alterations in cardiac substrate metabolism, implicated in the pathogenesis of type 2 diabetes and CVD. Several lines of evidence have shown that type 2 diabetic subjects exhibit elevated plasma NEFA levels. Diabetic hearts may adapt to these conditions by increasing the reliance on FA, with a concomitant decrease in carbohydrate utilisation. This is proposed to occur in order to meet energy (ATP) requirements for the maintenance of cardiac contractile function.[24,228,229] This metabolic switch is in agreement with the Randle hypothesis that enhanced FA utilisation is capable of inhibiting glucose uptake, glycolysis and glucose oxidation.[256]

A mismatch between the excessively high FA supply and mitochondrial FA β-oxidation can lead to myocardial lipid accumulation, increasing the intracellular pool of long-chain acyl-CoA within the cardiomyocytes, and result in the phenomenon termed lipotoxicity.[248,294,295] This in turn may have a number of detrimental clinical outcomes, including insulin resistance[233] and diabetic cardiomyopathy[266-269]. Reduced FA β-oxidation is proposed as a mechanism for mediating intramyocardial lipid accumulation. In fact, decreased FA β-oxidation was observed in the skeletal muscle of insulin-resistant offspring of type 2 diabetic patients[250,309], and isolated hearts of obese Zucker and ZDF rats perfused with oleate[249,248]. Activation of ACCβ results in the synthesis of malonyl-CoA, a potent inhibitor of CPT-1, which facilitates the import of long-chain acyl-CoA into the mitochondrion for FA β-oxidation.[63] Recent studies suggested that the decline of FA β-oxidation demonstrated in the skeletal muscle of type 2 diabetic subjects is attributed to increased ACCβ gene expression[232] and malonyl-CoA levels[251,252]. These studies raised the interesting possibility that elevating FA β-oxidation via inhibition of ACCβ within the diabetic context may be a potential mechanism of clearing damaging intramyocardial lipid accumulation. In support of this concept, the hearts of ACCβ-knock-out mice displayed reduced malonyl-CoA levels, elevated FA β-oxidation and decreased TG levels.[312,313] These mice were also protected from obesity and type 2 diabetes when fed a high fat/high carbohydrate diet.[314]

In light of this, we focussed on elucidating the pathway mediating the transcriptional repression of ACCβ in the heart. We employed a candidate transcription factor approach, namely NRF-1, a modulator of nuclear-encoded mitochondrial proteins involved in enhancing mitochondrial bioenergetic capacity, which is essential for high

FA β-oxidation rates.[190,191] Our selection was based on studies by Mootha et al[201] and Patti et al[202] reporting that the reduction of FA β-oxidation enzyme-encoding genes in the skeletal muscle of type 2 diabetic subjects may be due to the downregulation of NRF-1 and PGC-1α gene expression. Furthermore, NRF-1 was associated with upregulated CPT-1β gene expression and by implication higher FA β-oxidation rates.[200] Another study showed that activation of AMPK coincides with increased NRF-1 gene promoter binding activity and mitochondrial density in rat skeletal muscle.[199] Moreover, AMPK-mediated increases in FA β-oxidation correlated with reduced TG deposition in the skeletal muscle and liver.[110,119] Therefore, we hypothesised that NRF-1 inhibits ACCβ expression in the heart via AMPK activation, thereby lowering malonyl-CoA levels, which relieves the inhibition of CPT-1 activity, ultimately to enhance FA β-oxidation.

Our results show that human ACCβ gene promoter activity was markedly increased at baseline in neonatal rat cardiomyocytes. Moreover, overexpression of NRF-1 caused significant inhibition of ACCβ gene promoter activity. We also found that this effect was abolished when the cardiomyocytes were cotransfected with the dominant negative NRF-1 construct. This is consistent with a role of NRF-1 in regulating FA β-oxidation enzyme-encoding genes, since previous studies showed that NRF-1 was linked with increased CPT-1β gene expression.[200] In addition, an exercise-induced increase in skeletal muscle FA β-oxidation was associated with upregulated NRF-1 gene expression[203], as well as the inhibition of ACCβ activity and the lowering of malonyl-CoA levels[204]. We were unable to identify classic NRF-1 recognition elements (5'-(T/C)GCGCA(C/T)GCGC(A/G)-3')[187,190] in the ACCβ gene promoter. However, NRF-1 may bind to sequences on the gene promoter region that differs from the known consensus.[331] Collectively, these data clearly establishes the ability of NRF-1 to inhibit ACCβ gene promoter activity.

AMPK can phosphorylate and inhibit ACCβ activity, thereby lowering malonyl-CoA levels and stimulating FA β-oxidation.[329] Next, we investigated whether AMPK plays a role in directing the transcriptional regulation of ACCβ gene. Following AICAR treatment, we observed that AMPK activation dose-dependently reduced ACCβ gene promoter activity compared to the untreated control. These findings suggest that AMPK regulates ACCβ at both an enzyme and a transcriptional level. In support, Stoppani et al[138] demonstrated that *in vivo* infusion of AICAR into rat skeletal muscle accelerated CPT-1 gene transcription.

The NRF-1 attenuation of ACCβ gene promoter activity was independent of AICAR-induced AMPK activation. This finding was unexpected since Bergeron et al[199] showed that AMPK activation is associated with increased NRF-1 gene promoter binding activity. Moreover, serine phosphorylation within the NH_2-terminal domain of NRF-1 is known to enhance its DNA binding and transcriptional activity.[193,194] It is possible, however, that NRF-1 mediates its transcriptional response by serine/threonine kinases other than AMPK. Further work is necessary to elucidate this point. Surprisingly, metformin had no effect on ACCβ gene promoter activity at baseline or in response to NRF-1. Metformin is thought to indirectly activate AMPK through inhibition of respiratory chain complex 1, and this may explain our findings.[113,114] Also, extremely high concentrations of metformin (1 - 10 mM) are required to activate AMPK in cultured cells.[332] This experiment should therefore be repeated to reach statistical values and different doses of metformin must be employed in order to validate our findings.

Previously, USF1 was identified as a transactivator of the human ACCβ gene promoter.[169] To gain further insight into NRF-1-mediated inhibition of ACCβ gene promoter activity, we examined the response to USF1. In agreement with Makaula et al[169], USF1 augmented ACCβ gene promoter activity. However, overexpression of USF1 and USF2 in neonatal cardiomyocytes suppressed CPT-1β gene promoter activity.[170] It is likely that USF1-mediated ACCβ activation and CPT-1β inhibition may diminish FA β-oxidation (Figure 24). We also observed that NRF-1 suppressed the USF1-dependent induction of the ACCβ gene promoter and that this effect was reversed with dominant negative NRF-1. To further corroborate our findings, we then assessed the effect of NRF-1 on endogenous USF1 transcriptional activity. Our results indicate that NRF-1 inhibited endogenous USF1 transcriptional activity, an effect abrogated by dominant negative NRF-1. AICAR treatment did not alter the endogenous USF1 transcriptional activity in response to NRF-1.

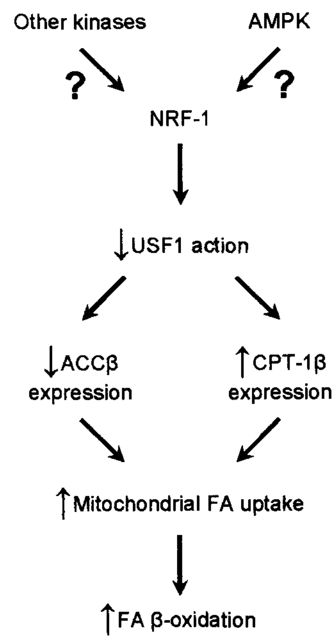


Figure 24. Proposed model for the regulation of cardiac FA β-oxidation. The DNA binding and transcriptional activity of NRF-1 may be controlled by AMPK and/or other unidentified kinases. This may lead to enhanced cardiac mitochondrial FA uptake and β-oxidation via NRF-1-induced inhibition of the USF1-mediated increase in ACCβ expression and decrease in CPT-1β expression. AMPK = 5'-AMP-activated protein kinase; NRF-1 = nuclear respiratory factor-1; USF1 = upstream stimulatory factor 1; ACCβ = acetyl-coenzyme A carboxylase beta; CPT-1β = carnitine palmitoyltransferase-1 beta; FA = fatty acids.

To further ascertain the mechanisms of the transcriptional regulation of ACCβ gene, we set out to determine endogenous ACCβ gene expression by performing qRT-PCR. Here the mRNA expression level of ACCβ gene was unaffected at baseline and by NRF-1 overexpression. A reason for these results is difficult to explain. We speculate that the quantity of RNA used was not sensitive enough to detect changes in ACCβ gene expression. These experiments should therefore be repeated with different amounts of RNA in order to improve the efficiency of the assay and to confirm our results. Although parallel experiments were performed on a separate set of cardiomyocytes to establish that the transfection was successful, it does not rule out that the transfection failed for the cardiomyocytes used in determining ACCβ gene expression. Increasing the number of experiments could possibly resolve this problem. In contrast, the ACCβ mRNA expression level was significantly reduced when exposed to AICAR, but unchanged when cotransfected with NRF-1, verifying our previous findings. We also attempted to determine endogenous ACCβ protein expression. However, insufficient amounts of total protein were obtained from transiently transfected cardiomyocytes and we were unable to detect ACCβ protein expression by Western blotting.

Although there is general consensus that FA utilisation is elevated in type 2 diabetic hearts, there are contradictory data for cardiac FA β-oxidation in type 2 diabetes. Studies on isolated hearts of db/db and ob/ob mice perfused with palmitate showed enhanced FA β-oxidation.[241,242] In contrast to these reports, reduced FA β-oxidation was demonstrated in isolated hearts of obese Zucker and ZDF rats perfused with oleate.[248,249] Magnetic resonance spectroscopy revealed that insulin-resistant offspring of type 2 diabetic patients also exhibited decreased skeletal muscle FA β-oxidation.[250] The reduction in FA β-oxidation may be due to defects in mitochondrial function. This is evidenced by reduced mitochondrial ATP synthesis[309] and mitochondrial density[253] in the skeletal muscle of insulin-resistant offspring of type 2 diabetic patients. Decreased mitochondrial oxidative and phosphorylation activity were also observed in elderly insulin-resistant subjects.[333] It is possible that cardiac FA β-oxidation in type 2 diabetes may related to the stage of disease progression. Elevated cardiac FA β-oxidation in type 2 diabetes may be an initial adaptation to high plasma NEFA levels. Increases in age-associated mtDNA mutations[334] or young subjects with inherited mtDNA defects may impair mitochondrial function thereby resulting in reduced cardiac FA β-oxidation. Thus whether cardiac FA β-oxidation is increased or decreased in type 2 diabetes may depend on the onset and the severity of the disease.

There are some caveats associated with increasing FA β-oxidation. Excessively high FA β-oxidation rates are proposed to increase ROS production, thereby leading to cardiomyocyte damage or apoptosis. As a result, this may contribute to the development of diabetic cardiomyopathy.[277-279] Augmented ROS production was indeed obtained in type 2 diabetic hearts.[248,277,278] Mitochondrial uncoupling is also suggested to occur due to elevated FA β-oxidation rates. Murray et al[335] observed increased cardiac UCP2 and UCP3 protein expression in db/db mice. Contrary to these findings, Boudina et al[287] found that UCP3 protein expression was unchanged in ob/ob hearts with high FA β-oxidation rates. Another study showed reduced muscle UCP3 protein expression in type 2 diabetic patients.[336] These differences may be due to variations in animal models and human studies.

It is important to note that there are controversies as to whether mitochondrial uncoupling is an adaptive or maladaptive mechanism.[337,338] Buchanan et al[254] reported that increased cardiac UCP2 and UCP3 protein expression in isolated working hearts from ob/ob and db/db mice was associated with reduced cardiac efficiency, which indicates a maladaptive effect. Others suggested that mitochondrial uncoupling is a protective mechanism against FA-induced ROS damage, countering the previous idea.[289,290] Consistent with this opinion, Himms-Hagen and Harper[291] postulated that UCP3 exports FA anions from the mitochondrial matrix and releases CoA, to promote FA β-oxidation when the mitochondrial FA supply is high, implying that these effects should decrease the proton-motive force and enhance the activity of the ETC, thereby reducing ROS production. In support, UCP3-deficient mice displayed increased ROS production.[339] However, further investigations are required to clarify the functional role of UCPs in type 2 diabetic hearts because of differences in its expression in various animal models and human studies. Of note, the degree of increasing cardiac FA β-oxidation is therefore important.

Despite these reservations, increasing FA β-oxidation may still represent a beneficial mechanism for type 2 diabetic hearts for a number of reasons. Increasing FA β-oxidation by overexpressing CPT-1 in L6E9 rat skeletal muscle cells was shown to protect against the accumulation of the toxic lipid metabolites ceramide and DAG, induced by palmitate.[340] CPT-1 overexpression also led to the attenuation of the activation of PKCθ and PKCζ, implicated in triggering insulin resistance[341,342]. Furthermore, Essop et al[313] reported that ACCβ-null mice exhibited lowered myocardial TG levels and maintained normal cardiac function even though FA β-oxidation rates were enhanced.

In summary, this study demonstrates that NRF-1 is a novel transcriptional inhibitor of the human ACCβ gene promoter in the heart that may promote FA β-oxidation. We have carefully reviewed the literature and taking into account the evolution of the type 2 diabetes disease process are of the opinion that this mechanism may be exploited for therapeutic purposes. We propose that prophylactic action by increasing FA β-oxidation relatively early in the diabetic process or in those with genetic defects could be beneficial in having as a secondary effect removal of harmful TG in the heart. An alternative approach is to lower the FA availability thereby increasing glucose utilisation and insulin sensitivity in type 2 diabetes. In support, Haemmerle et al[343] showed that lowering plasma NEFA levels by suppressing adipose triglyceride lipase in mice increased glucose uptake in the heart and skeletal muscle, as well as improved glucose tolerance and insulin sensitivity. Further experimental investigations are needed to explore these proposals put forward.

Limitations of the study

We relied on transfection-based experiments to explore our hypothesis, which may not reflect the physiological milieu of cells. However, we believe that the results provide firm evidence that the human ACCβ gene is transcriptionally inhibited by NRF-1 in the mammalian heart. We were not successful in determining endogenous ACCβ protein expression by Western blotting, despite many efforts to optimise the protocol. However, this can possibly be accomplished using higher numbers of transfected cardiomyocytes which is required to obtain sufficient protein for Western blotting. Unfortunately, due to the shortage of neonatal Wistar rats we were limited to the number of experiments performed and were unable to repeat some of the assays to reach statistical values.

F. CONCLUSION

We have identified, for the first time, to the best of our knowledge, that NRF-1 is a novel transcriptional inhibitor of the human ACCβ gene promoter in the heart. We propose that this finding may offer therapeutic utility by promoting mitochondrial FA uptake and β-oxidation in conditions where damaging intracellular lipids accumulate, such as the type 2 diabetic heart, thereby diminishing lipotoxic effects (Figure 25).

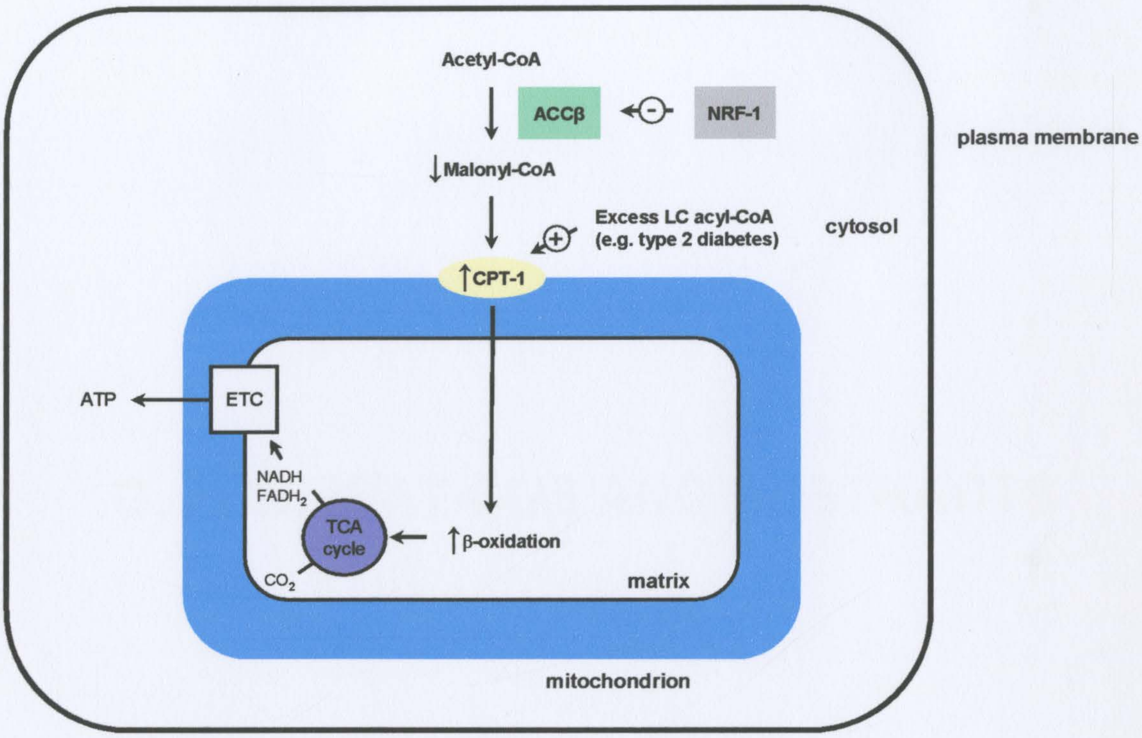


Figure 25. Identification of a novel pathway that may promote mitochondrial FA uptake and β-oxidation in the heart. ACCβ = acetyl-coenzyme A carboxylase beta; NRF-1 = nuclear respiratory factor-1; LC= long-chain; CPT-1 = carnitine palmitoyltransferase-1; TCA = tricarboxylic acid; ETC = electron transport chain; ATP = adenosine triphosphate.

Future direction

The results obtained in neonatal cardiomyocytes may not parallel the adult heart since the metabolic profile is different and this should indeed be explored. Direct measurement of NRF-1 binding to the ACCβ gene promoter using the HaloCHIP technique should be employed to strengthen our findings. Determining the functional effects of transcriptionally inhibiting ACCβ gene, such as FA β-oxidation, malonyl-CoA levels and CPT-1 activity would be advantageous in confirming our hypothesis. It would also be interesting to prove our concept in a model of intramyocardial lipid accumulation, such as ZDF rat hearts.

G. PUBLICATIONS AND ABSTRACTS

Publications and Abstracts

Makaula S, **Adam T**, Essop MF. Upstream stimulatory factor 1 transactivates the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase. Archives of Biochemistry and Biophysics. 2005; 446:91-100.

Adam T, Makaula S, and Essop MF. Nuclear respiratory factor-1: a novel inhibitor of the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase. Cardiovascular Drugs and Therapy. 2006; 20:396.

H. REFERENCES

1. Encyclopaedia of alternative medicine. The Gale Group, Inc. 2005.
2. World Health Organisation. http://www.who.int/cardiovascular_diseases/en/ 2008.
3. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006; 3:e442.
4. Yusuf S, Hawken S, Ounpuu S, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 2004; 364:937-52.
5. Yusuf S, Reddy S, Ounpuu S, Anand S. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanisation. Circ 2001; 104:2746-53.
6. World Health Organisation. The World Health Report 2002: Reducing risks, promoting healthy life. Geneva. 2002.
7. Rosengren A, Hawken S, Ounpuu S, et al. Association of psychosocial risk factors with risk of acute myocardial infarction in 11119 cases and 13648 controls from 52 countries (the INTERHEART study): case-control study. Lancet 2004; 364:953-62.
8. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001; 285:2489-97.
9. Hunt KJ, Resendez RG, Williams K, et al. National Cholesterol Education Program versus World Health Organisation metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. Circ 2004; 110:1251-7.
10. Bradshaw D, Groenewald P, Laubscher R, et al. Initial estimates from the South African National Burden of Disease Study, 2000. MRC Policy Brief 2003.
11. Steyn N, Bradshaw D, Norman R, et al. Dietary changes and the health transition in South Africa: implications for health policy. Cape Town. South African Medical Research Council 2006.
12. World Health Organisation. Definition, diagnosis and classification of diabetes mellitus and its complications. Report of a WHO consultation. Part 1: Diagnosis and classification of diabetes mellitus. Geneva. 1999.
13. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001; 414:782-7.

14. Amos AF, McCarty DJ, Zimmet P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 1997; 14 Suppl 5:S1-85.
15. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 1998; 21:1414-31.
16. International Diabetes Federation. http://www.idf.org/home/index.cfm?node=264 2007.
17. Wild S, Roglic G, Green A, et al. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047-53.
18. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. Jama 1979; 241:2035-8.
19. Howard BV, Best LG, Galloway JM, et al. Coronary heart disease risk equivalence in diabetes depends on concomitant risk factors. Diabetes Care 2006; 29:391-7.
20. Haffner SM, Lehto S, Ronnemaa T, et al. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. N Engl J Med 1998; 339:229-34.
21. Stone PH, Muller JE, Hartwell T, et al. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group. J Am Coll Cardiol 1989; 14:49-57.
22. Smith JW, Marcus FI, Serokman R. Prognosis of patients with diabetes mellitus after acute myocardial infarction. Am J Cardiol 1984; 54:718-21.
23. Hu FB, Stampfer MJ, Solomon CG, et al. The impact of diabetes mellitus on mortality from all causes and coronary heart disease in women: 20 years of follow-up. Arch Intern Med 2001; 161:1717-23.
24. Taegtmeyer H, McNulty P, Young ME. Adaptation and maladaptation of the heart in diabetes: Part I: general concepts. Circ 2002; 105:1727-33.
25. Schonekess BO. Competition between lactate and fatty acids as sources of ATP in the isolated working rat heart. J Mol Cell Cardiol 1997; 29:2725-33.
26. King KL, Okere IC, Sharma N, et al. Regulation of cardiac malonyl-CoA content and fatty acid oxidation during increased cardiac power. Am J Physiol Heart Circ Physiol 2005; 289:H1033-7.

27. Lopaschuk GD, Collins-Nakai RL, Itoi T. Developmental changes in energy substrate use by the heart. Cardiovasc Res 1992; 26:1172-80.
28. Opie L. Heart Physiology From Cell to Circulation: Lippincott Williams & Wilkins, 2004:306-11.
29. Henning SL, Wambolt RB, Schonekess BO, et al. Contribution of glycogen to aerobic myocardial glucose utilization. Circ 1996; 93:1549-55.
30. Luiken JJ, Coort SL, Koonen DP, et al. Regulation of cardiac long-chain fatty acid and glucose uptake by translocation of substrate transporters. Pflugers Arch 2004; 448:1-15.
31. Gould GW, Holman GD. The glucose transporter family: structure, function and tissue-specific expression. Biochem J 1993; 295 (Pt 2):329-41.
32. Wood IS, Trayhurn P. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br J Nutr 2003; 89:3-9.
33. Fischer Y, Thomas J, Sevilla L, et al. Insulin-induced recruitment of glucose transporter 4 (GLUT4) and GLUT1 in isolated rat cardiac myocytes. Evidence of the existence of different intracellular GLUT4 vesicle populations. J Biol Chem 1997; 272:7085-92.
34. Abel ED. Glucose transport in the heart. Front Biosci 2004; 9:201-15.
35. Kraegen EW, Sowden JA, Halstead MB, et al. Glucose transporters and in vivo glucose uptake in skeletal and cardiac muscle: fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4. Biochem J 1993; 295 (Pt 1):287-93.
36. Hayashi T, Wojtaszewski JF, Goodyear LJ. Exercise regulation of glucose transport in skeletal muscle. Am J Physiol 1997; 273:E1039-51.
37. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG. Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. Cardiovasc Res 1997; 33:243-57.
38. Russell RR, 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am J Physiol 1999; 277:H643-9.
39. Garcia CK, Goldstein JL, Pathak RK, et al. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell 1994; 76:865-73.
40. Pogson CI, Randle PJ. The control of rat-heart phosphofructokinase by citrate and other regulators. Biochem J 1966; 100:683-93.

41. Hue L, Rider MH. Role of fructose 2,6-bisphosphate in the control of glycolysis in mammalian tissues. Biochem J 1987; 245:313-24.
42. Marsin AS, Bertrand L, Rider MH, et al. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr Biol 2000; 10:1247-55.
43. Rider MH, van Damme J, Vertommen D, et al. Evidence for new phosphorylation sites for protein kinase C and cyclic AMP-dependent protein kinase in bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. FEBS Lett 1992; 310:139-42.
44. Rider MH, Bertrand L, Vertommen D, et al. 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem J 2004; 381:561-79.
45. Garland PB, Randle PJ, Newsholme EA. Citrate as an Intermediary in the Inhibition of Phosphofructokinase in Rat Heart Muscle by Fatty Acids, Ketone Bodies, Pyruvate, Diabetes, and Starvation. Nature 1963; 200:169-70.
46. Malloy CR, Sherry AD, Jeffrey FM. Analysis of tricarboxylic acid cycle of the heart using 13C isotope isomers. Am J Physiol 1990; 259:H987-95.
47. Kerbey AL, Randle PJ, Cooper RH, et al. Regulation of pyruvate dehydrogenase in rat heart. Mechanism of regulation of proportions of dephosphorylated and phosphorylated enzyme by oxidation of fatty acids and ketone bodies and of effects of diabetes: role of coenzyme A, acetyl-coenzyme A and reduced and oxidized nicotinamide-adenine dinucleotide. Biochem J 1976; 154:327-48.
48. Holness MJ, Sugden MC. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem Soc Trans 2003; 31:1143-51.
49. Augustus AS, Kako Y, Yagyu H, Goldberg IJ. Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. Am J Physiol Endocrinol Metab 2003; 284:E331-9.
50. Yagyu H, Chen G, Yokoyama M, et al. Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. J Clin Invest 2003; 111:419-26.
51. Augustus A, Yagyu H, Haemmerle G, et al. Cardiac-specific knock-out of lipoprotein lipase alters plasma lipoprotein triglyceride metabolism and cardiac gene expression. J Biol Chem 2004; 279:25050-7.
52. Augustus AS, Buchanan J, Park TS, et al. Loss of lipoprotein lipase-derived fatty acids leads to increased cardiac glucose metabolism and heart dysfunction. J Biol Chem 2006; 281:8716-23.

53. Musters MW, Bassingthwaighte JB, van Riel NA, van der Vusse GJ. Computational evidence for protein-mediated fatty acid transport across the sarcolemma. Biochem J 2006; 393:669-78.
54. Stremmel W, Strohmeyer G, Borchard F, et al. Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. Proc Natl Acad Sci USA 1985; 82:4-8.
55. Schaffer JE, Lodish HF. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell 1994; 79:427-36.
56. Abumrad NA, el-Maghrabi MR, Amri EZ, et al. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. J Biol Chem 1993; 268:17665-8.
57. Luiken JJ, Turcotte LP, Bonen A. Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. J Lipid Res 1999; 40:1007-16.
58. Brinkmann JF, Abumrad NA, Ibrahimi A, et al. New insights into long-chain fatty acid uptake by heart muscle: a crucial role for fatty acid translocase/CD36. Biochem J 2002; 367:561-70.
59. Luiken JJ, Coort SL, Willems J, et al. Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. Diabetes 2003; 52:1627-34.
60. Richards MR, Harp JD, Ory DS, Schaffer JE. Fatty acid transport protein 1 and long-chain acyl coenzyme A synthetase 1 interact in adipocytes. J Lipid Res 2006; 47:665-72.
61. Kerner J, Hoppel C. Fatty acid import into mitochondria. Biochim Biophys Acta 2000; 1486:1-17.
62. Kunau WH, Dommes V, Schulz H. beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. Prog Lipid Res 1995; 34:267-342.
63. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 1997; 244:1-14.
64. Stanley WC, Chandler MP. Energy metabolism in the normal and failing heart: potential for therapeutic interventions. Heart Fail Rev 2002; 7:115-30.
65. Lopaschuk GD. Metabolic abnormalities in the diabetic heart. Heart Fail Rev 2002; 7:149-59.

66. Hardie DG. Regulation of fatty acid synthesis via phosphorylation of acetyl-CoA carboxylase. Prog Lipid Res 1989; 28:117-46.
67. Thampy KG. Formation of malonyl coenzyme A in rat heart. Identification and purification of an isozyme of a carboxylase from rat heart. J Biol Chem 1989; 264:17631-4.
68. Dyck JR, Barr AJ, Barr RL, et al. Characterization of cardiac malonyl-CoA decarboxylase and its putative role in regulating fatty acid oxidation. Am J Physiol 1998; 275:H2122-9.
69. Abu-Elheiga L, Jayakumar A, Baldini A, et al. Human acetyl-CoA carboxylase: characterization, molecular cloning, and evidence for two isoforms. Proc Natl Acad Sci USA 1995; 92:4011-5.
70. Abu-Elheiga L, Almarza-Ortega DB, Baldini A, Wakil SJ. Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms. J Biol Chem 1997; 272:10669-77.
71. Abu-Elheiga L, Brinkley WR, Zhong L, et al. The subcellular localization of acetyl-CoA carboxylase 2. Proc Natl Acad Sci USA 2000; 97:1444-9.
72. Nakanishi S, Numa S. Purification of rat liver acetyl coenzyme A carboxylase and immunochemical studies on its synthesis and degradation. Eur J Biochem 1970; 16:161-73.
73. Allred JB, Reilly KE. Short-term regulation of acetyl CoA carboxylase in tissues of higher animals. Prog Lipid Res 1996; 35:371-85.
74. Munday MR. Regulation of mammalian acetyl-CoA carboxylase. Biochem Soc Trans 2002; 30:1059-64.
75. Kim KH. Regulation of mammalian acetyl-coenzyme A carboxylase. Annu Rev Nutr 1997; 17:77-99.
76. Kim TS, Freake HC. High carbohydrate diet and starvation regulate lipogenic mRNA in rats in a tissue-specific manner. J Nutr 1996; 126:611-7.
77. Bianchi A, Evans JL, Iverson AJ, et al. Identification of an isozymic form of acetyl-CoA carboxylase. J Biol Chem 1990; 265:1502-9.
78. Beaty NB, Lane MD. Kinetics of activation of acetyl-CoA carboxylase by citrate. Relationship to the rate of polymerization of the enzyme. J Biol Chem 1983; 258:13043-50.
79. Saha AK, Laybutt DR, Dean D, et al. Cytosolic citrate and malonyl-CoA regulation in rat muscle in vivo. Am J Physiol 1999; 276:E1030-7.

80. Nikawa J, Tanabe T, Ogiwara H, et al. Inhibitory effects of long-chain acyl coenzyme A analogues on rat liver acetyl coenzyme A carboxylase. FEBS Lett 1979; 102:223-6.

81. Chien D, Dean D, Saha AK, et al. Malonyl-CoA content and fatty acid oxidation in rat muscle and liver in vivo. Am J Physiol Endocrinol Metab 2000; 279:E259-65.

82. Thampy KG, Wakil SJ. Activation of acetyl-CoA carboxylase. Purification and properties of a Mn2+-dependent phosphatase. J Biol Chem 1985; 260:6318-23.

83. Gaussin V, Hue L, Stalmans W, Bollen M. Activation of hepatic acetyl-CoA carboxylase by glutamate and Mg2+ is mediated by protein phosphatase-2A. Biochem J 1996; 316 (Pt 1):217-24.

84. Mabrouk GM, Helmy IM, Thampy KG, Wakil SJ. Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine. J Biol Chem 1990; 265:6330-8.

85. Awan MM, Saggerson ED. Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation. Biochem J 1993; 295 (Pt 1):61-6.

86. Gamble J, Lopaschuk GD. Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. Metabolism 1997; 46:1270-4.

87. Kim KH, Lopez-Casillas F, Bai DH, et al. Role of reversible phosphorylation of acetyl-CoA carboxylase in long-chain fatty acid synthesis. Faseb J 1989; 3:2250-6.

88. Munday MR, Campbell DG, Carling D, Hardie DG. Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. Eur J Biochem 1988; 175:331-8.

89. Dyck JR, Kudo N, Barr AJ, et al. Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. Eur J Biochem 1999; 262:184-90.

90. Tipper JP, Bacon GW, Witters LA. Phosphorylation of acetyl-coenzyme A carboxylase by casein kinase I and casein kinase II. Arch Biochem Biophys 1983; 227:386-96.

91. Hardie DG, Carling D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 1997; 246:259-73.

92. Kudo N, Barr AJ, Barr RL, et al. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA

levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. J Biol Chem 1995; 270:17513-20.

93. Kudo N, Gillespie JG, Kung L, et al. Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. Biochim Biophys Acta 1996; 1301:67-75.

94. Vavvas D, Apazidis A, Saha AK, et al. Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. J Biol Chem 1997; 272:13255-61.

95. Hutber CA, Hardie DG, Winder WW. Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. Am J Physiol 1997; 272:E262-6.

96. Coven DL, Hu X, Cong L, et al. Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. Am J Physiol Endocrinol Metab 2003; 285:E629-36.

97. Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol 1996; 270:E299-304.

98. Wojtaszewski JF, Nielsen P, Hansen BF, et al. Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. J Physiol 2000; 528 Pt 1:221-6.

99. Itani SI, Saha AK, Kurowski TG, et al. Glucose autoregulates its uptake in skeletal muscle: involvement of AMP-activated protein kinase. Diabetes 2003; 52:1635-40.

100. Arad M, Seidman CE, Seidman JG. AMP-activated protein kinase in the heart: role during health and disease. Circ Res 2007; 100:474-88.

101. Suter M, Riek U, Tuerk R, et al. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J Biol Chem 2006; 281:32207-16.

102. Corton JM, Gillespie JG, Hardie DG. Role of the AMP-activated protein kinase in the cellular stress response. Curr Biol 1994; 4:315-24.

103. Stein SC, Woods A, Jones NA, et al. The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 2000; 345 Pt 3:437-43.

104. Hawley SA, Boudeau J, Reid JL, et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2003; 2:28.

105. Hawley SA, Pan DA, Mustard KJ, et al. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab 2005; 2:9-19.

106. Ponticos M, Lu QL, Morgan JE, et al. Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. Embo J 1998; 17:1688-99.

107. Davies SP, Helps NR, Cohen PT, Hardie DG. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett 1995; 377:421-5.

108. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995; 229:558-65.

109. Henin N, Vincent MF, Van den Berghe G. Stimulation of rat liver AMP-activated protein kinase by AMP analogues. Biochim Biophys Acta 1996; 1290:197-203.

110. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108:1167-74.

111. Saha AK, Avilucea PR, Ye JM, et al. Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue in vivo. Biochem Biophys Res Commun 2004; 314:580-5.

112. Fryer LG, Parbu-Patel A, Carling D. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem 2002; 277:25226-32.

113. Hawley SA, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes 2002; 51:2420-5.

114. El-Mir MY, Nogueira V, Fontaine E, et al. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem 2000; 275:223-8.

115. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 2000; 348 Pt 3:607-14.

116. Ha J, Daniel S, Broyles SS, Kim KH. Critical phosphorylation sites for acetyl-CoA carboxylase activity. J Biol Chem 1994; 269:22162-8.

117. Davies SP, Sim AT, Hardie DG. Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. Eur J Biochem 1990; 187:183-90.

118. Dean D, Daugaard JR, Young ME, et al. Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. Diabetes 2000; 49:1295-300.

119. Collier CA, Bruce CR, Smith AC, et al. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. Am J Physiol Endocrinol Metab 2006; 291:E182-9.

120. Chabowski A, Momken I, Coort SL, et al. Prolonged AMPK activation increases the expression of fatty acid transporters in cardiac myocytes and perfused hearts. Mol Cell Biochem 2006; 288:201-12.

121. Lee WJ, Kim M, Park HS, et al. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. Biochem Biophys Res Commun 2006; 340:291-5.

122. Henin N, Vincent MF, Gruber HE, Van den Berghe G. Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. Faseb J 1995; 9:541-6.

123. Woods A, Azzout-Marniche D, Foretz M, et al. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol Cell Biol 2000; 20:6704-11.

124. Foretz M, Carling D, Guichard C, et al. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. J Biol Chem 1998; 273:14767-71.

125. Sullivan JE, Brocklehurst KJ, Marley AE, et al. Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. FEBS Lett 1994; 353:33-6.

126. Yin W, Mu J, Birnbaum MJ. Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes. J Biol Chem 2003; 278:43074-80.

127. An D, Pulinilkunnil T, Qi D, et al. The metabolic "switch" AMPK regulates cardiac heparin-releasable lipoprotein lipase. Am J Physiol Endocrinol Metab 2005; 288:E246-53.

128. Dyck JR, Lopaschuk GD. AMPK alterations in cardiac physiology and pathology: enemy or ally? J Physiol 2006; 574:95-112.

129. Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 2006; 116:1776-83.

130. Fryer LG, Foufelle F, Barnes K, et al. Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. Biochem J 2002; 363:167-74.

131. Lehman JJ, Kelly DP. Gene regulatory mechanisms governing energy metabolism during cardiac hypertrophic growth. Heart Fail Rev 2002; 7:175-85.

132. Leff T. AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. Biochem Soc Trans 2003; 31:224-7.

133. Zheng D, MacLean PS, Pohnert SC, et al. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001; 91:1073-83.

134. Kawaguchi T, Osatomi K, Yamashita H, et al. Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. J Biol Chem 2002; 277:3829-35.

135. Ishii S, Iizuka K, Miller BC, Uyeda K. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. Proc Natl Acad Sci USA 2004; 101:15597-602.

136. da Silva Xavier G, Rutter GA, Diraison F, et al. ChREBP binding to fatty acid synthase and L-type pyruvate kinase genes is stimulated by glucose in pancreatic beta-cells. J Lipid Res 2006; 47:2482-91.

137. Lochhead PA, Salt IP, Walker KS, et al. 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. Diabetes 2000; 49:896-903.

138. Stoppani J, Hildebrandt A, Neufer P. Activation of AMP-activated protein kinase increases transcription of metabolic genes in rat gastrocnemius muscle (Abstract). FASEB J 2001; 15:A415.

139. Tae HJ, Luo X, Kim KH. Roles of CCAAT/enhancer-binding protein and its binding site on repression and derepression of acetyl-CoA carboxylase gene. J Biol Chem 1994; 269:10475-84.

140. Lopez-Casillas F, Kim KH. Heterogeneity at the 5' end of rat acetyl-coenzyme A carboxylase mRNA. Lipogenic conditions enhance synthesis of a unique mRNA in liver. J Biol Chem 1989; 264:7176-84.

141. Lopez-Casillas F, Ponce-Castaneda MV, Kim KH. In vivo regulation of the activity of the two promoters of the rat acetyl coenzyme-A carboxylase gene. Endocrinology 1991; 129:1049-58.

142. Lopez-Casillas F, Ponce-Castaneda MV, Kim KH. Acetyl-coenzyme A carboxylase mRNA metabolism in the rat liver. Metabolism 1992; 41:201-7.

143. Park K, Kim KH. Regulation of acetyl-CoA carboxylase gene expression. Insulin induction of acetyl-CoA carboxylase and differentiation of 30A5 preadipocytes require prior cAMP action on the gene. J Biol Chem 1991; 266:12249-56.

144. Park K, Pape M, Kim K-H. Sequence of acetyl-coA carboxylase promoter for tumour necrosis factor action. Mediat. Inflamm. 1993; 2:271-77.

145. Daniel S, Zhang S, DePaoli-Roach AA, Kim KH. Dephosphorylation of Sp1 by protein phosphatase 1 is involved in the glucose-mediated activation of the acetyl-CoA carboxylase gene. J Biol Chem 1996; 271:14692-7.

146. Oh SY, Lee MY, Kim JM, et al. Alternative usages of multiple promoters of the acetyl-CoA carboxylase beta gene are related to differential transcriptional regulation in human and rodent tissues. J Biol Chem 2005; 280:5909-16.

147. Lee JJ, Moon YA, Ha JH, et al. Cloning of human acetyl-CoA carboxylase beta promoter and its regulation by muscle regulatory factors. J Biol Chem 2001; 276:2576-85.

148. Kim JY, Lee JJ, Kim KS. Acetyl-CoA carboxylase beta expression mediated by MyoD and muscle regulatory factor 4 is differentially affected by retinoic acid receptor and retinoid X receptor. Exp Mol Med 2003; 35:23-9.

149. Park CE, Kim SM, Kim JM, et al. Rapid increase of cytosolic content of acetyl-CoA carboxylase isoforms in H9c2 cells by short-term treatment with insulin and okadaic acid. Exp Mol Med 1998; 30:73-9.

150. Foretz M, Pacot C, Dugail I, et al. ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. Mol Cell Biol 1999; 19:3760-8.

151. Oh SY, Park SK, Kim JW, et al. Acetyl-CoA carboxylase beta gene is regulated by sterol regulatory element-binding protein-1 in liver. J Biol Chem 2003; 278:28410-7.

152. Lefrancois-Martinez AM, Martinez A, Antoine B, et al. Upstream stimulatory factor proteins are major components of the glucose response complex of the L-type pyruvate kinase gene promoter. J Biol Chem 1995; 270:2640-3.

153. Shih H, Towle HC. Definition of the carbohydrate response element of the rat S14 gene. Context of the CACGTG motif determines the specificity of carbohydrate regulation. J Biol Chem 1994; 269:9380-7.

154. Foufelle F, Ferre P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem J 2002; 366:377-91.

155. Eberle D, Hegarty B, Bossard P, et al. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 2004; 86:839-48.

156. Sirito M, Lin Q, Maity T, Sawadogo M. Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. Nucleic Acids Res 1994; 22:427-33.

157. van Bilsen M, van der Vusse GJ, Reneman RS. Transcriptional regulation of metabolic processes: implications for cardiac metabolism. Pflugers Arch 1998; 437:2-14.

158. Vaulont S, Vasseur-Cognet M, Kahn A. Glucose regulation of gene transcription. J Biol Chem 2000; 275:31555-8.

159. Viollet B, Lefrancois-Martinez AM, Henrion A, et al. Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms. J Biol Chem 1996; 271:1405-15.

160. Girard J, Ferre P, Foufelle F. Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. Annu Rev Nutr 1997; 17:325-52.

161. Wang D, Sul HS. Upstream stimulatory factors bind to insulin response sequence of the fatty acid synthase promoter. USF1 is regulated. J Biol Chem 1995; 270:28716-22.

162. Casado M, Vallet VS, Kahn A, Vaulont S. Essential role in vivo of upstream stimulatory factors for a normal dietary response of the fatty acid synthase gene in the liver. J Biol Chem 1999; 274:2009-13.

163. Vallet VS, Henrion AA, Bucchini D, et al. Glucose-dependent liver gene expression in upstream stimulatory factor 2 -/- mice. J Biol Chem 1997; 272:21944-9.

164. Vallet VS, Casado M, Henrion AA, et al. Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose. J Biol Chem 1998; 273:20175-9.

165. Gopal-Srivastava R, Haynes JI, 2nd, Piatigorsky J. Regulation of the murine alpha B-crystallin/small heat shock protein gene in cardiac muscle. Mol Cell Biol 1995; 15:7081-90.

166. Navankasattusas S, Sawadogo M, van Bilsen M, et al. The basic helix-loop-helix protein upstream stimulating factor regulates the cardiac ventricular myosin light-chain 2 gene via independent cis regulatory elements. Mol Cell Biol 1994; 14:7331-9.

167. Xiao Q, Ojamaa K. Regulation of cardiac alpha-myosin heavy chain gene transcription by a contractile-responsive E-box binding protein. J Mol Cell Cardiol 1998; 30:87-95.
168. Ojamaa K, Samarel AM, Klein I. Identification of a contractile-responsive element in the cardiac alpha-myosin heavy chain gene. J Biol Chem 1995; 270:31276-81.
169. Makaula S, Adam T, Essop MF. Upstream stimulatory factor 1 transactivates the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase. Arch Biochem Biophys 2006; 446:91-100.
170. Moore ML, Park EA, McMillin JB. Upstream stimulatory factor represses the induction of carnitine palmitoyltransferase-Ibeta expression by PGC-1. J Biol Chem 2003; 278:17263-8.
171. Dowell RT. Mitochondrial component of the phosphorylcreatine shuttle is enhanced during rat heart perinatal development. Biochem Biophys Res Commun 1986; 141:319-25.
172. Kelly DP, Gordon JI, Alpers R, Strauss AW. The tissue-specific expression and developmental regulation of two nuclear genes encoding rat mitochondrial proteins. Medium chain acyl-CoA dehydrogenase and mitochondrial malate dehydrogenase. J Biol Chem 1989; 264:18921-5.
173. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J Biol Chem 1967; 242:2278-82.
174. Williams RS, Garcia-Moll M, Mellor J, et al. Adaptation of skeletal muscle to increased contractile activity. Expression nuclear genes encoding mitochondrial proteins. J Biol Chem 1987; 262:2764-7.
175. DiMauro S, Schon EA. Mitochondrial DNA mutations in human disease. Am J Med Genet 2001; 106:18-26.
176. Larsson NG, Oldfors A. Mitochondrial myopathies. Acta Physiol Scand 2001; 171:385-93.
177. Wallace DC. Mitochondrial diseases in man and mouse. Science 1999; 283:1482-8.
178. Vallance HD, Jeven G, Wallace DC, Brown MD. A case of sporadic infantile histiocytoid cardiomyopathy caused by the A8344G (MERRF) mitochondrial DNA mutation. Pediatr Cardiol 2004; 25:538-40.
179. Hug G, Bove KE, Soukup S. Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. N Engl J Med 1991; 325:1862-4.

180. Taroni F, Verderio E, Fiorucci S, et al. Molecular characterization of inherited carnitine palmitoyltransferase II deficiency. Proc Natl Acad Sci USA 1992; 89:8429-33.

181. Strauss AW, Powell CK, Hale DE, et al. Molecular basis of human mitochondrial very-long-chain acyl-CoA dehydrogenase deficiency causing cardiomyopathy and sudden death in childhood. Proc Natl Acad Sci USA 1995; 92:10496-500.

182. Rocchiccioli F, Wanders RJ, Aubourg P, et al. Deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase: a cause of lethal myopathy and cardiomyopathy in early childhood. Pediatr Res 1990; 28:657-62.

183. Bennett MJ, Rinaldo P, Strauss AW. Inborn errors of mitochondrial fatty acid oxidation. Crit Rev Clin Lab Sci 2000; 37:1-44.

184. Bennett MJ, Rinaldo P, Millington DS, et al. Medium-chain acyl-CoA dehydrogenase deficiency: postmortem diagnosis in a case of sudden infant death and neonatal diagnosis of an affected sibling. Pediatr Pathol 1991; 11:889-95.

185. Kelly DP, Strauss AW. Inherited cardiomyopathies. N Engl J Med 1994; 330:913-9.

186. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 2004; 18:357-68.

187. Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 2008; 88:611-38.

188. Evans MJ, Scarpulla RC. NRF-1: a trans-activator of nuclear-encoded respiratory genes in animal cells. Genes Dev 1990; 4:1023-34.

189. Virbasius CA, Virbasius JV, Scarpulla RC. NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. Genes Dev 1993; 7:2431-45.

190. Scarpulla RC. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochim Biophys Acta 2002; 1576:1-14.

191. Scarpulla RC. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. Gene 2002; 286:81-9.

192. Huo L, Scarpulla RC. Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. Mol Cell Biol 2001; 21:644-54.

193. Gugneja S, Scarpulla RC. Serine phosphorylation within a concise amino-terminal domain in nuclear respiratory factor 1 enhances DNA binding. J Biol Chem 1997; 272:18732-9.

194. Herzig RP, Scacco S, Scarpulla RC. Sequential serum-dependent activation of CREB and NRF-1 leads to enhanced mitochondrial respiration through the induction of cytochrome c. J Biol Chem 2000; 275:13134-41.

195. Virbasius JV, Scarpulla RC. Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc Natl Acad Sci USA 1994; 91:1309-13.

196. Wu Z, Puigserver P, Andersson U, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999; 98:115-24.

197. Lehman JJ, Barger PM, Kovacs A, et al. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest 2000; 106:847-56.

198. Chau CM, Evans MJ, Scarpulla RC. Nuclear respiratory factor 1 activation sites in genes encoding the gamma-subunit of ATP synthase, eukaryotic initiation factor 2 alpha, and tyrosine aminotransferase. Specific interaction of purified NRF-1 with multiple target genes. J Biol Chem 1992; 267:6999-7006.

199. Bergeron R, Ren JM, Cadman KS, et al. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab 2001; 281:E1340-6.

200. Xia Y, Buja LM, Scarpulla RC, McMillin JB. Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes governing mitochondrial proliferation and differentiation. Proc Natl Acad Sci USA 1997; 94:11399-404.

201. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003; 34:267-73.

202. Patti ME, Butte AJ, Crunkhorn S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci USA 2003; 100:8466-71.

203. Murakami T, Shimomura Y, Yoshimura A, et al. Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. Biochim Biophys Acta 1998; 1381:113-22.

204. Roepstorff C, Halberg N, Hillig T, et al. Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. Am J Physiol Endocrinol Metab 2005; 288:E133-42.

205. Onay-Besikci A. Regulation of cardiac energy metabolism in newborn. Mol Cell Biochem 2006; 287:1-11.
206. Makinde AO, Kantor PF, Lopaschuk GD. Maturation of fatty acid and carbohydrate metabolism in the newborn heart. Mol Cell Biochem 1998; 188:49-56.
207. Comline RS, Silver M. Some aspects of foetal and uteroplacental metabolism in cows with indwelling umbilical and uterine vascular catheters. J Physiol 1976; 260:571-86.
208. Girard J, Ferre P, Pegorier JP, Duee PH. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. Physiol Rev 1992; 72:507-62.
209. Lopaschuk GD, Spafford MA, Marsh DR. Glycolysis is predominant source of myocardial ATP production immediately after birth. Am J Physiol 1991; 261:H1698-705.
210. Wang C, Hu SM. Developmental regulation in the expression of rat heart glucose transporters. Biochem Biophys Res Commun 1991; 177:1095-100.
211. Postic C, Leturque A, Printz RL, et al. Development and regulation of glucose transporter and hexokinase expression in rat. Am J Physiol 1994; 266:E548-59.
212. Bristow J, Bier DM, Lange LG. Regulation of adult and fetal myocardial phosphofructokinase. Relief of cooperativity and competition between fructose 2,6-bisphosphate, ATP, and citrate. J Biol Chem 1987; 262:2171-5.
213. Lopaschuk GD, Witters LA, Itoi T, et al. Acetyl-CoA carboxylase involvement in the rapid maturation of fatty acid oxidation in the newborn rabbit heart. J Biol Chem 1994; 269:25871-8.
214. Makinde AO, Gamble J, Lopaschuk GD. Upregulation of 5'-AMP-activated protein kinase is responsible for the increase in myocardial fatty acid oxidation rates following birth in the newborn rabbit. Circ Res 1997; 80:482-9.
215. Blazquez E, Sugase T, Blazquez M, Foa PP. Neonatal changes in the concentration of rat liver cyclic AMP and of serum glucose, free fatty acids, insulin, pancreatic, and total glucagon in man and in the rat. J Lab Clin Med 1974; 83:957-67.
216. Haystead TA, Moore F, Cohen P, Hardie DG. Roles of the AMP-activated and cyclic-AMP-dependent protein kinases in the adrenaline-induced inactivation of acetyl-CoA carboxylase in rat adipocytes. Eur J Biochem 1990; 187:199-205.
217. Brown NF, Weis BC, Husti JE, et al. Mitochondrial carnitine palmitoyltransferase I isoform switching in the developing rat heart. J Biol Chem 1995; 270:8952-7.

218. Bartelds B, Takens J, Smid GB, et al. Myocardial carnitine palmitoyltransferase I expression and long-chain fatty acid oxidation in fetal and newborn lambs. Am J Physiol Heart Circ Physiol 2004; 286:H2243-8.

219. Goodwin CW, Mela L, Deutsch C, et al. Development and adaptation of heart mitochondrial respiratory chain function in fetus and in newborn. Adv Exp Med Biol 1976; 75:713-9.

220. Werner JC, Whitman V, Musselman J, Schuler HG. Perinatal changes in mitochondrial respiration of the rabbit heart. Biol Neonate 1982; 42:208-16.

221. Glatz JF, Veerkamp JH. Postnatal development of palmitate oxidation and mitochondrial enzyme activities in rat cardiac and skeletal muscle. Biochim Biophys Acta 1982; 711:327-35.

222. Fisher DJ, Heymann MA, Rudolph AM. Myocardial consumption of oxygen and carbohydrates in newborn sheep. Pediatr Res 1981; 15:843-6.

223. Rolph TP, Jones CT. Regulation of glycolytic flux in the heart of the fetal guinea pig. J Dev Physiol 1983; 5:31-49.

224. Montessuit C, Rosenblatt-Velin N, Lerch R. Metabolic changes in cardiac hypertrophy. Heart and Metabolism 2000; 9:3-8.

225. Allard MF. Energy substrate metabolism in cardiac hypertrophy. Curr Hypertens Rep 2004; 6:430-5.

226. Taegtmeyer H, King LM, Jones BE. Energy substrate metabolism, myocardial ischemia, and targets for pharmacotherapy. Am J Cardiol 1998; 82:54K-60K.

227. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. Heart Fail Rev 2007; 12:331-43.

228. Young ME, McNulty P, Taegtmeyer H. Adaptation and maladaptation of the heart in diabetes: Part II: potential mechanisms. Circ 2002; 105:1861-70.

229. Carley AN, Severson DL. Fatty acid metabolism is enhanced in type 2 diabetic hearts. Biochim Biophys Acta 2005; 1734:112-26.

230. Carley AN, Severson DL. What are the Biochemical Mechanisms Responsible for Enhanced Fatty Acid Utilization by Perfused Hearts from Type 2 Diabetic db/db Mice? Cardiovasc Drugs Ther 2008.

231. Reaven GM, Hollenbeck C, Jeng CY, et al. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. Diabetes 1988; 37:1020-4.

232. Debard C, Laville M, Berbe V, et al. Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of Type 2 diabetic patients. Diabetologia 2004; 47:917-25.

233. Schmitz-Peiffer C. Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply. Cell Signal 2000; 12:583-94.

234. Grundleger ML, Thenen SW. Decreased insulin binding, glucose transport, and glucose metabolism in soleus muscle of rats fed a high fat diet. Diabetes 1982; 31:232-7.

235. Storlien LH, James DE, Burleigh KM, et al. Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. Am J Physiol 1986; 251:E576-83.

236. Gilde AJ, van der Lee KA, Willemsen PH, et al. Peroxisome proliferator-activated receptor (PPAR) alpha and PPARbeta/delta, but not PPARgamma, modulate the expression of genes involved in cardiac lipid metabolism. Circ Res 2003; 92:518-24.

237. Jagasia D, Whiting JM, Concato J, et al. Effect of non-insulin-dependent diabetes mellitus on myocardial insulin responsiveness in patients with ischemic heart disease. Circ 2001; 103:1734-9.

238. Maki M, Nuutila P, Laine H, et al. Myocardial glucose uptake in patients with NIDDM and stable coronary artery disease. Diabetes 1997; 46:1491-6.

239. Carroll R, Carley AN, Dyck JR, Severson DL. Metabolic effects of insulin on cardiomyocytes from control and diabetic db/db mouse hearts. Am J Physiol Endocrinol Metab 2005; 288:E900-6.

240. Armoni M, Harel C, Bar-Yoseph F, et al. Free fatty acids repress the GLUT4 gene expression in cardiac muscle via novel response elements. J Biol Chem 2005; 280:34786-95.

241. Belke DD, Larsen TS, Gibbs EM, Severson DL. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. Am J Physiol Endocrinol Metab 2000; 279:E1104-13.

242. Mazumder PK, O'Neill BT, Roberts MW, et al. Impaired cardiac efficiency and increased fatty acid oxidation in insulin-resistant ob/ob mouse hearts. Diabetes 2004; 53:2366-74.

243. Coort SL, Hasselbaink DM, Koonen DP, et al. Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese zucker rats. Diabetes 2004; 53:1655-63.

244. Coort SL, Luiken JJ, van der Vusse GJ, et al. Increased FAT (fatty acid translocase)/CD36-mediated long-chain fatty acid uptake in cardiac myocytes from obese Zucker rats. Biochem Soc Trans 2004; 32:83-5.

245. Luiken JJ, Arumugam Y, Dyck DJ, et al. Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. J Biol Chem 2001; 276:40567-73.

246. Aasum E, Belke DD, Severson DL, et al. Cardiac function and metabolism in Type 2 diabetic mice after treatment with BM 17.0744, a novel PPAR-alpha activator. Am J Physiol Heart Circ Physiol 2002; 283:H949-57.

247. Christoffersen C, Bollano E, Lindegaard ML, et al. Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. Endocrinology 2003; 144:3483-90.

248. Zhou YT, Grayburn P, Karim A, et al. Lipotoxic heart disease in obese rats: implications for human obesity. Proc Natl Acad Sci USA 2000; 97:1784-9.

249. Young ME, Guthrie PH, Razeghi P, et al. Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. Diabetes 2002; 51:2587-95.

250. Befroy DE, Petersen KF, Dufour S, et al. Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes 2007; 56:1376-81.

251. Bavenholm PN, Kuhl J, Pigon J, et al. Insulin resistance in type 2 diabetes: association with truncal obesity, impaired fitness, and atypical malonyl coenzyme A regulation. J Clin Endocrinol Metab 2003; 88:82-7.

252. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM. Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects. Diabetes 2006; 55:2277-85.

253. Morino K, Petersen KF, Dufour S, et al. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest 2005; 115:3587-93.

254. Buchanan J, Mazumder PK, Hu P, et al. Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. Endocrinology 2005; 146:5341-9.

255. Sharma S, Adrogue JV, Golfman L, et al. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. Faseb J 2004; 18:1692-700.
256. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1963; 1:785-9.
257. Chatham JC, Seymour AM. Cardiac carbohydrate metabolism in Zucker diabetic fatty rats. Cardiovasc Res 2002; 55:104-12.
258. Dyck JR, Cheng JF, Stanley WC, et al. Malonyl coenzyme a decarboxylase inhibition protects the ischemic heart by inhibiting fatty acid oxidation and stimulating glucose oxidation. Circ Res 2004; 94:e78-84.
259. Saddik M, Gamble J, Witters LA, Lopaschuk GD. Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. J Biol Chem 1993; 268:25836-45.
260. Stanley WC, Hernandez LA, Spires D, et al. Pyruvate dehydrogenase activity and malonyl CoA levels in normal and ischemic swine myocardium: effects of dichloroacetate. J Mol Cell Cardiol 1996; 28:905-14.
261. Kennedy JA, Unger SA, Horowitz JD. Inhibition of carnitine palmitoyltransferase-1 in rat heart and liver by perhexiline and amiodarone. Biochem Pharmacol 1996; 52:273-80.
262. Lopaschuk GD, Spafford M. Response of isolated working hearts to fatty acids and carnitine palmitoyltransferase I inhibition during reduction of coronary flow in acutely and chronically diabetic rats. Circ Res 1989; 65:378-87.
263. Stephens TW, Higgins AJ, Cook GA, Harris RA. Two mechanisms produce tissue-specific inhibition of fatty acid oxidation by oxfenicine. Biochem J 1985; 227:651-60.
264. Broderick TL, Quinney HA, Lopaschuk GD. Carnitine stimulation of glucose oxidation in the fatty acid perfused isolated working rat heart. J Biol Chem 1992; 267:3758-63.
265. Kantor PF, Lucien A, Kozak R, Lopaschuk GD. The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. Circ Res 2000; 86:580-8.
266. An D, Rodrigues B. Role of changes in cardiac metabolism in development of diabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 2006; 291:H1489-506.

267. Poornima IG, Parikh P, Shannon RP. Diabetic cardiomyopathy: the search for a unifying hypothesis. Circ Res 2006; 98:596-605.

268. Boudina S, Abel ED. Diabetic cardiomyopathy revisited. Circulation 2007; 115:3213-23.

269. Shehadeh A, Regan TJ. Cardiac consequences of diabetes mellitus. Clin Cardiol 1995; 18:301-5.

270. Di Bonito P, Cuomo S, Moio N, et al. Diastolic dysfunction in patients with non-insulin-dependent diabetes mellitus of short duration. Diabet Med 1996; 13:321-4.

271. Zabalgoitia M, Ismaeil MF, Anderson L, Maklady FA. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. Am J Cardiol 2001; 87:320-3.

272. Fang ZY, Yuda S, Anderson V, et al. Echocardiographic detection of early diabetic myocardial disease. J Am Coll Cardiol 2003; 41:611-7.

273. Severson DL. Diabetic cardiomyopathy: recent evidence from mouse models of type 1 and type 2 diabetes. Can J Physiol Pharmacol 2004; 82:813-23.

274. Semeniuk LM, Kryski AJ, Severson DL. Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. Am J Physiol Heart Circ Physiol 2002; 283:H976-82.

275. Panagia M, Schneider JE, Brown B, et al. Abnormal function and glucose metabolism in the type-2 diabetic db/db mouse heart. Can J Physiol Pharmacol 2007; 85:289-94.

276. Aasum E, Hafstad AD, Severson DL, Larsen TS. Age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from db/db mice. Diabetes 2003; 52:434-41.

277. Vincent HK, Powers SK, Dirks AJ, Scarpace PJ. Mechanism for obesity-induced increase in myocardial lipid peroxidation. Int J Obes Relat Metab Disord 2001; 25:378-88.

278. Barouch LA, Gao D, Chen L, et al. Cardiac myocyte apoptosis is associated with increased DNA damage and decreased survival in murine models of obesity. Circ Res 2006; 98:119-24.

279. Kwon SH, Pimentel DR, Remondino A, et al. H(2)O(2) regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways. J Mol Cell Cardiol 2003; 35:615-21.

280. Ye G, Metreveli NS, Ren J, Epstein PN. Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. Diabetes 2003; 52:777-83.

281. Ye G, Metreveli NS, Donthi RV, et al. Catalase protects cardiomyocyte function in models of type 1 and type 2 diabetes. Diabetes 2004; 53:1336-43.

282. Shen X, Zheng S, Metreveli NS, Epstein PN. Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. Diabetes 2006; 55:798-805.

283. Kuo TH, Moore KH, Giacomelli F, Wiener J. Defective oxidative metabolism of heart mitochondria from genetically diabetic mice. Diabetes 1983; 32:781-7.

284. How OJ, Aasum E, Severson DL, et al. Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. Diabetes 2006; 55:466-73.

285. Wang P, Lloyd SG, Zeng H, et al. Impact of altered substrate utilization on cardiac function in isolated hearts from Zucker diabetic fatty rats. Am J Physiol Heart Circ Physiol 2005; 288:H2102-10.

286. Boudina S, Sena S, Theobald H, et al. Mitochondrial energetics in the heart in obesity-related diabetes: direct evidence for increased uncoupled respiration and activation of uncoupling proteins. Diabetes 2007; 56:2457-66.

287. Boudina S, Sena S, O'Neill BT, et al. Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. Circ 2005; 112:2686-95.

288. Rousset S, Alves-Guerra MC, Mozo J, et al. The biology of mitochondrial uncoupling proteins. Diabetes 2004; 53 Suppl 1:S130-5.

289. Schrauwen P, Saris WH, Hesselink MK. An alternative function for human uncoupling protein 3: protection of mitochondria against accumulation of nonesterified fatty acids inside the mitochondrial matrix. Faseb J 2001; 15:2497-502.

290. Echtay KS, Roussel D, St-Pierre J, et al. Superoxide activates mitochondrial uncoupling proteins. Nature 2002; 415:96-9.

291. Himms-Hagen J, Harper ME. Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. Exp Biol Med (Maywood) 2001; 226:78-84.

292. Murray AJ, Anderson RE, Watson GC, et al. Uncoupling proteins in human heart. Lancet 2004; 364:1786-8.

293. Wilson CR, Tran MK, Salazar KL, et al. Western diet, but not high fat diet, causes derangements of fatty acid metabolism and contractile dysfunction in the heart of Wistar rats. Biochem J 2007; 406:457-67.

294. Unger RH, Orci L. Diseases of liporegulation: new perspective on obesity and related disorders. Faseb J 2001; 15:312-21.

295. Szczepaniak LS, Victor RG, Orci L, Unger RH. Forgotten but not gone: the rediscovery of fatty heart, the most common unrecognized disease in America. Circ Res 2007; 101:759-67.

296. Bielawska AE, Shapiro JP, Jiang L, et al. Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. Am J Pathol 1997; 151:1257-63.

297. Thompson AL, Cooney GJ. Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. Diabetes 2000; 49:1761-5.

298. Chavez JA, Knotts TA, Wang LP, et al. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. J Biol Chem 2003; 278:10297-303.

299. Kim JK, Fillmore JJ, Sunshine MJ, et al. PKC-theta knockout mice are protected from fat-induced insulin resistance. J Clin Invest 2004; 114:823-7.

300. Richter C, Ghafourifar P. Ceramide induces cytochrome c release from isolated mitochondria. Biochem Soc Symp 1999; 66:27-31.

301. Wakasaki H, Koya D, Schoen FJ, et al. Targeted overexpression of protein kinase C beta2 isoform in myocardium causes cardiomyopathy. Proc Natl Acad Sci USA 1997; 94:9320-5.

302. Listenberger LL, Han X, Lewis SE, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci USA 2003; 100:3077-82.

303. McGavock JM, Lingvay I, Zib I, et al. Cardiac steatosis in diabetes mellitus: a 1H-magnetic resonance spectroscopy study. Circ 2007; 116:1170-5.

304. Szczepaniak LS, Dobbins RL, Metzger GJ, et al. Myocardial triglycerides and systolic function in humans: in vivo evaluation by localized proton spectroscopy and cardiac imaging. Magn Reson Med 2003; 49:417-23.

305. Chiu HC, Kovacs A, Ford DA, et al. A novel mouse model of lipotoxic cardiomyopathy. J Clin Invest 2001; 107:813-22.

306. Leone TC, Weinheimer CJ, Kelly DP. A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting

response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. Proc Natl Acad Sci USA 1999; 96:7473-8.

307. Finck BN, Han X, Courtois M, et al. A critical role for PPARalpha-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy: modulation by dietary fat content. Proc Natl Acad Sci USA 2003; 100:1226-31.

308. Yang J, Sambandam N, Han X, et al. CD36 deficiency rescues lipotoxic cardiomyopathy. Circ Res 2007; 100:1208-17.

309. Petersen KF, Dufour S, Befroy D, et al. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 2004; 350:664-71.

310. Watanabe K, Fujii H, Takahashi T, et al. Constitutive regulation of cardiac fatty acid metabolism through peroxisome proliferator-activated receptor alpha associated with age-dependent cardiac toxicity. J Biol Chem 2000; 275:22293-9.

311. Djouadi F, Brandt JM, Weinheimer CJ, et al. The role of the peroxisome proliferator-activated receptor alpha (PPAR alpha) in the control of cardiac lipid metabolism. Prostaglandins Leukot Essent Fatty Acids 1999; 60:339-43.

312. Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. Science 2001; 291:2613-6.

313. Essop MF, Camp HS, Choi CS, et al. Reduced Heart Size and Increased Myocardial Fuel Substrate Oxidation in ACC2 Mutant Mice. Am J Physiol Heart Circ Physiol 2008.

314. Abu-Elheiga L, Oh W, Kordari P, Wakil SJ. Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. Proc Natl Acad Sci USA 2003; 100:10207-12.

315. Savage DB, Choi CS, Samuel VT, et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. J Clin Invest 2006; 116:817-24.

316. Harary I, Farley B. In vitro studies of single isolated beating heart cells. Science 1960; 131:1674-5.

317. Harary I, Farley B. In vitro studies on single beating rat heart cells. II. Intercellular communication. Exp Cell Res 1963; 29:466-74.

318. Nelson TJ, Balza R, Jr., Xiao Q, Misra RP. SRF-dependent gene expression in isolated cardiomyocytes: regulation of genes involved in cardiac hypertrophy. J Mol Cell Cardiol 2005; 39:479-89.

319. Fu J, Gao J, Pi R, Liu P. An optimised protocol for culture of cardiomyocyte from neonatal rat. Cytotechnology 2005; 49:109-116.

320. Chlopcikova S, Psotova J, Miketova P. Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2001; 145:49-55.

321. Yamashita N, Nishida M, Hoshida S, et al. Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. J Clin Invest 1994; 94:2193-9.

322. Linssen MC, Vork MM, de Jong YF, et al. Fatty acid oxidation capacity and fatty acid-binding protein content of different cell types isolated from rat heart. Mol Cell Biochem 1990; 98:19-25.

323. Altschuld R, Gibb L, Ansel A, et al. Calcium tolerance of isolated rat heart cells. J Mol Cell Cardiol 1980; 12:1383-95.

324. Grasberger H, Ye H, Mashima H, Bell GI. Dual promoter structure of ZFP106: regulation by myogenin and nuclear respiratory factor-1. Gene 2005; 344:143-59.

325. Takahashi Y, Nakayama K, Itoh S, et al. Inhibition of the transcription of CYP1A1 gene by the upstream stimulatory factor 1 in rabbits. Competitive binding of USF1 with AhR.Arnt complex. J Biol Chem 1997; 272:30025-31.

326. Goetzl EJ, Lee H, Azuma T, et al. Gelsolin binding and cellular presentation of lysophosphatidic acid. J Biol Chem 2000; 275:14573-8.

327. Noah D, Blum M, Sherry B. Transfection of primary cardiomyocyte cultures with DNA and anti-sense oligonucleotides using fugene 6 transfection reagent. Biochemica 1998; 2:38-40.

328. Stavinoha MA, Rayspellicy JW, Hart-Sailors ML, et al. Diurnal variations in the responsiveness of cardiac and skeletal muscle to fatty acids. Am J Physiol Endocrinol Metab 2004; 287:E878-87.

329. Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997; 273:E1107-12.

330. World Health Statistics 2008. www.who.int/whosis/whostat/2008/en/index.html 2008.

331. Pfeifer K, Prezant T, Guarente L. Yeast HAP1 activator binds to two upstream activation sites of different sequence. Cell 1987; 49:19-27.

332. Hardie DG. Role of AMP-activated protein kinase in the metabolic syndrome and in heart disease. FEBS Lett 2008; 582:81-9.

333. Petersen KF, Befroy D, Dufour S, et al. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science 2003; 300:1140-2.

334. Michikawa Y, Mazzucchelli F, Bresolin N, et al. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science 1999; 286:774-9.

335. Murray AJ, Panagia M, Hauton D, et al. Plasma free fatty acids and peroxisome proliferator-activated receptor alpha in the control of myocardial uncoupling protein levels. Diabetes 2005; 54:3496-502.

336. Schrauwen P, Hesselink MK, Blaak EE, et al. Uncoupling protein 3 content is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 2001; 50:2870-3.

337. Harper ME, Dent RM, Bezaire V, et al. UCP3 and its putative function: consistencies and controversies. Biochem Soc Trans 2001; 29:768-73.

338. Harper ME, Gerrits MF. Mitochondrial uncoupling proteins as potential targets for pharmacological agents. Curr Opin Pharmacol 2004; 4:603-7.

339. Vidal-Puig AJ, Grujic D, Zhang CY, et al. Energy metabolism in uncoupling protein 3 gene knockout mice. J Biol Chem 2000; 275:16258-66.

340. Sebastian D, Herrero L, Serra D, et al. CPT I overexpression protects L6E9 muscle cells from fatty acid-induced insulin resistance. Am J Physiol Endocrinol Metab 2007; 292:E677-86.

341. Griffin ME, Marcucci MJ, Cline GW, et al. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes 1999; 48:1270-4.

342. Powell DJ, Turban S, Gray A, et al. Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. Biochem J 2004; 382:619-29.

343. Haemmerle G, Lass A, Zimmermann R, et al. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 2006; 312:734-7.

APPENDIX

1. CELL CULTURE

1.1. Cardiomyocyte isolation

1 x ADS buffer (pH 7.4)

NaCl	116.4 mM
KCl	5.4 mM
D-glucose	5.1 mM
$NaH_2PO_4.H_2O$	1.1 mM
$MgSO_4.7H_2O$	0.8 mM
HEPES	20.0 mM

The buffer was filter sterilised with a 0.22 micron filter (Microsep, South Africa).

Collagenase/pancreatin solution

Collagenase (394 U/mg)	0.0225 g
Pancreatin	0.0450 g

The solution was made up in 50 ml of 1 x ADS buffer, stirred for ~15 minutes to complete solubilisation, and filter sterilised with a 0.22 micron filter (Microsep, South Africa).

Percoll gradient

Percoll stock (10 ml):

Percoll	9 ml
10 x ADS buffer	1 ml

Dilutions:

(g/ml)	Percoll stock (ml)	1 x ADS buffer (ml)
1,050	2.25	4.00
1,062	2.89	3.36
1,082	3.95	2.30

Using a pasteur pipette, the Percoll solutions were added to the isolated cardiomyocytes as follows: bottom layer with 1,082 g/ml, middle layer with 1,062 g/ml and top layer with 1,050 g/ml. The tube was tilted and a pasteur pipette tip placed near the interface to prevent mixing of the different Percoll solutions.

1.2. Cell counting and viability

Trypan blue

5.2 mM

The solution was made up in PBS.

PBS (pH 7.4)

NaCl	136.9 mM
KCl	2.7 mM
Na_2HPO_4	4.0 mM
KH_2PO_4	1.8 mM

The solution was sterilised in an autoclave at 121°C for 20 minutes.

2. PLASMID DNA PREPARATION

2.1. Plasmid DNA isolation

2 x YT broth (1 litre)

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

The broth was sterilised in an autoclave at 121°C for 20 minutes.

2 x YT agar (1 litre)

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Agar	15 g

The agar was sterilised in an autoclave at 121°C for 20 minutes.

Ampicillin

100 mg/ml in sterile H_2O

The antibiotic was filter sterilised with a 0.22 micron filter (Microsep, South Africa), and added to 2 x YT broth or agar to a final concentration of 100 µg/ml.

1 x TE buffer (pH 7.4)

Tris	10 mM
EDTA	1 mM

The buffer was sterilised in an autoclave at 121°C for 20 minutes.

2.3. Plasmid DNA constructs

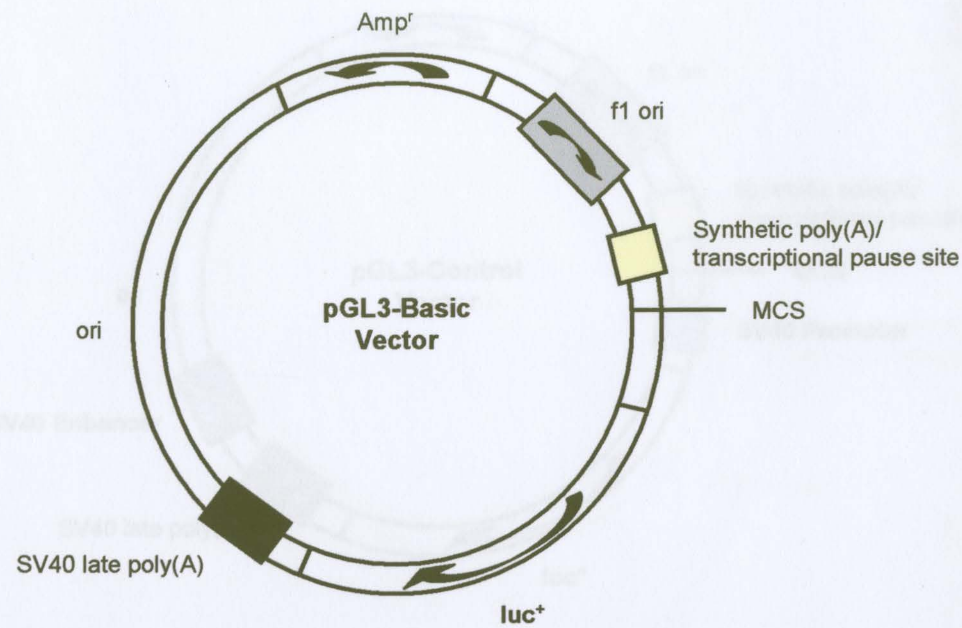


Figure 26. Circular map of pGL3-Basic vector. The pGL3-Basic vector is a reporter vector that does not contain promoter and enhancer sequences. luc$^+$ = firefly luciferase cDNA (arrow shows the direction of transcription); Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); MCS = multiple cloning site for insertion of DNA fragments; SV40 late poly(A) for effective termination of transcription and mRNA polyadenylation; synthetic poly(A) and transcriptional pause site for elimination of spurious transcription. Modified from Promega (Madison, WI, USA).

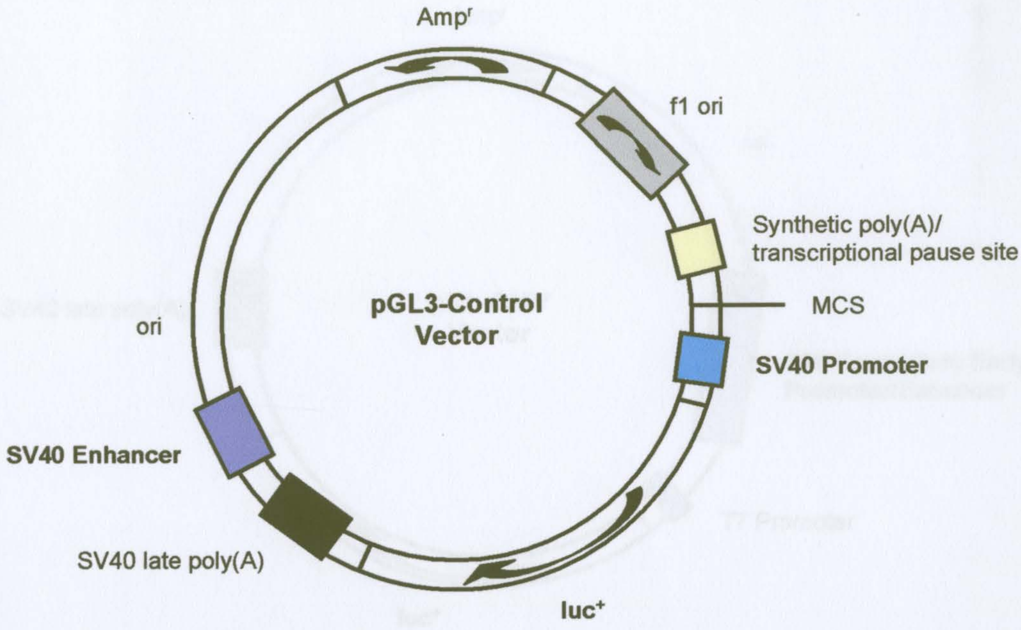


Figure 27. Circular map of pGL3-Control vector. The pGL3-Control vector contains the SV40 promoter and enhancer, which results in constitutive expression of luciferase gene. luc$^+$ = firefly luciferase cDNA (arrow shows the direction of transcription); Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); MCS = multiple cloning site for insertion of DNA fragments; SV40 late poly(A) for effective termination of transcription and mRNA polyadenylation; synthetic poly(A) and transcriptional pause site for elimination of spurious transcription. Modified from Promega (Madison, WI, USA).

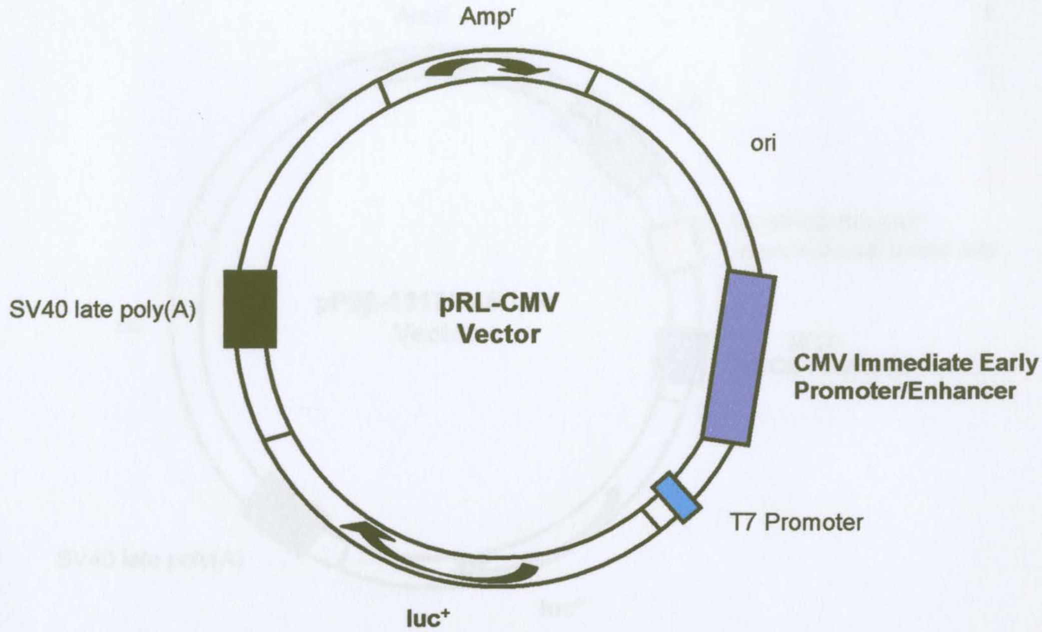


Figure 28. Circular map of pRL-CMV vector. The pRL-CMV vector contains the CMV immediate early promoter/enhancer for constitutive expression of *Renilla* luciferase gene. luc$^+$ = *Renilla* luciferase cDNA (arrow shows the direction of transcription); Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = origin of replication in *E. coli*; SV40 late poly(A) for effective termination of transcription and polyadenylation of mRNA; T7 promoter for synthesis of luciferase RNA transcripts. Modified from Promega (Madison, WI, USA).

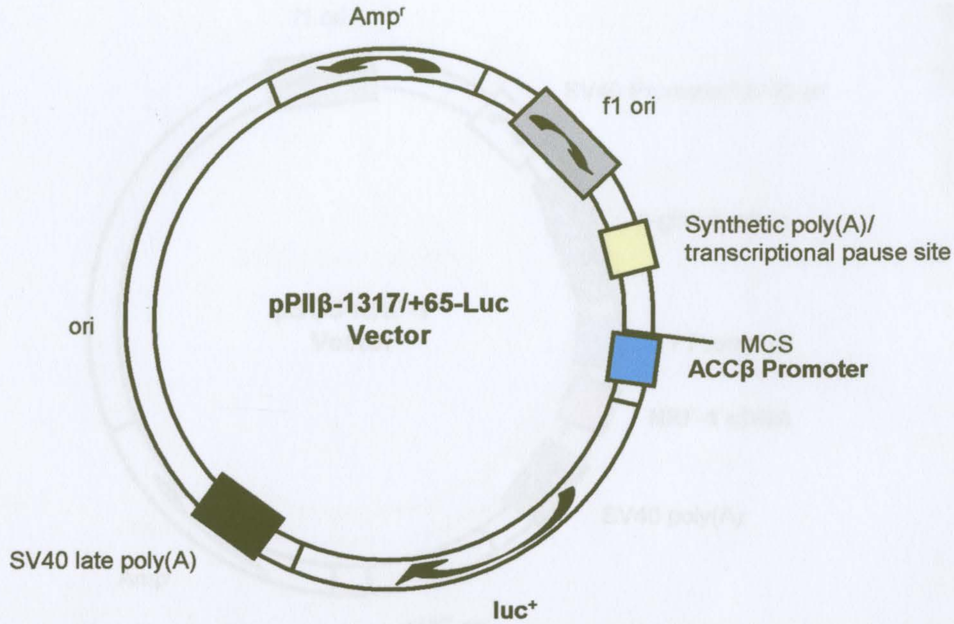


Figure 29. Circular map of pPIIβ-1317/+65-Luc vector. The 1400 bp (-1317 to +65) human ACCβ gene promoter was cloned into the *SmaI* site (located in MCS) of pGL3-Basic vector.[147] luc^+ = firefly luciferase cDNA (arrow shows the direction of transcription); Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); MCS = multiple cloning site for insertion of DNA fragments; SV40 late poly(A) for effective termination of transcription and mRNA polyadenylation; synthetic poly(A) and transcriptional pause site for elimination of spurious transcription.

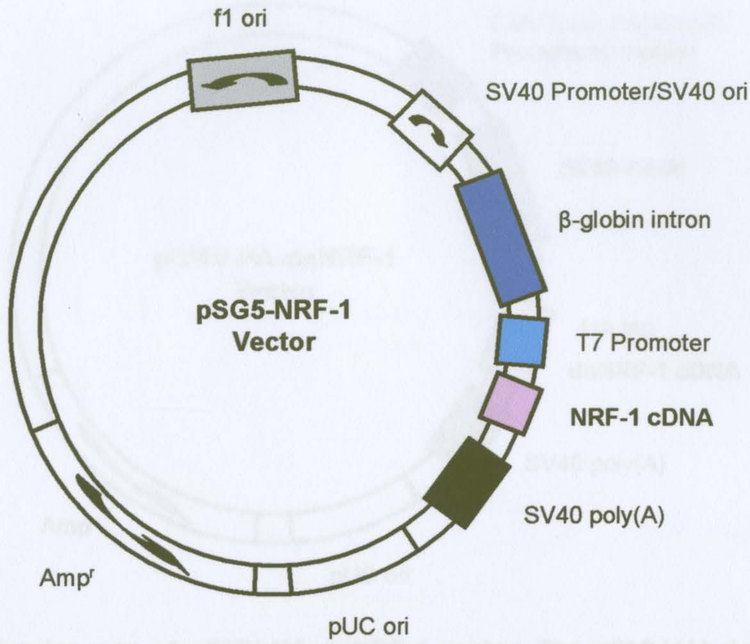


Figure 30. Circular map of pSG5-NRF-1 vector. The pSG5-NRF-1 vector is an expression vector that contains a 1543 bp (119 to 1662) NRF-1 cDNA sequence.[189] Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); pUC ori = pUC plasmid origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); SV40 poly(A) for effective termination of transcription and polyadenylation of mRNA; SV40 promoter and ori for elevated *in vivo* expression; T7 bacteriophage promoter for transcription of NRF-1 cDNA; β-globin intron for splicing of expressed transcripts.

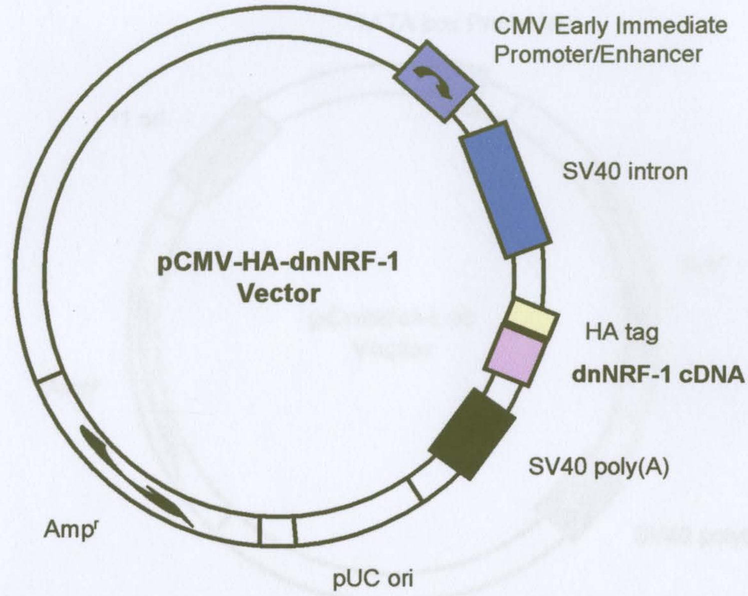


Figure 31. Circular map of pCMV-HA-dnNRF-1 vector. The pCMV-HA-dnNRF-1 vector is an expression vector that contains a 912 bp (1033 to 1944) NRF-1 cDNA sequence.[324] Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); pUC ori = pUC plasmid origin of replication in *E. coli*; SV40 poly(A) signal for effective termination of transcription and polyadenylation of mRNA; HA tag = hemagglutinin epitope tag; CMV early immediate promoter/enhancer for elevated *in vivo* expression; SV40 intron for splicing of expressed transcripts.

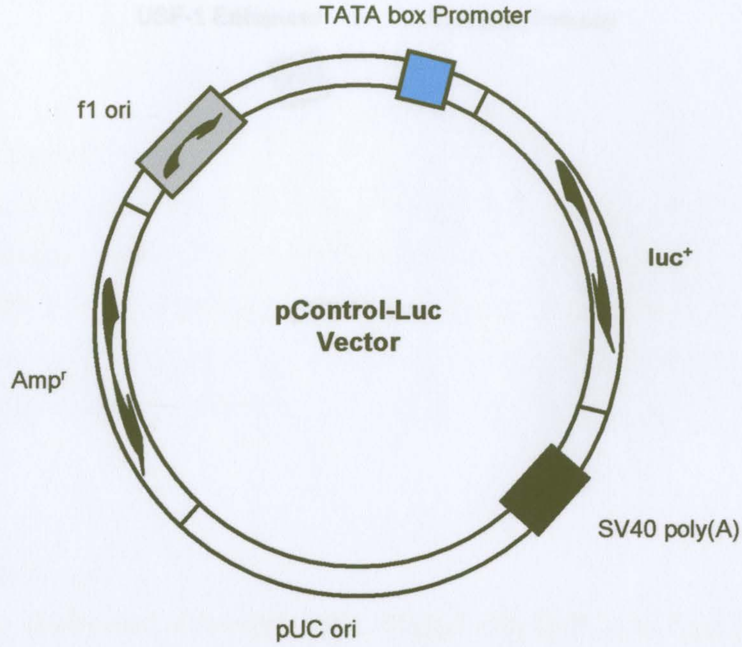


Figure 32. Circular map of pControl-Luc vector. The pControl-Luc vector is used to monitor endogenous transcriptional activity and lacks *cis*-acting enhancer elements. luc$^+$ = firefly luciferase cDNA (arrow shows the direction of transcription); Amp^r = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = pUC plasmid origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); SV40 poly(A) for effective termination of transcription and mRNA polyadenylation; TATA box promoter for expression of luciferase gene. Modified from Panomics (Redwood City, CA, USA).

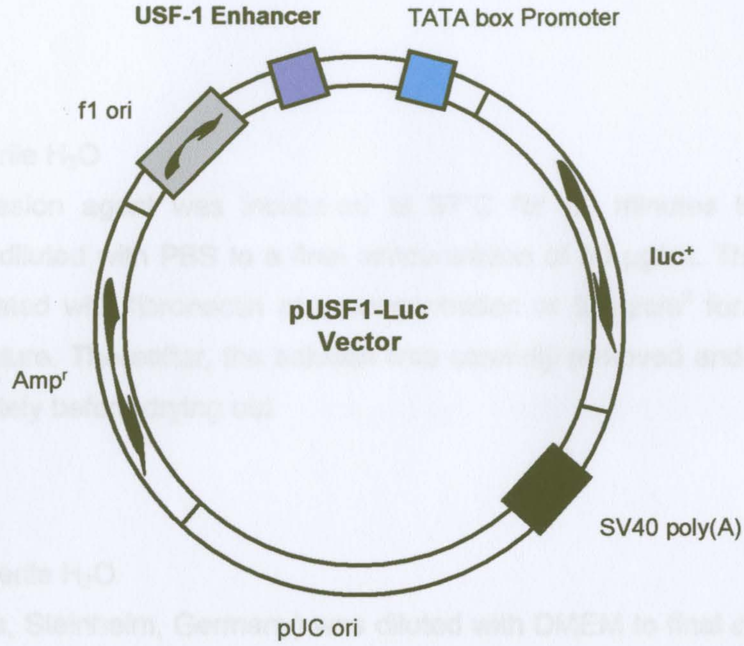


Figure 33. Circular map of pUSF1-Luc vector. The pUSF1-Luc vector contains multiple copies of USF1-specific enhancer elements. luc^{+} = firefly luciferase cDNA (arrow shows the direction of transcription); Amp^{r} = ampicillin resistance gene for selection in *E. coli* (arrow shows the direction of transcription); ori = pUC plasmid origin of replication in *E. coli*; f1 ori = filamentous phage origin of replication (arrow shows the direction of ssDNA synthesis); SV40 poly(A) for effective termination of transcription and polyadenylation of mRNA; TATA box promoter for expression of luciferase gene. Modified from Panomics (Redwood City, CA, USA).

3. TRANSIENT TRANSFECTION

Fibronectin

1 mg/ml in sterile H_2O

The cell adhesion agent was incubated at 37°C for 60 minutes to dissolve, and subsequently diluted with PBS to a final concentration of 50 µg/ml. The 12-well plates were then coated with fibronectin at a concentration of 5 µg/cm^2 for ~45 minutes at room temperature. Thereafter, the solution was carefully removed and the plates were used immediately before drying out.

AICAR

10 mg/ml in sterile H_2O

AICAR (Sigma, Steinheim, Germany) was diluted with DMEM to final concentrations of 0.5, 1 and 2 mM.

Metformin

100 mM in sterile H_2O

Metformin (Sigma, Steinheim, Germany) was diluted with DMEM to a final concentration of 1 mM.

5. ENDOGENOUS ACCβ GENE AND PROTEIN EXPRESSION

5.3. Quantitative Real-Time PCR

1% Agarose

1 g agarose D-1 LE (Hispanagar, Burgos, Spain) in 100 ml 1 x TAE buffer

The agarose was boiled until dissolved and then cooled before adding ethidium bromide to a final concentration of 0.5 µg/ml.

50 x TAE buffer (pH 8.0) (1 litre)

Tris	242 g
Glacial acetic acid	57.1 ml
500 mM EDTA	100 ml

The buffer was sterilised in an autoclave at 121°C for 20 minutes.

1 x TAE buffer

50 x TAE buffer	20 ml
H_2O	980 ml

Ethidium bromide

10 mg/ml solution (Promega, Madison, WI, USA)

Ethidum bromide was added to the 1% agarose solution to a final concentration of 0.5 µg/ml.